

Molecular basis for DNA cleavage by the mini-RNA-guided endonuclease Cas δ -1

Corresponding Author: Dr Yalan Zhu

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The manuscript presents the cryo-EM structure of the Cas δ -1-crRNA-DNA ternary complex, together with biochemical characterization of its nuclease activity. The structural data appear to be of generally good quality, and the study provides a useful structural description of a Cas δ effector complex.

However, overall the findings are highly reminiscent of previous studies on Cas12-family nucleases. The mechanistic insights appear largely consistent with what has already been established. The manuscript would benefit from a clearer positioning relative to prior Cas12 studies, with a more explicit discussion of both similarities and differences. Below are specific comments that I hope are helpful for the authors to improve their work.

1. In the concluding paragraph, some of the statements seem overstated. In particular, describing Cas δ -1 as “compact” or “miniature” is questionable given its length (~936 aa), especially considering that many Cas proteins or homologues reported in the literature are substantially smaller (e.g., ~400–600 aa). It would be better to revise this description to better reflect the current landscape of Cas protein sizes.
2. The manuscript would benefit from a clearer articulation of the key differences between Cas δ and other Cas12 proteins. The Introduction mentions that Cas δ is distinct, but I can hardly find specific differences; instead, I see many similarities. While the authors suggest that Cas δ -1 represents an evolutionary intermediate between Cas12n and canonical Type V systems, the specific features supporting this claim are not clearly described. Although a comparison with Cas12h is provided at the end, it would be beneficial to introduce these comparisons earlier to improve clarity.
3. The observation of a ssDNA substrate in the catalytic site has been reported multiple times in Cas9 and Cas12 proteins. These prior observations should be acknowledged and compared with the current structure.
4. In Fig. 4g, the cleavage assay compares WT and mutant proteins, but the differences in activity are quite subtle. Additional controls would help make the conclusions more convincing. In particular, it is unclear whether WT and mutant proteins were purified at comparable purity and used at similar amounts. It would be helpful to show protein purification results in a supplementary figure. In addition, all corresponding gel data should be presented.
5. Substrate DNA modeling in Fig. 4d: The density corresponding to the substrate DNA in Fig. 4d does not clearly support the modeled nucleotides, and the map appears to be displayed at a threshold that makes it difficult to assess the quality of the model fitting. The authors should provide clearer map–model fitting in this region, for example with close-up views at appropriate thresholds that demonstrate the fitting of individual nucleotides.
6. The Introduction could be more concise and focus more the specific features of Cas δ . In addition, the classification of CRISPR–Cas systems into six types is outdated, and the recently proposed Type VII systems should be acknowledged.

Reviewer #2

(Remarks to the Author)

* General comments:

In the present manuscript, Yin et al. investigated the biochemical characteristics and structural mechanisms of Cas δ -1, a recently reported V-type CRISPR enzyme. The authors elucidated the structure of the Cas δ -1–crRNA–target DNA triplex using cryo-electron microscopy at a resolution of 3.04 Å. Through biochemical experiments, it was confirmed that Cas δ -1 possesses double-stranded DNA (dsDNA) cleavage activity as an RNA-induced DNA endonuclease. Furthermore, by

identifying the precise cleavage sites of the target and non-target strands, it was revealed that staggered cleavages occur +16 nt downstream from the non-target strand (NTS) and +24 nt downstream from the target strand (TS). This study also explains the target DNA recognition mechanism of Cas δ -1 by presenting key amino acid residues involved in PAM (5'-ATG-3') recognition and crRNA scaffold binding. Overall, the manuscript is well-written and appears suitable for publication in the journal, Nature Communications. However, before publication, several experiments should be addressed to increase the value of this study.

* Specific comments:

1) I wonder if the Cas δ -1 system has pre-crRNA processing capabilities, similar to other type V Cas12 effector proteins. If so, multiplexed gene editing would also be feasible for Cas δ -1.

2) While the authors emphasize in main text that the Cas δ -1 system could be used for genome editing, it is surprising that there are no cell experiments at all. At least, it is necessary to show that gene editing is possible with Cas δ -1 and crRNA, as a proof-of-concept in human or mammalian cells.

3) It is interesting that TnpB-Cas δ -Cas12 is evolutionarily linked. I would appreciate it if the authors could illustrate this phylogenetically or diagrammatically to make it easier to understand, and kindly present the supporting evidences.

4) I wonder if Cas δ -1 can become a nickase or deactivated form by partially losing its activity.

Reviewer #3

(Remarks to the Author)

This manuscript by Yin et al. presents a comprehensive biochemical and structural characterization of Cas δ -1, a recently discovered compact RNA-guided endonuclease from the type V CRISPR-Cas family. The authors performed a biochemical characterization of Cas δ -1, demonstrating that this RNA-guided endonuclease exhibits robust double-stranded DNA cleavage activity and target-dependent trans-cleavage, and they determined its precise cleavage sites on both DNA strands. Additionally, the authors resolved a 3.04 Å cryo-EM structure of the Cas δ -1-crRNA-dsDNA ternary complex, which reveals molecular details of PAM recognition, crRNA architecture, and the catalytic site. This study provides structural insights into the mechanism of a compact type V CRISPR-Cas effector and may inform future development of genome editing tools. However, some concerns need to be addressed before the manuscript can be considered for publication.

1. Off-target effects represent an important aspect of genome editing tool characterization, but systematic evaluation of Cas δ -1's off-target activity is still lacking. Given the notable gene editing efficiency and application potential of Cas δ -1, it would be valuable for the authors to perform genome-wide off-target detection using in vivo methods (e.g. GUIDE-seq) or in vitro methods (e.g. Digenome-seq, CIRCLE-seq) to characterize the off-target profile of Cas δ -1.

2. Based on the cryo-EM structure, the authors identify several key residues involved in PAM recognition and catalytic activity, and validate their functional importance through alanine substitution followed by in vitro cleavage assays. However, this validation approach is relatively limited. For the PAM-interacting residues, alanine mutations only confirm that these residues are important for catalytic activity, but do not reveal whether they contribute specifically to PAM sequence discrimination. The authors should perform additional mutagenesis (e.g., charge-reversal or conservative mutations) on these residues and test cleavage activity on substrates with different PAM sequences to determine whether PAM specificity is altered. In addition, in vivo experiments (e.g., mammalian cell editing assays) comparing wild-type and key mutants would provide more direct validation of these residues' importance in living cells.

3. Structural analysis reveals that Cas δ -1 recognizes the PAM sequence through specific interactions. dG (-1*) forms a hydrogen bond with Ser111, while the phosphate groups of dT (-2*) and dA (-3*) interact with Tyr121, Leu124, Gln122, and Tyr114. Notably, these four residues only contact the phosphate backbones and do not interact with the nucleobases at positions -2* and -3*. Cas δ -1 has been reported to prefer an RYR PAM sequence, and this raises a critical question: how does Cas δ -1 achieve base discrimination at positions -2* and -3* when the identified interactions are only with the phosphate backbone. The authors should provide a mechanistic explanation for how Cas δ -1's PAM specificity is achieved.

4. There are many missing or improperly arranged characters across the figures, which hinder data interpretation. For example, in Fig. 3a, "crRNA Spacer" is incorrectly displayed as "crRNA S cer"; in Fig. 3d, "Arg592" appears as "Ar 592"; and in Fig. S4b, "crRNA" is shown as "c RNA". The authors should carefully review all figure labels to ensure they are complete, clear, and error-free.

5. In Fig. 3, Fig. 4, and Fig. S6, the authors indicate interactions between protein residues and nucleic acids with dashed lines. It would be helpful to also label the exact distances for these interactions, which would allow readers to better assess the strength and specificity of the binding.

6. In Fig. 3g and Fig. S6f, the authors illustrate base-flipping events between nucleotides C (-1) and G (1) in the crRNA, and between dC (-1) and dC (1) in the TS DNA. However, these panels display only a single strand, making it difficult to understand the base-flipping context. It would be helpful to also include the pairing strand (the complementary DNA or crRNA) in these panels, allowing readers to clearly visualize how the flipped bases are displaced from the duplex.

7. In Fig. 4g, the bar graph shows wild-type cleavage efficiency as 100%, but the corresponding gel image in the upper panel shows a clear uncleaved band, indicating that cleavage efficiency is not actually 100%. The authors should verify the quantification and ensure that the bar graph accurately reflects the gel data.

8. The authors position Cas δ -1 as an evolutionary transitional nuclease bridging Cas12n and canonical type V systems, and compare its structure with TnpB, Cas12h, Cas12i, and other Cas12 effectors. However, a structural comparison with Cas12n is notably absent. A recent study reported the cryo-EM structure of RdCas12n (Nat Commun 2025, 16(1):5667; PDB: 9J09). The authors could directly compare this structure with Cas δ -1 to better understand the evolutionary relationship within type V CRISPR systems.

9. The authors present numerous local interactions between protein residues and nucleic acids, but an overall protein-nucleic acid interaction map is lacking. It would be helpful to provide a schematic diagram summarizing these interactions, either by annotating the interacting residues on the existing schematic in Fig. S3a or by creating an additional figure that comprehensively shows the protein-RNA/DNA interaction networks.

10. In addition to Mg²⁺, the authors show that Mn²⁺ and Ni²⁺ also support Cas δ -1 cleavage activity. This is an interesting observation, as other type V systems such as Cas12f and Cas12n have been reported to exhibit cleavage activity only in the presence of Mg²⁺. It would be valuable for the authors to discuss the structural features that may enable Cas δ -1 to utilize Mn²⁺ and Ni²⁺, and to discuss the potential physiological significance of this broader metal tolerance.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

I appreciate the authors' careful responses to my previous comments. The revised manuscript has been substantially improved, and my previous concerns have been adequately addressed. I have no further comments.

Reviewer #2

(Remarks to the Author)

The authors responded to most of the issues I raised in the last review, and I felt that the revised edition was greatly improved. I would recommend publication of this revised version.

Reviewer #3

(Remarks to the Author)

In the revised manuscript, the authors have addressed most of the concerns raised in my previous review, including the addition of off-target analysis at predicted loci, conservative mutagenesis of PAM-interacting residues, in vivo gene editing assays in HEK293T cells, structural comparison with Cas12n, and a summarized protein-nucleic acid interaction map. I appreciate these efforts and am generally satisfied with the revisions, supporting publication in Nature Communications. However, before final acceptance, I would like the authors to address two remaining issues.

1. The authors evaluate off-target effects by examining only a limited number of computationally predicted top off-target loci that share high sequence homology with the on-target sites. While this approach provides an initial assessment of specificity, it is inherently limited and cannot exclude the possibility of off-target mutations at genome-wide unpredictable sites or at loci with lower sequence similarity. Therefore, the conclusion that Cas δ -1 "demonstrates its high-fidelity precision genome editing capability" (lines 105–106) is overstated. To maintain scientific rigor, I suggest that the authors should soften this claim.

In addition, in Fig. 1b, the control (CK) group shows detectable indel signals at both the on-target site and OFT-01. This result is rather strange and may raise concerns about background noise or sample contamination. The authors should provide an appropriate explanation.

2. Since deep sequencing was performed in the revised manuscript, the raw sequencing reads should be deposited in a public repository (e.g., NCBI SRA or GSA), and the corresponding accession number should be provided in the Data availability section to ensure data accessibility.

