nature portfolio

Peer Review File

Structural basis for target DNA cleavage and guide RNA processing by CRISPR-Casλ2

Corresponding Author: Professor Osamu Nureki

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 current manuscript by Omura et al., reported the cryo-EM structures of Casλ2 complex in five different states, revealing the structural architecture and dynamic conformational rearrangements during activation. The authors also provided biochemical assays showed that Casλ2 processes its precursor crRNA to a mature crRNA by ruler mechanism.
These results generate a deep insight into the mechanism of Casλ, as a potential genome editing tool. This manuscript is well written and illustrated. I have a few comments listed below.

1. The U-rich loop forms base-specific interactions with RuvC domain, whether the mutations of the residues K436 and N495 or U(-17) would reduce the DNA cleavage activity?

2. The residue K2 forms interaction with the first base pair, a detailed structural panel is needed for better understanding.

3. In Figs.3g and 4h, the sidechain of D680 seems far away from Mg ion, which is supposed to coordinate the MgA in NTS or TS cleaving state. It's better to check the density map carefully and confirm whether the residue is at the reasonable place.

4. Does the Casλ2 possess trans cleavage activity triggered by target DNA as observed in other Cas12 enzymes? If so, could Casλ2 cleave ssDNA or ssRNA in trans?

5. Casλ enzymes are promising genome editing tools, it would be highly beneficial to compared the editing efficiency of Casλ2 with Casλ1 or other Cas12 enzymes in mammalian cells.

6. Extended Data Fig. 4, the label "Casλ" should be "Casλ1".

7. Fig.5 Legend, the first "(d)" should be "(c)".

Reviewer #2

(Remarks to the Author)
This study provides a comprehensive biochemical and structural characterization of the CRISPR-Casλ2 system. The authors employ a combination of bioinformatics, biochemical, and structural biology approaches to present a complete mechanistic narrative, encompassing crRNA maturation, RNP assembly, crRNA recognition, PAM recognition, and target DNA cleavage. They elucidate a unique mechanism for crRNA maturation and resolved Casλ2 in distinct functional states, revealing a structural characteristic during activation that distinguishes Casλ2 from other type V effectors. Overall, the study is thorough and well-rounded, offering readers an in-depth understanding of the biochemical and structural features of the CRISPR-Casλ2 system.
However, I have some suggestions that could further improve the manuscript:
1. Fig. 1a, Line 84: The authors claim that Casλ2 contains a unique RuvC domain. Please provide a more detailed description of this domain, including its structural or biochemical features and how it differs from canonical RuvC domains in other CRISPR-Cas systems. A comparative analysis would enhance the clarity and significance of this statement.
2. All the target cleavage assays (Fig.1e-g, 2h) should include representative gel images that reflect results from at least

three independent replicates. This is essential to demonstrate the reproducibility and reliability of the experimental findings.
3. In the Results Section, the detailed descriptions of biochemical experimental procedures are overly lengthy and could benefit from simplification to improve readability and logical flow. For instance, Lines 116–120 could be condensed as follows: “We next tested the target DNA cleavage under different temperatures.” Similarly, other procedural details throughout the manuscript should be streamlined to enhance clarity and maintain focus on the main findings. A more concise presentation of the key results will make the article more accessible to readers.
4, Typically, CRISPR-Cas systems follow a sequential workflow beginning with crRNA processing and the RNP complex formation, followed by PAM recognition and target cleavage. Based on this established sequence, the last two parts of the Results—“Guide RNA processing by Casλ2” and “Cryo-EM structure of the Casλ2–crRNA binary complex”—would be more logically placed earlier in the manuscript, either at the beginning of the Results section or immediately following the biochemical characterization of the Casλ2 system.
Alternatively, the authors should provide a clear rationale for presenting these parts at the end of the Results section. Restructuring the manuscript in alignment with the natural progression of the system’s mechanism would improve the logical flow and readability.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In the revised manuscript, Omura et al. addressed all the reviewers' concerns and presented additional biochemical data. The authors also updated the figures and revised the PDB files. This manuscript is greatly improved and is acceptable for publication.
I only have a minor point listed below.
Line 915, “as in Figure S2A” should be “as in Extended Data Fig. 3a”.

Reviewer #2

(Remarks to the Author)
The authors have addressed the reviewers’ concerns in their revised manuscript. The responses to the review comments are comprehensive, with corresponding revisions clearly highlighted in the text. I believe the manuscript now meets the standards for publication in Communications Biology.

