

Structural Basis for RNA-guided DNA degradation by Cas5-HNH/Cascade complex

Corresponding Author: Mr Hongtao 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)

Zhu and colleagues present intriguing findings on a new type I effector complex. The results are interesting. The structures and science appear solid. In this reviewer's opinion, it is lacking some key information that could/should be gleaned from this system. The HNH domain seems to be the most interesting aspect of the paper, but there are several key experiments that are missing or not clearly described.

1. What are the dynamics required for the HNH domain to cleave both strands of the dsDNA?
2. It would be interesting to see additional bioinformatic analysis on the HNH domain, including not only comparisons to other bacteriophage HNH domains, but other type II nucleases. Comparing structural and sequence similarities and pathways of HNH acquisition could be informative.
3. HNH cleavage of dsDNA is a unique feature not observed in type II nucleases which require a RuvC domain to cleave the non-target strand of DNA. This seems like one of the most interesting features of the enzyme, but it is barely mentioned. It would be interesting to see the dynamics of the HNH rearrangements via structural methods or target and non-target strand cleavage kinetics.
4. The authors claim the Cas5-HNH/Cascade complex could be used as a potential gene-editing tool. While this paper shows that this enzyme efficiently targets dsDNA, there are no in vivo results that show this enzyme is active in mammalian cells. Therefore, I believe this statement is significant overstatement, and it needs to be removed from the manuscript.

Reviewer #2

(Remarks to the Author)

Liu et al., present a study of structural insights into the Cas5-HNH/Cascade complex from a *Candidatus Cloacimonetes* bacterium, a novel variant of the Type I-E CRISPR-Cas system. The authors report five cryo-EM structures of this complex, both in its unbound and DNA-bound states. Their findings reveal the interactions between the HNH nuclease domain of Cas5 and adjacent subunits (Cas6, Cas8, Cas11), as well as the conformational changes upon DNA binding.

These results provide the first structural evidence of how the Cas5-HNH/Cascade complex assembles and binds target DNA, advancing our understanding of the Type I-E CRISPR-Cas systems. However, the manuscript and figures are not well presented and need further improvement.

1. The manuscript needs to be reorganized for better logical flow. I suggest starting the results section with the apo Cas5-HNH/Cascade complex, followed by the target DNA binding assays, and then the Cas5-HNH/Cascade-ssDNA complex. Ensure that the figures are cited in the correct sequence.
2. The highlight of the manuscript is the activation and DNA cleavage mechanism of Cas5-HNH. However, the authors did not clearly elucidate this mechanism through structural analysis. Understanding the cleavage mechanism of the HNH domain is crucial for comprehending this novel I-E subtype.

It is recommended to provide structural data of the Cas5-HNH/Cascade complex that offers insights into how the HNH

domain interacts with dsDNA and facilitates strand cleavage. Specific structural features, such as the active site configuration and DNA binding interfaces, are essential for understanding this mechanism. Additionally, the HNH domain should be highlighted in the overall structures.

3. Previous studies have shown that the Cas5-HNH/Cascade complex can cleave dsDNA. It appears that Cas5-HNH cuts the target strand and the non-target strand in a sequence reminiscent of the RuvC domain in Cas12. It is of great significance to identify which strand is cleaved first by Cas5-HNH, as well as the specific cleavage sites in dsDNA. Can the structures in this study explain how the HNH domain cleaves these two strands?

4. As shown in Fig. 4, the “zinc finger like structure” is the typical zinc finger motif found in many Cas proteins, such as Cas10 and Cas12 families. The divalent ion coordinated by four cysteines should be a Zn²⁺. The zinc finger motif could not probably bind different metal ions. The metal ion for HNH activation is coordinated at catalytic center rather than zinc finger motif. The last part of “Divalent ions play important roles in regulating the cas5-HNH/Cascade complex” is misleading.

5. The sentence “As expected, the mutations involved with the Cas5-HNH and Cas6 interfaces can significantly reduce the efficiency of DNA digestion (Fig.2b).” cited the wrong figure.

6. Line 196 , what is the Z score between HNH domain and the PDB: 5H0M as shown in dali search?

7. Line 221, “Of note, these residues, including M338, F371, R275, D277 and Y278, are related to the interaction with Cas11 (Fig.2c,h).” The residues M338 and F371 are not shown in Fig. 2h.

8. Line 219, “Interestingly, a combined mutation of R275A, D277A and Y278A 219 in the linker domain resulted in a significant decrease in the nuclease activity.” It’s inconsistent with the results shown in Supplementary Fig.10d and 10h. The nuclease activity of mutant R275_D277_Y278 is only slightly decreased, whereas the ssDNA binding ability seems greatly increased.

9. The favored Ramachandran plot of the Apo_Conf2 is less than 90%, the structure needs further refinement.

10. Some typos, “ofβ-strand 1” is “of β-strand 1”, and “280nm” is “280 nm”.

Reviewer #3

(Remarks to the Author)

The manuscript “Structural Basis for RNA-guided DNA degradation by Cas5-HNH/Cascade complex” describes the structural, biochemical, and mutational analysis of the recently discovered variant of I-E CRISPR-Cas system, where Cas3 helicase/nuclease was replaced by HNH nuclease fused to Cas5 component of I-E effector complex. The authors solved five different conformation states of the effector complex with and without ssDNA target using Cryo-EM. Several mutations of amino acids in the HNH domain, including the Zn finger were reported and their effect on the activity of the nuclease was analyzed. Overall reported findings and most conclusions seem to be solid, and I think that this is a very valuable contribution to further understanding of how this interesting CRISPR-Cas system works. The text is mostly clear although the language still needs to be improved. I have several questions and suggestions outlined below:

1. In Han Altae-Tran et al. paper it has been mentioned that this system has a collateral activity cleaving ssDNA substrates stimulated by dsDNA and ssDNA targets in a PAM-dependent manner. Can these authors confirm this? And if yes, would it be possible to use the structures to explain this activity? This is obviously a very important question considering the potential applications of this system as a biotechnology tool.

2. Origin of the HNH nuclease from a phage requires more research including building a phylogenetic tree. DALI score cannot be considered as convincing evidence for this statement. So, I suggest simply drop the last part of the sentence in the lines 195-199.

3. Please clarify the experiment on metal dependence. It has been shown that HNH nuclease has two metal-binding sites (<https://doi.org/10.1046/j.1365-2958.2000.02010.x>). Is this the case for this HNH domain? If yes, the interpretation of metal dependence of nuclease activity and complex stability (lines 301-304) might not be entirely correct.

4. Lines 330-332. Give more details on these sequences (provided nucleotide and amino acid sequences accessions). The same is applied to Fig. 1A.

5. Why do two bands for the cleaved DNA have different intensities in the Figure 1B.

6. Why there are two bands on the Supplementary Fig.1A for Cas8?

Minor corrections:

1. Find a way to polish the language of the paper.

2. Line 21-22. “Cas” mean CRISPR-associated proteins or “cas” (italic) - CRISPR-associated genes

3. Line 106-109. Where was it shown? Please show this in a supplement or include a reference.

4. Lines 274-277. The presence of the Zn finger in HNH nucleases is well known (not discovered here for the first time), so please include references here. Eg. <https://doi.org/10.1046/j.1365-2958.2000.02010.x> and <https://onlinelibrary.wiley.com/doi/10.1110/ps.03115604>.