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We thank the reviewers for their valuable comments. In our point-by-point response below, we have specifically addressed the reviewers' concerns. The revised sections in the manuscript are highlighted in yellow. Our detailed responses to reviewers' concerns are presented below (reviewers' comments are in red and our responses are in black).

Reviewer #1 (Remarks to the Author):

The manuscript presents the cryo-EM structure of the Cas δ -1-crRNA-DNA ternary complex, together with biochemical characterization of its nuclease activity. The structural data appear to be of generally good quality, and the study provides a useful structural description of a Cas δ effector complex.

However, overall, the findings are highly reminiscent of previous studies on Cas12-family nucleases. The mechanistic insights appear largely consistent with what has already been established. The manuscript would benefit from a clearer positioning relative to prior Cas12 studies, with a more explicit discussion of both similarities and differences. Below are specific comments that I hope are helpful for the authors to improve their work.

We thank the reviewer for the thoughtful and constructive comments on our manuscript. We fully agree with the reviewer's assessment that our structural data are of good quality, but that the mechanistic novelty and the positioning relative to prior Cas12-family studies need to be substantially strengthened. In response to the reviewer's concerns, we have carefully revised the manuscript and deepened the discussion accordingly.

Specifically: 1) We have revised the Introduction section to include an updated classification of CRISPR-Cas systems, with the addition of the newly identified type VI subtypes of Class 2 CRISPR-Cas systems (lines 45-49). In addition, we have streamlined the text to make it more concise; in the revised manuscript, we have shortened the background descriptions of Cas9 and Cas12 to focus more specifically on the distinctive features of Cas δ (lines 52-69 and 74-84). For example, we now clearly highlight the distinct PAM specificity of Cas δ -1 (lines 77-81). At the same time, we have emphasized the structural retention of the C-terminal domain (CTD) in Cas δ , a domain that is highly conserved in both the ancestral TnpB nuclease and Cas12n, but is absent in other characterized Cas12 subtypes (lines 81-84). This feature serves as a key structural hallmark supporting Cas δ as an evolutionary transitional nuclease that bridges Cas12n and canonical type V systems.

2) In the Results section, we have added new data demonstrating the intracellular gene-editing activity of Cas δ -1, confirming that it mediates high-fidelity precise genome editing in human cells (lines 102-106, below figure, and Fig. 1a, b). Furthermore, we have included additional data validating the functional significance of key residues *in vivo*, including PAM-recognition residues and critical residues within the catalytic center (lines 227-232, 266-271, below figure, and Fig. 4h).

3) To further strengthen our Discussion, we incorporated a structure-guided phylogenetic analysis (lines 323-348 and Fig. 5f), which underscores the distinctive evolutionary status of Cas δ -1 and provides a clearer delineation of its phylogenetic placement within the Cas12 family.

We believe these revisions have significantly improved the clarity, depth, and perceived novelty of our work, and we hope they adequately address the reviewer's concerns.

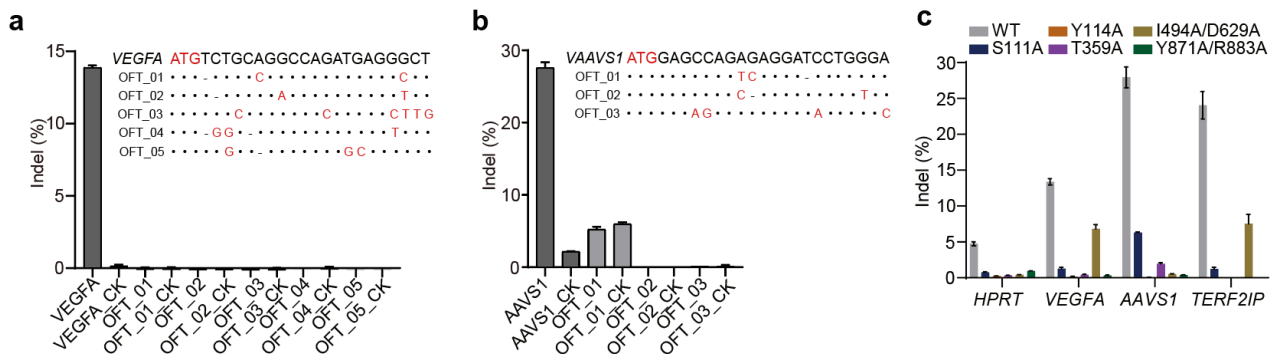


Figure Caption: Cas δ -1 mediates high-fidelity precise genome editing in human cells: a, b Indel activities of the engineered Cas δ -1 system on the VEGFA (a) and AAVS1 (b) genome loci in HEK293T cells and the top off-target loci predicted by Cas-OFFinder. Data are represented as mean $\pm$ SD (n = 3). **c** Indel activities of the Cas δ -1 and its PAM-recognized or catalytic site key amino acid residue alanine substitution mutants at HPRT, VEGFA and AAVS1 genome loci in HEK293T cells. Data are represented as mean $\pm$ SD (n = 3).

1. In the concluding paragraph, some of the statements seem overstated. In particular, describing Cas δ -1 as “compact” or “miniature” is questionable given its length (~936 aa), especially considering that many Cas proteins or homologues reported in the literature are substantially smaller (e.g., ~400-600 aa). It would be better to revise this description to better reflect the current landscape of Cas protein sizes.

We thank the reviewer for this suggestion. We agree that describing Cas δ -1 (936 aa) as “compact” or “miniature” is inaccurate, particularly given the existence of smaller homologues (e.g., 400-600 aa). We have removed the terms “compact” and “miniature” when referring to Cas δ -1 in the revised manuscript.

2. The manuscript would benefit from a clearer articulation of the key differences between Cas δ and other Cas12 proteins. The Introduction mentions that Cas δ is distinct, but I can hardly find specific differences; instead, I see many similarities.

We thank the reviewer for pointing out this issue. Compared with other Cas12 family members, the particularly critical differences of Cas δ lie in its distinct PAM specificity and the conserved the C-terminal domain (CTD). In our revised manuscript, we have restructured the Introduction to more prominently highlight these features (lines 74-84).

While the authors suggest that Cas δ -1 represents an evolutionary intermediate between Cas12n and canonical Type V systems, the specific features supporting this claim are not clearly described. Although a comparison with Cas12h is provided at the end, it would be beneficial to introduce these comparisons earlier to improve clarity.

Thank you, previous study has put forward the core concept that Cas δ -1 represents an evolutionary intermediate bridging Cas12n and canonical type V CRISPR-Cas systems (*Nucleic Acids Res* 53, doi:10.1093/nar/gkaf1358 (2025)). however, due to the absence of high-resolution structural data, the molecular basis underpinning this transitional role and its phylogenetic implications have remained largely unsubstantiated. In our revised manuscript, we have substantially restructured both the Introduction and Discussion sections. In the Introduction, we explicitly put forward the core concept that “Cas δ proteins represent an evolutionary transitional nuclease that bridges Cas12n and canonical type V systems” (lines 83-84). At the same time, we emphasize that although functional evidence supports this evolutionary position, “the structural basis underlying this bridging role remains largely unresolved, and key questions await further elucidation” (lines 86-88).

Leveraging the high-resolution Cas δ -crRNA-dsDNA ternary complex structures determined in our study, in the revised manuscript, we devote the Discussion section to a systematic structural analysis of the evolutionary relationships among Cas δ , TnpB, Cas12n, Cas12h, Cas12a, and Cas12b. Through comparative analyses of key structural elements, mainly the presence or absence of domains such as CTD, RuvC, REC2, and PI, we further reveal the transitional architectural features of Cas δ at the structural level (lines 323-348, below figure, and Fig. 5f). Collectively, these observations provide direct structural evidence that substantiates the unique position of Cas δ within the evolutionary lineage of the Cas12 family.

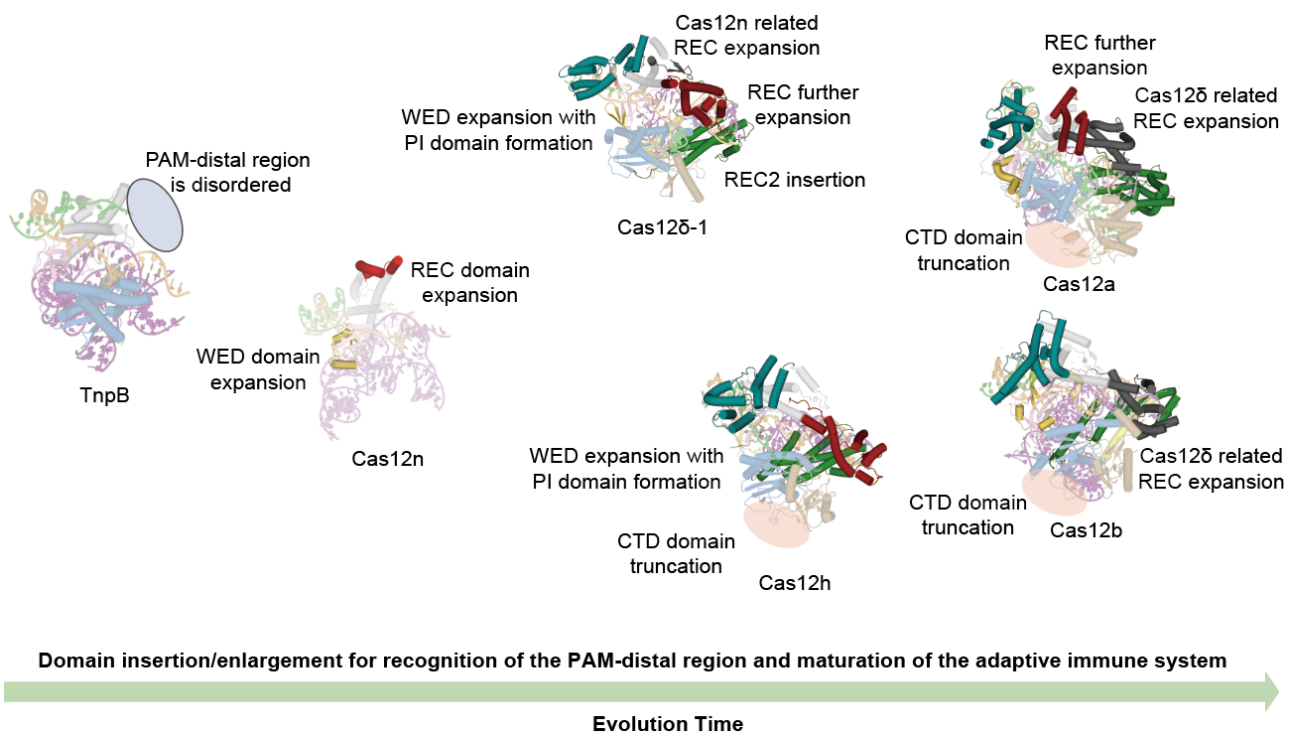


Figure Caption: Model of evolution from TnpB to Cas12 enzymes: Structural comparison of Cas δ -1

(PDB: 23RH) with TnpB (PDB:8H1J), RdCas12n (PDB: 9J09), Cas12h (PDB: 9L12), Cas12a (PDB: 8SFO), and AacCas12b (PDB: 5U33). The region corresponding to the REC expansion in Cas δ -1 that is already present in Cas12n, as well as the REC expansions in Cas12a and Cas12b that are already present in Cas δ -1, are highlighted in gray. REC1 expansion in Cas12n, Cas δ -1 and Cas12h and Cas12 are highlighted in red. The WED domain expansions, PI domain and REC2 domain insertion are highlighted in yellow, cyan and green, respectively. Other major differences are indicated by Elliptical Box.