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April 16, 2025

Joanna Timmins, Ph.D.
Editorial Board Member, *Communications Biology*

Dear Joanna,

First of all, we would like to thank you for the opportunity to revise our manuscript. We also appreciate the suggestions and concerns raised by the referees, which have significantly improved the manuscript. According to their comments, we revised the manuscript as follows, and hope that it is now acceptable for publication in *Communications Biology.*

Responses to reviewers' comments

Reviewer #1 (Remarks to the Author):

The current manuscript by Omura et al., reported the cryo-EM structures of Casλ2 complex in five different states, revealing the structural architecture and dynamic conformational rearrangements during activation. The authors also provided biochemical assays showed that Casλ2 processes its precursor crRNA to a mature crRNA by ruler mechanism. These results generate a deep insight into the mechanism of Casλ, as a potential genome editing tool. This manuscript is well written and illustrated. I have a few comments listed below.

We thank the reviewer for the positive comments. Our responses to the comments raised by the reviewer are as follows.

1. The U-rich loop forms base-specific interactions with RuvC domain, whether the mutations of the residues K436 and N495 or U(-17) would reduce the DNA cleavage activity?

We thank the reviewer for the important comment. To examine the importance of the Lys436 and Asn495 residues in the U-rich loop for Casλ2-mediated DNA cleavage, we purified the K436A and N495A mutants, and compared their *in vitro* DNA cleavage activities to that of wild-type Casλ2. Both mutants exhibited comparable DNA cleavage activities to that of wild-type Casλ2, suggesting that Lys436 and Asn495 alone do not play a critical role in target DNA cleavage by Casλ2 (Fig. L1). Indeed, the crRNA scaffold forms multiple contacts with Casλ2 across various regions (Extended Data Fig. 4a), likely compensating for the loss of base-specific interactions caused by the K436A and N495A mutations.

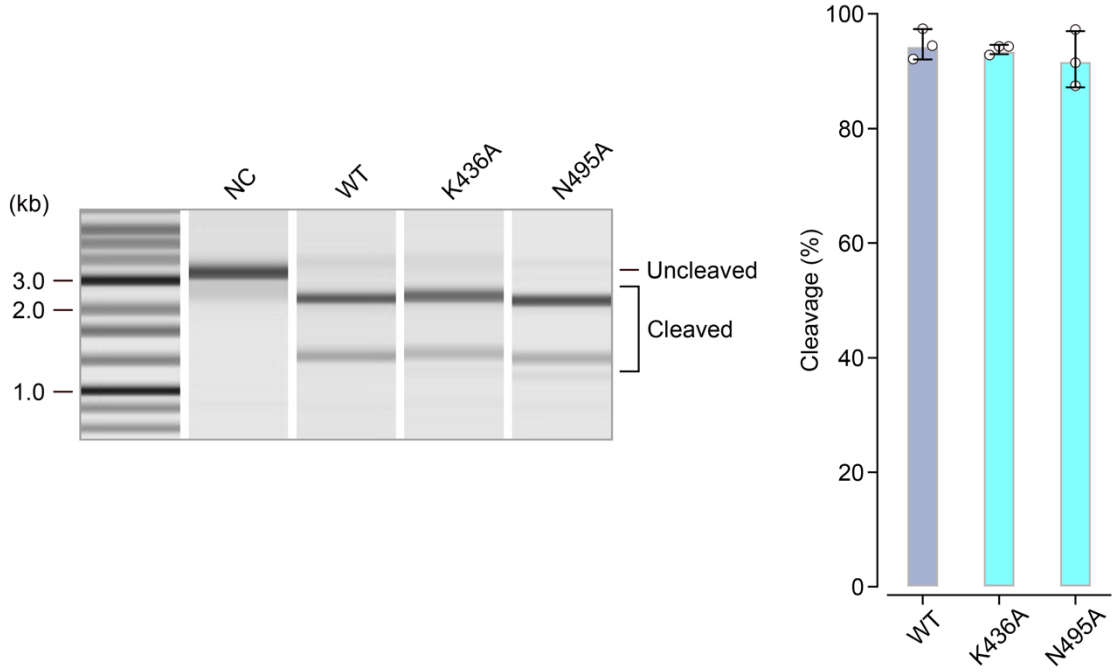

Fig. L1 | *In vitro* DNA cleavage activities of wild-type Casλ2 and Casλ2 mutants. Representative electropherogram of DNA cleavage products analyzed by MultiNA microchip electrophoresis (left) and corresponding quantification shown as a bar graph (right). Data represent mean ± s.d. (n = 3).