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have addressed some of my points. After a brief review of the recently published papers, those articles also couldn't address second strand cleavage. I recommend publication in Nature Communications, so this work can be contemporaneous with the other published articles.

David Taylor

Reviewer #2

(Remarks to the Author)

In the revised manuscript, Liu et al. have restructured the paper, fully addressed all reviewer comments, and added new in vitro cleavage data, resulting in substantial improvement.

Recently, two studies on the I-F and I-E HNH-Cascade complexes were published in Molecular Cell, which somewhat reduces the novelty of the current work. The authors should compare their structures with those in these recent publications and highlight the unique insights their findings provide that were not covered in the other studies. Additionally, they could propose a working model for the HNH-Cascade based on these comparisons.

In this revised version, the authors present evidence that the HNH domain cleaves the target strand more efficiently than the non-target strand. However, it appears that the dsDNA-bound Cas5-HNH Cascade complex has limited cleavage activity for random ssDNAs. Furthermore, the mechanism by which HNH cleaves the non-target strand and random ssDNAs remains unexplored.

Reviewer #3

(Remarks to the Author)

Overall, I am satisfied with the revised manuscript and have only two minor suggestions:

1. Include a reference after the sentence: "Previous research has established that the HNH nuclease contains two metal-binding sites, typically for zinc and magnesium"
2. When talking about sequence or structure comparison, use "similarity" but not "homology". Homology is an evolutionary term meaning common origin and is irrelevant to sequence or structure or any other characteristics of proteins.

made.

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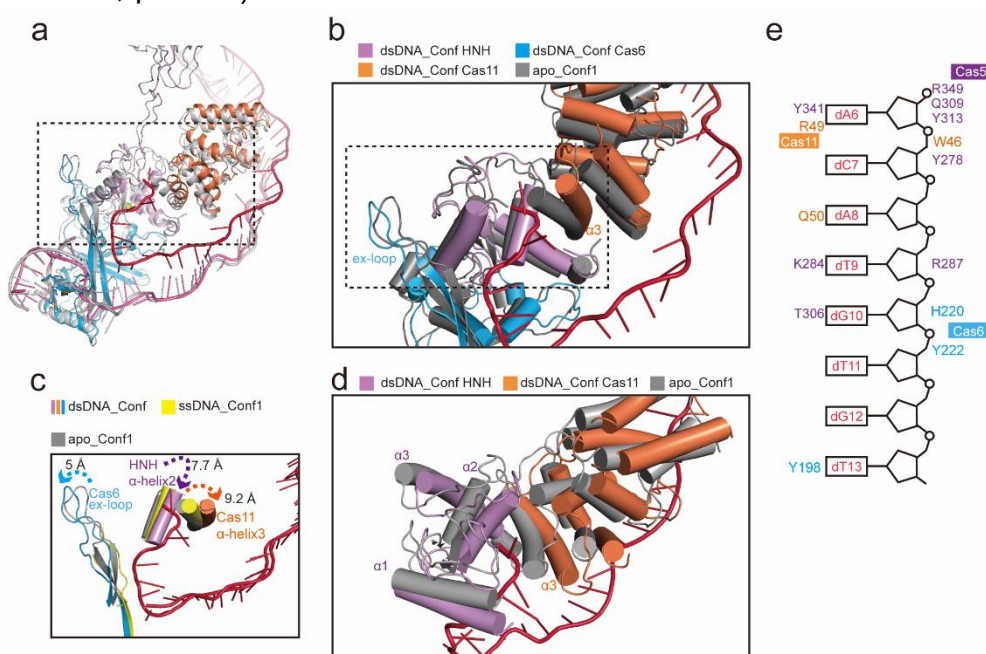
Reviewer #1 (Remarks to the Author):

1. What are the dynamics required for the HNH domain to cleave both strands of the dsDNA?

Reply:

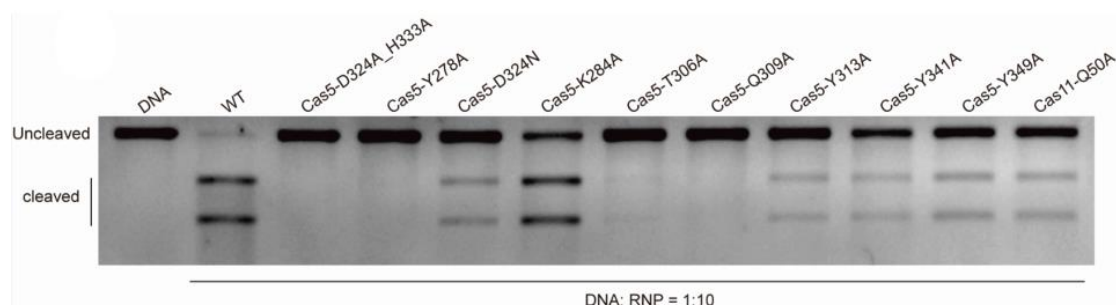
We appreciate your insightful comment. To investigate the DNA cleavage mechanism of Cas5-HNH, we collected two datasets comprising 10,379 micrographs and selected over 4 million particles (see Supplementary Fig. 9). Following a thorough 3D classification, we obtained one structure with the target strand inserted into the HNH domain. However, the density for the non-target strand was not clear, with only 6-7 base pairs of double-stranded DNA visible near the PAM recognition site, likely due to its flexibility.

To elucidate the cleavage mechanism of Cas5-HNH, we compared the dsDNA-bound state with the apo state. Significant movements were observed among Cas5, Cas6, and Cas11. Upon DNA binding, Cas6 and Cas11 shift away from the 5' end, opening the active channel for cleavage, while the HNH domain moves toward the substrate (see the figure below, panels a-d; see also Fig. 3 in the manuscript). The shift of the extension loop (residues 199-225, termed as ex-loop) between the two β -strands of Cas6 measures 5 Å, while the movements of the HNH domain and Cas11 range from 8 to 10 Å (see the figure below, panel c). Notably, α -helix 3 (α 3) of Cas11 undergoes a twisting conformational change upon DNA binding, with the C-terminus of Cas11- α 3 moving approximately 9 Å away from the DNA while the N-terminus remains nearly in its original position (see the figure below, panel d). Additionally, several residues that interact with the target DNA, such as Y278 (Cas5) and W46 (Cas11), are also involved in the interaction surface between subunits (see the figure below, panel e).



Dynamics of Cas5-HNH /Cascade complex upon binding target DNA. a-d, Movement of the HNH domain, Cas11, and Cas6. Cas6 and Cas11 shift away from the target DNA, while the HNH domain moves closer to the DNA. **e,** Residues of the complex involved in interactions with the 5'-end of DNA.

As anticipated, mutations in residues associated with the 5'-end of DNA significantly reduced nuclease activity, with specific mutations at Y278, T306, and Q309 in the HNH domain nearly abolishing the enzyme's function (see the figure below, see also Supplementary Fig. 14i).