3. The observation of a ssDNA substrate in the catalytic site has been reported multiple times in Cas9 and Cas12 proteins. These prior observations should be acknowledged and compared with the current structure.

We thank the reviewer for the suggestion. We have acknowledged those earlier findings and compared those structures with our current structure in the revised manuscript (lines 251-252, below figure, and Supplementary Fig. 8).

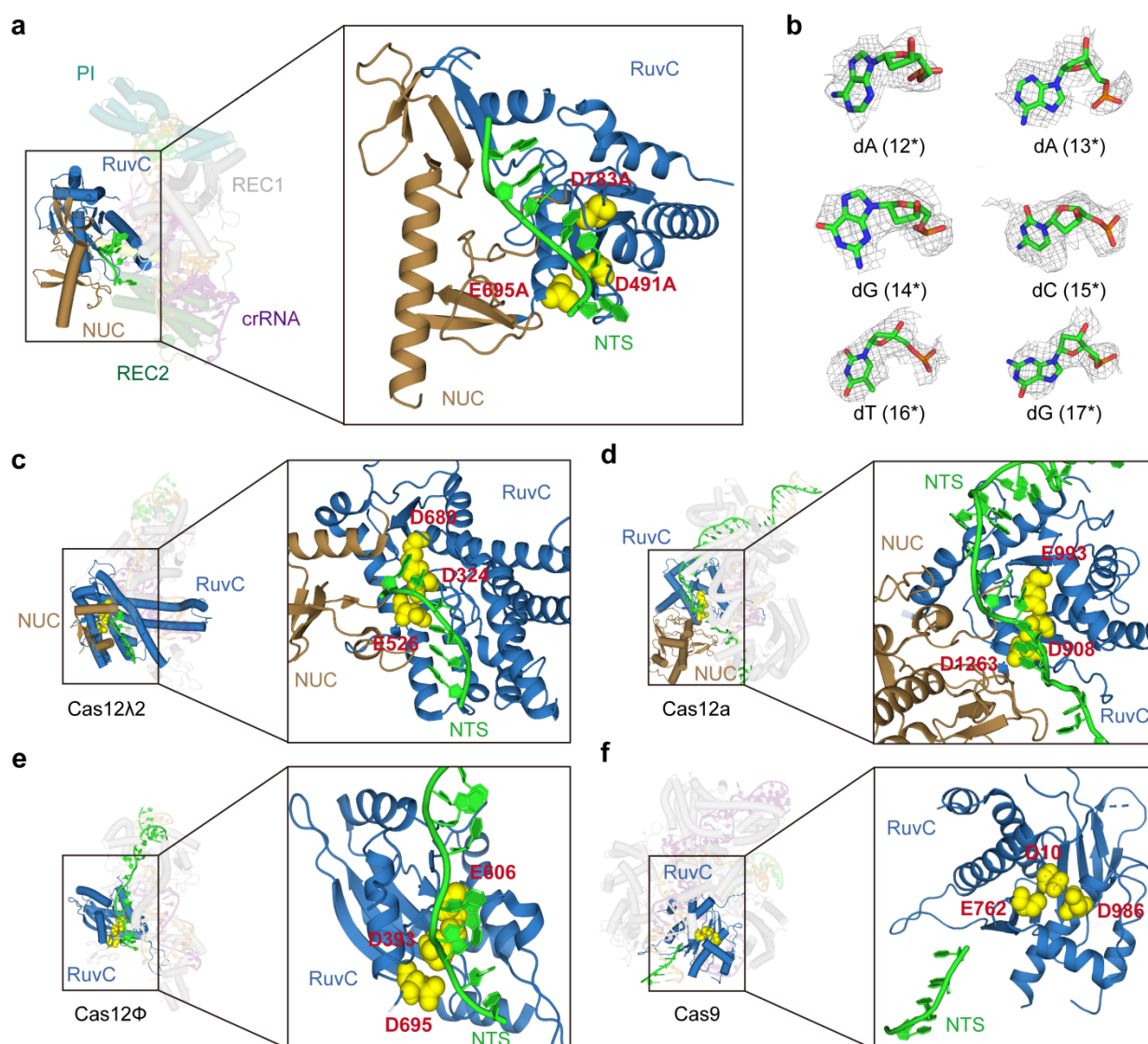


Figure Caption: Observation of the ssDNA substrate in the catalytic site in Cas δ -1^{WT}-crRNA-DNA complex: a The 6-nt ssDNA density bound observed within the groove between the RuvC and TNB

domains. RuvC domain is colored in skyblue, NUC domain domain is colored in sand and NTS are colored in green. The active residues are colored in yellow and shown as sphere models. **b** Electron densities for the 6-nt ssDNA density bound observed. Cryo-EM density maps contoured at 7σ . **c-f** The observation of a ssDNA substrate in the catalytic site in Cas12 λ 2 (PDB: 9IZR) (c), Cas12a (PDB: 8SFO) (d), Cas12 Φ (PDB: 7LYT) (e), or Cas9 (PDB: 6O0Y) (f).

4. In Fig. 4g, the cleavage assay compares WT and mutant proteins, but the differences in activity are quite subtle. Additional controls would help make the conclusions more convincing. In particular, it is unclear whether WT and mutant proteins were purified at comparable purity and used at similar amounts. It would be helpful to show protein purification results in a supplementary figure. In addition, all corresponding gel data should be presented.

We thank the reviewer for the suggestion. In our cleavage assays, the amounts of protein and substrate DNA were kept consistent between WT and mutant proteins across all reactions. Specifically, each reaction contained 100 nM of protein and 20 nM of substrate DNA, as detailed in the Methods section (lines 500-501). Furthermore, in this revised manuscript, we re-expressed and purified all WT and mutant proteins, and have included the purification results along with the corresponding gel data (Supplementary Fig. 7a-d). We further utilized this newly purified set of proteins to reassess the cleavage activities. To enhance the rigor of our experiments, we added a control lane containing substrate DNA only. The improved experimental setup not only yielded higher-quality result images but also allowed us to perform quantitative statistical analyses based on substrate depletion (below figure, Fig. 4g, and Supplementary Fig. 7e). The results demonstrate that the mutant proteins exhibit significantly reduced cleavage activities compared to the WT protein.

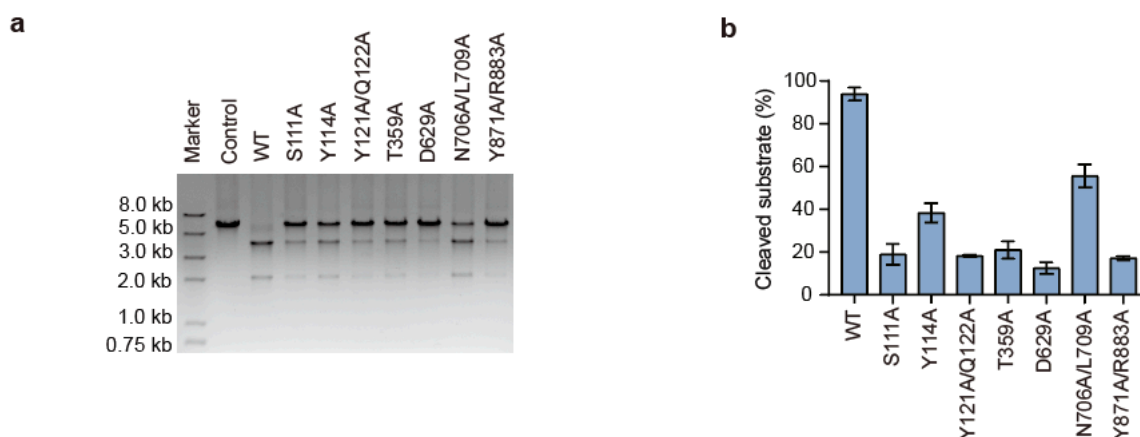


Figure Caption: Enzymatic activity analysis of target DNA-interacting residues mutants: **a** Alanine substitution of target DNA-interacting residues to analyze their effect on the DNA cleavage activity of Cas δ -1. **b** Cleavage activity statistics following alanine substitution of key amino acid residues responsible for PAM recognition and NTS chain cleavage. Data are represented as mean $\pm$ SD ($n = 3$).

5. Substrate DNA modeling in Fig. 4d: The density corresponding to the substrate DNA in Fig. 4d does not clearly support the modeled nucleotides, and the map appears to be displayed at a

threshold that makes it difficult to assess the quality of the model fitting. The authors should provide clearer map–model fitting in this region, for example with close-up views at appropriate thresholds that demonstrate the fitting of individual nucleotides.

We thank the reviewer for the suggestion. In the revised manuscript, we re-analyzed the density map for the substrate DNA region, increased the display threshold from the previous 6σ to 7σ , and provided close-up views of individual nucleotide densities (below figure, Fig. 4d, and Supplementary Fig. 8b). The revised figure demonstrates a good fit between the nucleotides and the electron density map, confirming the reliability of the model fitting.

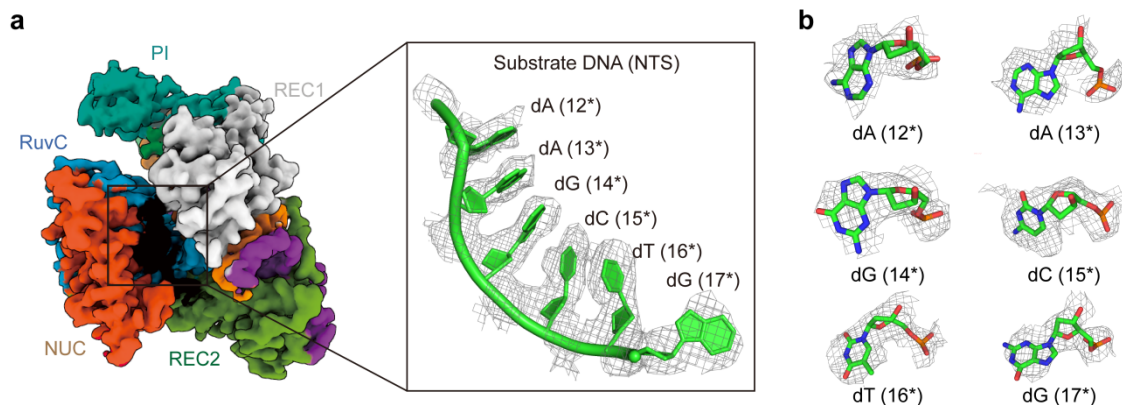


Figure Caption: Observation of the ssDNA substrate in the catalytic site in Cas δ -1^{WT}-crRNA-DNA

complex: **a** The 6-nt ssDNA density bound observed within the groove between the RuvC and TNB domains. RuvC domain is colored in skyblue, NUC domain domain is colored in sand and NTS are colored in green. The active residues are colored in yellow and shown as sphere models. **b** Electron densities for the 6-nt ssDNA density bound observed. Cryo-EM density maps contoured at 7σ .

