

Molecular Basis for the Calcium-Dependent Activation of the Ribonuclease EndoU

Corresponding Author: Dr Sebastien Campagne

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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)

In this paper Malard and colleagues describe a mechanism of enzyme activation for the human ribonuclease EndoU. They suggest that Ca^{2+} ions allosterically activate the enzyme by inducing conformational changes in the active site of the enzyme that result in an active arrangement of the catalytic triad.

The data is interesting for a wider readership which is concerned with enzyme mechanisms and RNase regulation. The paper is well written and suggests a tempting mechanism for EndoU activation. Yet, given that it is in part based on models and conjecture, and in part contradicts previous literature on *Xenopus* EndoU active site assignments, a few important controls and comparisons are missing that would reveal the relevance of the proposed models. Also some basic information is missing that would allow judgement of the evolutionary conservation of the proposed mechanism. In addition, illustrations of the structures should be improved to make it easier for readers to grasp, which residues are moving upon Ca^{2+} binding and how this relates to the active site location.

One major drawback is that the suggested conformational changes of the human enzyme are based on comparison to a homology model of the apo-structure from *Xenopus* EndoU. NMR data in presence and absence of Ca^{2+} is collected as well but here the residues that become ordered or change position are not correlated to the suggested conformational changes based on the structural models. Similarly, comparisons to active site conformations of related enzymes (e.g. RNase A, Nsp15) or sequence alignments with paralogs are currently missing, but should be made to reveal whether this model is likely to reflect a true and potentially conserved scenario. Finally, activation by Ca^{2+} could result from an increase in RNA affinity or an increase in the active population of the enzyme, yet this is not disentangled.

Detailed comments - Major points:

Identification of Ca^{2+} pockets - Ca^{2+} ions especially when not fully occupied in the crystal lattice are not easily distinguishable from other similar atoms (H_2O or other metal ions) in the electron density map. Without experimental verification, the nature of these atoms stands in question.

Did the authors perform any anomalous diffraction experiments to confirm the presence of the suggested metal ions in the four pockets? Eventhough the Ca K-edge is outside of reach of their synchrotron, they could still perform a S-SAD like experiment as close as possible to the K-edge and detect the weaker anomalous scattering of these metal ions in a highly redundant data collection.

Do the atom distances in the coordination sphere match literature values for calcium coordination (e.g. see Muller, Kopke, Sheldrick 2003 <https://doi.org/10.1107/S0907444902018000>)?

If these pockets are conserved in the *Xenopus* EndoU structure, what does the electron density map look like in these locations in the corresponding map?

Did the pocket residues become ordered or changed chemical shifts upon Ca^{2+} addition in NMR experiments?

Conformational changes upon Ca^{2+} binding - No apo-structure of human EndoU was obtained and putative conformational changes are deduced from a comparison of homology-modelled human apo-EndoU based on apo-*Xenopus* EndoU and calcium-bound human EndoU. This may be a good first hypothesis but needs further comparisons and experiments for verification. Especially, since the *XendoU* active site was suggested to be in a completely different position based on a

phosphate ion that was bound in the original crystal structure (Xenopus H162, N270, H272, T278). Further highlighting the problematic reliance on homology models: AlphaFold2 does actually predict human EndoU to be in the same "active" conformation even in the absence of Ca²⁺ ions, so no conformational change would be predicted if this model were chosen as reference point.

A more thorough comparison with available structures of homologous enzymes such as Nsp15, RNase A or others should be provided. Are the catalytic triad residues always in the same conformation? For RNase A, nucleotide-bound structures exist, which would allow comparison of the active spatial arrangement of the catalytic triad. Furthermore, it should be compared how well the proposed Ca²⁺ pockets are conserved between EndoU paralogs at sequence level and this sequence alignment should be part of the manuscript. If it is claimed (as currently in the discussion) that we can learn from the study about viral Nsp15 inhibition then Nsp15 should be included in the alignment.

The NMR spectra +/- Ca²⁺ should be analyzed and correlated to the proposed conformational changes. Are E290 and H285 shifting or becoming ordered upon Ca²⁺ addition? How about the loop that E290 is part of?

Mutations of some pocket residues were done but only two pockets seem to be important for nuclease activity, and only pocket 1 relevance was explained. Here, the E290D and E290A mutation was tested, but actually a better mutation to test the model for transmission of Ca²⁺ binding to the active site conformational change would be an E290Q mutation, because it would potentially uncouple Ca²⁺ binding from activation.

An open question is also still why pocket 3 mutation affects activity. Is this pocket more/less conserved than pockets 2/4? What does this pocket and its immediate vicinity look like in XendoU?

Basis of activating effect of Ca²⁺-ions - The binding of Ca²⁺ could increase RNA affinity of the enzyme by increasing the population of correctly arranged RNA binding sites, or it could increase the proportion of enzyme with correctly arranged active sites for efficient RNA cleavage. Currently, this aspect is not looked into which makes the mechanistic considerations a bit superficial. Given that the authors have all tools at their disposal to test RNA affinities and this is easily experimentally addressable, they should carry out RNA affinity measurements.

Relevance of RNA-binding model - In the manuscript a combination of AlphaFold3 and dynamics simulations is used to come up with a model for RNA substrate binding to EndoU. However this model is then not further tested or validated, which leaves the relevance of this model open. This is especially true since RNA-protein models of AlphaFold3 are poor (the program does not even know base pairing rules). NMR titrations of EndoU with RNA identify the same overall surface as the interaction site but allow no detailed structure calculation either. Based on their model the authors suggest that Y405 would be base stacking with the substrate RNA, so mutating this to Ala would be an easy thing to test minimally (effect on RNA affinity, and nuclease activity).

Control experiment for cellular EndoU rescue assays - Lack of rescue of RNase activity with an EndoU mutant should be added as a negative control for the assays in Fig. 1B.

Other points:

Visualization of structures and domains - In the overview figures (Fig. 2A, 4D/E, S6), the proposed active site should be visualized to allow judgement of the proximity of the Ca²⁺ ions.

I found the zoom views presented in Fig. 2B-D very difficult to understand. Residues are colored differently than their domains (pink, blue residues vs. grey, blue domains), they seem to float in empty space partially detached from the backbone and often missing the sidechains. Panel D is very busy with the superposition of three structures, here one (either the XendoU or the modelled human apo-structure) could be removed because they are redundant (SWISSPROT models are basically mimicking the existing structure and add very little new information).

In Fig. 1F the domain schemes are misleading in a way to suggest that XendoU has a different domain composition than the other eukaryotes. A consistent way of representing the domains should be used here and also numbers should be added to denote the domain boundaries.

Apparent differences in NMR spectra - Can the authors explain the differences between spectra in Figures S9 vs S5D? They should reflect almost the same conditions but S9 looks much better resolved and showing more peaks than S5D.

Information in figure legends and statistical analysis - For plots information in the figure legends is very sparse and no statistical analyses were carried out to denote significant differences. This should be amended. Figure legends should include what is plotted (e.g. means), number of replicates, what error bars represent, and which statistical test was carried out.

Methods - Units are written in an unusual way (e.g. mg.ml⁻¹) and should be changed to conventional annotation (e.g. mg/mL) instead.

Was TEV protease really used in 1:10 ratio? This is roughly 10-fold more than what is usually used.

Typos:

p.5 - dependence of EndoU for calcium > dependence of EndoU on calcium

p.9 - DNase (NEB) > DNase I (NEB)

p.12 - central tendencies are expressed as means, and variations as standard deviations > do you mean that "means of three replicates were plotted with error bars representing standard deviations"?

p.17 - that mainly cluster nearby protein loops > that mainly cluster in neighbouring protein loops

p.17 - The bonding correlates with a disorder-to-order transition in the loop carrying E290, forming a β -hairpin and stabilizing the catalytic site > This is misleading because the loop was already ordered before but in a different position.

p.18 - resulted in nearly half reduction of enzymatic activity > resulted in a reduction to nearly half of the enzymatic activity

Reviewer #2

(Remarks to the Author)

Eukaryotic EndoU is an endonuclease that shares the RNase-A family catalytic motif and has an N-terminal extension. Unlike RNase-A family nucleases, eukaryotic EndoU requires Ca^{2+} ions for catalysis. The mechanism by which Ca^{2+} ions are required for eukaryotic EndoU remains elusive. This manuscript describes the molecular basis of the activation of eukaryotic ribonuclease EndoU by calcium ions through structural and biochemical analysis. The authors determined the crystal structure of human EndoU in complex with Ca^{2+} at high resolution. In the structure, four Ca^{2+} ions were identified. Comparison of the Ca^{2+} -bound structure with the model apo structure (or apo XendoU) suggested that one of the Ca^{2+} ions binding to the enzyme allosterically changes the catalytic core structure (specifically conformation of one of the catalytic His residues) of EndoU, thereby activating it. The model was evaluated through biochemical analysis and molecular dynamics. The authors also built an RNA-binding model onto EndoU.

Major comment

This reviewer appreciates the solid structural and biochemical analysis of human EndoU in this manuscript. The manuscript is well organized and communicated. Having said that although eukaryotic EndoU might have acquired the N-terminal extension and evolutionarily adapted to cellular signaling in eukaryotes, the manuscript does not provide any mechanistic insight into the Ca^{2+} -specific activation of the enzyme. This reviewer feels that the structure of the EndoU-RNA complex (in the presence and absence of Ca^{2+}) would provide more mechanistic insights into the activation of EndoU and the cleavage of RNAs. Further, comparison of the structure of eukaryotic EndoU and those of Nidoviruses and bacteria EndoU would provide the more mechanistic insight into the Ca^{2+} dependent activation of eukaryotic EndoU.

The present form of the manuscript is suitable for other specific journals.

Minor comments

- 1) Page 16: Model of human EndoU: Why did the authors compute a homology model using SWISS-MODEL? The authors could have obtained the model via AlphaFold.
- 2) Page 18: Calcium binding sites: Sites (2) and (4) are not defined by residues from both the catalytic core and N-extension. Is this correct? Please confirm.
- 3) Figure 37: Figure 4D: Ca^{2+} sites should be specified (1–4) as in Figure 2A.
- 4) Figure S10: RNA-binding model: Did the authors try to determine the crystal structure of RNA-bound EndoU?
- 5) Structural comparison and amino acid alignments of EndoU and its homologs would help.

Reviewer #3

(Remarks to the Author)

In this manuscript, the authors explore the calcium-dependent activation mechanism of the human ribonuclease EndoU. By employing structural biology, molecular biology, and biophysical techniques, they demonstrate that calcium binding at specific sites induces allosteric changes that activate the catalytic triad essential for RNA cleavage. The study also provides insights into the structural basis of this activation and proposes a model where calcium binding is critical for the proper positioning of the catalytic residues. Although the study presents interesting findings, there are multiple questions that should be addressed first.

1. How does EndoU's specificity for calcium over other divalent cations, such as manganese, relate to its physiological function?
2. The mutagenesis results are critical for supporting the activation model. Could the authors provide more details on the rationale behind selecting specific residues, such as E290A, K343A, and others, for mutagenesis in the validation studies?
3. According to the authors' model, E290, with its long negatively charged side chain, should interact more readily with the catalytic H285 side chain in the active conformation. Could the authors clarify why the charge-conservative mutation E290D led to an increase in the enzymatic reaction rate, as observed in Figures 3A and 3C?
4. While the in vitro assays demonstrate calcium-dependent activity, how do these findings translate to in vivo conditions? Have any in vivo experiments been conducted to confirm the proposed activation mechanism?
5. The proposed model suggests allosteric activation involving specific residues. Have molecular dynamics simulations been employed to validate all the critical residues and proposed allosteric pathways? It appears that only D179A was validated in Figure 3D. The Methods section should clearly describe all the systems used in molecular dynamics simulations.
6. The manuscript identifies multiple calcium-binding sites. Could the authors elucidate the relative importance of each site for enzymatic activation and whether these sites exhibit cooperative binding?
7. The role of water-mediated interactions in the allosteric mechanism is mentioned. Can the authors provide more details on the involvement of water molecules and how their presence or absence influences the enzyme's activity?
8. The MD simulations describe conformational changes within 1 μs (the production of MD simulations was carried out for 1 μs). How does this conformational flexibility relate to enzyme activity, and does calcium binding stabilize specific conformations?
9. The EndoU structure with accession code 9FTW is not available in the PDB database and cannot be visualized now.
10. Given the enzyme's broad RNA substrate specificity, could the authors discuss the factors influencing substrate selection?

and the potential for catalytic promiscuity?

11. The manuscript mentions EndoU's relevance as a biomarker in various cancers. Could the authors expand on the potential clinical applications of these findings, particularly in diagnosing or treating diseases?

Reviewer #4

(Remarks to the Author)

Please note we participated in the Early Career Researchers initiative and this report represents a summary of our joint review.

In this manuscript the authors used structural and biophysical approaches to investigate the calcium-dependent activation of the human EndoU nuclease. This nuclease specifically cleaves RNA in polyU sequences and has been implicated in several cellular processes but the role that calcium plays in activating this nuclease has remained unclear. First the authors investigated mouseEndoU activity in VL3-3M2 cells. Analysis of cytoplasmic RNA from WT and mouseEndoU knock out cells revealed a strong calcium activation of cleavage. Next the authors investigated how calcium influences the structure of hEndoU. Using ¹⁵N NMR the authors were able to show that saturating calcium as well as RNA + sub-saturating calcium restore well-dispersed NMR peaks. These results suggest that binding of calcium and RNA lock the enzyme in a more fixed position. Next the authors solved a crystal structure of hEndoU with excess calcium, revealing four bound calcium ions, and a putative mechanism of allosteric activation leading to a disorder-to-order transition that stabilizes the active site. The authors confirmed the significance of the calcium binding sites by generating a series of mutants and determining the effect on hEndoU cleavage in vitro. Both in vitro assays and molecular dynamics suggest that sites 1 and 3 are responsible for allosteric activation. Finally, the authors used NMR, alphafold modeling, and molecular dynamics to probe the RNA-binding interface in hEndoU. Overall, the authors work reveals an intriguing mechanism of calcium mediated allosteric activation of hEndoU. While the allosteric mechanism is well supported by structures, in vitro assays and molecular dynamics, some additional work is needed to support the modeled RNA binding interface.

1. Figure S2 – Why do the authors not observe endonuclease cleavage products on the gels shown in panel A? Are the products too small to detect on an 8% denaturing gel? How many different cleavage products are expected for the different substrates? Will hEndoU cleave the RNA surrounding every U or only in UU regions?

2. Figure 6S – What type of density map is shown here? The authors must show unbiased experimental density, esp for the calcium ions.

3. It would be very useful for the authors to include a supplemental figure with a comprehensive sequence alignment of different eukaryotic EndoUs, in particular how well conserved are the calcium binding sites, residue E290, putative RNA binding interfaces, etc?

4. Figure 3 – The top cleavage gels should be re-sized and aligned with the mutants to allow readers to better compare WT-hEndoU to the mutants. Please indicate in the figure legend how many replicates were done and what RNA substrate was used for these experiments.

5. Is calcium required for RNA binding? The inclusion of some type of RNA binding experiment (with and without calcium) would add to the proposed model of allosteric activation. From the NMR experiments shown in Fig. S5 it is unclear if RNA can bind in the absence of calcium.

6. How many times were the molecular dynamics simulations run? The simulations should be repeated at least 3 times to ensure consistent results.

7. The authors NMR/modeling/dynamics suggests several putative RNA interfaces around the active site and a basic-beta sheet groove. The authors should confirm the significance of several of these residues through mutagenesis. Are these residues essential for RNA binding and/or cleavage activity? It would also be of interest if the authors could comment on if this work reveals new insight into Uracil specificity. In particular the authors show some base-specific interactions with U2 and U4 in Fig. S11.

8. The authors should cite and briefly discuss the recent work of Huang et al JBC, 2023 and Wang et al NAR, 2024 which suggests that divalent ion activation by Nsp15 is substrate dependent. This is a different mechanism from the allosteric activation model presented in this manuscript.

Minor Issues

1. Introduction, first paragraph – “RNases can be constitutively active (RNase A, angiogenin)”. New work from Korostelev and colleagues (Loveland et al Nature, 2024) suggest that angiogenin is activated by binding to the ribosome.

2. Introduction, second paragraph – “Xendo U cleaves single-stranded RNA preferentially at 5' of uridylates”. Ref 15 does not show cleavage is 5' of uridylates but rather that it requires two U residues.

3. The XendoU versus EndoU nomenclature was a bit hard to follow throughout the text. The authors should consider referring to human endoU as hEndoU (and mouse as mEndoU) throughout the manuscript to make it clear when they are referring to human EndoU and not another EndoU enzyme.
4. Page 16, first paragraph "mutation of two critical residues" – please specify which active site residues were mutated here.
5. Figure 1D – CD-HA Please define this in the figure legend including the residues that were mutated.
6. Figure 1 F. The comparison between different EndoUs is confusing and not to scale. For example, Nsp15 from coronaviruses has two additional domains (N-terminal and Middle domain) with no similarity to hEndoU but the overall length of the protein is almost the same size.
7. Page 16, second paragraph "we expressed the XendoU catalytic domain of human...." – This makes it sound like the construct used does not include the N-terminal extension important for calcium binding. Be specific with residue numbers in the results to make it clear this includes the N-terminal extension.
8. Fig S2B – What substrate was used for these cleavage assays? Please define this in the figure legend.
9. Fig. S3 – Please include the chi-squared value in panel C to indicate how well the experimental and theoretical curves overlap.
10. Fig 2A – It would be helpful to indicate the position of the active site in the top panel.

Reviewer #5

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In their revision, the authors have addressed many of the previous comments. Especially the added biochemical experiments are insightful.

However, some issues remain, were not addressed (e.g. due to experimental difficulties) or were sidelined in the discussion of the previous reviewers' comments. In particular, comparison with previous structures of EndoU homologs are not sufficient, especially structures with substrate (or analogs) were not compared, which might substantiate the proposed active site/RNA binding site placements.

Active site of EndoU and substrate binding region:

The proposed active site of human EndoU and the published active site of *Xenopus* EndoU are in different positions. This is not based - as the authors state - on a misunderstanding but rather the statements of a previous publication (PNAS Renzi et al. 2006) and on structural superposition of the deposited human EndoU structure of the authors (PDB 9ftw) and the published *Xenopus* EndoU structure (PDB 2c1w), which has a phosphate (i.e. substrate analog) bound. This means (as already stated in the first review) *Xenopus* EndoU active site = H162, N270, H272, T278 (see PNAS Renzi et al. 2006, where these residues were suggested as active site and some of them were also mutated to confirm importance for catalysis); while human EndoU = p.8 "catalytic triad (H285, H300, K343)". The only residue that is maintained between these two assignments both by location and by type is H162 (X.I.) vs. H285 (H.s.). This means, either the previous assignment in *Xenopus* EndoU is wrong or the current one is wrong.

The authors so far omit *Xenopus* EndoU-PO₄ from structural superpositions that show active site comparison with human EndoU (Fig. S16). They also omit structural comparisons of substrate/analog bound EndoU homologs (e.g. Nsp15 with dsRNA PDB 7tj2, *Xenopus* EndoU with phosphate PDB 2c1w, RNaseA with uridine vanadate PDB 6rsa). These comparisons should be included. They would be useful and could support their own data especially since their manuscript relies on modelling and broadly localized NMR peak shifts for identification of substrate binding sites.

Disorder to order transition:

Counter to what is currently mentioned in the rebuttal letter and text, disorder to order transitions do not mean that a protein region needs to take up an alpha-helical/beta-strand secondary structure. It rather means that it goes from high flexibility (i.e. many transitions between different conformational states) to a more stably populated conformational state. This can also happen in an ordered loop, a long peptide stretch without secondary structure elements or a region that goes from flexible random coil to secondary structure element.

The text in the beginning of p.9 is misleading and should be amended: "In apo-hEndoU, the loop containing E290 does not have a defined secondary structure and is likely flexible in solution. Thus, calcium binding correlates

with a disorder-to-order transition in this loop, forming a β -hairpin and stabilizing the catalytic site (Fig. 2 D)." The corresponding E290 residue (X.I. E167) in *Xenopus* EndoU is part of a well structured loop and forms multiple contacts with other residues, it is neither disordered nor flexible. Furthermore, based on which experimental evidence do the authors claim that it is "likely flexible in solution", especially if - as they state in their rebuttal letter - "Specifically, for residues E290 and H285, the corresponding crosspeaks are located in the central crowded region of the 1H-15N spectrum of calcium-bound EndoU, and we could not identify corresponding crosspeaks in the spectrum of the apo-state due to the limitations mentioned above."?

Figures:

Fig. 2C: orphan white box on blue helix in bottom left

Reviewer #2

(Remarks to the Author)

This reviewer greatly appreciates the revisions made by the authors. In particular, this reviewer agrees with the authors' assertion that "the activation mechanism of EndoU is calcium-dependent and does not involve RNA." However, as noted by this reviewer (and Reviewer #1) during the initial review process, the following critical aspect still requires further clarification.

The authors state that EndoU is specifically activated by calcium, but the molecular mechanism explaining why ONLY calcium triggers activation, while other metal ions do not, has not been demonstrated. This key point is not addressed explicitly in the manuscript at all..

The explanation provided in response to Reviewer #1 regarding the observed electron density in the structural analysis being attributed to calcium is satisfactory. However, this explanation does not shed light on the molecular mechanism underlying EndoU activation by calcium. Although the current manuscript title, "Molecular Basis for the Calcium-Dependent Activation of the Ribonuclease EndoU," implies that the molecular mechanism of calcium-specific activation has been elucidated, the reviewer feels that the manuscript does not provide sufficient molecular evidence to support this claim. Finally, while the discussion of intracellular calcium concentrations, provided in response to Reviewer #3, is appreciated, this reviewer believes that these details do not offer molecular evidence explaining the specificity of EndoU activation by calcium.

Reviewer #3

(Remarks to the Author)

The authors have addressed my initial comments and satisfactorily resolved my concerns. I recommend the revised manuscript for publication.

Reviewer #4

(Remarks to the Author)

Please note we participated in the Early Career Researchers initiative and this report represents a summary of our joint review.

The authors have adequately addressed our previous concerns and those of the other reviewers. We appreciate the authors careful and thorough responses in the rebuttal. The additional assays including RNA binding experiments and generating a series of RNA binding mutants help strengthen the proposed model of calcium dependent activation. The additional sequence and structural comparisons also further highlight how this mechanism is distinct from other EndoU enzymes such as Nsp15.

Minor Note – There appears to be an issue with black boxes in Fig. 5A but this is probably a file conversion error.

Reviewer #5

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have sufficiently addressed my comments and I congratulate them on their insightful study.

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Here is the response to reviewers. Reviewers' comments, authors' responses and additional text of the manuscript are presented in black, blue and green, respectively.

Reviewer #1 (Remarks to the Author):

In this paper Malard and colleagues describe a mechanism of enzyme activation for the human ribonuclease EndoU. They suggest that Ca^{2+} ions allosterically activate the enzyme by inducing conformational changes in the active site of the enzyme that result in an active arrangement of the catalytic triad.