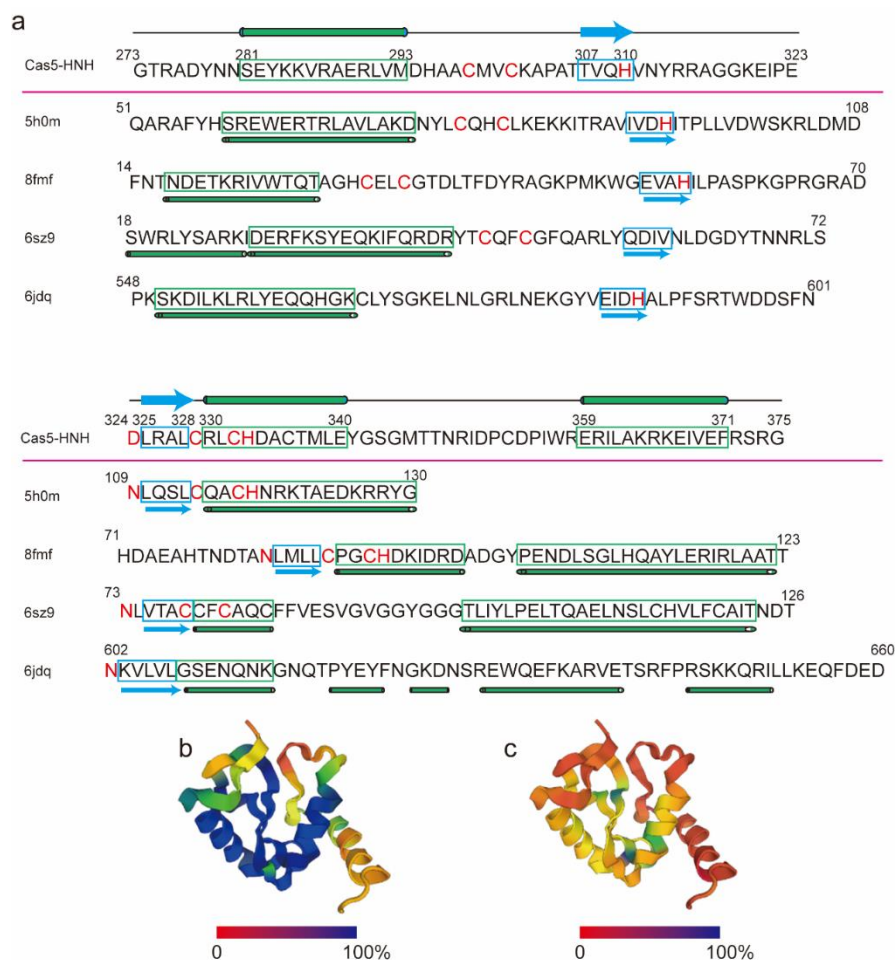
We have updated the manuscript to reflect these findings in Lines 226 to 244.

2. *It would be interesting to see additional bioinformatic analysis on the HNH domain, including not only comparisons to other bacteriophage HNH domains, but other type II nucleases. Comparing structural and sequence similarities and pathways of HNH acquisition could be informative.*

Reply:

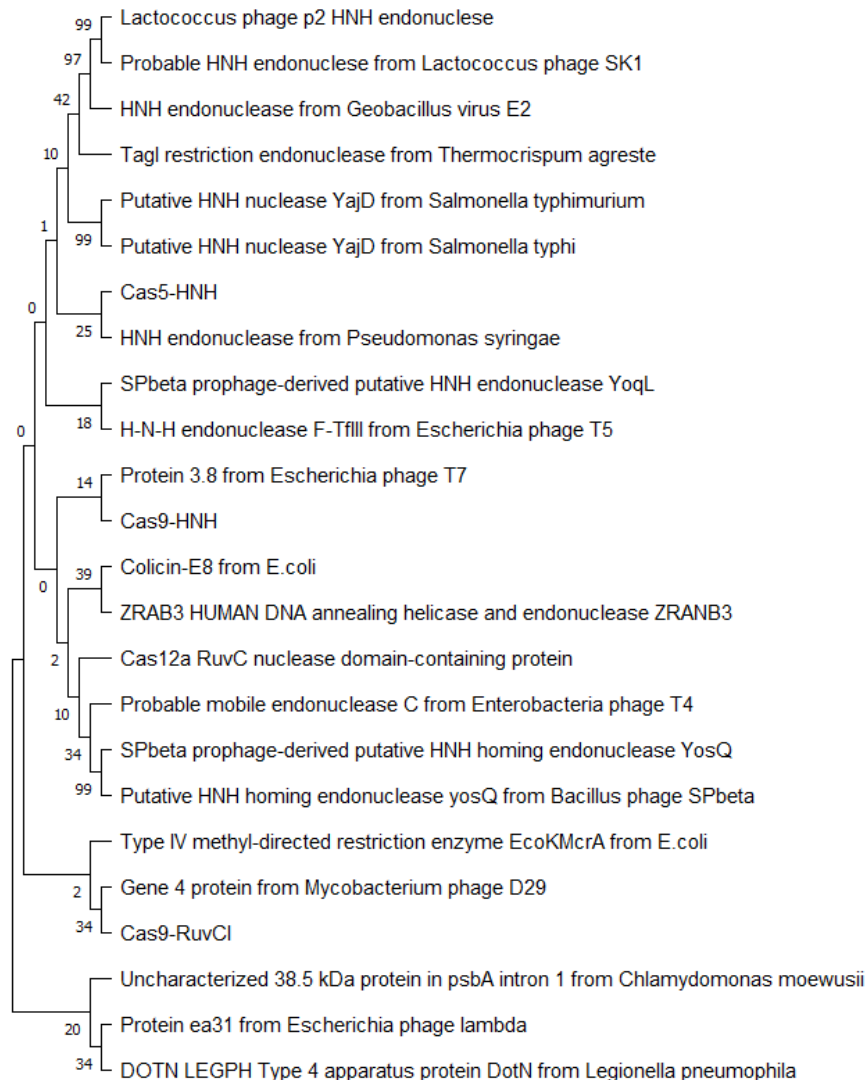
We appreciate your suggestions. For the structural comparisons with other bacteriophage HNH domains, we selected those with available structures. Structural comparisons using the DALI server revealed that the Cas5-HNH domain exhibits high similarity to the HNH endonuclease from the deep-sea thermophilic bacteriophage GVE2 (PDB: 5h0m), *Pseudomonas syringae* Cap5 (PDB: 8fmf), and the Type IV Coupling Complex (T4CC) from *Legionella pneumophila* (PDB: 6sz9), with z-scores of 8.6, 6.4 and 6.1, respectively. The HNH domain of type II nuclease Cas9 (PDB: 6jdq) also exhibits structural homology to Cas5-HNH, with a z-score of 2.7. Additionally, we isolated the C α atoms and calculated the RMSD values, yielding RMSD values ranging from 2.5 to 5.0 Å. However, these proteins exhibit low sequence similarities (see the figure below, panel a-c, see also Supplementary Fig.13), ranging from 7% to 26%. We have included these comparison results in Lines 190 to 198 to read "...Structural comparisons using the DALI server revealed that the Cas5-HNH domain shares strong structural similarity with the HNH endonuclease from the deep-sea thermophilic bacteriophage GVE2 (PDB: 5h0m), *Pseudomonas syringae* Cap5 (PDB: 8fmf), and the type IV Coupling Complex (T4CC) from *Legionella pneumophila* (PDB: 6sz9), with z-scores of 8.6, 6.4 and 6.1, respectively. The HNH domain of type II nuclease Cas9 (PDB: 6jdq) also

exhibits structural homology to Cas5-HNH, though with a lower z-score of 2.7 (Supplementary Fig.13a,b). Despite this structural similarity, these proteins display low sequence homology (Supplementary Fig.13c)”.