6. The Introduction could be more concise and focus more the specific features of Cas δ . In addition, the classification of CRISPR–Cas systems into six types is outdated, and the recently proposed Type VII systems should be acknowledged.

We thank the reviewer for the insightful suggestion. We have revised the Introduction section and streamlined the text to make it more concise; in the revised manuscript, we have shortened the background descriptions of Cas9 and Cas12 to focus more specifically on the distinctive features of Cas δ (lines 52-69 and 74-84). For example, we now clearly highlight the distinct PAM specificity of Cas δ -1: “Cas δ -1 specifically recognizes 5'-RYR-3' PAM sequences (where R = A or G, Y = T or C), with 5'-ATG conferring the highest editing efficiency. This feature markedly contrasts with the T-rich PAM motifs typically favored by canonical type V Cas12 family nucleases, such as Cas12a and Cas12b, as well as the A-rich PAM motifs by the earliest type V intermediate, Cas12n” (lines 77-81). At the same time, we have emphasized the structural retention of the C-terminal domain (CTD) in Cas δ , a domain that is highly conserved in both the ancestral TnpB nuclease and Cas12n, but is absent in other characterized Cas12 subtypes (lines 81-84). This feature serves as a key structural hallmark supporting Cas δ as an evolutionary transitional nuclease that bridges Cas12n and canonical type V systems. Additionally, we have updated the classification of CRISPR-Cas systems

to acknowledge the recently proposed Type VII systems (lines 45-49).

Reviewer #2 (Remarks to the Author):

* General comments:

In the present manuscript, Yin et al. investigated the biochemical characteristics and structural mechanisms of Cas δ -1, a recently reported V-type CRISPR enzyme. The authors elucidated the structure of the Cas δ -1–crRNA–target DNA triplex using cryo-electron microscopy at a resolution of 3.04 Å. Through biochemical experiments, it was confirmed that Cas δ -1 possesses double-stranded DNA (dsDNA) cleavage activity as an RNA-induced DNA endonuclease. Furthermore, by identifying the precise cleavage sites of the target and non-target strands, it was revealed that staggered cleavages occur +16 nt downstream from the non-target strand (NTS) and +24 nt downstream from the target strand (TS). This study also explains the target DNA recognition mechanism of Cas δ -1 by presenting key amino acid residues involved in PAM (5'-ATG-3') recognition and crRNA scaffold binding. Overall, the manuscript is well-written and appears suitable for publication in the journal, Nature Communications. However, before publication, several experiments should be addressed to increase the value of this study.

We sincerely thank the reviewer for summarizing the main contributions of this study and for recommending it that “*the manuscript is well-written and appears suitable for publication in the journal, Nature Communications*”. In response, we have provided detailed explanations to address the reviewer’s concerns.

* Specific comments:

1) I wonder if the Cas δ -1 system has pre-crRNA processing capabilities, similar to other type V Cas12 effector proteins. If so, multiplexed gene editing would also be feasible for Cas δ -1.

We thank the reviewer for the insightful and valuable suggestion. During the revision, we assessed whether Cas δ -1 possesses pre-crRNA processing capability in *vitro* and in *vivo* levels. We first evaluated the in *vitro* pre-crRNA processing activity of Cas δ -1 using purified recombinant protein and a synthetic pre-crRNA mimic generated by extending the crRNA sequence at its 3' end. The results demonstrated that Cas δ -1 cleaves the pre-crRNA substrate, producing a stable crRNA species consistent with the expected mature length (below figure). We next examined whether such processing occurs in *vivo*. Following a previously established method (*Cell Rep* 44, 115718, doi: 10.1016/j.celrep.2025.115718 (2025)), we co-expressed the pre-crRNA with Cas δ -1. Critically, urea denaturing gel electrophoresis revealed that the RNA co-purified with Cas δ -1 was shorter than the pre-crRNA and corresponded in size to the expected mature crRNA (below figure). These results indicate that Cas δ -1 possesses pre-crRNA processing capability. However, the detailed mechanism,

including which domain is responsible for this activity and the precise cleavage site within the pre-crRNA, remains to be fully elucidated.

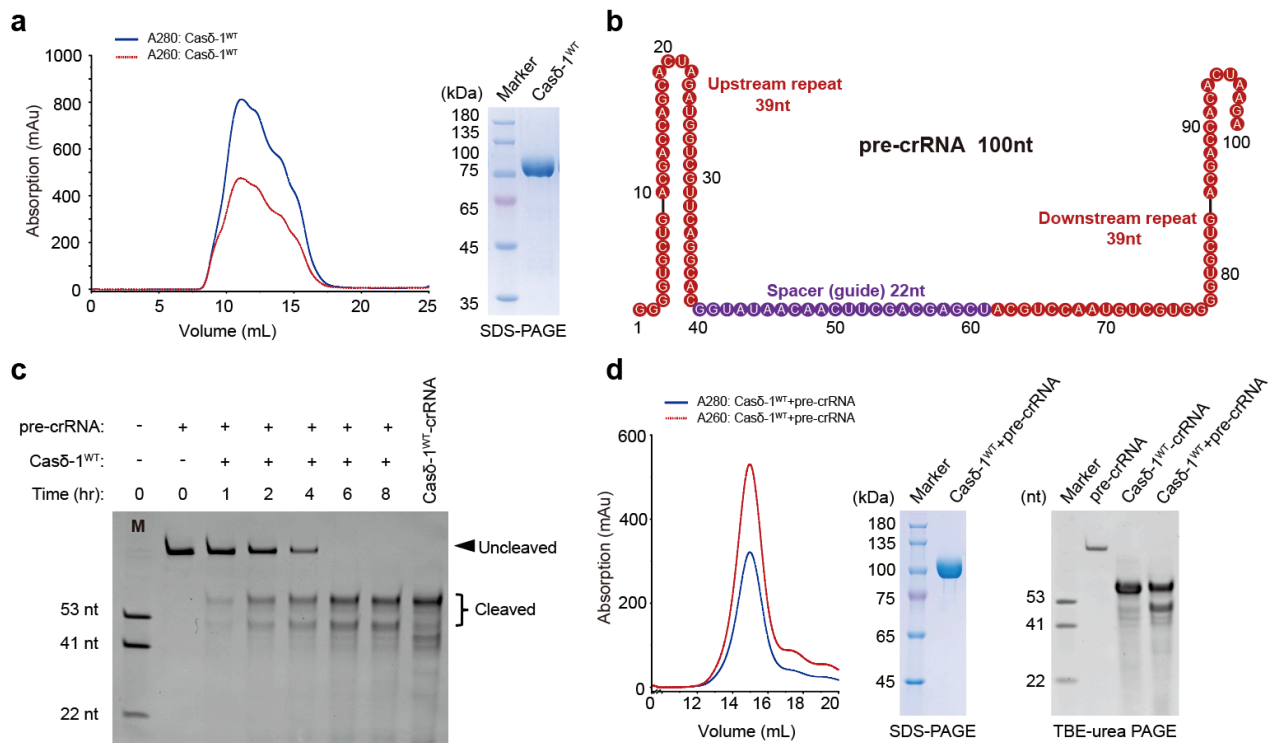


Figure Caption: Processing of pre-crRNA by Cas δ -1^{WT}: **a** Purification and identification of the Cas δ -1^{WT} via gel filtration chromatography. The Cas δ -1^{WT} was fractionated using a Superdex 200 Increase 10/300 column, and the peak fraction was analyzed by SDS-PAGE. **b** The schematic of the pre-crRNA. **c** In vitro processing of pre-crRNA by Cas δ -1^{WT}. **d** In vivo (h) processing of pre-crRNA by Cas δ -1^{WT}. The Cas δ -1^{WT}-pre-crRNA complex was fractionated using a Superdex 200 Increase 10/300 column, and the peak fraction was analyzed by SDS-PAGE and TBE-Urea-PAGE gels.

2) While the authors emphasizes in main text that the Cas δ -1 system could be used for genome editing, it is surprising that there are no cell experiments at all. At least, it is necessary to show that gene editing is possible with Cas δ -1 and crRNA, as a proof-of-concept in human or mammalian cells.

We thank the reviewer for the insightful suggestion. In the revised manuscript, we have added gene editing experiments with Cas δ -1 in HEK293T cells to the Results section and systematically evaluated its editing fidelity. Our results data demonstrate that Cas δ -1 enables high-fidelity precise genome editing in human cells (lines 102-106, below figure, and Fig. 1a, b). Furthermore, based on the structural information we resolved, we introduced site-directed mutations at key residues and validated the gene editing capabilities of these mutants in HEK293T cells (lines 227-232, 266-271, below figure, and Fig. 4h). Collectively, these experimental results provide reliable proof-of-concept data supporting the feasibility of Cas δ -1-mediated gene editing in human and mammalian cells.

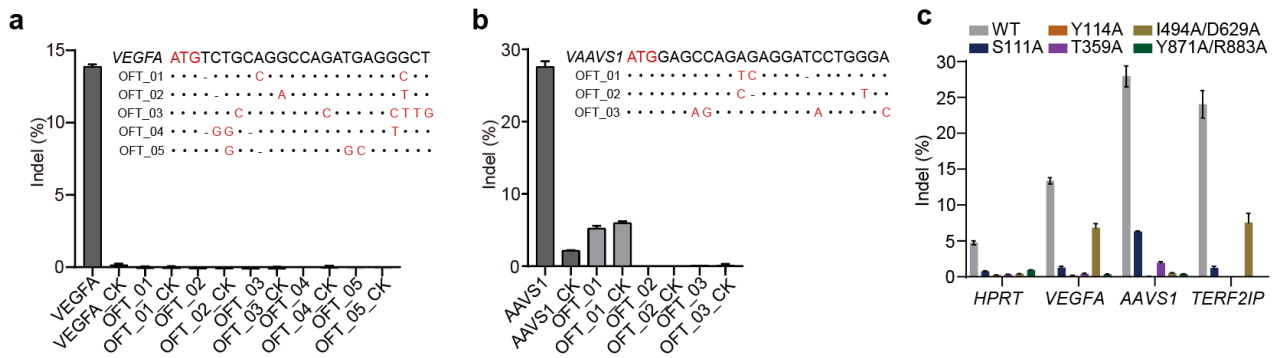


Figure Caption: Cas δ -1 mediates high-fidelity precise genome editing in human cells: a, b Indel activities of the engineered Cas δ -1 system on the VEGFA (a) and AAVS1 (b) genome loci in HEK293T cells and the top off-target loci predicted by Cas-OFFinder. Data are represented as mean $\pm$ SD ($n = 3$). **c** Indel activities of the Cas δ -1 and its PAM-recognized or catalytic site key amino acid residue alanine substitution mutants at HPRT, VEGFA and AAVS1 genome loci in HEK293T cells. Data are represented as mean $\pm$ SD ($n = 3$).

3) It is interesting that TnpB-Cas δ -Cas12 is evolutionarily linked. I would appreciate it if the authors could illustrate this phylogenetically or diagrammatically to make it easier to understand, and kindly present the supporting evidences.