The data is interesting for a wider readership which is concerned with enzyme mechanisms and RNase regulation. The paper is well written and suggests a tempting mechanism for EndoU activation. Yet, given that it is in part based on models and conjecture, and in part contradicts previous literature on *Xenopus* EndoU active site assignments, a few important controls and comparisons are missing that would reveal the relevance of the proposed models. Also some basic information is missing that would allow judgment of the evolutionary conservation of the proposed mechanism. In addition, illustrations of the structures should be improved to make it easier for readers to grasp which residues are moving upon Ca^{2+} binding and how this relates to the active site location.

We thank reviewer #1 for the careful review of our manuscript. The overall feedback effectively captures the essence of our work. Although we will discuss this point further below, we must clarify at this point that the remark "and in part contradicts previous literature on *Xenopus* EndoU active site assignments" likely stems from a misunderstanding by reviewer #1. Our data are, in fact, highly consistent with the hypothesized active site for *Xenopus* EndoU. Overall, we clearly state in our responses below that we have considered and implemented most of the remarks made by reviewer #1. Accordingly, we are grateful to reviewer #1 for helping us to strengthen our claims and enhance the overall quality of the paper.

One major drawback is that the suggested conformational changes of the human enzyme are based on comparison to a homology model of the apo-structure from *Xenopus* EndoU. NMR data in presence and absence of Ca^{2+} is collected as well but here the residues that become ordered or change position are not correlated to the suggested conformational changes based on the structural models. Similarly, comparisons to active site conformations of related enzymes (e.g. RNase A, Nsp15) or sequence alignments with paralogs are currently missing, but should be made to reveal whether this model is likely to reflect a true and potentially conserved scenario. Finally, activation by Ca^{2+} could result from an increase in RNA affinity or an increase in the active population of the enzyme, yet this is not disentangled.

We have taken note of the summary regarding the major drawbacks mentioned by reviewer #1, which will be discussed in detail in the following paragraphs of this review document. To echo this summary, we have justified the relevance of using a homology model for apo-EndoU in the context of our work, especially in comparison with AlphaFold models. We have also explained why we could not use NMR spectroscopy to compare the apo- and calcium-bound states of EndoU at the residue level, due to the particular dynamics of apo-EndoU that lead to signal

extinction and impair the recording of 3D data of appropriate quality for backbone assignment. Additionally, we have responded in depth to the remark related to the impact of calcium on RNA affinity or enzyme activation, stating that both phenomena are downstream events following calcium binding. Finally, we have implemented structural comparisons for active site conformations of related enzymes, and sequence alignments, to support the conservation of EndoU activation by calcium, as suggested by reviewer #1 (Fig. 5B, Fig. S8, Fig. S16).

Detailed comments - Major points:

Identification of Ca^{2+} pockets - Ca^{2+} ions especially when not fully occupied in the crystal lattice are not easily distinguishable from other similar atoms (H_2O or other metal ions) in the electron density map. Without experimental verification, the nature of these atoms stands in question.

We thank reviewer #1 for this highly relevant comment, which we had already considered prior to analyzing the electron density map of calcium-activated EndoU. As reviewer #1 mentioned, Ca^{2+} ions are not easily distinguishable from other similar atoms, especially when they are not fully occupied in the crystal lattice. In our case, each Ca^{2+} ion (five per monomer) was refined with an occupancy of 100%. Then, we expect a coordination number of seven for Ca^{2+} ions (hepta-coordinated). We demonstrated that in the structure of calcium-activated EndoU, each Ca^{2+} ion was indeed coordinated by seven oxygen atoms, originating either from protein residues or water molecules. For Na^+ and Cl^- ions, hexa-coordination would have been expected, while water molecules exhibit a maximum of divalent coordination. Furthermore, we verified that all coordinated Ca^{2+} ions have a bond-length distance ranging from 2.16 Å to 2.61 Å, consistent with expected values. Ultimately, as suggested by reviewer #1, the bond-valence method is applicable at a resolution of 1.7 Å for Ca^{2+} ions (Müller et al., Acta D, 2003; see below).

Did the authors perform any anomalous diffraction experiments to confirm the presence of the suggested metal ions in the four pockets? Even though the Ca K-edge is outside of reach of their synchrotron, they could still perform a S-SAD like experiment as close as possible to the K-edge and detect the weaker anomalous scattering of these metal ions in a highly redundant data collection.

We agree with reviewer #1 that performing anomalous diffraction experiments could have provided additional evidence to confirm the presence of Ca^{2+} . Following the suggestion, we proceeded with new crystallization assays in an attempt to perform an SAD experiment. Unfortunately, the crystals we produced were not of comparable quality to the original assays, resulting in poor diffraction data. Therefore, we have not yet been able to provide this additional element to confirm the presence of calcium in the five pockets.

Do the atom distances in the coordination sphere match literature values for calcium coordination (e.g. see Müller, Kopke, Sheldrick 2003 <https://doi.org/10.1107/S0907444902018000>)?

We agree with Reviewer #1 that the atom distances in the coordination sphere of calcium must match literature values in order to provide a strong element confirming the nature of the bound ions. Therefore, we have carefully checked all distances for each Ca²⁺ atom and found that the distances range from 2.16 Å to 2.61 Å, consistent with expected, published data. To further confirm the nature of the bound ions, we used the CheckMyMetal server (<https://cmm.minorlab.org/>) on the PDB code 9FTW. We show the summary table below:

Nr	ID	Res.	Metal	Occupancy	Bfactor(env.) ¹	Atomic contacts	Valence ²	nVECSUM ³	Geometry ^{1,4}	gRMSD(°) ¹	Vacancy ¹	Bidentate
1	A:502	CA	Ca	1.0	20.0 (20.8)	O ₇	2.1	0.05	Octahedral	10.1°	16%	1.0
2	A:503	CA	Ca	1.0	17.4 (22.1)	O ₇	2.2	0.1	Octahedral	10.4°	16%	1.0
3	A:504	CA	Ca	1.0	20.0 (22.1)	O ₇	2.0	0.041	Octahedral	8.9°	16%	1.0
4	A:505	CA	Ca	1.0	24.2 (27.6)	O ₆	1.4	0.38	Octahedral	17.8°	50%	1.0
5	A:506	CA	Ca	1.0	16.7 (19.8)	O ₇	2.1	0.018	Octahedral	11.8°	16%	1.0
6	B:501	CA	Ca	1.0	20.5 (22.0)	O ₇	2.0	0.03	Octahedral	8.3°	16%	1.0
7	B:502	CA	Ca	1.0	23.3 (25.8)	O ₇	2.4	0.13	Octahedral	10.9°	16%	1.0
8	B:503	CA	Ca	1.0	20.4 (22.5)	O ₇	2.1	0.049	Octahedral	13.7°	16%	1.0
9	B:504	CA	Ca	1.0	24.2 (25.0)	O ₇	2.2	0.12	Octahedral	11.0°	16%	1.0
10	B:505	CA	Ca	1.0	23.1 (32.1)	O ₆	1.7	0.33	Octahedral	10.2°	33%	1.0

The analysis indicates that all Ca²⁺ binding sites are geometrically compatible with calcium coordination. For 8 out of 10 sites, all test outcomes were deemed "acceptable" or "borderline," collectively suggesting that the calcium assignment at these sites aligns well with expectations. For the remaining 2 out of 10 sites, "dubious" ratings were assigned to two test outcomes. However, these particular calcium binding sites are involved in crystal packing, and the 9FTW PDB only includes the unit cell. This limitation explains why the quality of calcium assignment assessed by CheckMyMetal yields ambiguous results for these sites. Altogether, we think that this analysis confirms our interpretation of the electron density map.

For reference, CheckMyMetal is based on the following publications:

- (1) CheckMyMetal: a macromolecular metal-binding validation tool. Zheng,H., Cooper,D.R., Porebski,P.J., Shabalin,I.G., Handing,K.B., Minor,W. (2017) Acta crystallographica. Section D, Structural biology, 73, 223-233.
- (2) Zheng H, Chordia MD, Cooper DR, Chruszcz M, Müller P, Sheldrick GM, Minor W (2014) Nature Protocols, 9(1), 156-70.
- (3) Brown ID (2009) Chem. Rev., 109, 6858-6919.
- (4) Müller P, Köpke S, Sheldrick GM (2003) Acta Crystallogr. D Biol. Crystallogr., 59, 32-37.
- (5) Kuppuraj G, Dudev M, Lim C (2009) J. Phys. Chem. B, 113, 2952-2960.
- (6) CSD: Cambridge Structural Database

We added the following sentences in the methods section, and structure determination subsection:

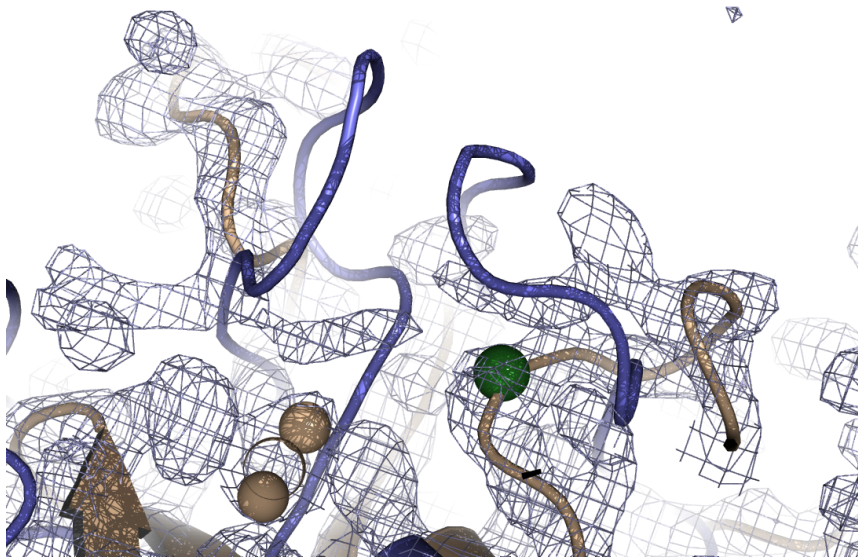
“Each calcium ion was refined with an occupancy of 100 % and exhibited a coordination number of seven. We used the bond-valence method to verify the nature of the divalent calcium ions, which is suitable given the 1.7 Å resolution of the structure. All coordinated calcium ions showed

bond-length distances ranging from 2.16 Å to 2.61 Å, consistent with expected values (Müller et al. Acta Crystallogr. D Biol. Crystallogr., 2003). Furthermore, we used the CheckMyMetal webserver to confirm that the geometry of ion coordination corresponded to the expected geometry for calcium coordination (Zheng et al. Acta Crystallogr. Sect. D, 2017)."

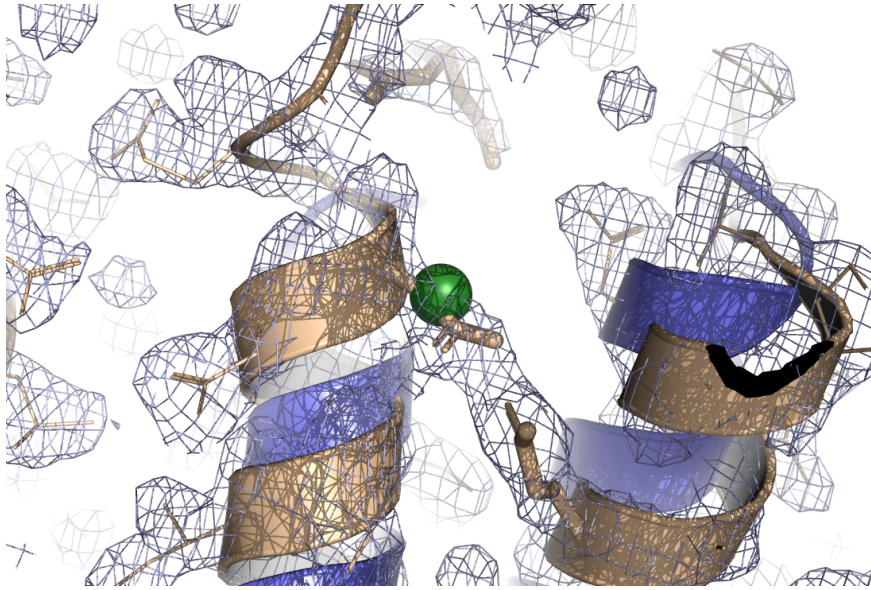
If these pockets are conserved in the *Xenopus* EndoU structure, what does the electron density map look like in these locations in the corresponding map?

Inspection of the different Ca²⁺ binding sites from the XEndoU structure is described below. Superimposed models of XEndoU and hEndoU are shown in addition to a 2Fo-Fc map of XEndoU contoured at 1 sigma. For clarity, no electron density is shown for hEndoU.

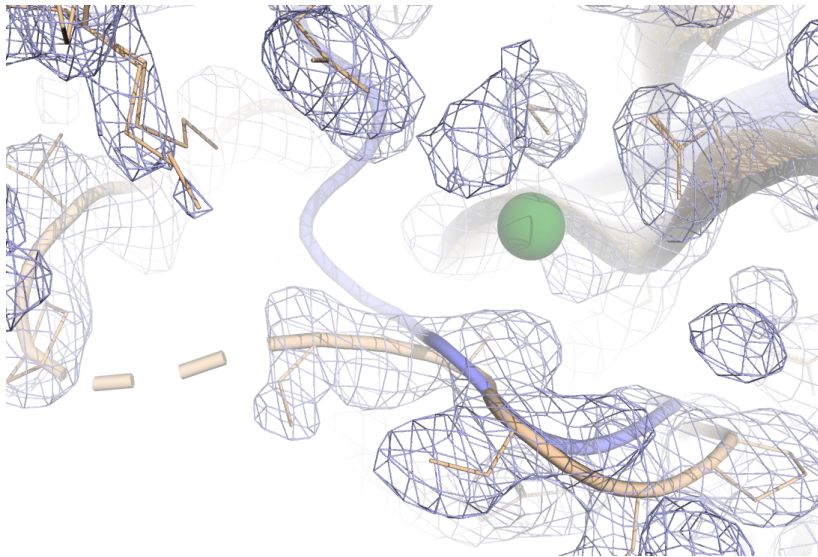
Site 1: Loops are in different orientation in the apo XEndoU (in brown) in comparison to hEndoU (in blue). Calcium is green.



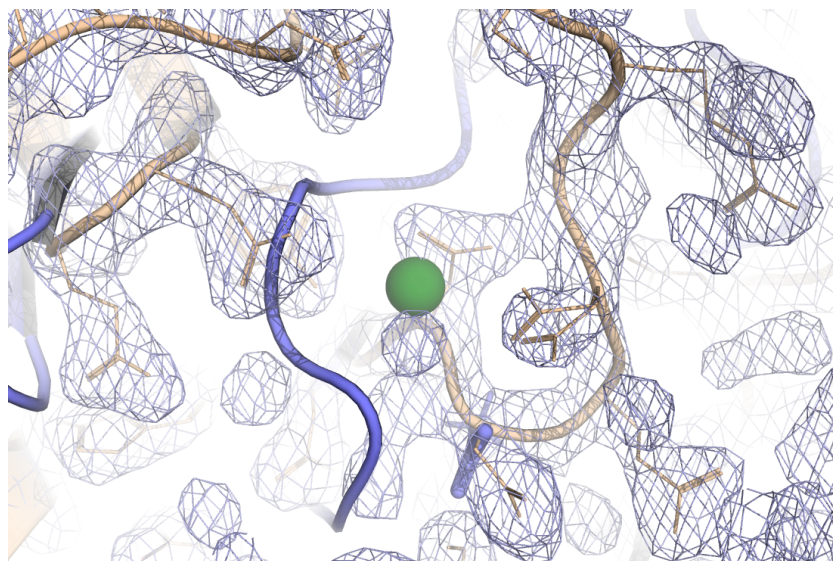
Site 2: Overall similar but Ca²⁺ (in green) position in hEndoU (in blue) is buried by N83 & E101 side chains making H-bonds in XEndoU (in brown).



Site 3: Only partially visible loop in XEndoU (in brown).



Site 4: Loops are visible in XEndoU (in brown) but at a different position in hEndoU (in blue).



Did the pocket residues become ordered or changed chemical shifts upon Ca^{2+} addition in NMR experiments?

As mentioned in the original manuscript, the ^1H - ^{15}N spectrum of apo-EndoU exhibited intermediate exchange on the NMR timescale, resulting in signal broadening and the loss of a significant fraction of expected crosspeaks. Moreover, the specific dynamics of apo-EndoU impaired our ability to record 3D NMR data of sufficient quality for backbone assignment of the apo protein. Furthermore, the calcium-bound form of EndoU shifts to the slow exchange regime upon calcium titration, which prevents tracking or extrapolating the positions of crosspeaks related to the calcium-binding pocket in the apo state of EndoU. Therefore, we are unable to use NMR to precisely monitor changes in chemical shifts and dynamics at the residue level upon calcium binding to EndoU. However, we can reasonably hypothesize that calcium-binding residues, particularly those located in flexible loops, become more ordered in the presence of calcium due to the stability required to maintain coordination networks with calcium ions. For instance, calcium is coordinated by the backbone oxygen of Q210 at site (1). In the ^1H - ^{15}N spectrum of calcium-activated hEndoU, the corresponding crosspeaks appeared at ~ 10.4 ppm (^1H) and ~ 123.5 ppm (^{15}N) (see Fig. S12). In apo-hEndoU, we could not detect any signal in this area (see Fig. S3B), and Q210 likely becomes ordered upon calcium binding, hence the appearance of a crosspeak in a region corresponding to more ordered residues.

To make explicit that we could not assign the backbone of apo-hEndoU, we added a mention in the result section, structural basis for hEndoU activation by calcium ions subsection:

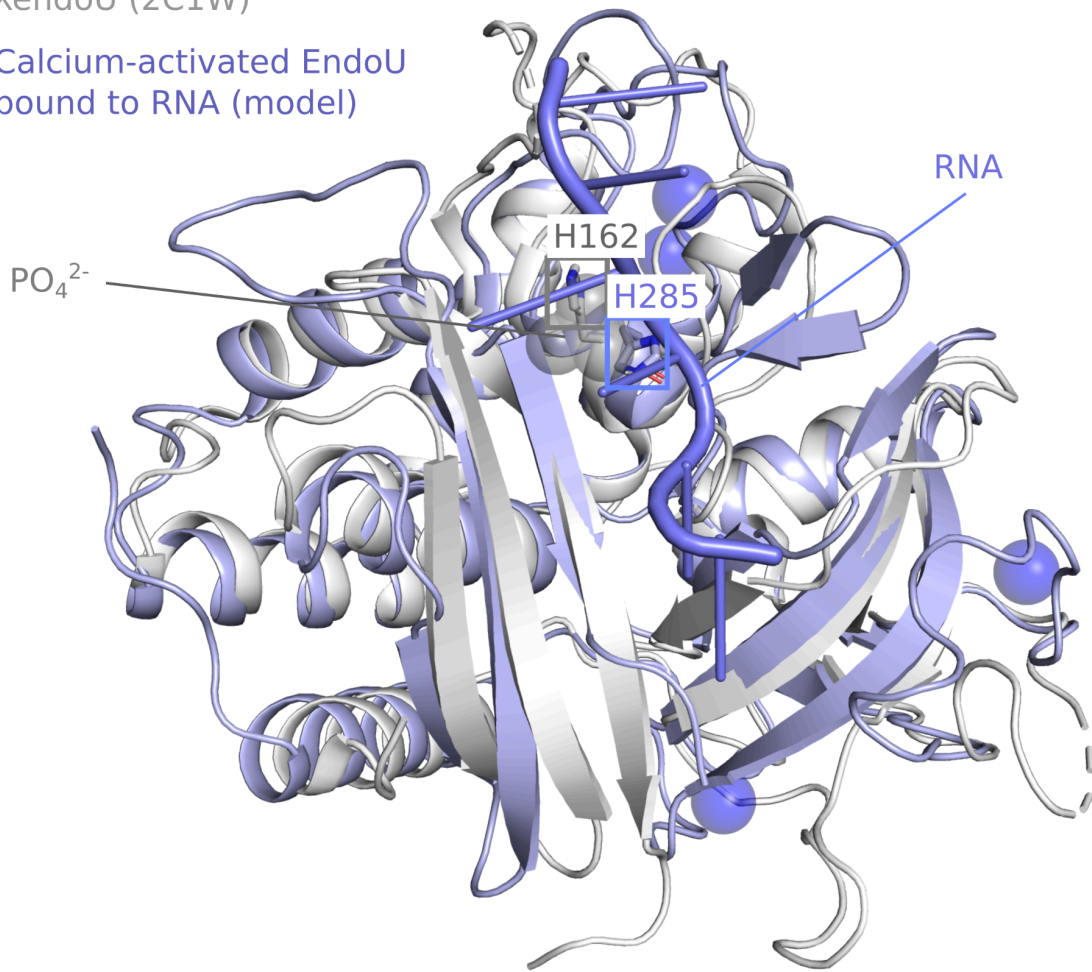
“In the ^{15}N SOFAST-HMQC spectrum of apo-hEndoU, we observed well-dispersed, but broader and fewer signals than expected, suggesting conformational exchange in the μs -ms range (Fig. S3 B). Accordingly, we could not record suitable triple-resonance NMR experiments to reliably assign the backbone of apo-hEndoU.”

Conformational changes upon Ca^{2+} binding - No apo-structure of human EndoU was obtained and putative conformational changes are deduced from a comparison of homology-modelled human apo-EndoU based on apo-Xenopus EndoU and calcium-bound human EndoU. This may be a good first hypothesis but needs further comparisons and experiments for verification. Especially since the XendoU active site was suggested to be in a completely different position based on a phosphate ion that was bound in the original crystal structure (Xenopus H162, N270, H272, T278). Further highlighting the problematic reliance on homology models: AlphaFold2 does actually predict human EndoU to be in the same "active" conformation even in the absence of Ca^{2+} ions, so no conformational change would be predicted if this model were chosen as a reference point.