Structural and sequence alignment of HNH domains. **a**, α -helices are represented by green cylinders, and β -sheets are depicted as cyan arrows. **b-c**, Diagram of structure similarities (**b**) and sequence conservation (**c**). The conservation gradually decreases from blue (100%) to red (0%).

Regarding the pathways of HNH acquisition, we selected structurally homologous proteins identified by the DALI server, along with representative HNH endonucleases from various species, to construct a phylogenetic tree using MEGA (see the figure below). According to the results, these proteins show limited homology with Cas5-HNH, suggesting that it is not reliable to claim the origin of Cas5-HNH. Additionally, as suggested by Reviewer #3, we have removed the sentence stating “hinting that the bacteria probably captured the HNH domain from bacteriophage during evolution” from the manuscript to avoid the misleading.

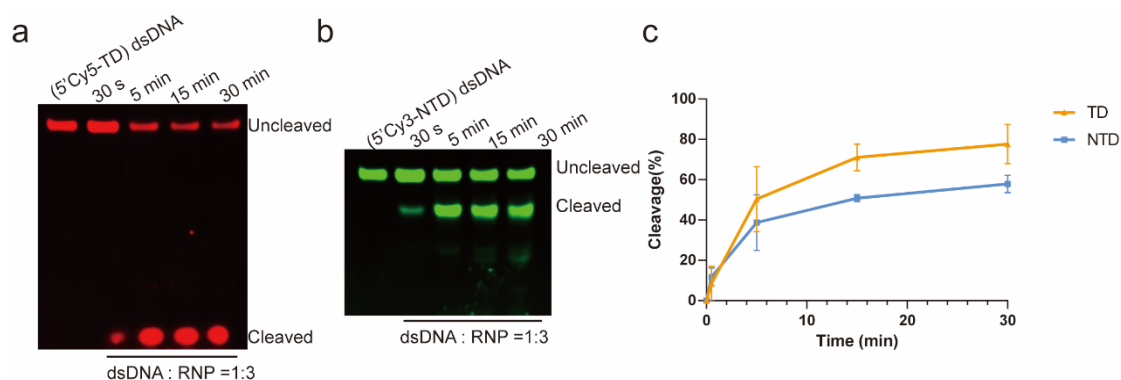


3. HNH cleavage of dsDNA is a unique feature not observed in type II nucleases which require a RuvC domain to cleave the non-target strand of DNA. This seems like one of the most interesting features of the enzyme, but it is barely mentioned. It would be interesting to see the dynamics of the HNH rearrangements via structural methods or target and non-target strand cleavage kinetics.

Reply:

We appreciate this comment. As mentioned earlier, the density of the non-target strand remains unresolved due to its flexibility, making it unreliable to use the structures to elucidate the HNH cleavage of dsDNA. Instead, we conducted a cleavage order test on the complex. For this, we labeled the target stranded DNA (TD) with Cy5 and the non-target stranded DNA (NTD) with Cy3. Our findings indicate that NTD cleavage occurs after TD digestion (see the figure below; also see Supplementary Fig.15). The corresponding data has been updated on page 11, Lines 245-250, to read "...Given the unique dsDNA-

cleavage mechanism of Cas5-HNH, which differs from type II CRISPR-Cas systems that require a RuvC domain to cleave the non-target strand, we conducted cleavage order experiments. The results indicated that NTD cleavage occurs following TD digestion (Supplementary Fig.15a-b). The non-target strand was cleaved less efficiently than the target strand (Supplementary Fig.15c)".



Cleavage order test of Cas5-HNH/Cascade complex. a-b, EMSA detected the cleavage progression over 30 minutes. The target strand DNA (TD) was labeled with Cy5 (**a**) and the non-target strand DNA (NTD) was labeled with Cy3 (**b**) at both 5'-end. **c,** The complex cleaves TD first, followed by NTD.

4. The authors claim the Cas5-HNH/Cascade complex could be used as a potential gene-editing tool. While this paper shows that this enzyme efficiently targets dsDNA, there are no in vivo results that show this enzyme is active in mammalian cells. Therefore, I believe this statement is significant overstatement, and it needs to be removed from the manuscript.

Reply:

Thank you for the advice. We have removed the sentence from the manuscript.

Reviewer #2 (Remarks to the Author):

1. The manuscript needs to be reorganized for better logical flow. I suggest starting the results section with the apo Cas5-HNH/Cascade complex, followed by the target DNA binding assays, and then the Cas5-HNH/Cascade-ssDNA complex. Ensure that the figures are cited in the correct sequence.

Reply:

Thank you for the suggestion. We have re-organized the manuscript as suggested and the citations of the figures have been updated. The revised manuscript in the first part of *Results* read " ...Prior to the cryo-EM studies, digestion assays were performed to validate enzymatic activity, showing that the wild-type Cas5-HNH/Cascade complex efficiently cleaves target DNA

(Supplementary Fig.1c). Three conformations of the target-free complex were resolved: two intact complexes (apo_Conf1, apo_Conf2) and one partially assembled complex (apo_Conf3), with resolutions of 2.47 Å, 3.10 Å and 2.55 Å, respectively (Supplementary Figs.2,3).

Previous studies have demonstrated that the Cas5-HNH/Cascade complex can cleave both single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA). To further investigate the interaction of target DNA with the complex, fluorescence-detection size-exclusion chromatography (FSEC) was used. A fluorophore group, FAM, was attached to the 3' end of the target strand of dsDNA (TD), while Cy3 was labeled at the 3' end of the non-target strand of dsDNA (NTD). FSEC data showed peak migration for both TD and NTD, indicating that the Cas5-HNH/Cascade complex targets both ssDNA and dsDNA (Supplementary Fig.4). To capture stable structures bound to target DNA, we incubated a catalytic mutant ribonucleoprotein (RNP) complex with target ssDNA or dsDNA, followed by cryo-EM analysis.

For the ssDNA-bound complex, two conformations were observed: an intact complex (ssDNA_Conf1) and a partially assembled complex (ssDNA_Conf2), with resolutions of 3.00 Å and 2.81 Å, respectively (Supplementary Figs.5-8). Additionally, we collected 10,379 micrographs to resolve the structure of the complex bound to dsDNA, achieving a resolution of 2.93 Å with partial density of the NTD (dsDNA_Conf, Fig.1, Supplementary Fig.9)."