We thank the reviewer for the suggestion. In the revised manuscript, we devote the Discussion section to a systematic structural analysis of the evolutionary relationships among Cas δ , TnpB, Cas12n, Cas12h, Cas12a, and Cas12b. Through comparative analyses of key structural elements, mainly the presence or absence of domains such as CTD, RuvC, REC2, and PI, we further reveal the transitional architectural features of Cas δ at the structural level (lines 323-348). To present this evolutionary relationship easier to understand, we have summarized and illustrated it in a schematic diagram (below figure and Fig. 5f). Collectively, these observations provide direct structural evidence that substantiates the unique position of Cas δ within the evolutionary lineage of the Cas12 family.

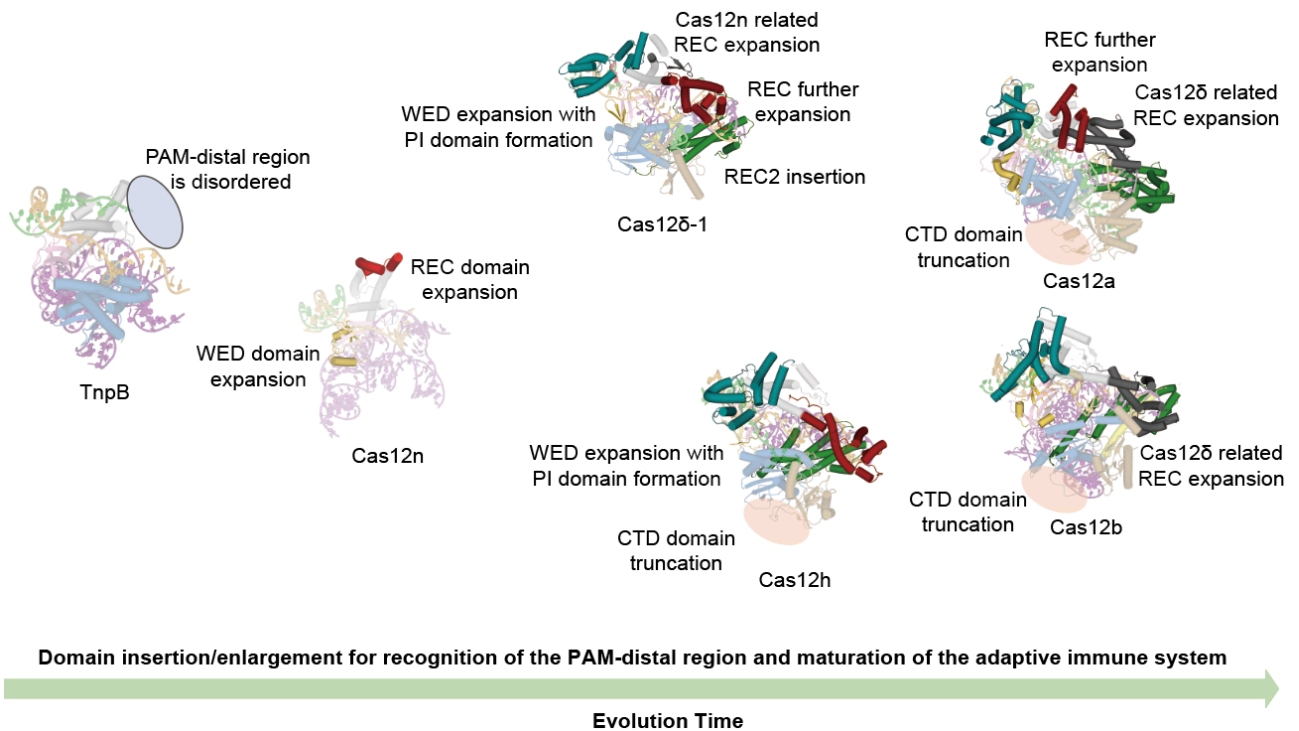


Figure Caption: Model of evolution from TnpB to Cas12 enzymes: Structural comparison of Cas δ -1 (PDB: 23RH) with TnpB (PDB: 8H1J), RdCas12n (PDB: 9J09), Cas12h (PDB: 9L12), Cas12a (PDB: 8SFO), and AacCas12b (PDB: 5U33). The region corresponding to the REC expansion in Cas δ -1 that is already present in Cas12n, as well as the REC expansions in Cas12a and Cas12b that are already present in Cas δ -1, are highlighted in gray. REC1 expansion in Cas12n, Cas δ -1 and Cas12h and Cas12 are highlighted in red. The WED domain expansions, PI domain and REC2 domain insertion are highlighted in yellow, cyan and green, respectively. Other major differences are indicated by Elliptical Box.

4) I wonder if Cas δ -1 can become a nickase or deactivated form by partially losing its activity.

We thank the reviewer for the suggestion. During the revision process, we sought to experimentally determine whether Cas δ -1 could be converted into a nickase or a catalytically deactivated form. Previous studies have shown that mutagenesis of the lid sequence within the catalytic pocket of certain Cas12 proteins can convert the enzyme into a nickase or a deactivated form (*Cell* 175, doi: 10.1016/j.cell.2018.10.045 (2018); *Nat Commun* 15, 1473, doi:10.1038/s41467-024-45762-6 (2024)). Following this rationale, we first adopted the nickase engineering strategy established for Cas12a and introduced glycine substitutions at the corresponding positions in Cas δ -1, generating mutant Mut1 (N707G/R711G). Cleavage assays performed with this mutant revealed a marked reduction in enzymatic activity (below figure); however, no detectable single-strand-specific cleavage activity was observed (below figure).

We next replaced the lid sequence of Cas δ -1 with the counterpart from Cas12h (*Cell Rep* 44, 115718, doi: 10.1016/j.celrep.2025.115718 (2025)), which shares the highest structural similarity with Cas δ -1 among type V effectors and is known to cleave only single-stranded DNA. This yielded mutant Mut2, in which residues 698-701 were changed from STGF to DGPSD. Unfortunately, this mutant retained the capacity to cleave double-stranded DNA (below figure). We further generated a double mutant combining both sets of mutations; nonetheless, cleavage assays again failed to detect single-

strand-specific cleavage activity (below figure). Collectively, under the mutagenesis strategies tested thus far, we were unable to convert Cas δ -1 into a nickase or a catalytically dead form.

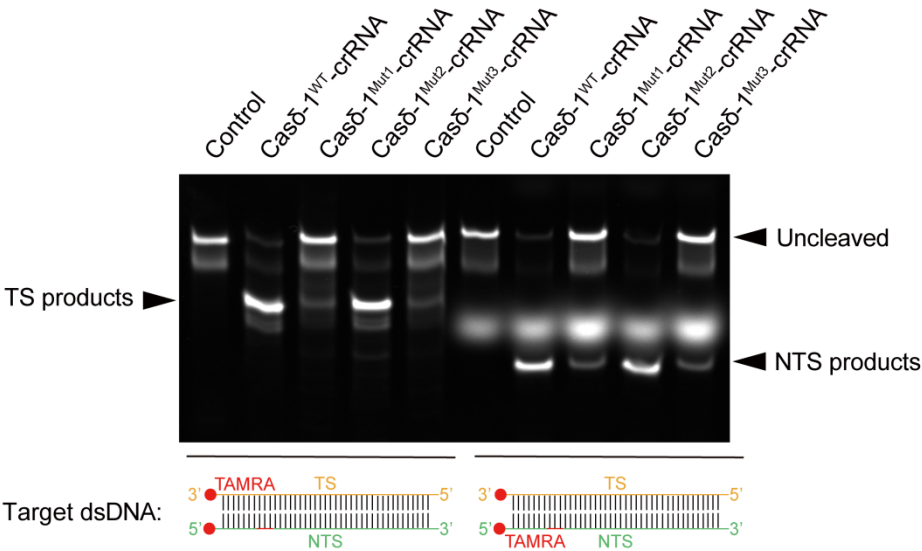


Figure Caption: *In vitro* cleavage assays investigating the dsDNA cleavage ability of pre-designed nickase or deactivated mutants. T-strand (TS), NT-strand (NTS) and their products are marked through fluorescently labeled oligonucleotide. Mut1: N707G/R711G. Mut2: residues 698-701 were changed from STGF to DGPSD. Mut3: Mut1 + Mut2.

Reviewer #3 (Remarks to the Author):

This manuscript by Yin et al. presents a comprehensive biochemical and structural characterization of Cas δ -1, a recently discovered compact RNA-guided endonuclease from the type V CRISPR-Cas family. The authors performed a biochemical characterization of Cas δ -1, demonstrating that this RNA-guided endonuclease exhibits robust double-stranded DNA cleavage activity and target-dependent trans-cleavage, and they determined its precise cleavage sites on both DNA strands. Additionally, the authors resolved a 3.04 Å cryo-EM structure of the Cas δ -1-crRNA-dsDNA ternary complex, which reveals molecular details of PAM recognition, crRNA architecture, and the catalytic site. This study provides structural insights into the mechanism of a compact type V CRISPR-Cas effector and may inform future development of genome editing tools. However, some concerns need to be addressed before the manuscript can be considered for publication.

We thank the reviewer for summarizing the key contributions of our work and referred our work

“provides structural insights into the mechanism of a compact type V CRISPR-Cas effector and may inform future development of genome editing tools”. In response, we have provided detailed explanations to address the reviewer’s concerns.

1. Off-target effects represent an important aspect of genome editing tool characterization, but systematic evaluation of Cas δ -1's off-target activity is still lacking. Given the notable gene editing efficiency and application potential of Cas δ -1, it would be valuable for the authors to perform genome-wide off-target detection using in vivo methods (e.g. GUIDE-seq) or in vitro methods (e.g. Digenome-seq, CIRCLE-seq) to characterize the off-target profile of Cas δ -1.

We thank the reviewer for the insightful suggestion. In our revised manuscript, we performed genome-wide off-target detection in HEK293T cells. We selected two representative human genomic loci, *VEGFA* and *AAVS1*, and replaced the on-target sites in HEK293T cells with computationally predicted potential off-target sites, an approach that has been validated in previous studies (*Nucleic Acids Res* 53, doi:10.1093/nar/gkaf1358 (2025)). These off-target sites shared high sequence homology with the original target sequences and were all flanked by the 5'-ATG PAM. Our results showed that almost no detectable indel mutations occurred at any of the predicted off-target sites, suggesting that Cas δ -1 enables high-fidelity precise genome editing in human cells (below figure and Fig. 1a, b). We have incorporated these results into the Results section of the revised manuscript (lines 102-106).

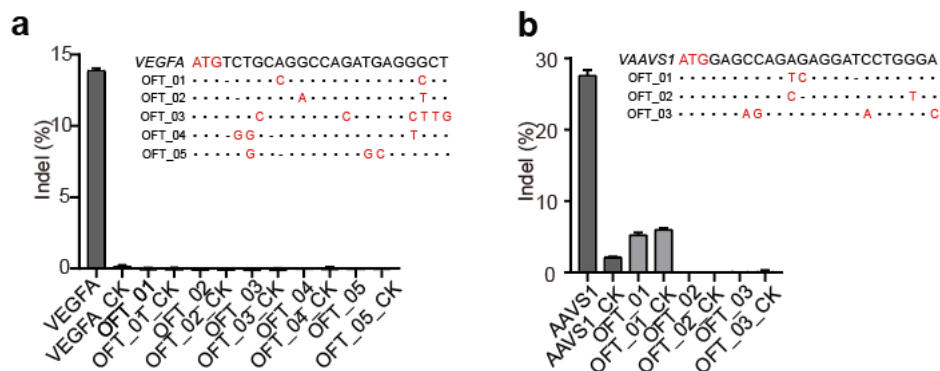


Figure Caption: Cas δ -1 mediates high-fidelity precise genome editing in human cells: **a, b** Indel activities of the engineered Cas δ -1 system on the *VEGFA* (**a**) and *AAVS1* (**b**) genome loci in HEK293T cells and the top off-target loci predicted by Cas-OFFinder. Data are represented as mean $\pm$ SD ($n = 3$).