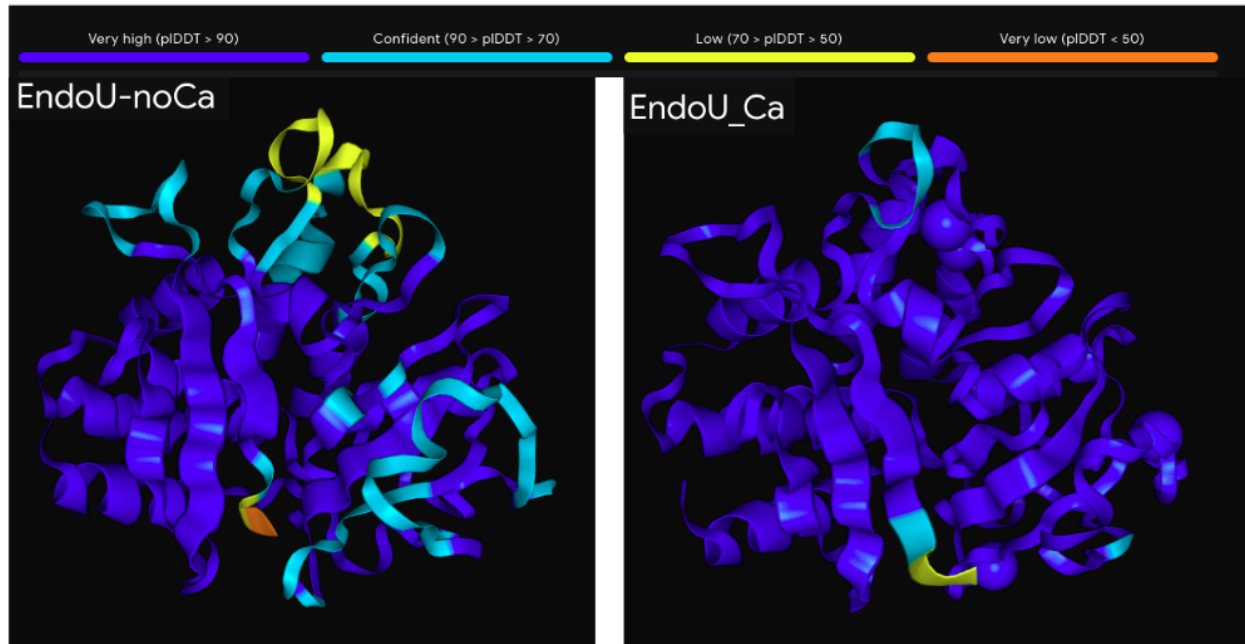
We thank reviewer #1 for the insightful comment. However, the statement "since the XendoU active site was suggested to be in a completely different position" made by reviewer #1 is not correct. This error likely originates from differences in protein lengths and residue numbering between Xenopus EndoU (292 amino acids long) and human EndoU (410 amino acids long). For example, the XendoU residue H162, mentioned by reviewer #1, corresponds to residue H275 in human EndoU, which is a catalytic histidine in both cases. Consequently, the active site we identified in our work is located in the same position in both human and Xenopus EndoU. To avoid this confusion and to increase the clarity of our manuscript, we now included a sequence alignment (Fig. S8). Below, we also present a structural alignment between apo-XendoU (PDB ID: 2C1W) and one model representative of the ensemble for calcium-activated, RNA-bound EndoU, corresponding to Fig. 4D in the revised manuscript. This alignment unambiguously shows that our findings are consistent with previous XendoU studies.

XendoU (2C1W)

Calcium-activated EndoU
bound to RNA (model)

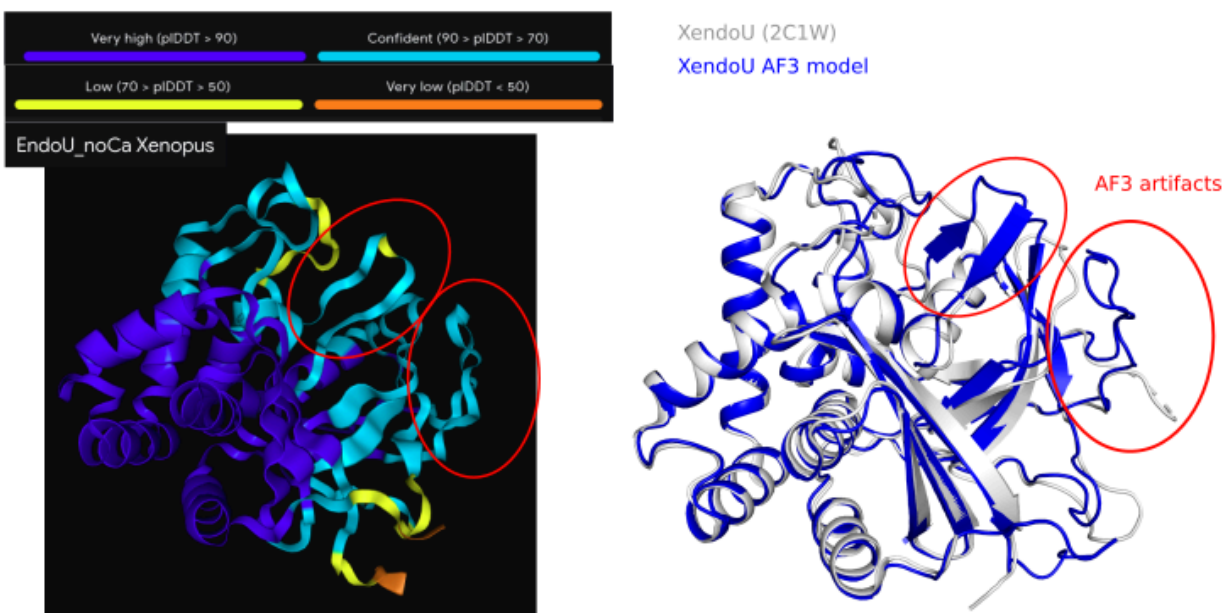


Then, reviewer #1 stated “AlphaFold2 does actually predict human EndoU to be in the same “active” conformation even in the absence of Ca²⁺ ions, so no conformational change would be predicted if this model were chosen as a reference point.” Below, we present the AlphaFold3 models of apo-EndoU and calcium-activated EndoU:



As reviewer #1 mentioned, the EndoU-noCa (apo) model reproduces the crystal structure of calcium-activated EndoU, which we present in our work, extremely well. This might suggest that the conformational change we observed in comparison with a homology model might be biased by the homology model itself. Moreover, computing a model of calcium-activated EndoU with AlphaFold3 yields a structure seemingly identical to the apo condition (RMSD = 0.2 Å). Nevertheless, we still observe a major difference between the two AlphaFold3 models, specifically in the confidence scores in the loop regions, including those we experimentally identified as being involved in conformational changes upon calcium binding. As reviewer #1 knows, AlphaFold models do not directly sample intramolecular dynamics when predicting a protein model. Instead, empirically, AlphaFold models intramolecular dynamics through confidence scores. This can be easily observed by computing a model of an Intrinsically Disordered Protein (IDP), which outputs a structure with very low confidence. Considering the side-by-side comparison above, we can then observe that confidence scores are strikingly different between the apo and calcium-bound conditions for human EndoU. This demonstrates that AlphaFold detects a clear difference between the two conditions, which can be interpreted as a significant reduction in protein dynamics upon calcium binding. Finally, choosing the AlphaFold model of apo-EndoU as a reference, rather than a homology model, was not the preferred option, precisely because this AlphaFold model shows lower confidence in critical regions. In this context, there is no rationale for preferring a model that does not directly rely on an experimental basis, as opposed to homology models.

To further discuss the biases associated with considering AlphaFold models, we present below a comparison between the crystal structure of *Xenopus* EndoU (2C1W) and its corresponding AlphaFold3 model. Of note, we used the crystal structure of *Xenopus* EndoU (2C1W) to build the homology model of human EndoU.



Herein, it can be observed that some of the regions with lower confidence in the AF3 model of XendoU represent artifacts when compared to the corresponding crystal structure. Specifically, the beta-hairpin circled in red is a feature we identified upon calcium binding, which is part of the conformational change we describe. However, this beta-hairpin is present in the AF3 model of apo-XendoU, while it is absent in the corresponding crystal structure. This clearly demonstrates that we should rely on AF models with caution, and that these models should not be considered in the absence of the highest level of confidence, a threshold not reached by modeling either Xenopus or human EndoUs in their apo state.

Of note, these observations are also valid when comparing the crystal structure of XendoU (2C1W) to the AlphaFold2 model (<https://alphafold.ebi.ac.uk/entry/Q8JFY9>).

In summary, we have addressed the comments of reviewer #1 by clarifying that the active site we identified in human EndoU is identical to that of Xenopus EndoU. Additionally, we provided a rebuttal concerning the use of AlphaFold models in the specific context of modeling apo-EndoUs. According to reviewer #1 comments, we believe these arguments are adequate verifications, validating the use of a homology model for human apo-EndoU based on the experimental crystal structure of Xenopus EndoU. It is important to note that in the original manuscript, we reported that theoretical SAXS data were computed from this homology model, and they show significant alignment with experimental SAXS data recorded on human apo-EndoU. We also specify that crystallization attempts of human apo-EndoU were unsuccessful, likely due to protein dynamics as highlighted earlier. In conclusion, we have no evidence suggesting that using this homology model is not the best choice in the specific context of this study. The conformational change we identified upon calcium binding is otherwise highly consistent with NMR spectroscopy data, which strengthens even more the rationale to use this homology model as reference.

A more thorough comparison with available structures of homologous enzymes such as Nsp15, RNase A or others should be provided. Are the catalytic triad residues always in the same conformation? For RNase A, nucleotide-bound structures exist, which would allow comparison of the active spatial arrangement of the catalytic triad. Furthermore, it should be compared how well the proposed Ca²⁺ pockets are conserved between EndoU paralogs at sequence level and this sequence alignment should be part of the manuscript. If it is claimed (as currently in the discussion) that we can learn from the study about viral Nsp15 inhibition then Nsp15 should be included in the alignment.

We agree that sequence alignment and structure comparison providing a more thorough analysis with homologous enzymes should be provided, and we implemented the changes accordingly (Fig. S8, Fig. 5B, Fig. S16). Regarding the orientation of the catalytic triad residues, we aligned the catalytic triad of RNase A, bacterial EndoU-like toxin, Nsp15, and calcium-activated hEndoU (Fig. S16). We observed that H12 and K41 in RNase A align well with the corresponding residues in EndoU-related proteins. However, we observed that H119 in RNase A does not align well with the corresponding residue in EndoU-related proteins. Spatially, H187 from bacterial EndoU-like toxin is the closest to H119 in RNase A, while H285 (hEndoU) and H234 (Nsp15) are more distant. These differences might be consistent with the ancient evolutionary connection that has been reported between RNase A and members of the EndoU-like family. The precise comparison between the nucleotide-bound catalytic site of RNase A and the catalytic site of calcium-activated hEndoU bound to RNA would be appealing, but the latter structure does not exist and we consider that our AI/MD-based model is not appropriate to draw reliable conclusions at such high resolution. Hence, we limit catalytic site comparison to what we already described (Fig. S16). Nevertheless, and according to the reviewer comment, we also clarified how well the calcium binding pockets are conserved between EndoU paralogs at sequence level, including the viral Nsp15 in the alignment (Fig. S8).

In reference to Fig. S8, S16, we added this mention in the discussion section of the manuscript, first paragraph:

“The endonucleolytic activity of RNase A and EndoU-like domains relies on a conserved catalytic triad composed of two histidines and one lysine (Fig. S8). The catalytic residues H12 and K41 in RNase A align well with corresponding residues in EndoU-related proteins, whereas residue H119 is spatially shifted (Fig. S16). The closest spatial equivalent to H119 in RNase A is H187 in a bacterial EndoU-like toxin, while H285 in hEndoU and H234 in Nsp15 are more distantly located. This spatial rearrangement likely reflects evolutionary divergence while preserving a metal-independent catalytic mechanism.”

In reference to Fig. S8, we added this mention in the results section, experimental validation of hEndoU activation model subsection, end of first paragraph:

“Moreover, the side chains of residues E217 and E284 directly contribute to calcium coordination at site (1), as do D179 and D315 at site (3) (Fig. S8). All show identity

conservation, with the exception of D315, which can present a semi-conservative substitution to asparagine across eukaryotic EndoUs (Fig. S8)."

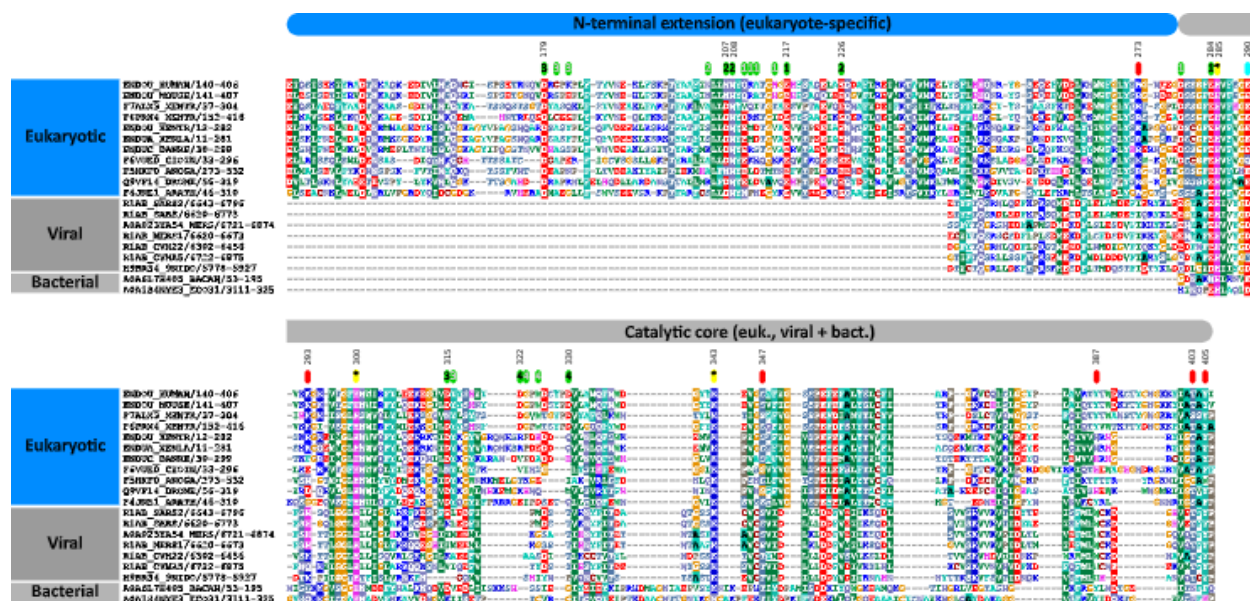
In reference to Fig. S8, we added this mention in the discussion section, second paragraph:

"Indeed, we identified calcium binding sites (1) and (3) as critical for hEndoU activation, and they are highly conserved in eukaryotic EndoUs, supporting a shared activation mechanism (Fig. S8). In addition, we have shown that calcium-coordinating residues at these sites originate from both the conserved catalytic core and the eukaryote-specific N-terminal extension. Although not essential for activation, site (2) is also broadly conserved, suggesting a putative function awaiting characterization. In contrast, site (4) is poorly conserved, consistent with its nonessential role for activation."

In reference to Nsp15, Fig. S8, and Fig. 5B, discussion section, fourth paragraph:

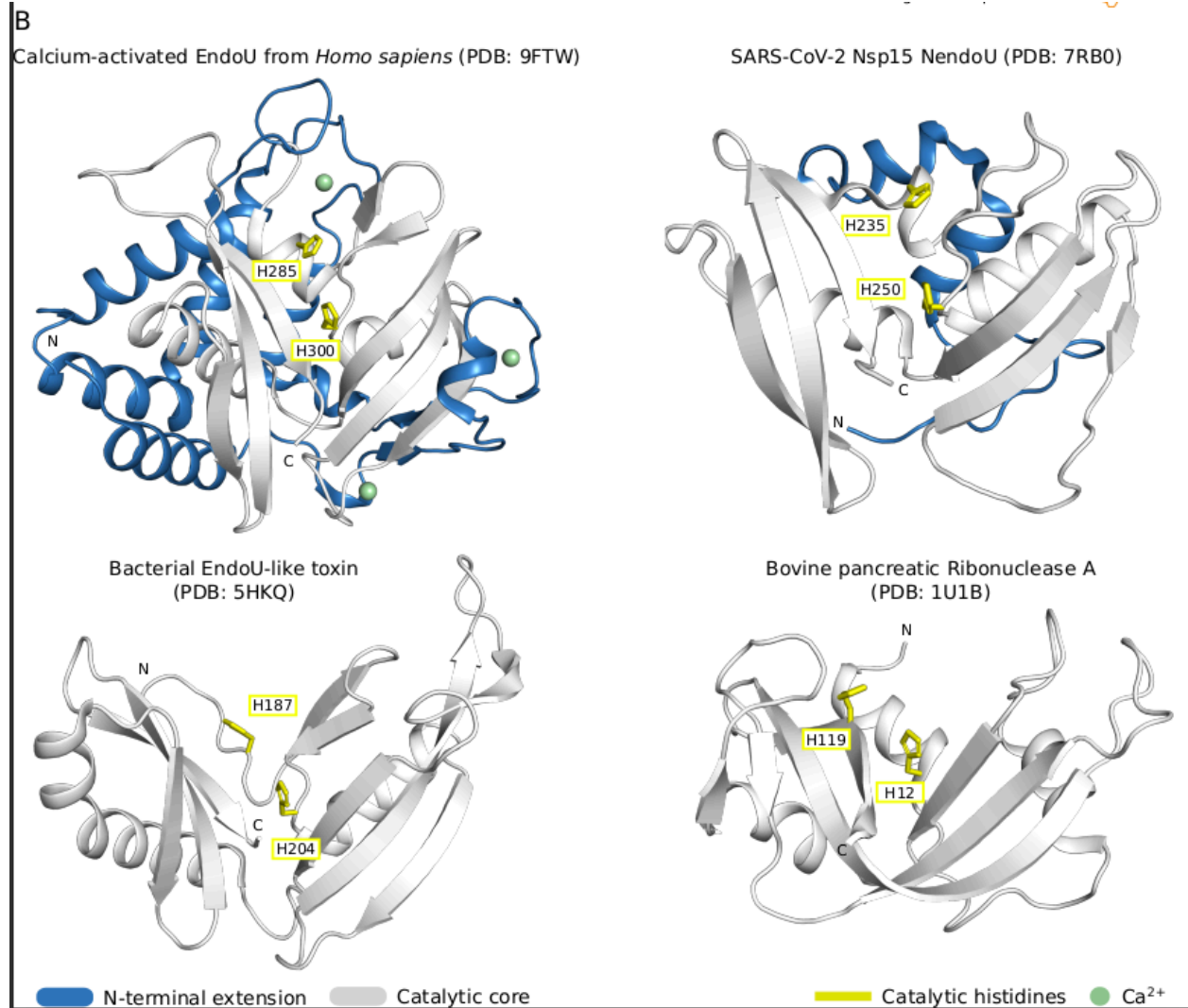
"However, the conserved metal-coordination sites found in eukaryotic EndoUs are absent in Nsp15 and related viral homologs (Fig. 5B, S8), suggesting that manganese might enhance enzymatic activity by promoting Nsp15-RNA interaction, mirroring the observed effect of calcium in promoting hEndoU binding to RNA. Consistently, recent in vitro studies demonstrated that Nsp15 retains some activity on single-stranded RNA without divalent metal ions, although manganese increases this activity in a substrate-dependent manner (Huang et al. JBC, 2023; Wang et al. NAR, 2024). Other reports also found enhanced RNA binding by Nsp15 in the presence of manganese (Bhardwaj et al. J. Mol. Biol. 2006) and suggest that manganese may provide charge shielding to promote Nsp15 interactions with the RNA (Frazier et al. NAR, 2021), although it has also been proposed that manganese stabilizes an alternative enzymatic state with a higher cleavage rate (Huang et al. JBC, 2023). Insights from Nsp15 studies also highlight the significance of tipiracil, a uridine analog that binds the Nsp15 uridine binding site and inhibits its activity (Kim et al. Communications Biology, 2021). Determining whether tipiracil can similarly bind and inhibit eukaryotic EndoUs would provide a valuable tool for probing their cellular and extracellular functions."

Fig. S8 (see high resolution figure in the manuscript):



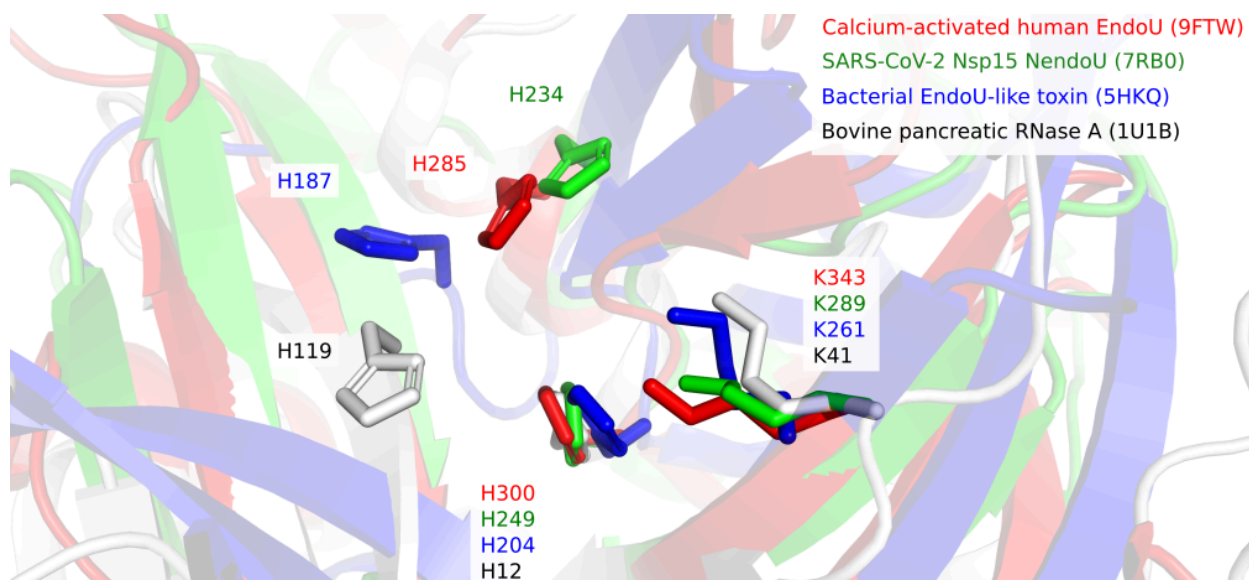
A structure-based sequence alignment of eukaryotic, viral and bacterial EndoU homologs. The human EndoU structure from this study, together with the following existing structures of eukaryotic, viral, and bacterial EndoU homologs (2c1wC, 4s1tF, 2gthA, 5yvdB, 2gtiA, 2ozkD, 6vwvA, 5hkqA), were structurally aligned (all-against-all) using the DALI server. The eukaryote-specific N-terminal extensions of the EndoU catalytic domain were aligned separately from the shorter viral N-terminal extensions. The resulting sequence alignment was manually adjusted and annotated to highlight calcium-coordinating residues (green), catalytic residues (yellow, asterisk), and selected RNA-binding residues (red). Residue E290 in hEndoU, which mediates communication between calcium-binding site (1) and the catalytic residues, is also highlighted (cyan). For the calcium-coordinating residues, the numbers 1–4 refer to the calcium-binding site numbering used in this study. Residues that coordinate calcium via their side chains (black numbers) or via their backbone (grey numbers) are distinctly highlighted. Residue numbers refer to the hEndoU sequence.

Fig. 5B:



(B) Structural comparison between calcium-activated human EndoU, SARS-CoV-2 Nsp15 NendoU, bacterial EndoU-like toxin, and bovine pancreatic ribonuclease A.