2. The highlight of the manuscript is the activation and DNA cleavage mechanism of Cas5-HNH. However, the authors did not clearly elucidate this mechanism through structural analysis. Understanding the cleavage mechanism of the HNH domain is crucial for comprehending this novel I-E subtype. It is recommended to provide structural data of the Cas5-HNH/Cascade complex that offers insights into how the HNH domain interacts with dsDNA and facilitates strand cleavage. Specific structural features, such as the active site configuration and DNA binding interfaces, are essential for understanding this mechanism. Additionally, the HNH domain should be highlighted in the overall structures.

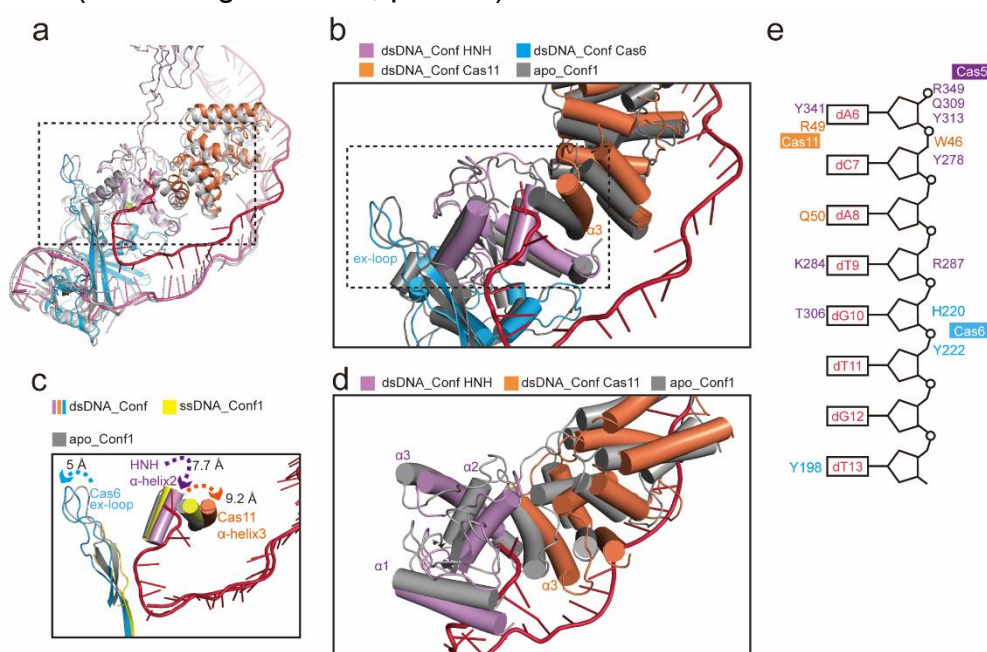
Reply:

We appreciate this comment. To investigate the DNA cleavage mechanism of Cas5-HNH, we collected two datasets, comprising 10,379 micrographs and over 4 million selected particles (see Supplementary Fig.9). Following a thorough 3D classification, we captured one structure with the target strand inserted into the HNH domain. However, the density for the most of non-target strand was unclear, with only 6-7 base pairs of double-stranded DNA visible near the PAM recognition site, likely due to its flexibility.

We compared the dsDNA-bound and apo states. Significant movements were observed among Cas5, Cas6, and Cas11. Upon binding to the target DNA,

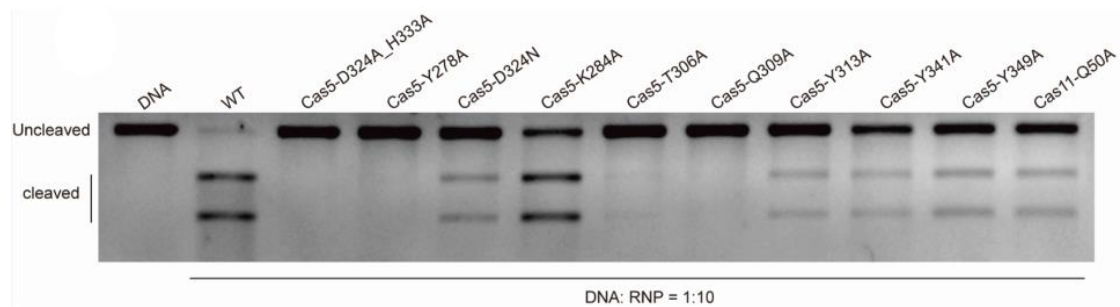
Cas6 and Cas11 shift away from the 5' end, opening the active channel for cleavage, while the HNH domain moves toward the substrate (see the figure below, panels a-d; see also Fig.3 in the manuscript). The shift distance of the extension loop (ex-loop) between the two β -strands of Cas6 measures 5 Å, while the movements of the HNH domain and Cas11 range from 8 to 10 Å (see the figure below, panel c). Notably, α -helix 3 (α 3) of Cas11 undergoes a twisting conformational change upon DNA binding, with the C-terminus of Cas11- α 3 moving approximately 9 Å away from the DNA, while the N-terminus remains nearly in its original position (see the figure below, panel d).

Additionally, several residues that interact with the target DNA, such as Y278 (Cas5) and W46 (Cas11), are also involved in the interaction surface between subunits (see the figure below, panel e).



Dynamics of Cas5-HNH /Cascade complex upon binding target DNA. a-d, Movement of HNH domain, Cas11 and Cas6. Cas6 and Cas11 move away from target DNA, while HNH domain move towards the DNA. **e,** Residues of the complex involved in interactions with the 5'-end of DNA.

As anticipated, mutations in residues associated with the 5'-end of DNA significantly reduced nuclease activity, with specific mutations at Y278, T306, and Q309 in the HNH domain nearly abolishing the enzyme's function (see the figure below, see also Supplementary Fig. 14i).

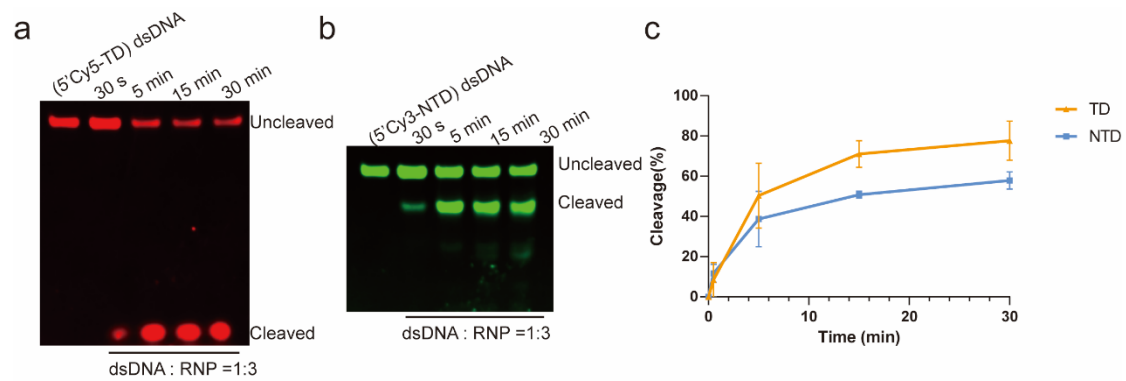


We have updated the manuscript to reflect these findings in Lines 226 to 244.