2. The residue K2 forms interaction with the first base pair, a detailed structural panel is needed for better understanding.

According to the reviewer's helpful comment, we have added a panel showing a close-up view around Lys2 to better illustrate its interaction with the first base pair in the revised manuscript (Fig. 3e).

3. In Figs.3g and 4h, the sidechain of D680 seems far away from Mg ion, which is supposed to coordinate the MgA in NTS or TS cleaving state. It's better to check the density map carefully and confirm whether the residue is at the reasonable place.

We appreciate the reviewer for the helpful comment. We have revisited the structural refinement using adjusted parameters in the phenix.real_space_refine process, and updated the PDB files, in which the Mg_A^{2+} ion is now appropriately coordinated by Asp680 (Fig. L2, Figs. 3h, 4f, and 4h).

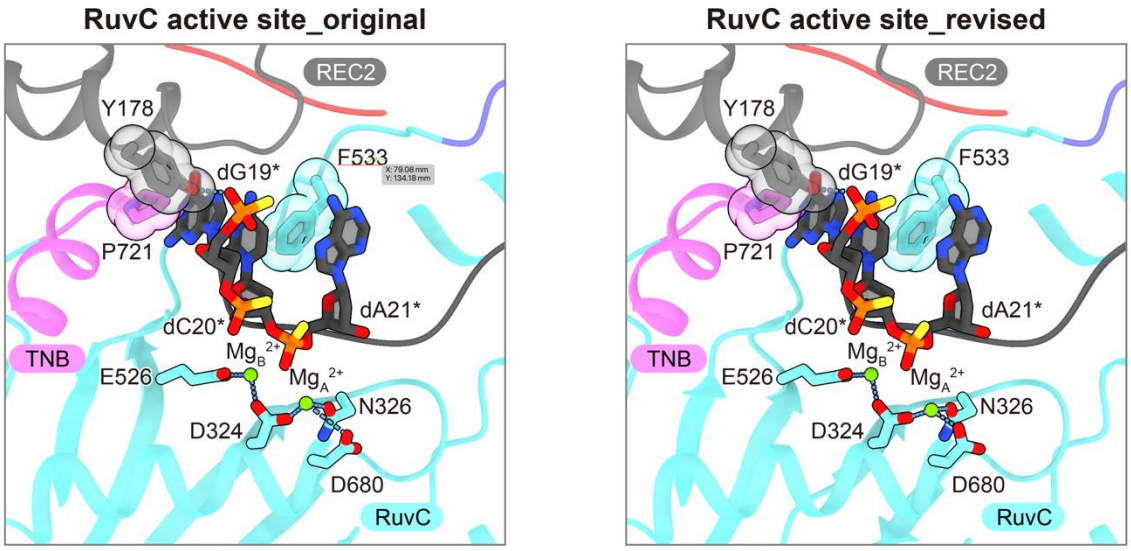

Fig. L2 | Close-up views of the RuvC active site.
Close-up views of the RuvC active site in the original structural model (left) and revised structural model (right).

4. Does the Casλ2 possess trans cleavage activity triggered by target DNA as observed in other Cas12 enzymes? If so, could Casλ2 cleave ssDNA or ssRNA in trans?

We thank the reviewer for the important comment. A previous study revealed that Casλ1 exhibits a low but detectable level of ssDNA or ssRNA cleavage in *trans* upon DNA recognition in *cis*, as observed in other Cas12 enzymes[1]. Given the similar biochemical properties shared between Casλ1 and Casλ2, it is reasonable to speculate that Casλ2 may also possess collateral cleavage activity. However, a thorough biochemical characterization would be required to conclusively determine whether Casλ2 exhibits such trans-cleavage activity. In the present study, our primary focus is to elucidate the detailed structural mechanism underlying Casλ2-mediated DNA cleavage. We believe that expanding the scope to include an in-depth analysis of potential collateral activity could shift the emphasis away from the central theme of this manuscript. Therefore, we respectfully prefer to defer this line of investigation to a future study

and proceed with the current manuscript without additional biochemical data on trans-cleavage activity.

5. Casλ enzymes are promising genome editing tools, it would be highly beneficial to compared the editing efficiency of Casλ2 with Casλ1 or other Cas12 enzymes in mammalian cells.