Fig. S16:



Structural alignment of catalytic residues for human EndoU and homologs. The catalytic His-His-Lys triad of calcium-activated human EndoU (9FTW, red), SARS-CoV-2 Nsp15 NendoU (7RB0, green), and bacterial EndoU-like toxin (5HKQ, blue) were aligned on the corresponding His-His-Lys catalytic triad from bovine pancreatic ribonuclease A (1U1B, white).

The NMR spectra +/- Ca^{2+} should be analyzed and correlated to the proposed conformational changes. Are E290 and H285 shifting or becoming ordered upon Ca^{2+} addition? How about the loop that E290 is part of?

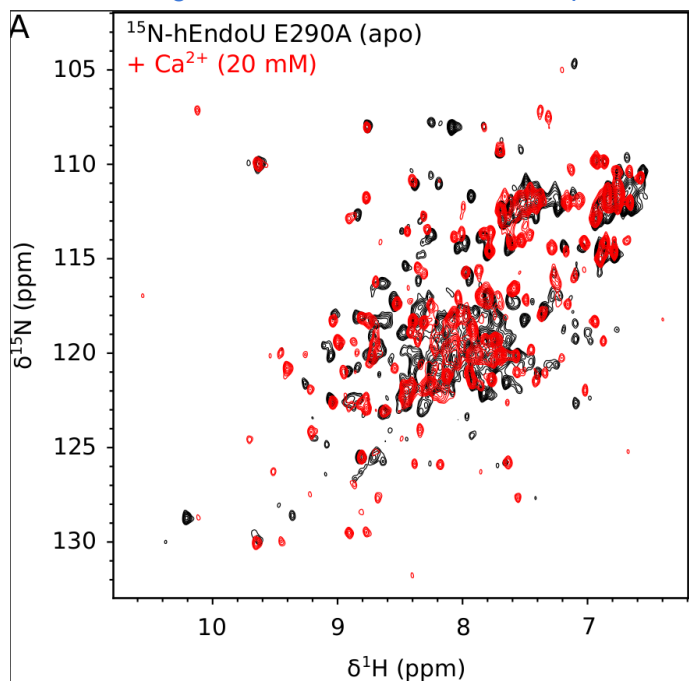
As mentioned in the original manuscript, the ^1H - ^{15}N spectrum of apo-EndoU exhibits significant intermediate exchange on the NMR timescale, resulting in signal broadening and the loss of a substantial fraction of the expected crosspeaks. We attempted to record 3D backbone spectra on apo-EndoU, but the data quality was insufficient to perform even a partial backbone assignment of apo-EndoU with acceptable confidence. On the other hand, the subset of crosspeaks that recovers during EndoU titration by calcium appears in the slow exchange regime, which does not allow tracking or extrapolating the positions of crosspeaks in the ^1H - ^{15}N spectrum of apo-EndoU. Specifically, for residues E290 and H285, the corresponding crosspeaks are located in the central crowded region of the ^1H - ^{15}N spectrum of calcium-bound EndoU, and we could not identify corresponding crosspeaks in the spectrum of the apo-state due to the limitations mentioned above. Although of significant interest, the suggestion raised by reviewer #1 cannot be implemented, due to the particular dynamics of apo-EndoU, which strongly impair the ability to record appropriate NMR data for the backbone assignment of the apo-protein.

Mutations of some pocket residues were done but only two pockets seem to be important for nuclease activity, and only pocket 1 relevance was explained. Here, the E290D and E290A mutation was tested, but actually a better mutation to test the model for transmission of Ca^{2+}

binding to the active site conformational change would be an E290Q mutation, because it would potentially uncouple Ca^{2+} binding from activation.

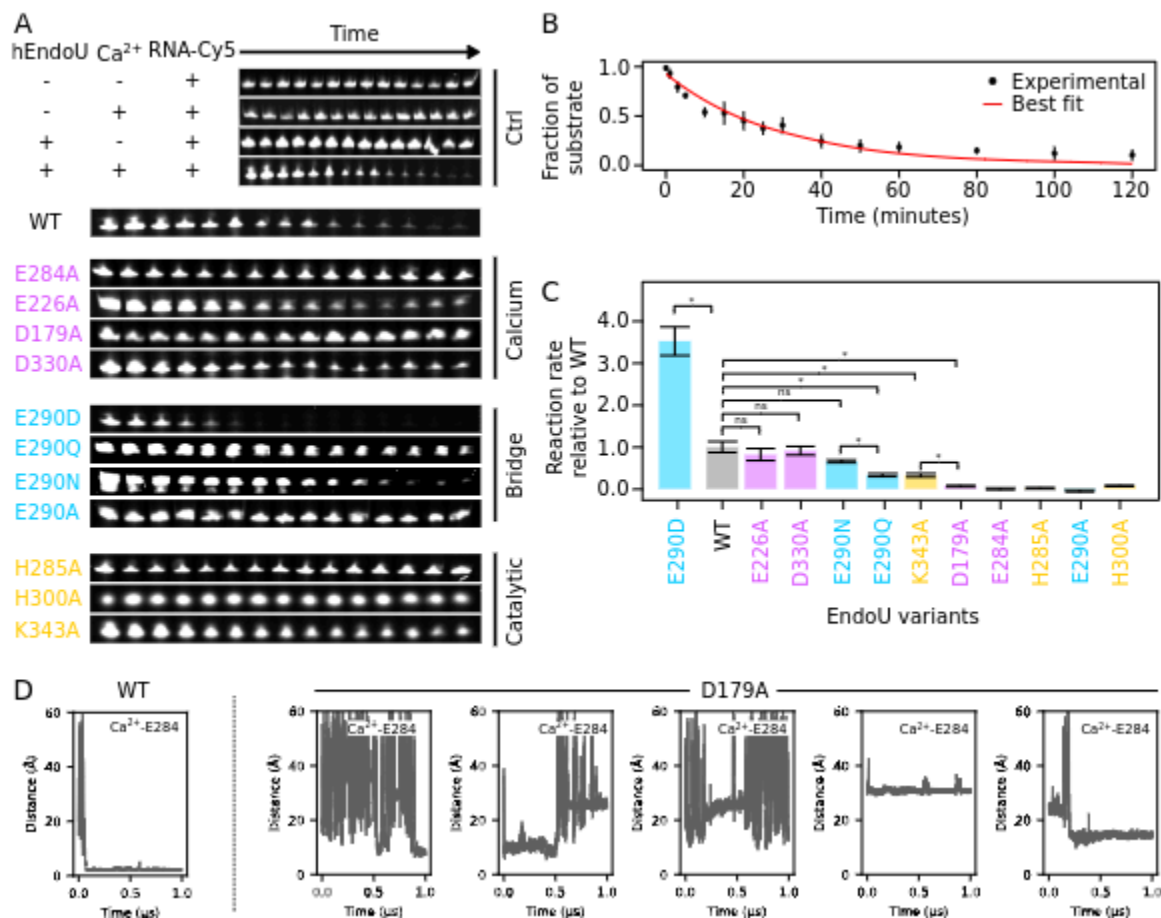
We thank Reviewer #1 for this highly relevant suggestion. First and foremost, the E290 residue is not directly involved in calcium binding, but rather in the transduction of the signal from pocket 1 to the catalytic triad upon calcium binding. Therefore, the E290A mutation is already thought to uncouple calcium binding from activation. We demonstrated this, as the E290A mutant shows no detectable RNase activity (Fig. 2), while still able to bind calcium, as seen from the spectral signatures in the 1H-15N spectrum of EndoU E290A in the presence of calcium (Fig. S9A).

We added Fig. S9A to the revised manuscript:



(A) Overlay of ^{15}N SOFAST-HMQC spectra from isolated ^{15}N -hEndoU E290A (100- μM , black) and upon addition of saturating calcium ions (Ca^{2+} , 20 mM, red).

Yet, we cloned and produced two new mutants of EndoU, namely E290N and E290Q. As predicted by the reviewer, RNA degradation assays revealed that the E290Q mutation significantly reduced the reaction rate compared to the wild type (~50%), suggesting that this mutation partially uncouples calcium binding from catalytic activation, consistent with the semi-conservative nature of the mutation. Interestingly, we had previously shown that E290D was significantly more active compared to the wild type (WT), indicating that D290, with its shorter side chain relative to E290, enhances enzymatic activity. Therefore, we introduced the E290N mutation for comparison with E290Q, predicting that a shorter side chain with the asparagine mutation would preserve more of the catalytic activity. With the mutant E290N, we observed no significant difference compared to the wild type, although this mutant was significantly more active than its E290Q counterpart. Notably, E290N remained significantly less active than E290D, indicating that the carboxylate terminal group is more effective in sensing calcium binding and transducing the signal to the catalytic triad. Consequently, substituting a



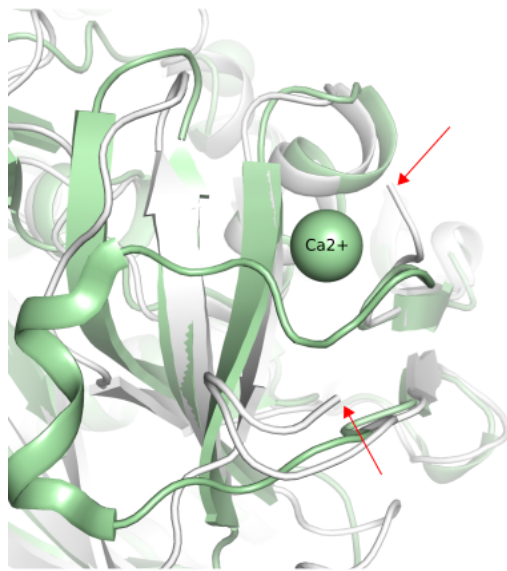
Enzymatic activity of hEndoU and its variants. (A) RNA degradation assays. Comparison of mutants for calcium-binding sites (magenta), the bridging residue E290 (cyan), and catalytic residues (yellow) with wild-type hEndoU over a 2~hrs degradation assay. The RNA was labeled with Cy5 at the 3' end, with the sequence 5'-CAGGUUCCCCAACGAAAAAAAAA-3'. (B) Enzymatic progress curve. Example of fit for a first-order reaction model $A \cdot \exp(-k \cdot t)$ with wild-type hEndoU. (C) Relative reaction rates of hEndoU variants compared to wild-type. Experiments were done in triplicate. The fitted reaction rate is expressed relative to the wild-type (plotted value), and the error in the fitted reaction rate was propagated to be expressed consistently (error bars). Statistical significance was assessed by computing a z-score for the difference between each pair of reaction rates, adjusting for their respective errors, and then these z-score were converted into two-tailed p-values adjusted for multiple comparisons using Bonferroni correction. Statistical significance (*, $p \sim < 0.05$) and non-significance (ns) are highlighted for a representative set of pairwise comparisons. (D) Calcium binding to hEndoU WT or D179A monitored through Molecular Dynamics. Each plot displays the distance between the E284 side-chain carboxylate and a calcium ion throughout the simulation. Five calcium ions were introduced in the simulation box.

An open question is also still why pocket 3 mutation affects activity. Is this pocket more/less conserved than pockets 2/4? What does this pocket and its immediate vicinity look like in XendoU?

We have added a multiple sequence alignment for bacterial, viral, and eukaryotic EndoUs in Fig. S8. Consistently with the requirement of eukaryotic EndoU for divalent metal ions, the next part of this answer refers to eukaryotic sequences only. Pocket 1 is the most conserved calcium-binding site. In Pocket 1, E284 and E217 contribute to calcium binding, both showing strict conservation across species. Pockets 2 and 3 also exhibit a high degree of conservation, although conservative substitutions can be observed. For instance, D315 in Pocket 3 can be substituted by an asparagine residue in some species. Finally, Pocket 4 shows less conservation, with sequence gaps or non-conservative substitutions for residues D322 and D330 across the aligned sequences.

Regarding apo-XendoU, an overlay with calcium-activated hEndoU at site (3) gives the following:

apo-XendoU (2C1W)
Calcium-activated hEndoU



Here, the red arrows indicate the location of a gap in the electronic density from the crystal structure of apo-XendoU. In contrast, the missing loop is clearly visible in the density map of calcium-activated hEndoU, consistent with the fact that it contributes to calcium coordination. From RNA degradation assays and molecular dynamics experiments, we have identified an interplay between site (1) and site (3). We are currently developing plans to investigate this question separately, which should be the subject of an independent report in the future.

Basis of activating effect of Ca^{2+} -ions - The binding of Ca^{2+} could increase RNA affinity of the enzyme by increasing the population of correctly arranged RNA binding sites, or it could increase the proportion of enzyme with correctly arranged active sites for efficient RNA cleavage. Currently, this aspect is not looked into which makes the mechanistic considerations a bit superficial. Given that the authors have all tools at their disposal to test RNA affinities and this is easily experimentally addressable, they should carry out RNA affinity measurements.

We thank Reviewer #1 for these highly relevant suggestions. For the sake of clarity, we will refer to parts of the comment by introducing (H1) The binding of Ca^{2+} could increase the RNA affinity of the enzyme by increasing the population of correctly arranged RNA binding sites; (H2) it could increase the proportion of enzymes with correctly arranged active sites for efficient RNA cleavage. Reviewer #1 suggests that (H1) and (H2) represent mutually exclusive hypotheses, meaning that if (H1) is true, (H2) is false, and vice versa. According to our data, we have evidence to support that (H1) and (H2) are not mutually exclusive and that they both represent downstream events upon calcium addition. Below, we will provide arguments to support that both hypotheses are true.

As Reviewer #1 stated, (H1) can be easily tested. Therefore, we performed EMSA assays using a catalytic EndoU mutant (H285A) and a phosphodiester RNA, in the presence of increasing

calcium concentrations (Fig. S11A). In the absence of calcium, no stable complex was observed, while increasing calcium concentration clearly populated a stable complex between EndoU and the RNA. From this simple experiment, it is clear that (H1) is true, and that calcium does increase the RNA affinity of the enzyme, possibly by increasing the population of correctly arranged RNA binding sites. To further support this, we have shown that the addition of 2'F RNA to wild-type EndoU in the absence of calcium triggers limited perturbations on the 1H-15N spectrum of the enzyme (Fig. S5D), while dramatic changes were observed in the presence of calcium (Fig. S5C), corroborating the EMSA results and supporting that (H1) is true.

Regarding (H2), it must first be stated that no RNA degradation was observed over the course of a 2-hour kinetics experiment performed in the absence of calcium (Fig. 3). Yet, we could still detect a weak interaction between a phosphodiester RNA and wild-type EndoU by NMR spectroscopy, even though no degradation occurred (Fig. S10A, Fig. 3). Moreover, we also repeated the EMSA experiment using EndoU E284A, which shows no catalytic activity while still being sensitive to calcium for RNA binding (Fig. S11C). Relying on the crystal structure of calcium-activated EndoU (Fig. 2), we described an intramolecular signaling network originating from pocket 1, which leads to the activation of the catalytic triad through allostery. These high-resolution data explain why calcium is required for catalysis and are highly consistent with the fact that no RNA degradation is observed in the absence of calcium, despite the formation of a weak complex with the enzyme. Therefore, we also consider (H2) to be true.

To conclude our response to reviewer #1, we can discuss the degree of coupling between (H1) and (H2). We have shown that calcium binding triggers an allosteric network that results in enzyme stabilization and local structure changes in the neighboring RNA binding pocket (H1), along with the activation of the catalytic triad (H2). Strictly speaking, (H1) and (H2) are downstream events upon the addition of calcium, which does not preclude a strict coupling between (H1) and (H2). For instance, the impact of calcium on (H1) could be observed by EMSA using a catalytic mutant of EndoU (H285A). This means that the activation of the catalytic triad (H2) is not necessary to observe the positive impact of calcium on RNA binding (H1). Whether or not the changes in EndoU structure and dynamics that increase the affinity for RNA (H1) are required for catalytic activation (H2) remains an open question that would be difficult to address due to the intricate nature of allosteric transition networks.

Regarding the impact of calcium on EndoU binding to RNA, we added a paragraph in the result section, calcium-activated hEndoU in complex with RNA subsection:

“We established that calcium activates hEndoU by triggering an allosteric network that remotely activates the catalytic triad. Without calcium, binding of 2'F RNA showed limited impact on the 15N SOFAST-HMQC spectrum of hEndoU (Fig. S5D). Repeating the experiment with phosphodiester RNA yielded similar results, indicating weak interaction in the absence of calcium (Fig. S10A). Consistent with our Ca²⁺-dependent activation model, this weak binding did not lead to RNA degradation (Fig. 2, 3). To assess the impact of calcium on RNA binding, we used EMSA with the catalytically dead hEndoU variant H285A, which still binds calcium, and a phosphodiester RNA containing a single uridylate (Fig. S9B, S11A). Without calcium, EMSA

did not detect the weak interaction observed by NMR, likely due to its inability to detect complexes with fast dissociation rates. However, increased calcium concentrations strongly populated a low molecular weight (LMW) complex and weakly populated a high molecular weight (HMW) complex (Fig. S11A). A control experiment, in which the single uridylate was replaced by another nucleotide, resulted in a significant increase in free RNA and a weakly populated LMW complex (Fig. S11B). Interestingly, we observed Ca^{2+} -dependent RNA binding to the hEndoU E284A mutant, despite its lack of catalytic activity (Fig. S11C, 3A,C). This indicates that enzyme activation does not result from the Ca^{2+} -dependent enhancement of hEndoU interaction with RNA, consistent with our model (Fig. 2C). Altogether, we conclude that calcium promotes the formation of an uridylate-specific complex, but also of higher order complexes due to non-specific secondary binding to other nucleotides.”

Fig. S11:

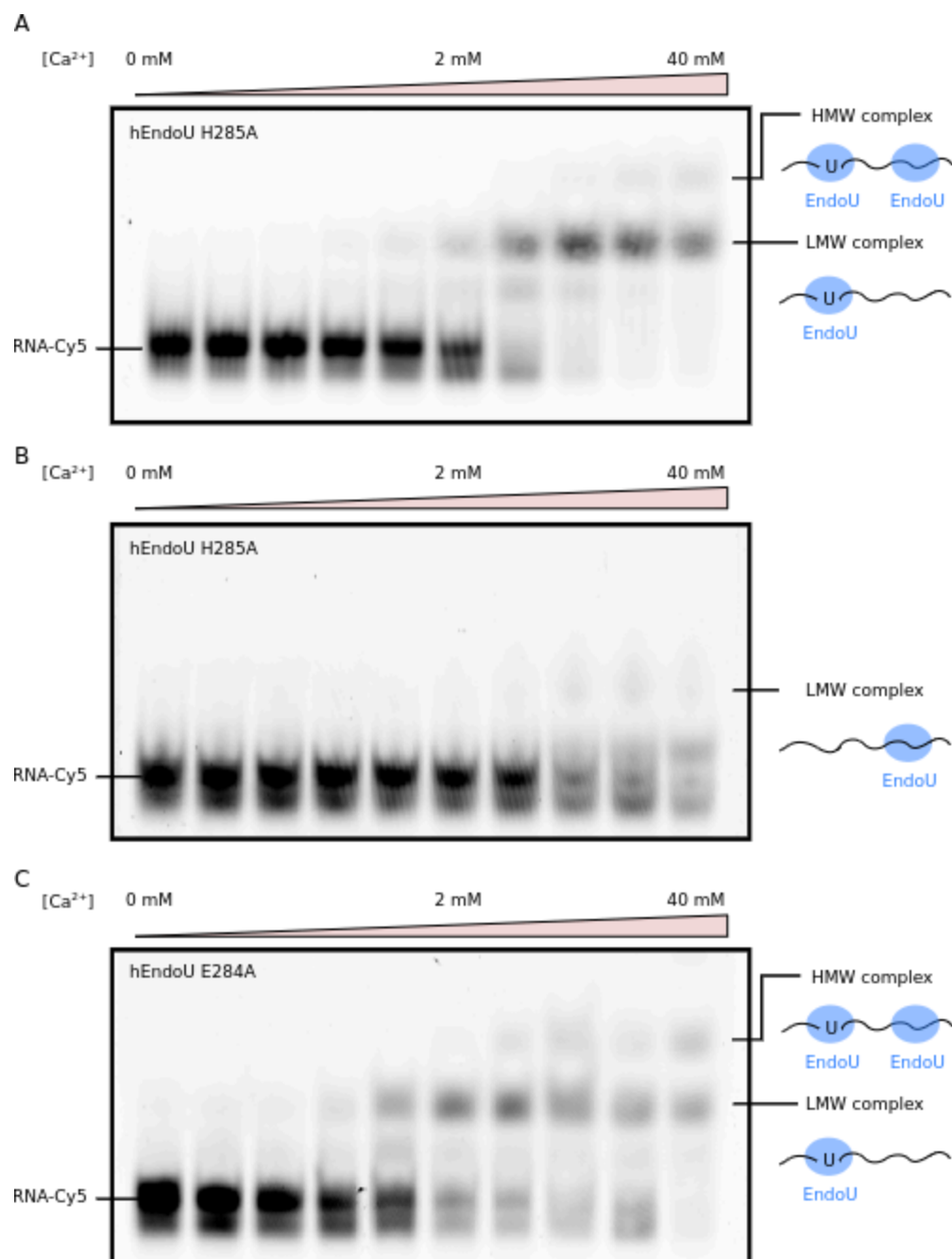
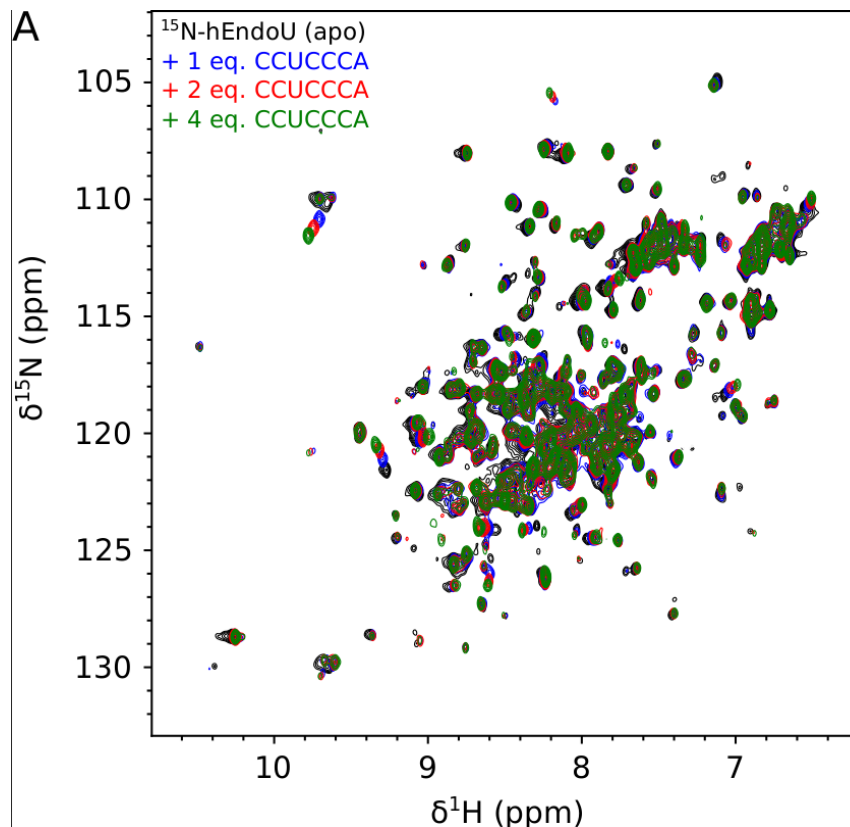


Figure S11: Impact of calcium on RNA binding monitored by EMSA. (A) Binding of a catalytically dead hEndoU mutant (H285A) to a 3'-labeled Cy5 RNA containing a single uridylate with the sequence 5'-CACGAGUACCAGAAAACCAAAA-3'. (B) Control experiment to monitor the binding of hEndoU H285A to a 3'-labeled Cy5 RNA that does not contain an uridylate in the sequence 5'-CACGAAAACCAGAAAACCAAAA-3'. (C) Binding of a calcium-binding hEndoU mutant (E284A) to a 3'-labeled Cy5 RNA containing a single uridylate with the sequence 5'-CACGAGUACCAGAAAACCAAAA-3'. The samples were incubated for 2~hours at room temperature before being loaded onto the gel. For (A, C), the low molecular weight (LMW) complex refers to specific hEndoU binding to uridylate, while the high molecular weight (HMW) complex refers to non-specific secondary binding of hEndoU to a non-uridylate base. For (B),

the LMW complex refers to non-specific hEndoU binding to a non-uridylate base, since the control RNA does not contain an uridylate.