3. Previous studies have shown that the Cas5-HNH/Cascade complex can cleave dsDNA. It appears that Cas5-HNH cuts the target strand and the non-target strand in a sequence reminiscent of the RuvC domain in Cas12. It is of great significance to identify which strand is cleaved first by Cas5-HNH, as well as the specific cleavage sites in dsDNA. Can the structures in this study explain how the HNH domain cleaves these two strands?

Reply:

We appreciate this comment. As mentioned above, we found that the non-target strand is extremely flexible, preventing us from resolving the density associated with it. Consequently, we believe it is not reliable to use our structures to determine which strand is cleaved first. Instead, we performed cleavage kinetics experiments to elucidate how the HNH domain cleaves the target and non-target strands. We labeled the target stranded DNA (TD) with Cy5 and the non-target stranded DNA (NTD) with Cy3, and then conducted a cleavage order test on the complex. Our findings indicate that NTD cleavage occurs after TD digestion (see the figure below; also see Supplementary Fig.15). The corresponding data has been updated on page 11 Lines 245-250, to read "...Given the unique dsDNA-cleavage mechanism of Cas5-HNH, which differs from type II CRISPR-Cas systems that require a RuvC domain to cleave the non-target strand, we conducted cleavage order experiments. The results indicated that NTD cleavage occurs following TD digestion (Supplementary Fig.15a-b). The non-target strand was cleaved less efficiently than the target strand (Supplementary Fig.15c)".



Cleavage order test of Cas5-HNH/Cascade complex. a-b, EMSA detected the cleavage progression over 30 minutes. The target strand DNA (TD) was labeled with Cy5 (a) and the non-target strand DNA (NTD) was labeled with Cy3 (b) at both 5'-end. **c,** The complex cleaves TD first, followed by NTD.

4. As shown in Fig. 4, the “zinc finger like structure” is the typical zinc finger motif found in many Cas proteins, such as Cas10 and Cas12 families. The divalent ion coordinated by four cysteines should be a Zn^{2+} . The zinc finger motif could not probably bind different metal ions. The metal ion for HNH activation is coordinated at catalytic center rather than zinc finger motif. The last part of “Divalent ions play important roles in regulating the cas5-HNH/Cascade complex” is misleading.

Reply:

Thanks for this comment. We deleted the part related in which metal ions bound by cysteines. The change can be found in Page 12 Lines 288-292. We have moved the zinc-finger panels to Supplementary Fig.12e to avoid the misleading.

5. The sentence “As expected, the mutations involved with the Cas5-HNH and Cas6 interfaces can significantly reduce the efficiency of DNA digestion (Fig.2b).” cited the wrong figure.

Reply:

Thank you for pointing this out. We have corrected the citation of the figures. Please see the Lines 208-210 to read “As expected, some of mutations affecting the HNH-Cas6 interface resulted in a significant reduction in DNA cleavage efficiency (Supplementary Fig.14a-c)”.

6. Line 196, what is the Z score between HNH domain and the PDB: 5H0M as shown in dali search?

Reply:

After structural alignment using the DALI server, the Z score between HNH domain and the thermophilic bacteriophage GVE2 (PDB: 5H0M) is 8.6. The value of the Z-score was added in the line 194 to read “...the Cas5-HNH

domain exhibits high similarity to the HNH endonuclease from the deep-sea thermophilic bacteriophage GVE2 (PDB: 5h0m), ...with z-score of 8.6...".

7. Line 221, *"Of note, these residues, including M338, F371, R275, D277 and Y278, are related to the interaction with Cas11 (Fig.2c,h)."The residues M338 and F371 are not shown in Fig. 2h.*

Reply:

We appreciate your comment. The residues M338 and F371 has been included in Fig.2i. The sentence read as "Interestingly, these residues are associated with interaction with Cas11 (Fig. 2h), and mutating the interacting partners (W46, R49, and Q53) of Cas11 completely abolished the enzyme's activity (Supplementary Fig. 14d)" in Lines 218-221.

8. Line 219, *"Interestingly, a combined mutation of R275A, D277A and Y278A in the linker domain resulted in a significant decrease in the nuclease activity." It's inconsistent with the results shown in Supplementary Fig.10d and 10h. The nuclease activity of mutant R275_D277_Y278 is only slightly decreased, whereas the ssDNA binding ability seems greatly increased.*

Reply:

We appreciate your comment. We have revised the text in Line 216-218 to read "...A combined mutation of R275A, D277A, and Y278A in the linker region resulted in a slight reduction in nuclease activity (Supplementary Fig. 14d).....".

9. *The favored Ramachandran plot of the Apo_Conf2 is less than 90%, the structure needs further refinement.*

Reply:

We have refined the structure Apo_Conf2 and the favored Ramachandran plot now reached to 91.51%. Please see the Supplementary Table 1 for more details.

10. *Some typos, "ofβ-strand 1" is "of β-strand 1", and "280nm" is "280 nm".*

Reply:

Thanks for pointing this out. These kinds of typos have been corrected in the main text (see Line 170 and 373).

Reviewer #3 (Remarks to the Author):

1. *In Han Altae-Tran et al. paper it has been mentioned that this system has a collateral activity cleaving ssDNA substrates stimulated by dsDNA and ssDNA*

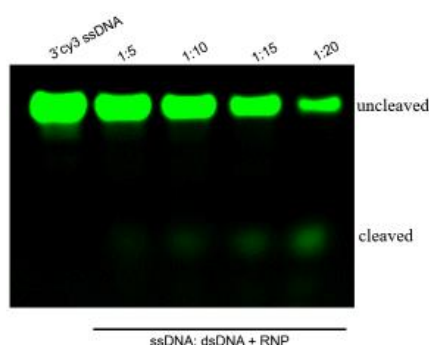
targets in a PAM-dependent manner. Can these authors confirm this? And if yes, would it be possible to use the structures to explain this activity? This is obviously a very important question considering the potential applications of this system as a biotechnology tool.

Reply:

We have confirmed that Cas5/HNH Cascade complex has collateral activity. To confirm this, a random ssDNA was labeled with Cy3, while the dsDNA remained unlabeled. The sequence of the random ssDNA is as follows:

AGCTGGTAAGGATTGCCGTCAGGAAATTAGGTGCGCTTAGCACACATGTC
AAGCTCTCCG.