We thank the reviewer for this important comment. In response to the suggestion, we evaluated the genome-editing efficiencies of Casλ1 and Casλ2 at endogenous *VEGFA*, *EMX1*, and *DYRK1A* loci in HEK293T cells, using plasmid transfection. However, under our experimental conditions, neither Casλ1 nor Casλ2 induced detectable indel formation (Fig. L3). In a previous study, Al-Shayeb et al. demonstrated the genome-editing activity of Casλ1 using RNP nucleofection rather than plasmid-based transfection[1], suggesting that efficient genome editing by Casλ enzymes may be highly dependent on the mode of delivery. Due to current technical limitations in our lab, we are unable to perform RNP-based nucleofection for a direct comparison of editing efficiencies. Therefore, we respectfully defer this line of investigation to a future study and wish to proceed with the current manuscript's focus on the structural and biochemical characterization of Casλ2.

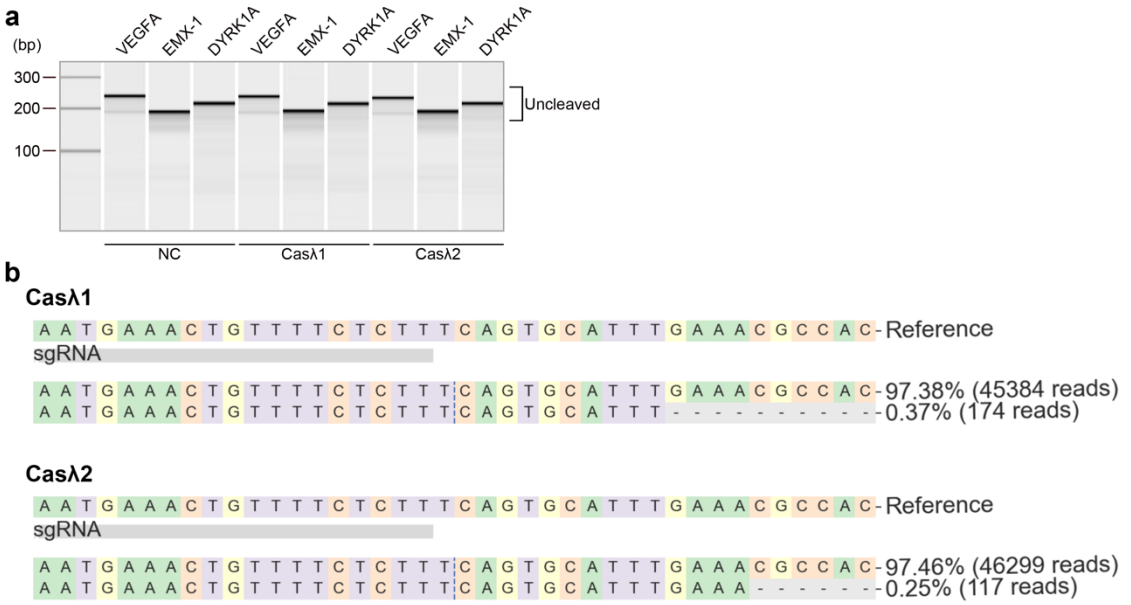

Fig. L3 | Genome-editing analysis.

(**a**) T7 endonuclease assay performed on endogenous VEGFA, EMX-1, and DYRK1A loci in HEK293T cells to assess genome-editing activities of Casλ1 and Casλ2.

(**b**) Deep sequencing analysis of PCR-amplified DYRK1A target sites.

6. Extended Data Fig. 4, the label "Casλ" should be "Casλ1".

We have corrected the term.

7. Fig.5 Legend, the first "(d)" should be "(c)".

We have corrected the term.

Reviewer #2 (Remarks to the Author):

This study provides a comprehensive biochemical and structural characterization of the CRISPR-Casλ2 system. The authors employ a combination of bioinformatics, biochemical, and structural biology approaches to present a complete mechanistic narrative, encompassing crRNA maturation, RNP assembly, crRNA recognition, PAM recognition, and target DNA cleavage. They elucidate a unique mechanism for crRNA maturation and resolved Casλ2 in distinct functional states, revealing a structural characteristic during activation that distinguishes Casλ2 from other type V effectors. Overall, the study is thorough and well-rounded, offering readers an in-depth understanding of the biochemical and structural features of the CRISPR-Casλ2 system. However, I have some suggestions that could further improve the manuscript:

We thank the reviewer for the positive comments. Our responses to the comments raised by the reviewer are as follows.

1. Fig. 1a, Line 84: The authors claim that Casλ2 contains a unique RuvC domain. Please provide a more detailed description of this domain, including its structural or biochemical features and how it differs from canonical RuvC domains in other CRISPR-Cas systems. A comparative analysis would enhance the clarity and significance of this statement.