Fig. S10A:

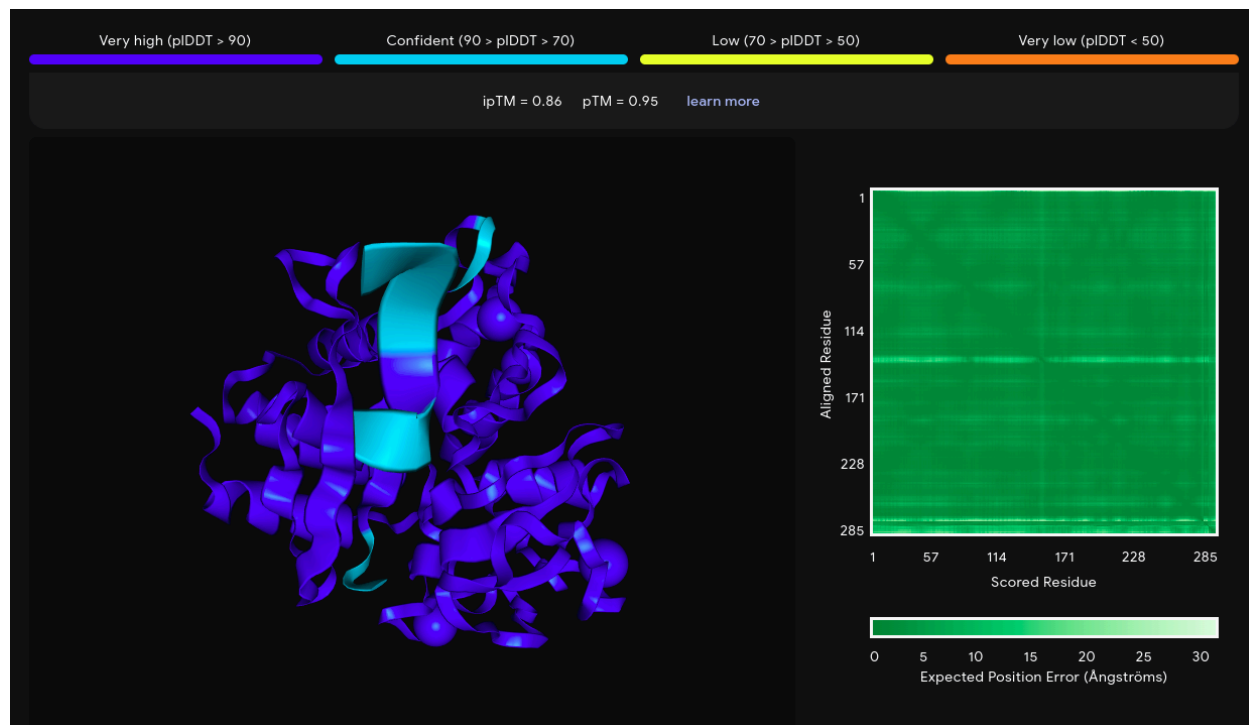


(A) Overlay of ^{15}N SOFAST-HMQC spectra from ^{15}N -apo-hEndoU (200 μM , black) and upon successive addition of phosphodiester RNA.

Relevance of RNA-binding model - In the manuscript a combination of AlphaFold3 and dynamics simulations is used to come up with a model for RNA substrate binding to EndoU. However this model is then not further tested or validated, which leaves the relevance of this model open. This is especially true since RNA-protein models of AlphaFold3 are poor (the program does not even know base pairing rules). NMR titrations of EndoU with RNA identify the same overall surface as the interaction site but allow no detailed structure calculation either. Based on their model the authors suggest that Y405 would be base stacking with the substrate RNA, so mutating this to Ala would be an easy thing to test minimally (effect on RNA affinity, and nuclease activity).

We acknowledge that a model of RNA-bound, calcium-activated EndoU, obtained via AlphaFold 3 and refined through molecular dynamics, although in agreement with NMR data, might not be

sufficiently substantiated to be considered a plausible hypothesis. First, we will discuss the original AlphaFold 3 model, computed with EndoU (135-410), U6 RNA, and four calcium ions.



As seen from the AF3 output above, it is important to note that the confidence in the protein region is very high for nearly all the structure, except for a short loop region near the RNA. However, as mentioned by reviewer #1, AF3 does not recognize the rules of base pairing. The single-stranded RNA used to model the complex shown above is not expected to base pair with itself, which the AF3 model accurately represents. Regarding the confidence in the RNA region, the base U2 is labeled with very high confidence, representing the anchoring point of the RNA near the active site of the enzyme. Since the rest of the RNA is labeled with medium confidence, we interpret this as indicative of potential dynamics, which at the time was consistent with the line broadening observed upon the addition of 2'F RNA in the 1H-15N NMR spectrum of calcium-activated EndoU. This interpretation prompted us to perform molecular dynamics simulations using the AF3 model as input, to further cluster the trajectory using an RMSD cutoff applied to the RNA segment, leading to the model ensemble proposed in the original manuscript.

In addition to the considerations mentioned above, we followed Reviewer #1 suggestion and expanded upon it by cloning and producing putative RNA-binding mutants of EndoU. We used the catalytic mutant H285A as a background to prepare the following mutants for RNA binding: K343A, S347A, R385A, R385K, Y387A_Y405A. We used EMSA to monitor the interaction (Fig. 4F). Overall, the impact of the mutations confirmed the involvement of these residues in RNA binding, thus supporting the model we proposed in the manuscript. We show below the corresponding text added to the main manuscript.

In the result section, Calcium-Activated hEndoU in Complex with an RNA Analog subsection, fourth paragraph:

To further validate our model ensemble, we conducted mutagenesis experiments targeting residues interacting with U2 near the active site (Fig. 4E, S14, S15), which showed consistent contacts across models with an RMSD of 0.73 +/- 0.35 Å (Fig. 4D, E). We selected K343, S347, R385, Y387, and Y405, and generated mutants in the catalytically dead hEndoU H285A background (Fig. 2A, C), evaluating RNA binding via EMSA in the presence of calcium (Fig. 4F). The hEndoU H285A variant showed consistent RNA binding, with a stoichiometric complex corresponding to uridylate-specific binding, and higher order complexes corresponding to non-specific secondary binding. Mutations S347A and K343A were expected to disrupt electrostatic interactions with U2, reducing RNA binding. Consistent with our model, double mutants H285A/S347A and H285A/K343A showed dramatically reduced RNA binding. Similarly, the triple mutant H285A/Y387A/Y405A exhibited weak RNA binding, possibly explained by loss of π -stacking between Y405 and U2 and destabilization of R385 conformation due to the absence of the Y387 hydroxyl group (Fig. 4E). The conservative mutation R385K (H285A/R385K) had limited impact on RNA binding. Unexpectedly, the double mutant H285A/R385A, which should reduce RNA binding, showed increased secondary binding at high protein:RNA ratios, suggesting enhanced binding to non-uridylate bases. Our findings demonstrate that specific residues at the hEndoU–RNA interface are essential for substrate binding, providing further experimental evidence supporting our AI/MD-derived model.

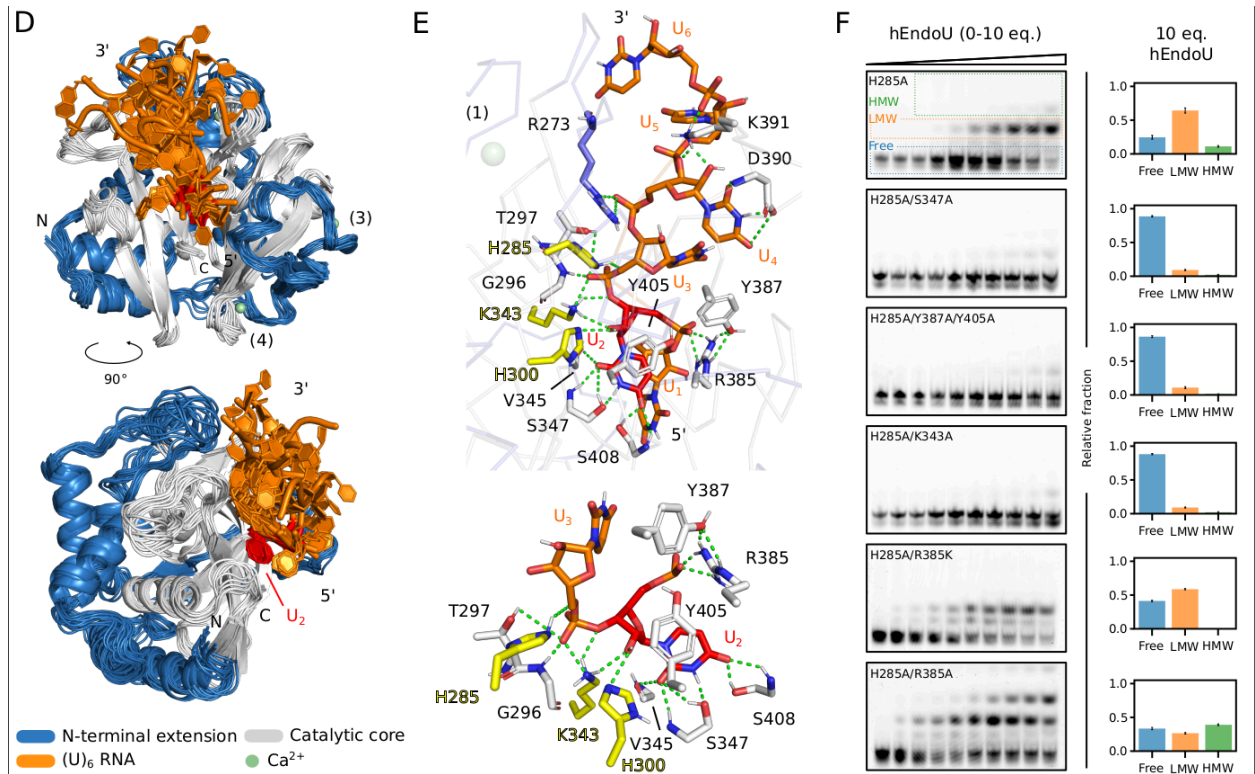
Regarding the EMSA experiments to assess either the impact of calcium or mutations to RNA binding, we added a subsection in the Methods of the manuscript:

“Electrophoretic Mobility Shift Assay

Electrophoretic Mobility Shift Assays (EMSA) were conducted to assess the impact of calcium and mutations on RNA binding to EndoU. We prepared 8% acrylamide/bisacrylamide (19:1) non-denaturing gels in 0.5X TBE (44.5 mM Tris base, 44.5 mM boric acid, 1 mM EDTA) supplemented with 5 % (v/v) glycerol. Since calcium was used in our assays, we initially attempted to use an EDTA-free 0.5X TB buffer, but this resulted in inconsistent results, possibly due to trace amounts of heavy metals in the molecular biology-grade glycerol (Sigma-Aldrich G5516) and Tris base (ThermoFisher 17926). Consequently, we used 0.5 X TBE. For the EMSA experiments, samples were prepared in a 20 μ L reaction mix with the following buffer composition: 10 mM Tris base (pH 7), 50 mM NaCl, 0.5 mM TCEP. We used a 3'-labeled Cy5 RNA probe containing a single uridylate with the sequence 5'-CACGAGUACCAGAAAACCAAAA-3', at a concentration of 250 nM. To prevent enzymatic degradation of the probe, we used a catalytically inactive hEndoU mutant (H285A), which also served as a background to establish RNA-binding mutants of hEndoU. To assess the impact of calcium on RNA binding, we prepared an equimolar mix of the RNA and hEndoU (H285A), supplemented with increasing concentrations of calcium as follows (mM): 0, 0.1, 0.3, 0.5, 1, 2, 5, 10, 20, 40. To monitor the impact of mutations in hEndoU on RNA-binding activity, we

prepared samples containing the RNA probe (250 nM) and calcium ions (2 mM), supplemented with increasing amounts of hEndoU variant as follows (eq.): 0, 0.25, 0.5, 0.75, 1, 2, 3, 5, 10. For all experiments, 20 μ L of loading buffer (50% glycerol v/v, orange G) were added to each sample prior to loading on the gel. All samples were prepared at room temperature and loaded on the gel immediately upon preparation unless specified otherwise. Electrophoresis was conducted at 100~V for 45 minutes at room temperature. Each gel was subsequently scanned with a fluorescence scanner. We used GelAnalyzer software to integrate band intensities to assess the impact of hEndoU mutations on RNA binding. To this end, bands on the lane corresponding to a 1:10 RNA:protein ratio were integrated and expressed as relative fractions of signal for the free RNA, the low molecular weight complex indicating specific binding to uridylate, and the high molecular weight complexes indicating non-specific binding to other bases.”

We refactored Fig. 4 and added results of EMSA experiments to monitor the impact of mutations on RNA binding, in order to validate our model:



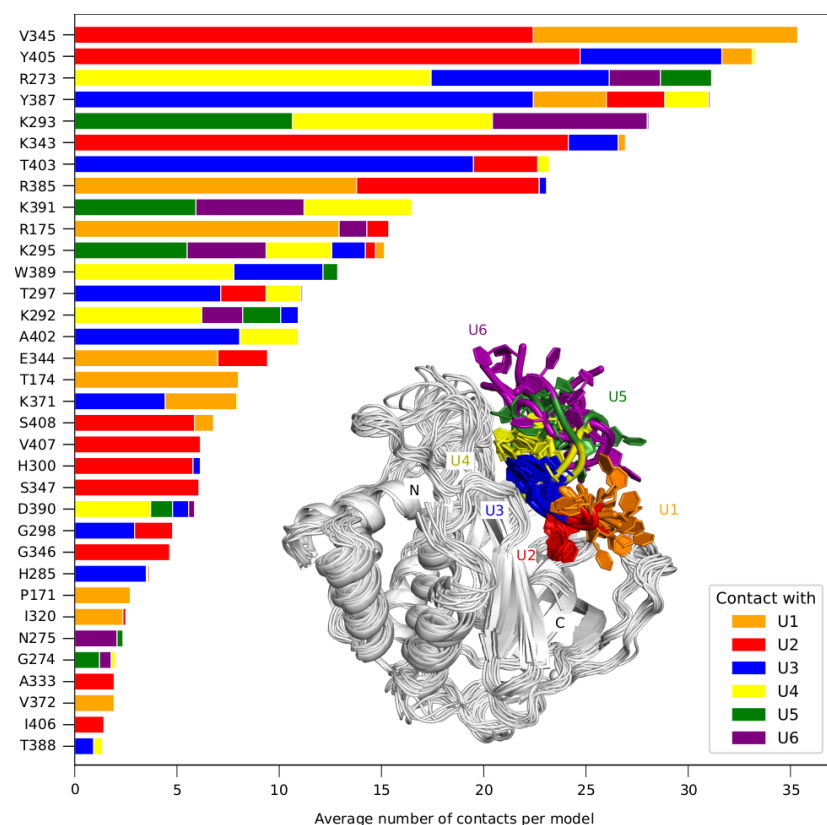
(D) Structure alignment of the ensemble of models (N=14) for calcium-activated hEndoU in complex with RNA. The alignment was performed on the protein region.

(E) Detailed view of a representative model of the ensemble with highlights on protein-RNA interactions. A close-up view of interactions at U2-U3 is also shown.

(F) RNA binding to calcium-activated hEndoU variants monitored by EMSA. The regions corresponding to the free Cy5-labeled RNA, the low molecular weight complex (LMW), and the higher molecular weight complexes (HMW), are highlighted. Experiments were done in triplicate.

For each experiment, the relative fraction of Free RNA, LMW, and HMW complexes were computed for each replicate, and the resulting means (plotted values) and standard deviations (error bars) were plotted at 10 eq. hEndoU.

In line, we also added Fig. S15 which ranks residues showing the greater numbers of contacts with the RNA, given our ensemble of models and each base on the RNA:



Analysis of intermolecular contacts within the ensemble of calcium-activated hEndoU in complex with RNA. For each model of calcium-activated hEndoU in complex with (U)6 RNA, we measured the distance between each hydrogen atom of the protein and each hydrogen atom of the RNA, defining intermolecular contact as a distance less than 5.5~Å. We averaged the number of contacts per model (N=14) and represented the value for each protein residue toward each base of the RNA. Residues showing fewer than one contact per model with the RNA are not shown. For ease of analysis, we display the ensemble of models for calcium-activated hEndoU in complex with RNA, highlighting bases U1-6, consistent with the plot legend.

Control experiment for cellular EndoU rescue assays - Lack of rescue of RNase activity with an EndoU mutant should be added as a negative control for the assays in Fig. 1B.

To address this point, we generated a number of EndoU mutants in a retroviral backbone and made the corresponding virus for VL3-3M2 cell line transduction. However, unfortunately due to

issues with a lab move, tissue culture hood malfunction, and ultimately bacterial contamination, we were unable to select and test the necessary stable VL3-3M2 EndoU KO rescue lines by the submission deadline.

Other points:

Visualization of structures and domains - In the overview figures (Fig. 2A, 4D/E, S6), the proposed active site should be visualized to allow judgment of the proximity of the Ca^{2+} ions.

We have updated Fig. 2A, 4D (now 4B), and S6 to highlight the proposed active site, allowing for an assessment of the proximity of Ca^{2+} ions as requested by reviewer 1. We did not update Fig. 4E, because the electrostatic surface we present is not appropriate to highlight specific residues.

Fig. 2A:

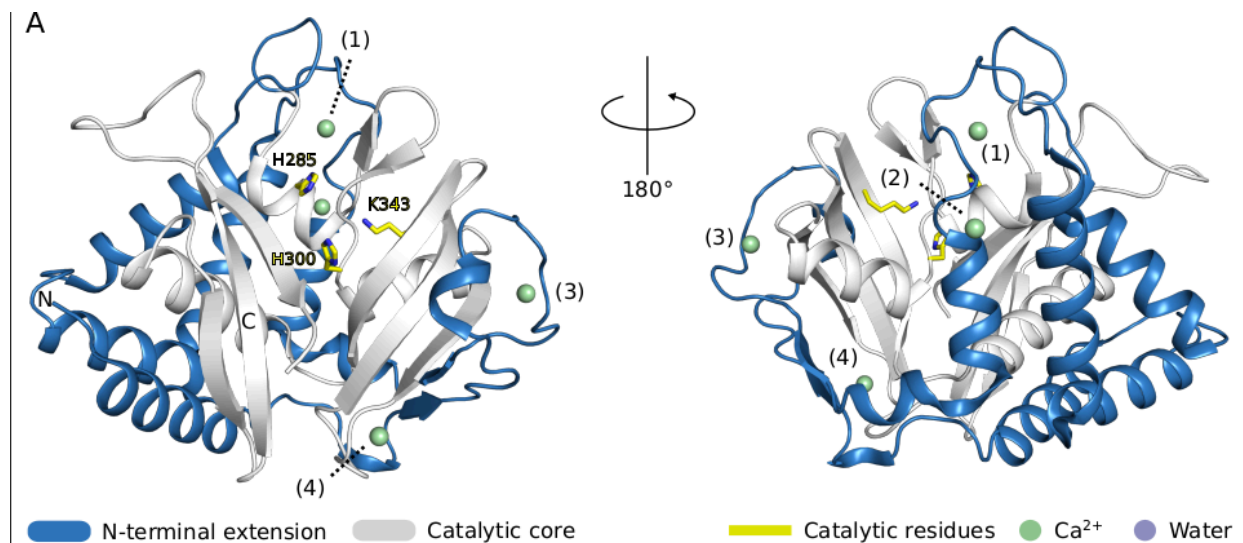


Fig. 4B (now 4D):

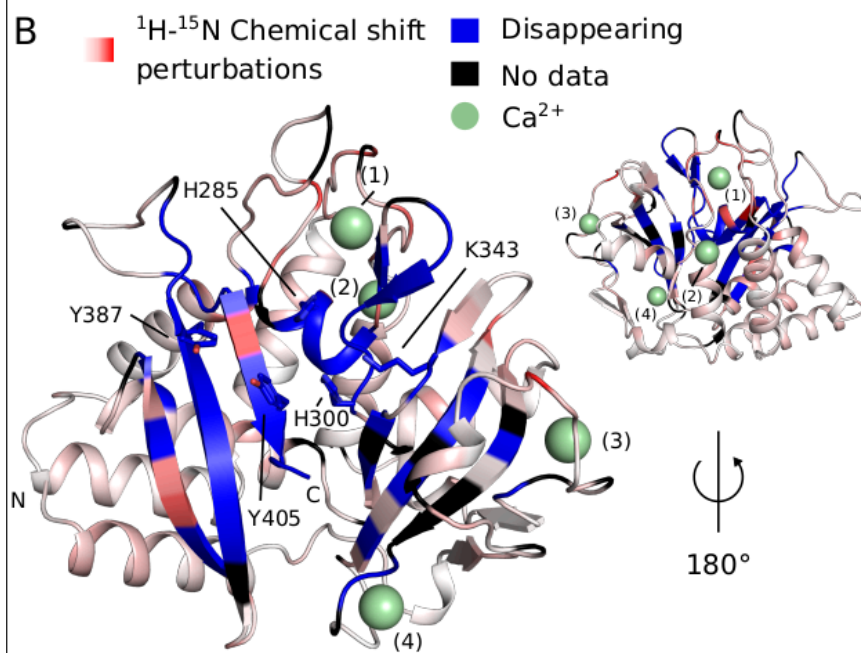


Fig. S6:

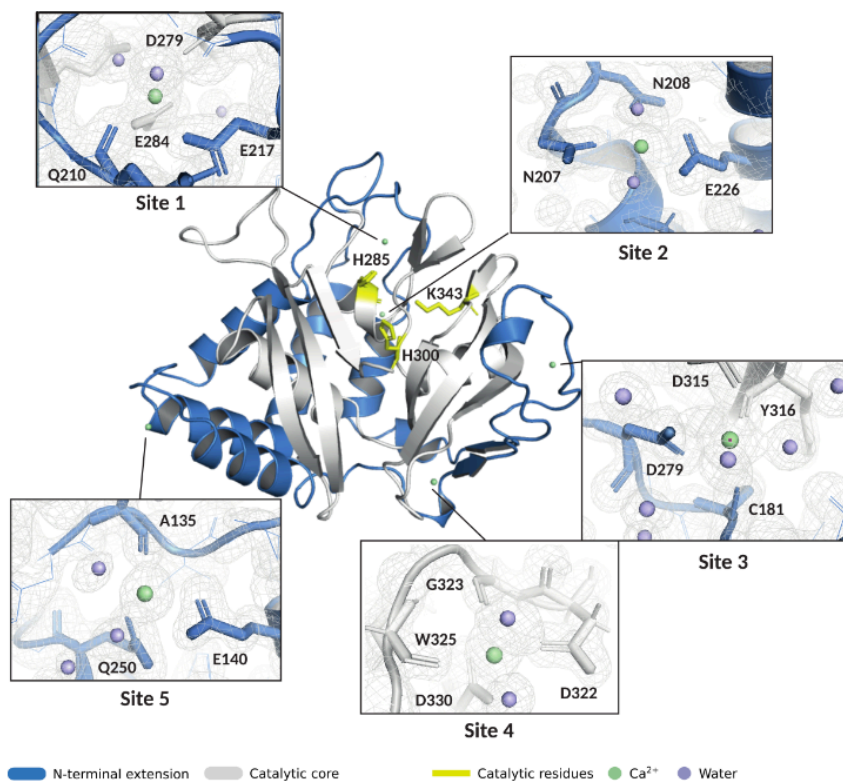
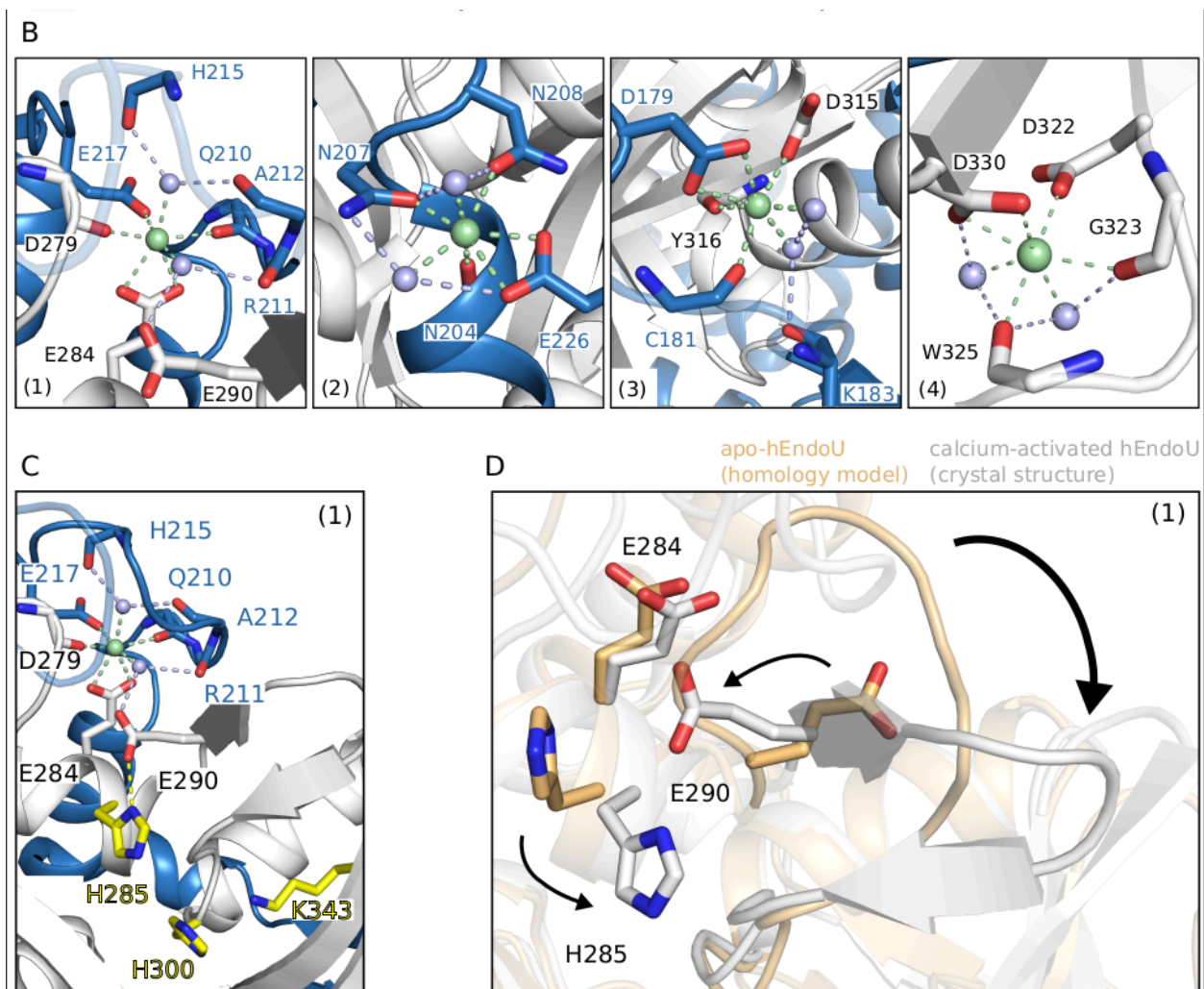


Figure S6: **Crystal structure of hEndoU.** For each calcium binding site, the neighboring electronic density is shown. Calcium ions (green spheres) and water molecules (blue spheres) are shown.

I found the zoom views presented in Fig. 2B-D very difficult to understand. Residues are colored differently than their domains (pink, blue residues vs. gray, blue domains), they seem to float in empty space partially detached from the backbone and often missing the sidechains.

We refactored Fig. 2B-D to enhance the clarity of the panels as suggested by reviewer #1. For Fig. 2B-C, we colored the residues according to the domain to which they belong. In Fig. 2C, we maintained the same color scheme for the region corresponding to the calcium binding site, but we highlighted the catalytic residues in yellow. Regarding the residues with missing side-chains, these were intentionally not shown because they do not contribute to calcium binding (e.g., H215 at site 1). Regarding the residues that appear partially detached from the backbone, reviewer #1 was referring to the PyMOL cartoon transparency effect that we used to reduce crowding in the figure, especially in the foreground. We have decreased the use of this effect across the panels in agreement with the comment from reviewer #1.

Figure 2B-D:



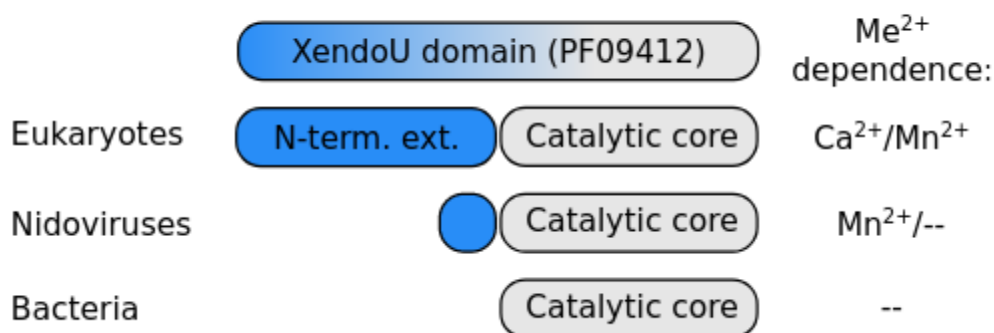
Panel D is very busy with the superposition of three structures, here one (either the XendoU or the modelled human apo-structure) could be removed because they are redundant (SWISSPROT models are basically mimicking the existing structure and add very little new information).

We have removed one structure from panel D (the XendoU crystal structure), as suggested by reviewer #1, leaving only the apo-EndoU model and the crystal structure of calcium-activated EndoU (Fig. 2D, see above).

In Fig. 1F the domain schemes are misleading in a way to suggest that XendoU has a different domain composition than the other eukaryotes. A consistent way of representing the domains should be used here and also numbers should be added to denote the domain boundaries.

We have refactored the figure to address the ambiguities noted by reviewer #1 (Fig. 1F). However, we did not add numbers to denote the domain boundaries, as the length of EndoUs varies significantly across phyla. This variation could confuse readers if we were to choose an arbitrary sequence as a reference.

Figure 1F:



Apparent differences in NMR spectra - Can the authors explain the differences between spectra in Figures S9 vs S5D? They should reflect almost the same conditions but S9 looks much better resolved and shows more peaks than S5D.

Fig. S9 in the original submission now corresponds to Fig. S10B in the revised manuscript.

The caption for Fig. S5D reads: “(D) Overlay of 15N SOFAST-HMQC spectra from **isolated 15N-EndoU** (170 μM, black) and upon the addition of 2' fluorinated RNA (1.5 eq., red),” while the caption for Fig. S10B states: “Overlay of 15N SOFAST-HMQC spectra from **calcium-bound 15N-EndoU** (200 μM, green) and upon the addition of 2' fluorinated (U)6 RNA (1 eq., red).”

Therefore, in Fig. S5D, we observe the interaction between **apo-EndoU** and a 2'F RNA, while in Fig. S10B, we observe the interaction between **calcium-bound EndoU** and a 2'F RNA. The

difference between the two spectra thus lies in the absence (Fig. S5D) or presence (Fig. S10B) of calcium, which indeed has a significant impact on RNA binding.

Of note, we updated the caption of Fig. S5 to stick with the “apo” notation instead of “isolated”. The caption for Fig. S5B-D now reads: “(B, C, D) Overlay of 15N SOFAST-HMQC spectra from 15 N-apo-hEndoU (170 μ M, black) and (B) upon addition of sub-saturating calcium ions (2 mM, blue) or (C) upon addition of sub-saturating calcium ions (2 mM, blue) and 2'-F RNA (1.5 eq., red) or (D) upon addition of 2'-F RNA (1.5 eq., red). The sequence of the 2'-F RNA is as follows: 5'-AAGUCC-3'.” We updated the caption of all figures accordingly.

Information in figure legends and statistical analysis - For plot information in the figure legends is very sparse and no statistical analyses were carried out to denote significant differences. This should be amended. Figure legends should include what is plotted (e.g. means), number of replicates, what error bars represent, and which statistical test was carried out.

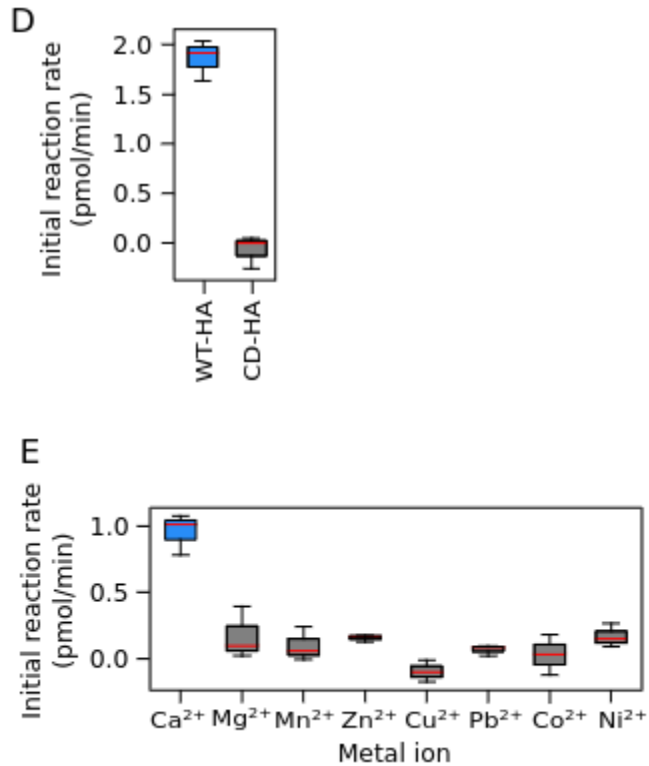
We have updated the figures and caption, along with the methods, and performed statistical analysis when appropriate.

In the method section of the revised manuscript, we added a subsection related to statistical tests and procedures:

“Statistical tests and procedures

Statistical tests and procedures were performed using in-house python scripts, relying on widely recognized *scipy* (Virtanen et al. Nat Methods. 2020) and *statsmodels* libraries. Comparison between groups of data was achieved through pairwise t-tests (*scipy.stats.ttest_ind*), ensuring data normality (*scipy.stats.shapiro*) and variance homogeneity (*scipy.stats.levene*). Computed p-values were adjusted for multiple comparisons using the Benjamini/Hochberg method (*statsmodels.stats.multitest.multipletests*). Comparison between parameters with associated errors was achieved as follows: Z-scores were calculated pairwise as the difference between parameters divided by the standard error of the difference. Two-tailed p-values were then computed from the z-score using a normal distribution survival function (*scipy.stats.norm.sf*). Resulting p-values were adjusted for multiple comparisons using the Bonferroni correction method (*statsmodels.stats.multitest.multipletests*). “

Updated Fig. 1D-E with caption (including histogram to boxplot change):

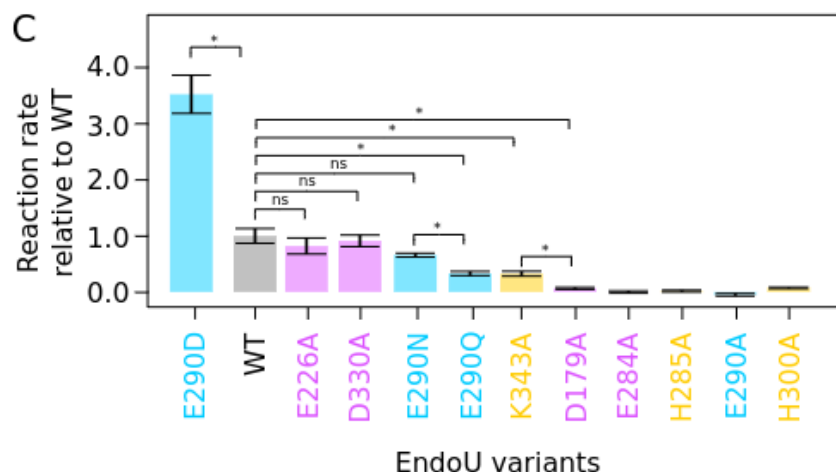


(D) Cleavage activity of WT mEndoU (WT-HA) and the catalytically dead (CD-HA) mEndoU double mutant E285A-H286A. The initial reaction rate was significantly higher for WT-HA compared to CD-HA ($p = 0.0002$).

(E) mEndoU RNase activity is specifically activated by Ca²⁺-ions. The initial reaction rate was significantly higher for Ca²⁺ compared to Mg²⁺ ($p = 0.02$), Mn²⁺ ($p = 0.01$), Zn²⁺ ($p = 0.01$), Cu²⁺ ($p = 0.01$), Pb²⁺ ($p = 0.01$), Co²⁺ ($p = 0.01$), and Ni²⁺ ($p = 0.01$).

For (C) and (D), experiments were done in triplicate. Initial reaction rates were computed from linear regression at early time points for each replicate and they are plotted as boxplot. Checks for normality and equal variance across the data were performed using the Shapiro-Wilk and Levene's tests, respectively. Group comparisons were achieved with pairwise independent two-sample t-tests. The p-values were adjusted using the Benjamini-Hochberg method for multiple testing correction.

Updated Fig. 3C with caption:



(C) Relative reaction rates of hEndoU variants compared to wild-type. Experiments were done in triplicate. The fitted reaction rate is expressed relative to the wild-type (plotted value), and the error in the fitted reaction rate was propagated to be expressed consistently (error bars). Statistical significance was assessed by computing a z-score for the difference between each pair of reaction rates, adjusting for their respective errors, and then these z-score were converted into two-tailed p-values adjusted for multiple comparisons using Bonferroni correction. Statistical significance (*, $p < 0.05$) and non-significance (ns) are highlighted for a representative set of pairwise comparisons.

Updated Fig. S2C-F caption:

(C) Quantification of initial rates of cleavage activity with different divalent metal ions. Experiments were done in triplicate. For each experiment, replicates were averaged for each time point (plotted values) and the associated standard deviation was computed (error bars).
 (D, E, F) K^+ , Na^+ , and pH dependence of mEndoU cleavage activity. Experiments were done in triplicate. For each experiment, replicates were averaged, and the associated standard deviation was computed. The initial reaction rate was determined by linear regression of the averaged values and their associated error for each time point. The fitted slope (plotted value) and associated error (error bars) are represented.

Methods - Units are written in an unusual way (e.g. mg.ml-1) and should be changed to conventional annotation (e.g. mg/mL) instead.

We have updated all text and figures in the manuscript, including the Supplementary Information, to use the conventional notation (e.g., mg/mL) as suggested by reviewer #1.

Was TEV protease really used in 1:10 ratio? This is roughly 10-fold more than what is usually used.

A previous batch of poorly active TEV was used at a 1:10 ratio. We corrected this to a 1:100 ratio, as our usual batches of TEV are used under these conditions for complete cleavage between the tag and the protein.

Typos:

p.5 - dependence of EndoU for calcium > dependence of EndoU on calcium

Corrected.

p.9 - DNase (NEB) > DNase I (NEB)

Corrected.

p.12 - central tendencies are expressed as means, and variations as standard deviations > do you mean that "means of three replicates were plotted with error bars representing standard deviations"?

We updated this vocabulary when answering to the "Information in figure legends and statistical analysis" point.

p.17 - that mainly cluster nearby protein loops > that mainly cluster in neighbouring protein loops

Corrected.

p.17 - The bonding correlates with a disorder-to-order transition in the loop carrying E290, forming a β -hairpin and stabilizing the catalytic site > This is misleading because the loop was already ordered before but in a different position.

In the sentence, we mention a disorder-to-order transition in the loop carrying E290. Reviewer #1 suggests that the loop was already ordered, albeit in a different position. We do not clearly understand which reference reviewer #1 is considering. In both the crystal structure of apo-XendoU and our homology model, the corresponding loop does not form any secondary structure. In the crystal structure of calcium-activated EndoU presented in this work, we observed a transition from disordered to ordered, as a β -hairpin was identified. Such transitions from a loop to a stable β -hairpin are generally recognized as disorder-to-order transitions. If reviewer #1 is referring to the AlphaFold model of apo-EndoU, we have already explained earlier why this model is inappropriate for the context of this study. If reviewer #1 considers that visible loops in a crystal structure reflect ordered conformations present in solution, we unfortunately disagree, based on the comparison of many X-ray vs NMR protein structures.

Yet, we acknowledge that we do not have direct experimental evidence that the loop carrying E290 is indeed disordered in solution, even this can be largely expected. Therefore, we modified the corresponding text section as follows:

“In apo-hEndoU, the loop containing E290 does not have a defined secondary structure and is likely flexible in solution. Thus, calcium binding correlates with a disorder-to-order transition in this loop, forming a β -hairpin and stabilizing the catalytic site.”

p.18 - resulted in nearly half reduction of enzymatic activity > resulted in a reduction to nearly half of the enzymatic activity

Corrected.

Reviewer #2 (Remarks to the Author):

Eukaryotic EndoU is an endonuclease that shares the RNase-A family catalytic motif and has an N-terminal extension. Unlike RNase-A family nucleases, eukaryotic EndoU requires Ca^{2+} ions for catalysis. The mechanism by which Ca^{2+} ions are required for eukaryotic EndoU remains elusive. This manuscript describes the molecular basis of the activation of eukaryotic ribonuclease EndoU by calcium ions through structural and biochemical analysis. The authors determined the crystal structure of human EndoU in complex with Ca^{2+} at high resolution. In the structure, four Ca^{2+} ions were identified. Comparison of the Ca^{2+} -bound structure with the model apo structure (or apo XendoU) suggested that one of the Ca^{2+} ions binding to the enzyme allosterically changes the catalytic core structure (specifically conformation of one of the catalytic His residues) of EndoU, thereby activating it. The model was evaluated through biochemical analysis and molecular dynamics. The authors also built an RNA-binding model onto EndoU.

We thank reviewer #2 for the careful review of our manuscript. We appreciate that the summary provided by reviewer #2 accurately captures the essence of our work on the activation of eukaryotic EndoU by calcium. We have considered all remarks made by reviewer #2 and have implemented most of the suggestions, as discussed below.

Major comment

This reviewer appreciates the solid structural and biochemical analysis of human EndoU in this manuscript. The manuscript is well organized and communicated. Having said that although eukaryotic EndoU might have acquired the N-terminal extension and evolutionarily adapted to cellular signaling in eukaryotes, the manuscript does not provide any mechanistic insight into the Ca^{2+} -specific activation of the enzyme. This reviewer feels that the structure of the EndoU-RNA complex (in the presence and absence of Ca^{2+}) would provide more mechanistic insights into the activation of EndoU and the cleavage of RNAs. Further, comparison of the structure of eukaryotic EndoU and those of Nidoviruses and bacteria EndoU would provide the more mechanistic insight into the Ca^{2+} dependent activation of eukaryotic EndoU.

The present form of the manuscript is suitable for other specific journals.

We are grateful to reviewer #2 for recognizing the solid structural and biochemical analysis of human EndoU presented in our work. However, we have provided experimental evidence in the manuscript showing that human and mouse EndoUs are specifically activated by calcium,

potentially extending to other eukaryotic EndoU family members, consistent with previously published literature. Additionally, we solved the crystal structure of calcium-bound EndoU and provided mechanistic insights into EndoU activation by calcium, identifying an intramolecular signaling network that activates the catalytic triad through allostery. We further validated this model through mutagenesis and RNA degradation assays, thereby providing solid evidence of how calcium binding to EndoU leads to catalytic activation of the enzyme. Although we acknowledge that reviewer #2 considers that the manuscript does not provide any mechanistic insight into the Ca^{2+} -specific activation of the enzyme, we believe this assessment may be somewhat overstated, which limits our ability to precisely understand the underlying issues prompting this comment. While we regret the perception of reviewer #2, we lack the necessary details to address the concerns more precisely.