2. Based on the cryo-EM structure, the authors identify several key residues involved in PAM recognition and catalytic activity, and validate their functional importance through alanine substitution followed by in vitro cleavage assays. However, this validation approach is relatively limited. For the PAM-interacting residues, alanine mutations only confirm that these residues are important for catalytic activity, but do not reveal whether they contribute specifically to PAM sequence discrimination. The authors should perform additional mutagenesis (e.g., charge-reversal or conservative mutations) on these residues and test cleavage activity on substrates with different PAM sequences to determine whether PAM specificity is altered.

We thank the reviewer for the suggestion. We agree with the reviewer that alanine mutations only

confirm that these residues are important for catalytic activity, but do not fully reveal whether they contribute specifically to PAM sequence discrimination. To address the reviewer's concern, we performed additional site-directed mutagenesis on these residues as recommended. Specifically, we introduced synonymous mutations at key residues implicated in PAM recognition, S111, Y114, Y121, and T359, generating four mutants: S111T, Y114F, Y121F, and T359S. We then assessed the cleavage activity of these mutants on various substrates. The results showed that, compared with the wild-type protein, the S111T and T359S mutants exhibited no significant changes in enzymatic activity, whereas the S111A and T359A mutants displayed markedly reduced activity (below figure). Structural analysis revealed that S111 and T359 primarily interact with the PAM via hydrogen bonds formed by their side-chain hydroxyl groups. When each was mutated to a synonymous residue bearing a hydroxyl group with a similar side-chain length, the ability to form hydrogen bonds with the PAM was preserved, and thus PAM recognition remained unaffected. In contrast, when Y114 and Y121 were separately mutated to phenylalanine, both mutants showed activity profiles comparable to those of the alanine-substituted variants (below figure). This is likely because phenylalanine lacks a hydroxyl group in its side chain compared with tyrosine, thereby disrupting hydrogen-bonding interactions with the PAM and impairing PAM recognition. Collectively, these findings suggest that the key residues involved in PAM recognition are not restricted to specific amino acid types; rather, any residue capable of mediating interactions with the PAM can support effective recognition.

Furthermore, in addition to the 5'-ATG PAM sequence used in our study, we also tested several alternative PAM sequences, including TTG, AAG, ATC, and TAC. Our cleavage assays revealed that none of these PAM sequences could be recognized by Cas δ -1, further demonstrating the high specificity of Cas δ -1 for PAM recognition (below figure and Supplementary Fig. 7f). We have incorporated these results into the Results section of the revised manuscript (lines 225-227).

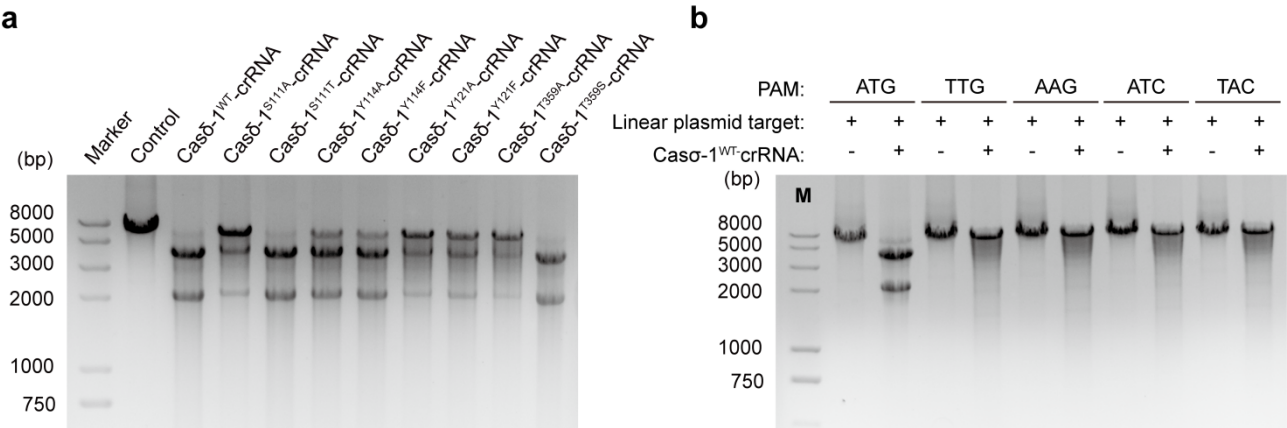


Figure Caption: functional importance of PAM-interacting residues in Cas δ -1: a Alanine substitution or conservative mutations of PAM-interacting residues to analyze their effect on the DNA cleavage activity of Cas δ -1. **b** The detailed preference of Cas δ -1 on substrates with different PAM sequences.

In addition, *in vivo* experiments (e.g., mammalian cell editing assays) comparing wild-type and key mutants would provide more direct validation of these residues' importance in living cells.

We thank the reviewer for the suggestion. To validate the *in vivo* functional significance of these

PAM-recognition residues, we performed gene editing assays in HEK293T cells. The results demonstrated that, compared with wild-type Cas δ -1, the S111A, Y114A, and T359A mutants almost completely abolished gene editing activity (below figure and Fig. 4h). These findings provide direct evidence for the essential role of these residues in living cells. We have incorporated these results into the Results section of the revised manuscript (lines 227-232).

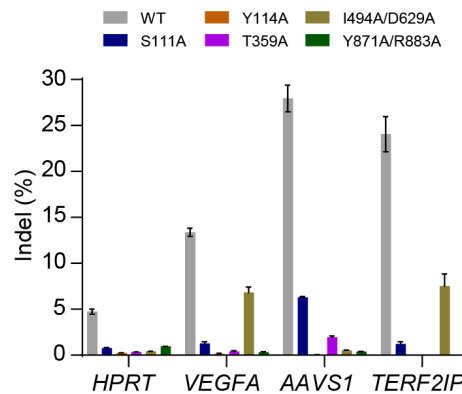


Figure Caption: Indel activities of the Cas δ -1 and its PAM-recognized or catalytic site key amino acid residue alanine substitution mutants at HPRT, VEGFA and AAVS1 genome loci in HEK293T cells. Data are represented as mean $\pm$ SD ($n = 3$).

3. Structural analysis reveals that Cas δ -1 recognizes the PAM sequence through specific interactions. dG (-1*) forms a hydrogen bond with Ser111, while the phosphate groups of dT (-2*) and dA (-3*) interact with Tyr121, Leu124, Gln122, and Tyr114. Notably, these four residues only contact the phosphate backbones and do not interact with the nucleobases at positions -2* and -3*. Cas δ -1 has been reported to prefer an RYR PAM sequence, and this raises a critical question: how does Cas δ -1 achieve base discrimination at positions -2* and -3* when the identified interactions are only with the phosphate backbone. The authors should provide a mechanistic explanation for how Cas δ -1's PAM specificity is achieved.

We thank the reviewer for the insightful and valuable suggestion. We performed a systematic analysis of the structural data, which revealed that Tyr114 not only participates in hydrogen bonding with the phosphate backbone of dA(-3), but also forms a hydrogen bond with the base of dA(-3) (below figure and Fig. 4b). We have corrected this description in the revised manuscript (lines 214-218). Furthermore, to verify the functional significance of the base specificity of dA(-3) in the PAM sequence, we replaced dA(-3) with T to generate a 5'-TTG PAM sequence and performed cleavage assays. Our results showed that Cas δ -1 could not recognize this mutant PAM sequence (below figure and Supplementary Fig. 7f), further indicating that the hydrogen bond between Tyr114 and the base of dA(-3) plays a critical role in PAM recognition.

Regarding the specificity of dT (-2*) recognition, it is primarily determined by the specificity conferred by the complementary strand (below figure and Fig. 4c). Based on our structural analysis, dA(2) on the target strand (which forms Watson-Crick pairing with the PAM nucleotide dT(-2*)) also establishes a hydrogen bond through its base with Thr359 in the WED domain, thereby further

ensuring the specificity of dT(-2*) recognition (below figure and Fig. 4c). In the revised manuscript, we have updated and rephrased this section to enhance clarity (lines 218-221).

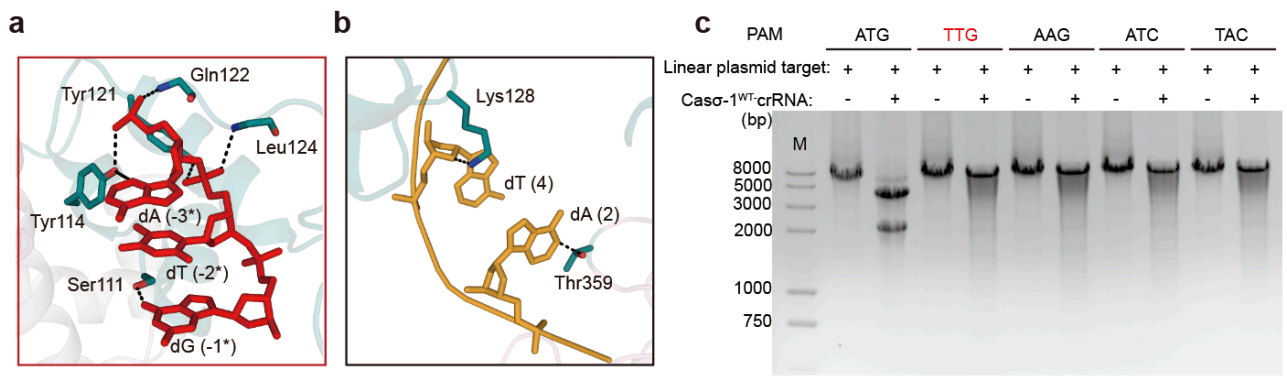


Figure Caption: PAM-recognition specificity of Casδ-1: *a,b* Detailed views of PAM motif (*a*) and PAM-complementary (*b*) sequence recognition by Casδ-1. Hydrogen bonds are shown as dashed lines. *c* The detailed preference of Casδ-1 on substrates with different PAM sequences.

4. There are many missing or improperly arranged characters across the figures, which hinder data interpretation. For example, in Fig. 3a, "crRNA Spacer" is incorrectly displayed as "crRNA S cer"; in Fig. 3d, "Arg592" appears as "Ar 592"; and in Fig. S4b, "crRNA" is shown as "c RNA". The authors should carefully review all figure labels to ensure they are complete, clear, and error-free.

We thank the reviewer for pointing out this issue. We have corrected these formatting mistakes in the revised manuscript.

5. In Fig. 3, Fig. 4, and Fig. S6, the authors indicate interactions between protein residues and nucleic acids with dashed lines. It would be helpful to also label the exact distances for these interactions, which would allow readers to better assess the strength and specificity of the binding.

We thank the reviewer for the suggestion. To enable a more rigorous evaluation of the binding strength and specificity, we have added a supplementary figure to the revised manuscript that systematically lists the relevant interactions between protein residues and nucleic acids, with the specific distances annotated for each contact (below figure and Supplementary Fig. 4).