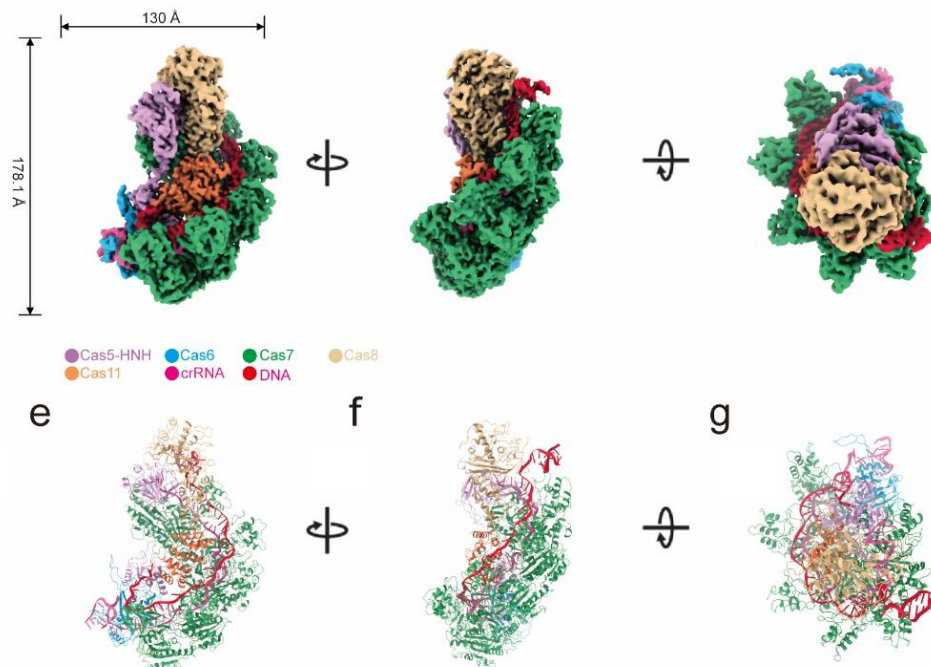
We observed a decrease in the uncleaved band as the RNP concentration increased (Please see the figure below).



In vitro collateral cleavage activity assay of the dsDNA-bound Cas5-HNH Cascade complex.

We sincerely agree that it is essential to use structural data to elucidate the collateral activity which requires us to obtain the dsDNA bound structures. To do this, we collected 10,379 micrographs and picked over 4 million particles (Supplementary Fig.9). We are able to observe that the target strand is inserted into the HNH domain (Please see the figure below, or Fig.1 in the manuscript). However, the density for the non-target strand was not clear, with only 6-7 base pairs of double-stranded DNA visible near the PAM recognition site, likely due to its flexibility. We have updated the manuscript to reflect these findings in Lines 159 to 164.

Our current structures do not provide sufficient evidence to clarify this mechanism. We believe that the presence of random ssDNA may further complicate the situation, requiring significant effort to address it. It is our next plan to resolve the associated structures to enhance our understanding of the collateral activity.



Cryo-EM maps and model of the complex bound with dsDNA.

2. Origin of the HNH nuclease from a phage requires more research including building a phylogenetic tree. DALI score cannot be considered as convincing evidence for this statement. So, I suggest simply drop the last part of the sentence in the lines 195-199.

Reply:

Thanks for your suggestion. We completely agree with your suggestion. Considering the comments proposed by Reviewer #1 and Reviewer #2, the sentence in Line 190-198 has been revised as suggested which reads "...Structural comparisons using the DALI server revealed that the Cas5-HNH domain shares strong structural similarity with the HNH endonuclease from the deep-sea thermophilic bacteriophage GVE2 (PDB: 5h0m), *Pseudomonas syringae* Cap5 (PDB: 8fmf), and the type IV Coupling Complex (T4CC) from *Legionella pneumophila* (PDB: 6sz9), with z-scores of 8.6, 6.4 and 6.1, respectively. The HNH domain of type II nuclease Cas9 (PDB: 6jdq) also exhibits structural homology to Cas5-HNH, though with a lower z-score of 2.7 (Supplementary Fig.13a-b). Despite this structural similarity, these proteins display low sequence homology (Supplementary Fig.13c)."

3. Please clarify the experiment on metal dependence. It has been shown that HNH nuclease has two metal-binding sites (<https://doi.org/10.1046/j.1365-2958.2000.02010.x>). Is this the case for this HNH domain? If yes, the interpretation of metal dependence of nuclease activity and complex stability (lines 301-304) might not be entirely correct.

Reply:

We appreciate for your comments. In our cryo-EM map, we didn't find the density for the second metal-binding site except the one coordinated by the zinc finger. We have moved the panel of Zinc-finger to Supplementary Fig.12b to avoid the misleading. The text is revised in Lines 288-292 to read "...Previous research has established that the HNH nuclease contains two metal-binding sites, typically for zinc and magnesium. We tested various metal ions, including magnesium, manganese, calcium, cesium, cobalt, nickel and zinc, to determine their effects on the enzyme efficiency of the Cas5-HNH/Cascade complex. The findings demonstrated that, in the presence of 0.5 mM magnesium, manganese and calcium, the enzymatic efficiency of the Cas5-HNH/Cascade complex was comparable across these ions (Fig.5b). However, the adding cesium, cobalt, nickel and zinc significantly reduced DNA digestion efficiency. Especially for zinc and cobalt, the activity of the enzyme was nearly abolished (Fig.5b)". The reference as suggested is cited in Lines 286 and 289.

4. Lines 330-332. Give more details on these sequences (provided nucleotide and amino acid sequences accessions). The same is applied to Fig. 1A.

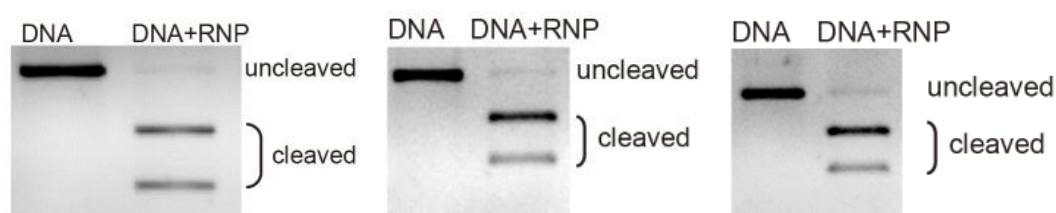
Reply:

We appreciate this comment. The Uniprot IDs are added in lines 331-333 to read "...The Cas genes encoding full-length Cas5-HNH (Uniprot ID: A0A1V6F8C5), Cas6 (Uniprot ID: A0A1V6F8C4), Cas7 (Uniprot ID: A0A1V6F8B5), Cas11 (Uniprot ID: A0A1V6F8C9) and Cas8 (Uniprot ID: A0A1V6F8D1) proteins of the type I-E Cas5-HNH system were sourced from *Candidatus Cloacimonetes* bacterium ADurb.Bin088....". We also added the accession numbers in the figure legend for Fig.1A.