Nevertheless, we considered the two suggestions raised by Reviewer #2 to provide more mechanistic insights into the Ca^{2+} -specific activation of the enzyme. First, solving the structure of the EndoU-RNA complex in the absence of Ca^{2+} via NMR spectroscopy is not feasible due to the particular dynamics of apo-EndoU, which results in intermediate exchange on the NMR timescale, leading to the loss of many signals and overall poor data quality. Although a weak interaction between apo-EndoU and RNA can be detected using NMR spectroscopy, this interaction cannot be detected through EMSA, which stands for the very low affinity of the complex that forms in the absence of calcium. Therefore, crystallogenesis approaches using a dynamic apo-EndoU forming a barely detectable complex with RNA are likely not to succeed. Yet, we still attempted to prepare crystals of calcium-activated EndoU in complex with 2'F RNA. As the NMR data suggest, the intermolecular interface shows intermediate exchange dynamics, which is interpreted in our model of RNA-bound calcium-activated EndoU through multiple conformations that can be taken by the RNA tails flanking the base well buried in the vicinity of the catalytic triad. We were unable to obtain crystals of RNA-bound, calcium-activated EndoU, and we believe that the dynamics of the RNA at the molecular interface with EndoU is a strong factor explaining why crystallogenesis attempts were unsuccessful. Referring to reviewer #2 comment, we must state that mechanistic insights into RNA cleavage are not the focus of our manuscript, which is centered on the activation mechanism by calcium, not the catalytic mechanism leading to RNA cleavage, which is calcium-independent. Since the activation mechanism we proposed relies on EndoU and calcium, without involving RNA, we are also uncertain how a structure of RNA-bound, calcium-activated EndoU could have significantly improved our model of EndoU activation by calcium, which again does not involve RNA. Yet, we fully agree with reviewer #2 that such structures would be of significant interest to understand how RNA binds to calcium-activated EndoU and to gain mechanistic insights into RNA cleavage, which might constitute a future piece of work suitable for an independent publication.

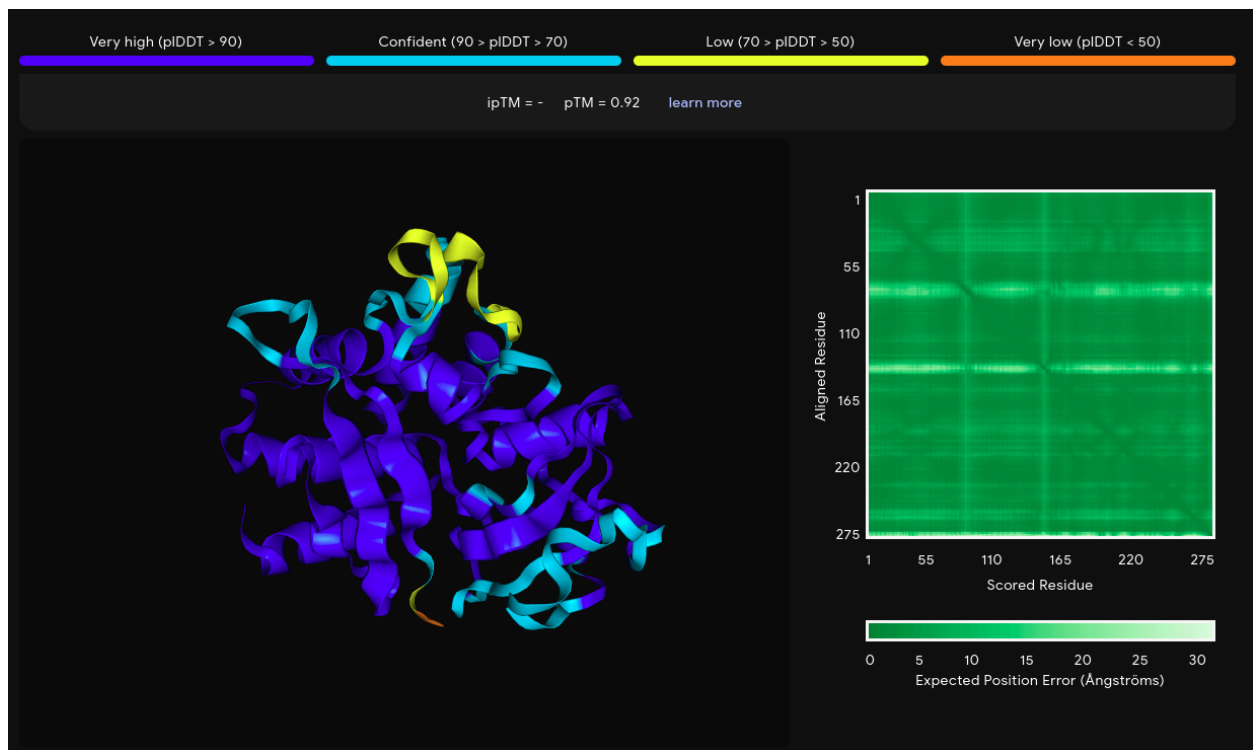
Additionally, reviewer #2 suggests that comparing the structure of eukaryotic EndoU with those of Nidoviruses and bacteria would offer more mechanistic insights into the Ca^{2+} dependent activation of eukaryotic EndoU. In response, we have provided structural alignments and discussed the point raised by reviewer #2 to offer further insights and hypotheses regarding the evolution of eukaryotic and certain viral EndoUs that resulted in a divalent metal ion dependence for catalytic activity (Fig. 5B, Fig. S8, Fig. S16).

Ultimately, we acknowledge the last comment of reviewer #2, which mentions that the current form of the manuscript is suitable for other specific journals. Accordingly, we have significantly revised this original manuscript, considering all the comments and suggestions made by reviewers. We placed particular emphasis on clearly and precisely addressing each concern raised by all reviewers. We sincerely hope that these substantial improvements will satisfy the reviewers and demonstrate our commitment to the highest standards.

Minor comments

1) Page 16: Model of human EndoU: Why did the authors compute a homology model using SWISS-MODEL? The authors could have obtained the model via AlphaFold.

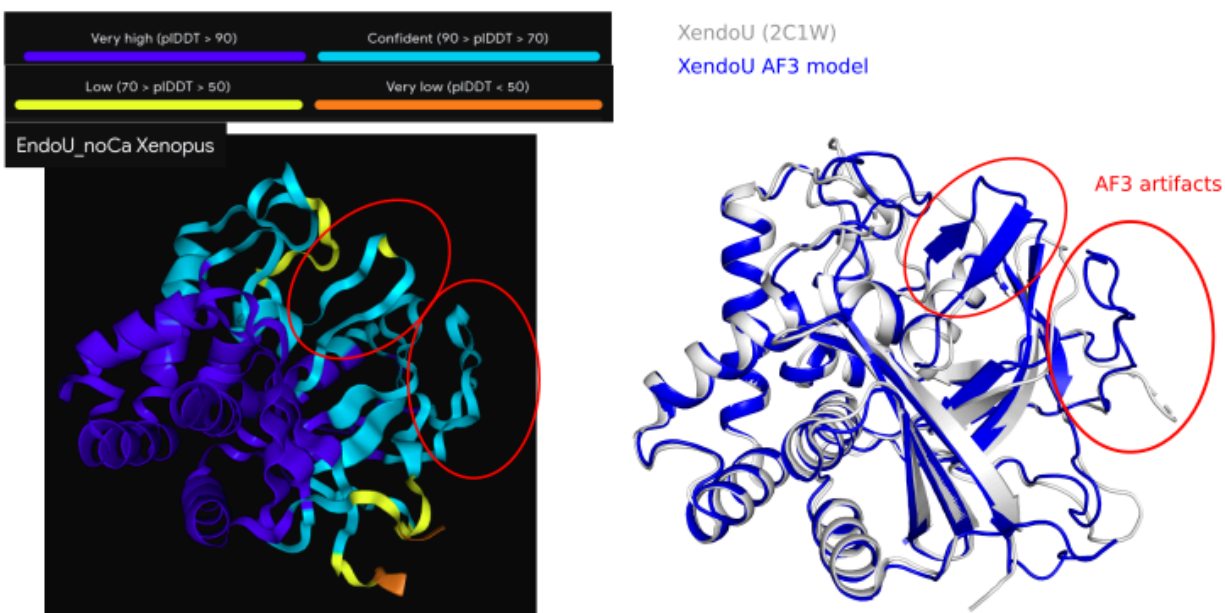
Like all tools, AlphaFold must be used with caution. Below, we provide the output for a structural model of apo-EndoU obtained using AlphaFold3:



In this model of apo-EndoU, we observe that the majority of the protein structure is predicted with very high confidence. However, a significant fraction of the protein, which is enriched in calcium-binding regions, is predicted with medium to low confidence. AlphaFold users might generally interpret lower confidence regions as flexible or disordered, suggesting that the static structure provided for these regions may not stand up to experimental validation. Even though very high confidence models from AlphaFold3 should not be considered equivalent to experimental data, extreme caution should be taken when functionally important regions are labeled with medium to low confidence. This is why we did not select this AlphaFold model as a

reference, and we will go one step further to demonstrate why, in our case, this decision is likely a highly relevant choice.

Below, we present an AlphaFold model of apo-EndoU from *Xenopus*, with confidence labels, and compare it to the corresponding crystal structure of apo-EndoU from *Xenopus*:



As observed for human apo-EndoU, the AlphaFold3 model of *Xenopus* apo-EndoU predicts a significant fraction of functionally important regions with medium to low confidence. Upon comparison with the actual crystal structure of *Xenopus* apo-EndoU, structural artifacts modeled by AlphaFold are easily identifiable. These artifacts are predictably located in medium to low confidence regions, which demonstrates unequivocally that AlphaFold3 is not a reliable tool for accurately modeling *Xenopus* apo-EndoU. In the absence of an experimentally determined structure of human apo-EndoU, we postulate that medium to low confidence regions in the corresponding AlphaFold model might also result in structural artifacts, similarly to what we have shown for *Xenopus* apo-EndoU.

Altogether, these elements justify using the experimentally determined *Xenopus* apo-EndoU structure as a template for homology modeling of human apo-EndoU. Although a lower-resolution method, we also highlight that we used SEC-SAXS experiments to confirm that recombinantly produced human apo-EndoU exhibits diffusion profiles and subsequent structural properties well in agreement with theoretical data computed from the homology model of human apo-EndoU.

2) Page 18: Calcium binding sites: Sites (2) and (4) are not defined by residues from both the catalytic core and N-extension. Is this correct? Please confirm.

This is indeed correct.

3)Figure 37: Figure 4D: Ca²⁺ sites should be specified (1–4) as in Figure 2A.

Labels for Ca²⁺ sites were added in figure 4D as in figure 2A.

4)Figure S10: RNA-binding model: Did the authors try to determine the crystal structure of RNA-bound EndoU?

As detailed in the comments section of reviewer #2, we attempted crystallogenesis assays of calcium-activated EndoU bound to 2'F RNA. Unfortunately, we were unable to obtain crystals of the complex. We believe that the conformational exchange of the RNA at the molecular interface with EndoU, evident from the loss of corresponding signals in the 1H-15N spectrum of the calcium-activated, RNA-bound EndoU, may be responsible for impairing the formation of stable crystal packing. This limitation hinders our ability to obtain materials suitable for X-ray diffraction. On the other hand, NMR spectroscopy is unsuitable for solving the structure of this complex due to the aforementioned signal loss from intermediate exchange on the NMR timescale, which prevents the recording of data required for NOE determination and structure calculation. Although the binding of RNA to calcium-activated EndoU was not the main focus of this manuscript, we still explored RNA binding by NMR and computed a compatible model of calcium-activated, RNA-bound EndoU, further validated through mutagenesis assays as suggested by comments from other reviewers.

5) Structural comparison and amino acid alignments of EndoU and its homologs would help.

Based on comments from reviewer #2, we have implemented structural and sequence alignments of EndoU and its homologs (Fig. S8, Fig. S16, Fig. 5B).

In reference to Fig. S8, S16, we added this mention in the discussion section of the manuscript, first paragraph:

“The endonucleolytic activity of RNase A and EndoU-like domains relies on a conserved catalytic triad composed of two histidines and one lysine (Fig. S8). The catalytic residues H12 and K41 in RNase A align well with corresponding residues in EndoU-related proteins, whereas residue H119 is spatially shifted (Fig. S16). The closest spatial equivalent to H119 in RNase A is H187 in a bacterial EndoU-like toxin, while H285 in hEndoU and H234 in Nsp15 are more distantly located. This spatial rearrangement likely reflects evolutionary divergence while preserving a metal-independent catalytic mechanism.”

In reference to Fig. S8, we added this mention in the results section, experimental validation of hEndoU activation model subsection, end of first paragraph:

“Moreover, the side chains of residues E217 and E284 directly contribute to calcium coordination at site (1), as do D179 and D315 at site (3) (Fig. S8). All show identity conservation, with the exception of D315, which can present a semi-conservative substitution to asparagine across eukaryotic EndoUs (Fig. S8).”

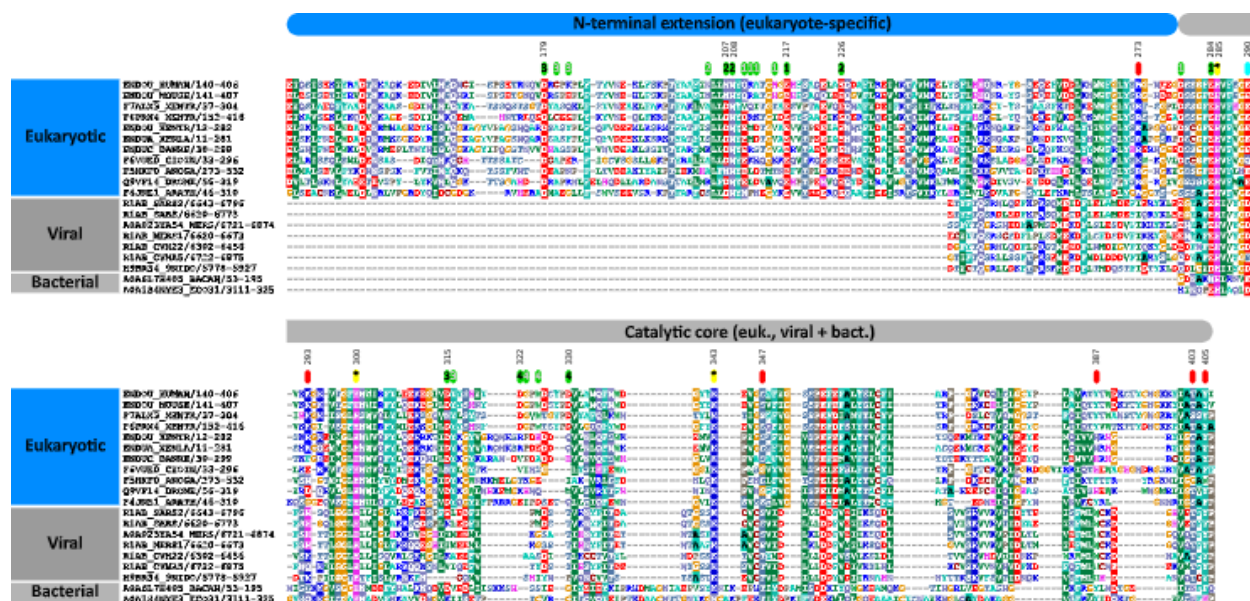
In reference to Fig. S8, we added this mention in the discussion section, second paragraph:

“Indeed, we identified calcium binding sites (1) and (3) as critical for hEndoU activation, and they are highly conserved in eukaryotic EndoUs, supporting a shared activation mechanism (Fig. S8). In addition, we have shown that calcium-coordinating residues at these sites originate from both the conserved catalytic core and the eukaryote-specific N-terminal extension. Although not essential for activation, site (2) is also broadly conserved, suggesting a putative function awaiting characterization. In contrast, site (4) is poorly conserved, consistent with its nonessential role for activation.”

In reference to Nsp15, Fig. S8, and Fig. 5B, discussion section, fourth paragraph:

“However, the conserved metal-coordination sites found in eukaryotic EndoUs are absent in Nsp15 and related viral homologs (Fig. 5B, S8), suggesting that manganese might enhance enzymatic activity by promoting Nsp15-RNA interaction, mirroring the observed effect of calcium in promoting hEndoU binding to RNA. Consistently, recent in vitro studies demonstrated that Nsp15 retains some activity on single-stranded RNA without divalent metal ions, although manganese increases this activity in a substrate-dependent manner (Huang et al. JBC, 2023; Wang et al. NAR, 2024). Other reports also found enhanced RNA binding by Nsp15 in the presence of manganese (Bhardwaj et al. J. Mol. Biol. 2006) and suggest that manganese may provide charge shielding to promote Nsp15 interactions with the RNA (Frazier et al. NAR, 2021), although it has also been proposed that manganese stabilizes an alternative enzymatic state with a higher cleavage rate (Huang et al. JBC, 2023). Insights from Nsp15 studies also highlight the significance of tipiracil, a uridine analog that binds the Nsp15 uridine binding site and inhibits its activity (Kim et al. Communications Biology, 2021). Determining whether tipiracil can similarly bind and inhibit eukaryotic EndoUs would provide a valuable tool for probing their cellular and extracellular functions.”

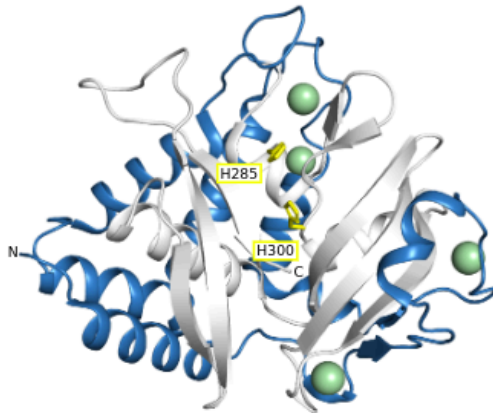
Fig. S8 (see high resolution figure in the manuscript):



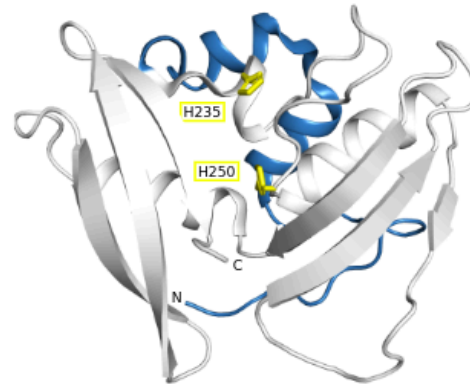
A structure-based sequence alignment of eukaryotic, viral and bacterial EndoU homologs. The human EndoU structure from this study, together with the following existing structures of eukaryotic, viral, and bacterial EndoU homologs (2c1wC, 4s1tF, 2gthA, 5yvdB, 2gtiA, 2ozkD, 6vwwA, 5hkqA), were structurally aligned (all-against-all) using the DALI server. The eukaryote-specific N-terminal extensions of the EndoU catalytic domain were aligned separately from the shorter viral N-terminal extensions. The resulting sequence alignment was manually adjusted and annotated to highlight calcium-coordinating residues (green), catalytic residues (yellow, asterisk), and selected RNA-binding residues (red). Residue E290 in hEndoU, which mediates communication between calcium-binding site (1) and the catalytic residues, is also highlighted (cyan). For the calcium-coordinating residues, the numbers 1–4 refer to the calcium-binding site numbering used in this study. Residues that coordinate calcium via their side chains (black numbers) or via their backbone (grey numbers) are distinctly highlighted. Residue numbers refer to the hEndoU sequence.

Fig. 5B:

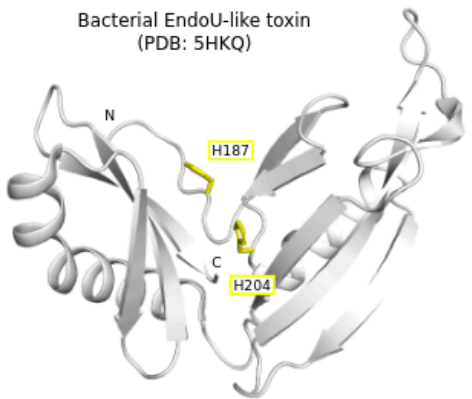
Calcium-activated EndoU from *Homo sapiens* (PDB: 9FTW)



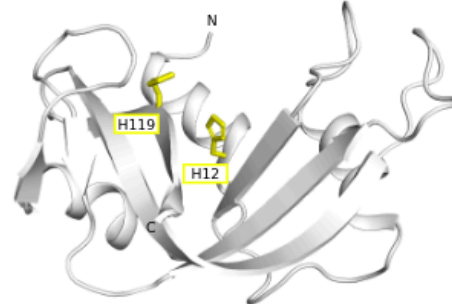
SARS-CoV-2 Nsp15 NendoU (PDB: 7RB0)



Bacterial EndoU-like toxin (PDB: 5HKQ)



Bovine pancreatic Ribonuclease A (PDB: 1U1B)

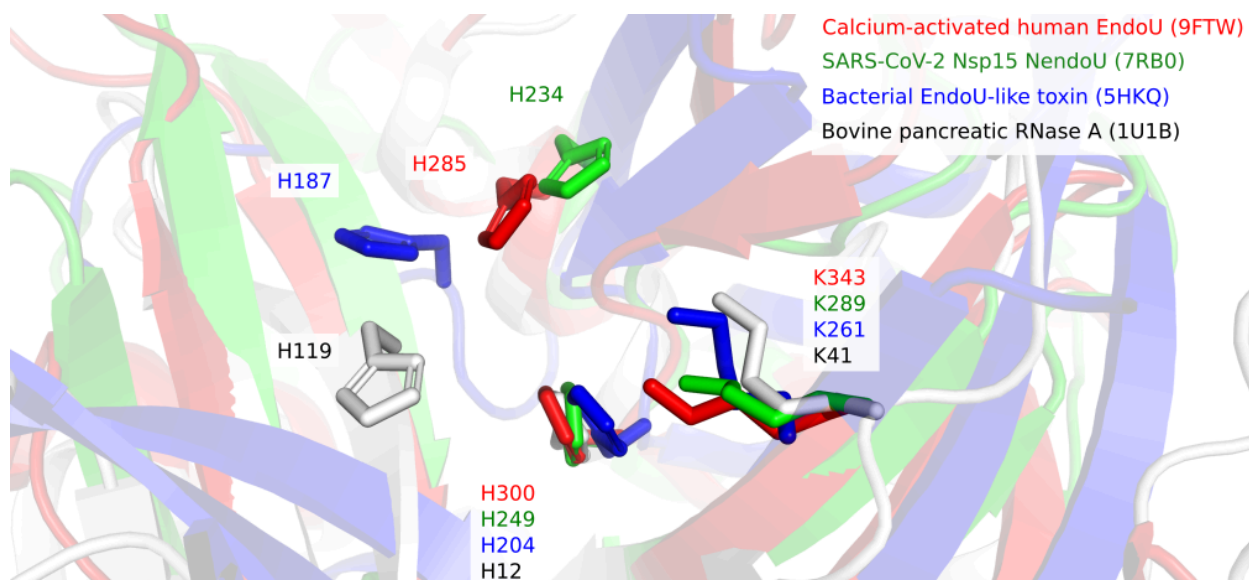


■ N-terminal extension ■ Catalytic core

■ Catalytic histidines ● Ca^{2+}

(B) Structural comparison between calcium-activated human EndoU, SARS-CoV-2 Nsp15 NendoU, bacterial EndoU-like toxin, and bovine pancreatic ribonuclease A.

Fig. S16:



Structural alignment of catalytic residues for human EndoU and homologs. The catalytic His-His-Lys triad of calcium-activated human EndoU (9FTW, red), SARS-CoV-2 Nsp15 NendoU (7RB0, green), and bacterial EndoU-like toxin (5HKQ, blue) were aligned on the corresponding His-His-Lys catalytic triad from bovine pancreatic ribonuclease A (1U1B, white).