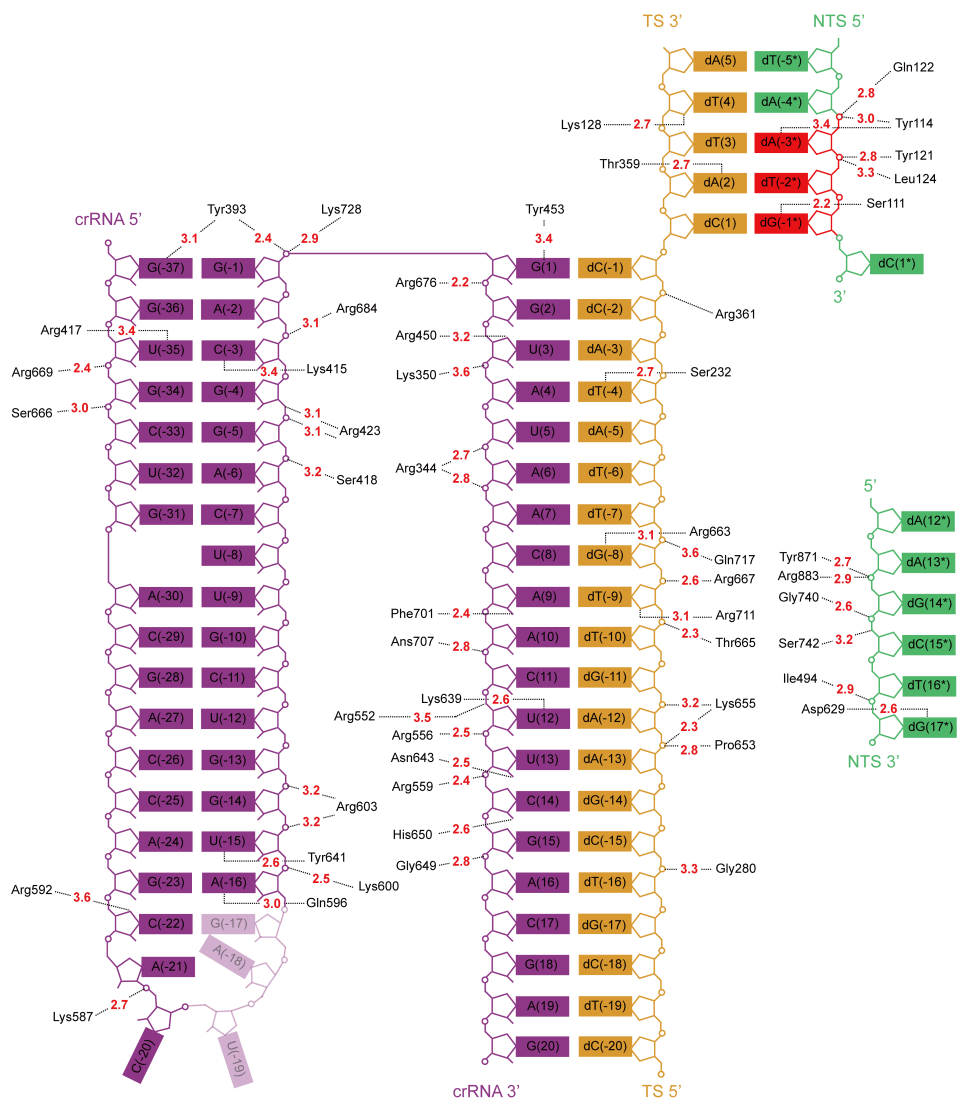


Figure Caption: Schematic of nucleic-acid recognition: Bond lengths are indicated by red numbers. Transparent pane denotes the nucleotides that are not resolved in the density map.

6. In Fig. 3g and Fig. S6f, the authors illustrate base-flipping events between nucleotides C (-1) and G (1) in the crRNA, and between dC (-1) and dC (1) in the TS DNA. However, these panels display only a single strand, making it difficult to understand the base-flipping context. It would be helpful to also include the pairing strand (the complementary DNA or crRNA) in these panels, allowing readers to clearly visualize how the flipped bases are displaced from the duplex.

We thank the reviewer for pointing out this issue. To better illustrate the structural context of the base-flipping events, we have revised Figure 3g and Figure S6f in the manuscript by incorporating the pairing strands into each panel, specifically the complementary bases in the crRNA or the complementary strand of the target DNA. In the updated figures, the flipped bases are highlighted in green, and corresponding explanatory notes have been added to the figure legends (lines 625-626 and 713-714, below figure, Fig. 3g, and Supplementary Fig. 6f).

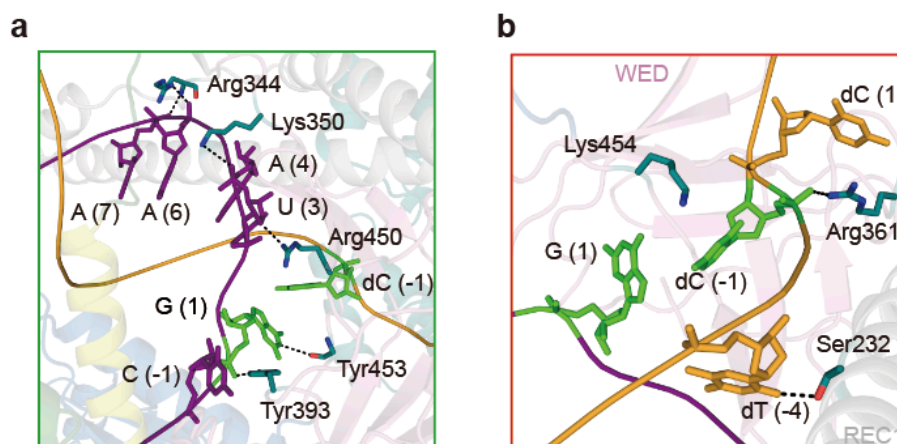


Figure Caption: *The base-flipping events between nucleotides C (-1) and G (1) in the crRNA, and between dC (-1) and dC (1) in the TS DNA: a Detailed view of crRNA spacer recognition within WED and REC1 domains. b The detailed interactions of Casδ-1 with nucleotides dC (-1) to dA (-5) within TS. Specially, the flips of dC (-1) in TS DNA and G (1) in crRNA is colored in green.*

7. In Fig. 4g, the bar graph shows wild-type cleavage efficiency as 100%, but the corresponding gel image in the upper panel shows a clear uncleaved band, indicating that cleavage efficiency is not actually 100%. The authors should verify the quantification and ensure that the bar graph accurately reflects the gel data.

We thank the reviewer for pointing out this issue and for the valuable suggestion. We acknowledge that the Figure 4g was indeed insufficient in terms of experimental rigor. In the revised manuscript, we re-expressed and purified all wild-type and mutant proteins, and have included the purification results along with the corresponding gel electrophoresis data (Supplementary Fig. 7a-d). We further utilized this newly purified set of proteins to reassess their cleavage activities. To enhance experimental rigor, we added a control lane containing substrate DNA only. The improved experimental setup not only yielded higher-quality imaging results but also enabled us to perform quantitative statistical analyses based on substrate depletion. The results demonstrate that the mutant proteins exhibit significantly reduced cleavage activities compared with the wild-type protein (Fig. 4g and Supplementary Fig. 7e). We have updated the Results section accordingly to incorporate these new results (lines 221-225 and 263-266).

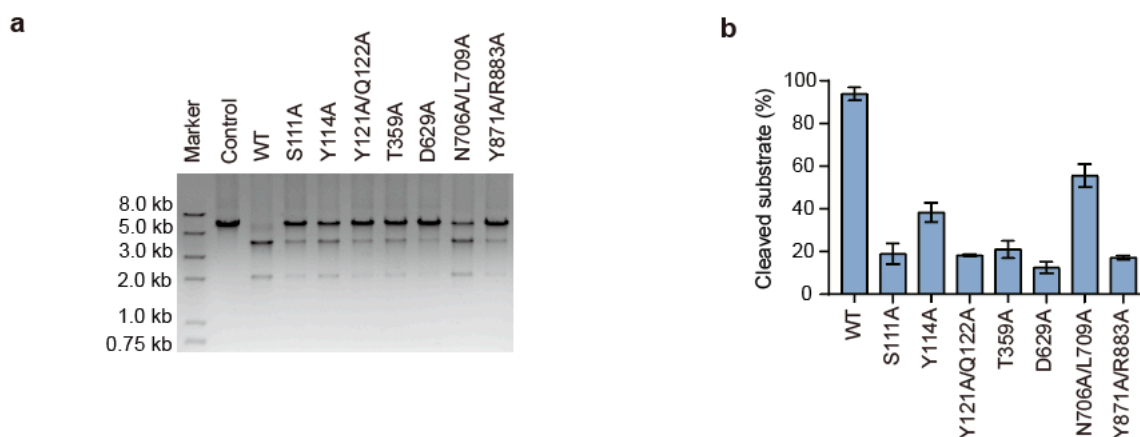


Figure Caption: Enzymatic activity analysis of target DNA-interacting residues mutants: a Alanine substitution of target DNA-interacting residues to analyze their effect on the DNA cleavage activity of Cas δ -1. **b** Cleavage activity statistics following alanine substitution of key amino acid residues responsible for PAM recognition and NTS chain cleavage. Data are represented as mean $\pm$ SD ($n = 3$).

8. The authors position Cas δ -1 as an evolutionary transitional nuclease bridging Cas12n and canonical type V systems, and compare its structure with TnpB, Cas12h, Cas12i, and other Cas12 effectors. However, a structural comparison with Cas12n is notably absent. A recent study reported the cryo-EM structure of RdCas12n (Nat Commun 2025, 16(1):5667; PDB: 9J09). The authors could directly compare this structure with Cas δ -1 to better understand the evolutionary relationship within type V CRISPR systems.

We thank the reviewer for pointing out this issue and for the valuable suggestion. In the revised manuscript, we have incorporated a structural comparison with Cas12n and systematically analyzed the evolutionary relationships among Cas δ , TnpB, Cas12n, Cas12h, Cas12a, and Cas12b in the Discussion section (lines 323-348). Through comparative analyses of key structural elements, specifically the presence or absence of domains such as CTD, RuvC, REC2, and PI, we further reveal the transitional architectural features of Cas δ at the structural level. These structural comparisons help us to better understand the evolutionary relationships within type V CRISPR systems. In addition, to facilitate a more intuitive understanding of this evolutionary relationship, we have summarized and illustrated it in a schematic diagram (below figure and Fig. 5f).