5. Why do two bands for the cleaved DNA have different intensities in the Figure 2B?

Reply:

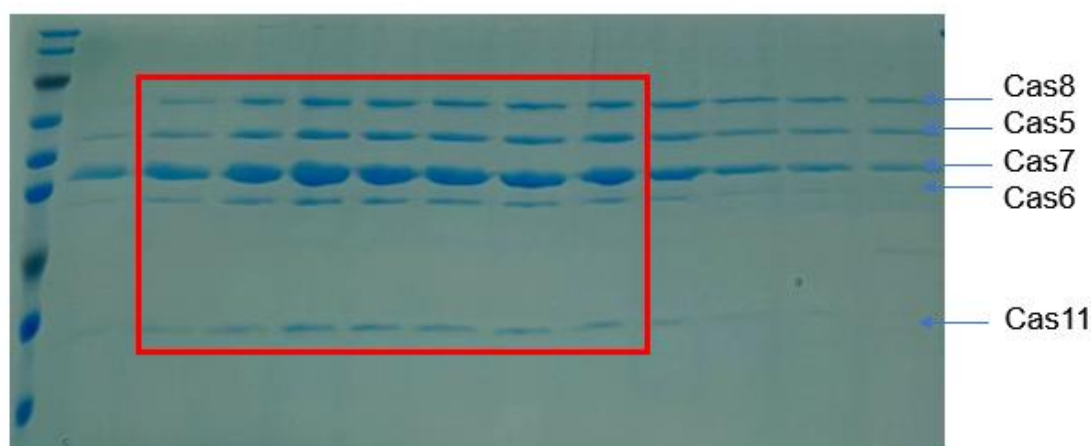
We repeated the cleavage assay (Please see the figure below), and the cleaved DNA fragments displayed varying intensities. This could be due to the lower band being further degraded into smaller fragments. We have replaced Fig.2B by the gel with similar intensities.



6. Why there are two bands on the Supplementary Fig.1A for Cas8?

Reply:

By shorting the time to purify the complex, the lower band was disappeared (Please see the figure below), so we believe that the lower band is probably caused by the degradation of Cas8. We have updated the Supplementary Fig.1 with the latest SDS-PAGE result.



Minor corrections:

1. Find a way to polish the language of the paper.

Reply:

We polished the manuscript by ChatGPT. The grammar errors have been corrected.

2. Line 21-22. “Cas” mean CRISPR-associated proteins or “cas” (*italic*) - CRISPR-associated genes.

Reply:

We appreciate this comment. The sentence in Line 22 now reads “...Type I-E CRISPR (clustered regularly interspaced short palindromic repeats)–Cas (CRISPR-associated proteins) system is one of the most extensively studied RNA-guided adaptive immune systems in prokaryotes...”.

3. Line 106-109. Where was it shown? Please show this in a supplement or include a reference.

Reply:

Thank you for this comment. In lines 105-107, the digestion assay was shown in the Supplementary Fig.1c.

4. Lines 274-277. The presence of the Zn finger in HNH nucleases is well known (not discovered here for the first time), so please include references here. Eg. <https://doi.org/10.1046/j.1365->

[2958.2000.02010.x](#) and <https://onlinelibrary.wiley.com/doi/10.1110/ps.03115604>.

Reply:

We appreciate your comment. The references were cited in line 286.

Reviewer #1 (Remarks to the Author):

The authors have addressed some of my points. After a brief review of the recently published papers, those articles also couldn't address second strand cleavage. I recommend publication in Nature Communications, so this work can be contemporaneous with the other published articles.

Reply:

Thank you very much for your valuable comments. We completely agree that addressing the mechanism of the second strand cleavage is crucial, and it is the focus of our next research plan.

Reviewer #2 (Remarks to the Author):

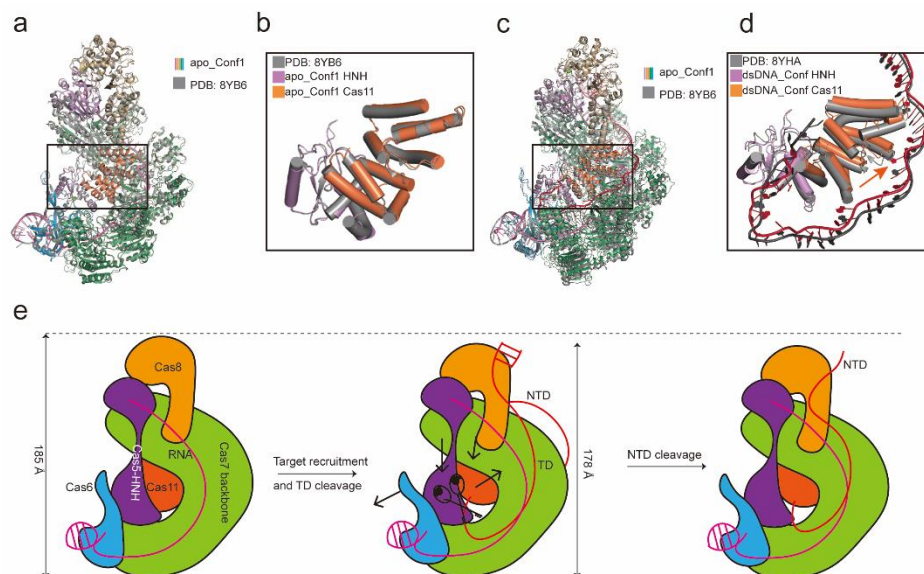
In the revised manuscript, Liu et al. have restructured the paper, fully addressed all reviewer comments, and added new in vitro cleavage data, resulting in substantial improvement.

Recently, two studies on the I-F and I-E HNH-Cascade complexes were published in Molecular Cell, which somewhat reduces the novelty of the current work. The authors should compare their structures with those in these recent publications and highlight the unique insights their findings provide that were not covered in the other studies. Additionally, they could propose a working model for the HNH-Cascade based on these comparisons.

In this revised version, the authors present evidence that the HNH domain cleaves the target strand more efficiently than the non-target strand. However, it appears that the dsDNA-bound Cas5-HNH Cascade complex has limited cleavage activity for random ssDNAs. Furthermore, the mechanism by which HNH cleaves the non-target strand and random ssDNAs remains unexplored.

Reply:

Thank you for your valuable suggestions. We compared our structures with the structures published by Zhang *et al.* We found that the target-free structure (apo_Conf1) aligns perfectly with the structure 8YB6 in apo state (see the figure below, panel a-b, see also Supplementary Fig.18a-b). Interestingly, when compared to 8YHA in target-bound state, the subunit Cas11 of our dsDNA-bound structure exhibits a noticeable displacement that away from the nuclease center (see figure below, panel c-d, see also Supplementary Fig.18c-d). As suggested, we proposed a working model for the Cas5-HNH/Cascade based on our analysis (see figure below, panel e, see also Fig.5e). We have updated the manuscript accordingly to reflect these findings in Lines 319-329.



Comparison of Cas5-HNH/Cascade complex with published structures and proposed model

We sincerely agree that it is essential to use structural data to elucidate the collateral activity. However, our current structures do not provide sufficient evidence to clarify this mechanism. It is our next plan to resolve the associated structures to enhance our understanding of the collateral activity.

Reviewer #3 (Remarks to the Author):

Overall, I am satisfied with the revised manuscript and have only two minor suggestions:

1. Include a reference after the sentence: “Previous research has established that the HNH nuclease contains two metal-binding sites, typically for zinc and magnesium”

Reply:

We appreciated your comments. We have included the references as suggested.

2. When talking about sequence or structure comparison, use “similarity” but not “homology”. Homology is an evolutionary term meaning common origin and is irrelevant to sequence or structure or any other characteristics of proteins.

Reply:

We have replaced the word “homology” by “similarity”. Changes can be found in Lines 196 and 198.