We thank the reviewer for this important comment. Structurally, Casλ family enzymes possess a distinct RuvC domain characterized by insertional helices located between strands β2 and β3, as well as between strand β3 and helix α1. These structural insertions expand the overall size of the RuvC domain relative to that of other Cas12 enzymes (Extended Data Fig. 3a,b). However, at the sequence level, the RuvC domain of Casλ remains broadly consistent with the canonical RuvC fold. To avoid potential confusion arising from this distinction, we have removed the term “unique” from the corresponding sentence in the revised manuscript.

2. All the target cleavage assays (Fig.1e-g, 2h) should include representative gel images that reflect results from at least three independent replicates. This is essential to demonstrate the reproducibility and reliability of the experimental findings.

According to the reviewer’s suggestion, we have included representative gel images of all target DNA cleavage assays analyzed by MultiNA microchip electrophoresis in the revised manuscript (Extended Data Fig. 1 and Extended Data Fig. 4b).

3. In the Results Section, the detailed descriptions of biochemical experimental procedures are overly lengthy and could benefit from simplification to improve readability and logical flow. For instance, Lines 116–120 could be condensed as follows: "We next tested the target DNA cleavage under different temperatures." Similarly, other procedural details throughout the manuscript should be streamlined to enhance clarity and maintain focus on the main findings. A more concise presentation of the key results will make the article more accessible to readers.

We thank the reviewer for the helpful comment. According to the reviewer's suggestion, we have rephrased the sentences as "We next examined the *in vitro* DNA cleavage activity of Casλ2 under various biochemical conditions." in the revised manuscript (Lines 116–118). In addition, we have simplified some sentences in the initial characterization section in the revised manuscript.

4, Typically, CRISPR-Cas systems follow a sequential workflow beginning with crRNA processing and the RNP complex formation, followed by PAM recognition and target cleavage. Based on this established sequence, the last two parts of the Results—"Guide RNA processing by Casλ2" and "Cryo-EM structure of the Casλ2–crRNA binary complex"—would be more logically placed earlier in the manuscript, either at the beginning of the Results section or immediately following the biochemical characterization of the Casλ2 system. Alternatively, the authors should provide a clear rationale for presenting these parts at the end of the Results section. Restructuring the manuscript in alignment with the natural progression of the system's mechanism would improve the logical flow and readability.

We thank the reviewer for this thoughtful and constructive comment. As noted, Cas12 enzymes typically follow a sequential mechanism—beginning with crRNA processing and RNP complex formation, followed by PAM recognition and target DNA cleavage. We agree that restructuring the Results section to reflect this established biological sequence is a valid and logical approach. However, in this study, Casλ2 was initially identified and characterized based on its DNA interference activity in the *E. coli* negative screening. Accordingly, prior to confirming its pre-crRNA processing activity, we first performed a detailed biochemical analysis of its *in vitro* DNA cleavage activity, which was followed by the structural analysis focused on the Casλ2–crRNA–target DNA ternary complex to elucidate the DNA cleavage mechanism. The investigation into crRNA processing and the structure of the binary complex came at a later stage of the study. Therefore, the current structure of the Results section reflects the experimental progression of our work—beginning with functional characterization of DNA

cleavage, followed by structural elucidation of the cleavage mechanism, and subsequently addressing the upstream steps of crRNA processing. We believe this order best preserves the logical flow of our research and respectfully prefer to retain it in the current manuscript.
As an alternative to restructuring the Results section, and to improve clarity for the reader, we have added a clarifying statement at the beginning of the biochemical characterization section in the revised manuscript (Lines 114–115): “Before examining whether Casλ2 processes its pre-crRNA prior to DNA recognition (described in detail later)…”.

Thank you again for your thoughtful consideration, and we hope that the revised manuscript is now acceptable for publication in *Communications Biology.*

Sincerely,
Osamu

Reference

1. Al-Shayeb, B. *et al.* Diverse virus-encoded CRISPR-Cas systems include streamlined genome editors. *Cell* **185**, 4574-4586.e16 (2022).

Department of Biological Sciences

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May 15, 2025

Laura Rodriguez Perez, Ph.D.
Editorial Board Member, *Communications Biology*

Dear Laura,

First of all, we would like to thank you for the opportunity to revise our manuscript. We also appreciate the suggestions and concerns raised by the referees, which have significantly improved the manuscript. According to their comments, we revised the manuscript as follows, and hope that it is now acceptable for publication in *Communications Biology.*

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