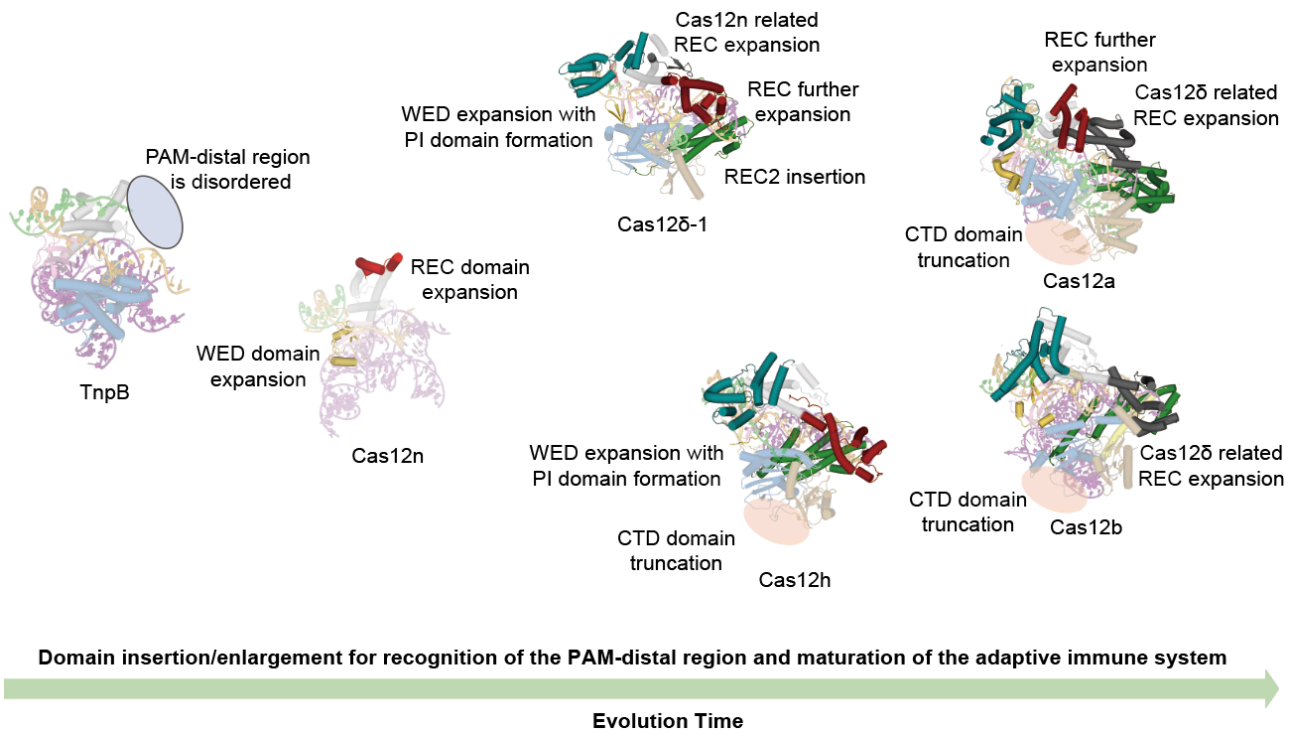
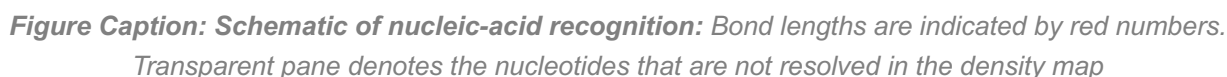


Figure Caption: Model of evolution from TnpB to Cas12 enzymes: Structural comparison of Cas δ -1 (PDB: 23RH) with TnpB (PDB: 8H1J), RdCas12n (PDB: 9J09), Cas12h (PDB: 9L12), Cas12a (PDB: 8SFO), and AacCas12b (PDB: 5U33). The region corresponding to the REC expansion in Cas δ -1 that is already present in Cas12n, as well as the REC expansions in Cas12a and Cas12b that are already present in Cas δ -1, are highlighted in gray. REC1 expansion in Cas12n, Cas δ -1 and Cas12h and Cas12 are highlighted in red. The WED domain expansions, PI domain and REC2 domain insertion are highlighted in yellow, cyan and green, respectively. Other major differences are indicated by Elliptical Box.

9. The authors present numerous local interactions between protein residues and nucleic acids, but an overall protein-nucleic acid interaction map is lacking. It would be helpful to provide a schematic diagram summarizing these interactions, either by annotating the interacting residues on the existing schematic in Fig. S3a or by creating an additional figure that comprehensively shows the protein-RNA/DNA interaction networks.

We thank the reviewer for the valuable suggestion. For clarity, we have added a supplementary figure to the revised manuscript, which systematically lists relevant interactions between protein residues and nucleic acids (below figure and Supplementary Fig. 4).



We thank the reviewer for the valuable suggestion. In most Cas12 proteins, the RuvC domain contains a canonical DED catalytic motif (three conserved acidic amino acids: Asp, Glu, Asp), which coordinates two Mg²⁺ ions at the active site. These two metal ions work synergistically to catalyze the hydrolysis of DNA phosphodiester bonds. Many Cas12 proteins, similar to Casδ-1, can be activated by multiple metal ions in addition to Mg²⁺. For example, Cas12a (PDB: 8SFO), Cas12b (PDB: 5U33), and Cas12h (PDB: 9L12) can be activated by Mg²⁺ and Mn²⁺, while Cas12i2 (PDB:

6LTU) can be activated by Mg^{2+} , Ni^{2+} , and Co^{2+} . However, the underlying mechanisms by which these Cas12 family members utilize metal ions beyond Mg^{2+} remain unclear.

To explore the possible structural basis, we performed a comparative structural analysis of Cas δ -1 with other Cas12 family members whose structures have been resolved, including Cas12a, Cas12b, and Cas12h (which can be activated by Mg $^{2+}$ and Mn $^{2+}$), Cas12i2 (which can be activated by Mg $^{2+}$, Ni $^{2+}$, and Co $^{2+}$), as well as Cas12i1 (PDB: 6W5C) and Cas12f (PDB: 7L49) (which are reported to be activated only by Mg $^{2+}$). Cas12n was not included in this comparison because its full-length protein containing the RuvC domain has not yet been structurally resolved. Structural alignment revealed that the DED catalytic triad of Cas δ -1 exhibits side-chain conformations similar to those all Cas12 members (below figure). Therefore, it is difficult to deduce the underlying mechanisms of broader metal tolerance among these Cas12 family members from structural comparisons alone. Based on our current analyses, we propose that the ability of Cas δ -1 to be activated by multiple metal ions may arise from the sufficient spatial flexibility of its RuvC active-site metal-binding pocket, which can accommodate these distinct ions. Furthermore, this broader ion tolerance of Cas δ -1 may represent a compensatory strategy under magnesium-limited environmental conditions. More importantly, the differential metal dependence of Cas δ -1 provides a conceptual foundation for engineering ion-sensitive CRISPR platforms capable of monitoring cellular metal homeostasis. We have incorporated this discussion on the potential physiological significance of the broader metal tolerance of Cas δ -1 into the Discussion section of the revised manuscript (lines 317-322).

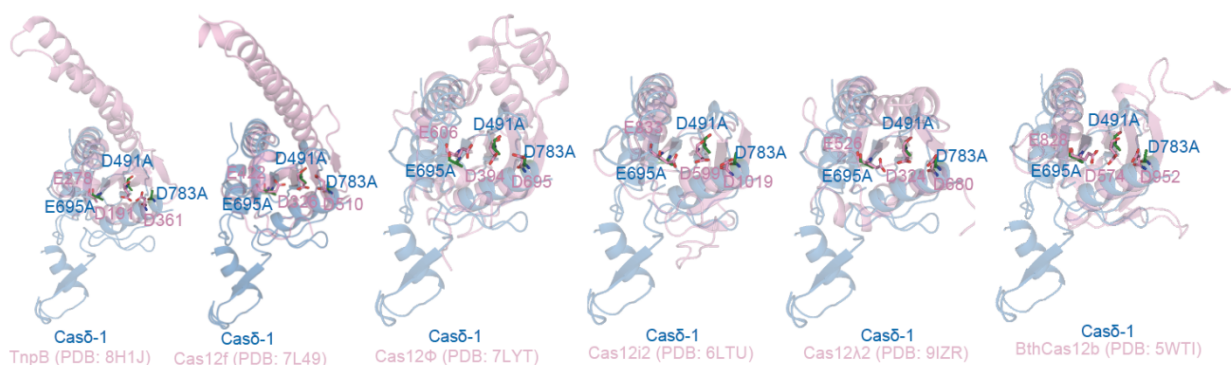


Figure Caption: Structural superposition of the RuvC catalytic residues in Cas δ -1 and other Cas12 proteins: RuvC catalytic residues superposition of Cas δ -1 and TnpB (PDB: 8H1J), Cas12f (PDB: 7I49), Cas12 Φ (PDB: 7LYT), Cas12i2 (PDB: 6LTU), Cas12 λ 2 (PDB: 9IZR) or BthCas12b (PDB: 5WTI).

We thank the reviewers for their valuable comments. In response to their suggestions and the editorial requests, we have made necessary revisions to the manuscript. Our detailed responses to reviewers' concerns are presented below.

Reviewer #1 (Remarks to the Author):

I appreciate the authors' careful responses to my previous comments. The revised manuscript has been substantially improved, and my previous concerns have been adequately addressed. I have no further comments.

We thank the reviewer for the positive feedback on our manuscript.

Reviewer #2 (Remarks to the Author):

The authors responded to most of the issues I raised in the last review, and I felt that the revised edition was greatly improved. I would recommend publication of this revised version.

We thank the reviewer for the positive feedback on our manuscript.

Reviewer #3 (Remarks to the Author):

In the revised manuscript, the authors have addressed most of the concerns raised in my previous review, including the addition of off-target analysis at predicted loci, conservative mutagenesis of PAM-interacting residues, in vivo gene editing assays in HEK293T cells, structural comparison with Cas12n, and a summarized protein-nucleic acid interaction map. I appreciate these efforts and am generally satisfied with the revisions, supporting publication in Nature Communications. However, before final acceptance, I would like the authors to address two remaining issues.

We thank the reviewer for summarizing the key insights of our work and the positive comments.

1. The authors evaluate off-target effects by examining only a limited number of computationally predicted top off-target loci that share high sequence homology with the on-target sites. While this approach provides an initial assessment of specificity, it is inherently limited and cannot exclude the possibility of off-target mutations at genome-wide unpredictable sites or at loci with lower sequence similarity. Therefore, the conclusion that Cas δ -1 "demonstrates its high-fidelity precision genome editing capability" (lines 105–106) is overstated. To maintain scientific rigor, I suggest that the authors should soften this claim.

We thank the reviewer for this suggestion. We agree that our off-target analysis was limited to predicted high-homology sites and that genome-wide off-target events cannot be excluded. Accordingly, we have softened our conclusion from "demonstrating its high-fidelity precision genome editing capability" to "indicating that Cas δ -1 maintains high on-target activity with low off-target signals at the loci examined" (lines 102-103) in the revised manuscript.

In addition, in Fig. 1b, the control (CK) group shows detectable indel signals at both the on-target site and OFT-01. This result is rather strange and may raise concerns about background noise or sample contamination. The authors should provide an appropriate explanation.

We thank the reviewer for pointing out this issue. We agree that the indel signals observed in the control group in Fig. 1b are unusual. To exclude the possibility of background noise or sample contamination, we repeated this experiment in HEK293T cells. Based on the results of this repeat experiment, we have updated Fig. 1b in the revised manuscript.

2. Since deep sequencing was performed in the revised manuscript, the raw sequencing reads should be deposited in a public repository (e.g., NCBI SRA or GSA), and the corresponding accession number should be provided in the Data availability section to ensure data accessibility.

We thank the reviewer for this suggestion. We have deposited the raw sequencing data in the NCBI database under the accession number PRJNA1526349, and have included this accession number in the Data availability section of the revised manuscript.