Reviewer #3 (Remarks to the Author):

In this manuscript, the authors explore the calcium-dependent activation mechanism of the human ribonuclease EndoU. By employing structural biology, molecular biology, and biophysical techniques, they demonstrate that calcium binding at specific sites induces allosteric changes that activate the catalytic triad essential for RNA cleavage. The study also provides insights into the structural basis of this activation and proposes a model where calcium binding is critical for the proper positioning of the catalytic residues. Although the study presents interesting findings, there are multiple questions that should be addressed first.

We thank reviewer #3 for the careful review of the manuscript and note that the reviewer effectively summarized the essence of the work presented. We considered all comments from the reviewer and implemented most suggestions, thereby strengthening the body of evidence used to support our model for the activation of EndoU by calcium ions.

1. How does EndoU's specificity for calcium over other divalent cations, such as manganese, relate to its physiological function?

Although the physiological function of EndoU is not the focus of this manuscript, we discuss the possibilities in the fourth paragraph of the Discussion. Conflicting reports have attributed the specificity of EndoU to either calcium or manganese. In the discussion section of the manuscript, we explained that manganese concentrations in mammalian tissues are very low, reaching at most dozens of micromolars in brain tissues. In cells, manganese concentrations

are typically maintained in the nanomolar to low micromolar range, which is far from the millimolar range required for optimal enzymatic activity. In contrast, calcium concentrations may vary significantly, with levels reaching up to 100 μM inside the cell during signaling and millimolar levels in the extracellular space. As discussed in the manuscript, the *C. elegans* homolog endu-2 and the *D. melanogaster* homolog CG2145 have both been shown to be secreted outside the cells. This makes calcium-dependent activation of EndoU highly relevant in this context, since extracellular calcium concentrations are typically in the 1-2 mM range. Of note, animal EndoUs contain an N-terminal signal peptide, and mammalian EndoUs possess two somatomedin B (SmB) domains rich in disulfide bonds, suggesting secretion or ER association. Inside the cell, human and *Xenopus* EndoUs were shown to be ER-associated, and a model of XendoU nuclease activity on membranes was published as follows (Schwarz & Blower, Journal of Cell Biology, 2014):

[REDACTED]

In their work, Schwartz & Blower observed that XendoU is active on the surface of ER membranes and speculated that RNAs targeted by XendoU are likely determined by their proximity to the nuclease upon calcium release from the ER. Their data support the hypothesis

that XendoU acts by locally cleaving membrane-bound mRNAs and rRNAs, followed by the release of a subpopulation of ribosomes and other RNA-associated proteins from the ER. Schwartz & Blower then speculated that RNA cleavage functions to clear a patch of ER membrane, possibly to remove complexes that could sterically hinder the vesicle fusion machinery, allowing for tubule growth and fine-tuning of network remodeling. Accordingly, it was shown in *Xenopus* egg extracts that the loss of XendoU leads to a failure of vesicle fusion and a lack of a tubular ER network. Similar observations were made from the loss of EndoU in HeLa cells, which leads to a loss of tubular ER and an expansion of ER sheets. Altogether, this interesting work by Schwartz & Blower explains how calcium-mediated activation of EndoU could regulate ribosome occupancy on the surface of the ER, thus impacting ER morphology as a whole.

In the context of our work, we discussed that in mammals, EndoU expression is limited to specific cell types. Based on the analysis of immunological genome project data, we identified that mouse EndoU is highly expressed in developing thymocytes up to the double-positive (DP) stage, after which EndoU is absent in later stages, specifically in single-positive thymocytes that survive selection and in circulating T cells. Using the mouse thymic lymphoma cell line VL3-3M2, which closely resembles DP thymocytes with high EndoU expression, we have shown that stimulation with PMA/ionomycin, anti-CD3/CD28, or anti-CD3/CD4 results in cells showing further maturation, correlated with the downregulation of EndoU expression. We then demonstrated that detectable calcium-dependent RNase activity in VL3-3M2 cell lines depends solely on EndoU expression, based on CRISPR/Cas9 knockout of the protein. Although not assayed in our work, which focuses on the mechanisms by which calcium activates EndoU, it is tempting to speculate that EndoU upregulation during thymocyte maturation might be required for rearrangement of the ER that would be important for the transition to later maturation stages, hence situating our findings in the context of the interesting work published by Schwartz & Blower, as discussed above.

2. The mutagenesis results are critical for supporting the activation model. Could the authors provide more details on the rationale behind selecting specific residues, such as E290A, K343A, and others, for mutagenesis in the validation studies?

In the original version of the manuscript, we cloned and produced nine mutants of EndoU. Four were calcium-binding mutants. For each calcium-binding site, we selected a calcium-coordinating residue, either aspartate or glutamate, and replaced it with alanine to prevent calcium from binding to this site. Through this approach, we aimed to identify which calcium-binding sites were essential for catalytic activity. This methodology enabled us to determine that binding sites 1 and 3 are crucial for activity, as the corresponding E284A and D179A mutations inactivated the enzyme. We then chose to mutate each of the presumably catalytic residues that are part of the His-His-Lys catalytic triad. This confirmed the importance of residues H285 and H300 for catalysis, as their substitution with alanine abolished enzymatic activity. The mutation of K343 to alanine resulted in a nearly fifty percent reduction of the reaction rate, consistent with the role of K343 in stabilizing reaction intermediates. Additionally, we observed in the crystal structure of calcium-activated EndoU that the residue E290 acts as a

bridge between pocket 1 and the remote catalytic triad. Upon calcium binding to pocket 1, a water molecule participates in the calcium coordination network and is also engaged by E290, which is part of the allosteric network leading to catalytic activation. Consequently, we mutated E290 to alanine to disrupt the allosteric network, resulting in the loss of catalytic activity, consistent with our model. Interestingly, the charge-conservative E290D mutation resulted in increased catalytic activity, indicating that the negative charge of the acidic side chain plays a crucial role in maintaining productive interactions that lead to the allosteric activation of catalytic residues.

In addition to the original mutants, we conducted further mutagenesis experiments in our revised manuscript to validate the proposed model of RNA-bound, calcium-activated EndoU. We used the catalytic mutant H285A as a background to prepare the following mutants for RNA binding: K343A, S347A, R385A, R385K, Y387A_Y405A. We used EMSA to monitor the interaction (Fig. 4F). Overall, the impact of the mutations confirmed the involvement of these residues in RNA binding, thus supporting the model we proposed in the manuscript.

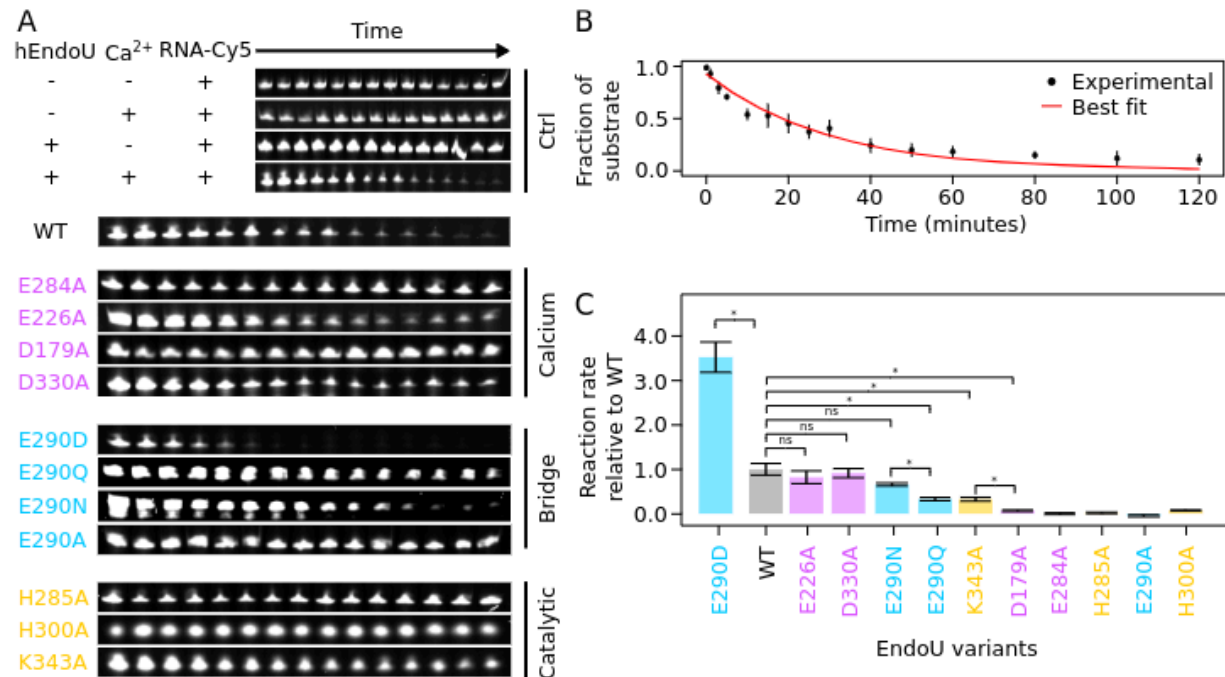
3. According to the authors' model, E290, with its long negatively charged side chain, should interact more readily with the catalytic H285 side chain in the active conformation. Could the authors clarify why the charge-conservative mutation E290D led to an increase in the enzymatic reaction rate, as observed in Figures 3A and 3C?

Based on the crystal structure of calcium-activated EndoU, we agree that the long, negatively charged side chain of E290 should more readily interact with the catalytic H285 side chain than the charge-conservative mutation E290D. The residue at position 290 is situated on a beta-hairpin, which is believed to form upon calcium binding. Prior to this event, residue 290 is part of a loop, as indicated by the crystal structure of *Xenopus* apo-EndoU and the homology model of human apo-EndoU. It should first be noted that the disorder-to-order transition observed upon calcium binding may slightly differ in terms of the final conformation, whether in the context of the wild-type glutamate or the aspartate mutant. Furthermore, the crystal structure of calcium-activated EndoU reveals that E290 not only maintains polar contact with H285 but also with R273 and S280, both of which are parts of flexible loop segments. Therefore, the potential for structural plasticity near E290 is high. We speculate that shortening the side chain of E290 by one bond in the E290D mutation could trigger slight local structural changes upon calcium binding, overall accommodating the charge-conservative mutation well.

Although we do not have a definitive explanation for what we observe, we were able to reproduce this side-chain length effect using new EndoU mutants, specifically E290N and E290Q, both assessed by RNA degradation assays. The substitution of the terminal carboxylate with an amide (E → Q) was intended to partially uncouple calcium binding from catalytic activation. Consistently, we observed nearly a 50% reduction in enzymatic activity with E290Q compared to WT. When shortening the side-chain of Q290 through the mutation E290N, we observed an enzymatic activity that showed no statistically relevant difference from the wild type. The enhancing effect of a shorter side-chain with either a carboxylate or amide terminal

group remains to be investigated. However, this effect shows some consistency upon study of four distinct EndoU constructs.

Updated Fig. 3A, C with E290N and E290Q mutants:



4. While the *in vitro* assays demonstrate calcium-dependent activity, how do these findings translate to *in vivo* conditions? Have any *in vivo* experiments been conducted to confirm the proposed activation mechanism?

As mentioned by reviewer #3, the RNA degradation assays performed *in vitro* with recombinantly produced human EndoU have demonstrated that the RNase activity is dependent on calcium. The endogenous ribonuclease activity of EndoU was assayed in VL3-3M2 cell extracts, as reported in the results section of the paper (Fig 1B). By performing a CRISPR/Cas9 knockout of EndoU in the VL3-3M2 cell line, we were able to demonstrate that the calcium-dependent ribonuclease activity in cell extracts solely depended on the presence or absence of EndoU. In this context, immunoprecipitation of the endogenous EndoU from VL3-3M2 cell extracts confirmed the calcium-dependent ribonuclease activity of EndoU.

As discussed above, we aimed to test the corresponding mouse EndoU mutations in the extract assay as in Fig 1B. However, unanticipated malfunctions and culture contamination prevented us from completing the experiments in time.

5. The proposed model suggests allosteric activation involving specific residues. Have molecular dynamics simulations been employed to validate all the critical residues and

proposed allosteric pathways? It appears that only D179A was validated in Figure 3D. The Methods section should clearly describe all the systems used in molecular dynamics simulations.

In the original version of the manuscript, the MD simulations we presented were related to only two experiments aimed at monitoring the binding of calcium to pocket 1 in either wild-type or D179A mutant conditions. For these experiments, as stated in the methods section, we used the crystal structure of EndoU bound to calcium as the initial model, from which we removed the calcium ions. Calcium ions were reintroduced into the simulation box as solvent ions. Intriguingly, we observed that calcium binding to pocket 1 was not detected following mutation D179A in pocket 3. Consistent with RNA degradation assays, which showed that mutations in either pocket 1 or pocket 3 abolish enzymatic activity, we concluded that both binding sites are essential for catalytic function. We thoroughly explained how pocket 1 is essential, by describing an allosteric activation network that originates in pocket 1 and extends to the remote catalytic triad of the enzyme.

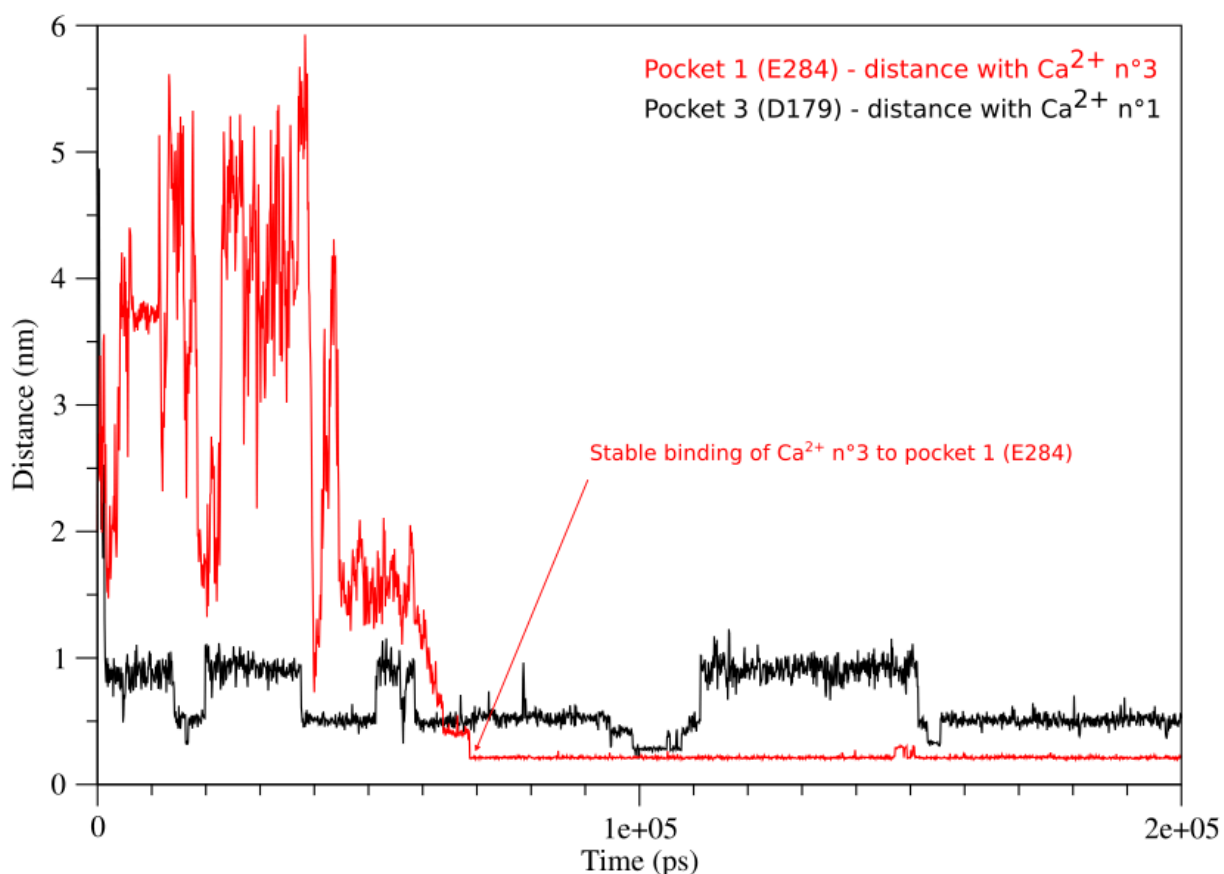
To provide a more in-depth response to reviewer #3, the MD simulations we presented were not conducted to validate the proposed allosteric pathway. Instead, they were designed to initiate an investigation into the role of the D179 residue at pocket 3 in enzyme activation. By doing so, we were able to correlate RNA degradation assays, which showed no activity with the D179A mutation, to MD simulations, where no binding of calcium at pocket 1 with the D179A mutation was observed. Although these experiments did not offer substantial explanatory power, they presented an intriguing hypothesis for further investigation: Could there be some degree of cooperativity between pocket 1 and pocket 3? This question remains to be explored in future work.

6. The manuscript identifies multiple calcium-binding sites. Could the authors elucidate the relative importance of each site for enzymatic activation and whether these sites exhibit cooperative binding?

In the crystal structure of calcium-activated EndoU, we identified five calcium-binding sites, including one involved in crystal packing that was not considered in the study. Therefore, four calcium-binding sites were relevant, and each was tested through mutagenesis experiments and RNA degradation assays. We demonstrated that inactivating calcium-binding sites 2 or 4, with mutations E226A or D330A, respectively, was conservative with respect to the calcium-dependent activation of EndoU and catalytic activity. We concluded that pockets 2 and 4 were not necessary for EndoU to be activated by calcium and to cleave RNA. In contrast, we identified that mutating either pocket 1 or 3 abolished enzymatic activity. From these experiments, we conclude that pockets 1 and 3 are essential for catalytic activity, while pockets 2 and 4 are not.

While we could explain well the importance of pocket 1 for the catalytic activation of the enzyme, we were surprised to observe that mutating pocket 3 resulted in a loss of enzymatic activity. Consequently, we performed MD simulations in triplicate and observed that the integrity

of pocket 3 was necessary for calcium binding at pocket 1, suggesting an interplay between the two sites. Indeed, MD simulations showed that calcium binds to pocket 1 within about 100 ns in the context of the wild-type protein. In contrast, no calcium binding could be observed at pocket 1 during a 1 μ s simulation when using the pocket 3 mutant D179A. Strict cooperativity between pockets 1 and 3 would, therefore, require calcium to bind to pocket 3 first to enable calcium binding to pocket 1. Below we show the profiles of calcium binding to pocket 1 and 3 over time through the first 200 ns of one MD simulation using the wild-type EndoU:



Herein, we examine the distances between the carboxylate group of E284 (red curve) and D179 (black curve) and Ca^{2+} ions n°3 or n°1, respectively. We observe that stable calcium binding to pocket 1 is preceded by either fuzzy binding or spatial proximity between Ca^{2+} n°1 and D179 within a range of 3-5 Å, but without stable binding. Mutating D179 to alanine prevents calcium binding to E284, as demonstrated by three distinct 1 μ s simulations. Therefore, the degree of cooperativity appears to be limited to the presence of calcium in the direct vicinity of D179, even without stable binding, which seems to enable the binding of calcium to pocket 1. We are investigating the interplay between site (1) and (3) as part of an independent work.

7. The role of water-mediated interactions in the allosteric mechanism is mentioned. Can the authors provide more details on the involvement of water molecules and how their presence or absence influences the enzyme's activity?

Considering the whole structure of calcium-activated EndoU, water molecules play a role in both calcium coordination and for the transmission of calcium binding signal from pocket 1 to the remote catalytic triad. For each of the four coordinated calcium, we indeed observed in the crystal structure of calcium-activated EndoU that two water molecules were directly involved in each calcium coordination network, making water likely essential for calcium binding. Regarding the allosteric activation of the catalytic triad, we have shown that one of the two calcium-coordinating water molecules in pocket 1 is also engaged by residue E290, which acts as a bridge with the catalytic histidine H285. We concluded that an intramolecular, water-mediated signaling network starting from pocket 1 was responsible for the allosteric activation of the remote catalytic triad. To support this claim, we rely on a crystal structure obtained at high resolution (1.7 Å), and the high-quality electron density map unambiguously enables the assignment of water molecules. We verified the role of water through mutating the residue E290 with an alanine, which was anticipated to break the water-mediated signaling network from calcium pocket 1 to the catalytic triad. Accordingly, we observed the loss of catalytic activity for this EndoU mutant.

8. The MD simulations describe conformational changes within 1 μ s (the production of MD simulations was carried out for 1 μ s). How does this conformational flexibility relate to enzyme activity, and does calcium binding stabilize specific conformations?

The MD simulations were conducted over a duration of 1 μ s to monitor the binding of calcium to wild-type EndoU and to observe the effects of mutating the D179 residue to alanine. According to the methods section of our manuscript, the starting model was based on the crystal structure of EndoU bound with calcium. The calcium ions were removed and reintroduced as solvent ions randomly placed within the simulation box. Therefore, the conformational changes described by comparing the homology model of human apo-EndoU to the calcium-bound crystal structure of the enzyme were not sampled in the simulation, as our focus was solely on monitoring calcium binding events to pre-formed calcium binding sites. Consequently, the MD simulations were not designed to assess whether calcium stabilizes specific conformations, which would have required using the homology model of apo-EndoU as the starting model. Furthermore, observing the stabilization or folding of specific EndoU regions upon calcium binding, as described by comparing the homology model of apo-EndoU to the calcium-bound crystal structure, would likely require significantly more than 1 μ s of classical MD simulation, or other methodologies such as REST2 or gaussian-accelerated MD. This is because protein folding events, including allosteric regulation, typically occur in the μ s-ms range. In contrast, the 1 μ s classical MD simulation was sufficient to monitor the binding of calcium ions to pre-formed binding sites, as demonstrated by the simulations. In conclusion, the MD simulations were not performed to describe intermediate-slow conformational changes, but rather to monitor the binding of calcium. Therefore, we are unable to describe how conformational changes observed in the MD simulations might relate to enzyme activity or whether calcium binding stabilizes specific conformations throughout the MD simulation.

9. The EndoU structure with accession code 9FTW is not available in the PDB database and cannot be visualized now.

We have released the EndoU structure with the accession code 9FTW, now accessible at <https://www.rcsb.org/structure/9FTW>

10. Given the enzyme's broad RNA substrate specificity, could the authors discuss the factors influencing substrate selection and the potential for catalytic promiscuity?

The topic of substrate selection and the potential for catalytic promiscuity is beyond the scope of this manuscript; however, some speculation can be raised based on the model of calcium-activated, RNA-bound EndoU included in the supplementary information. Specifically, we appended a schematic of the intermolecular contacts observed in the model of the complex (Fig. S14). We observed that base-specific contacts with EndoU were limited to U2 and U4 in the model considered. In the ensemble of models we proposed, base-specific contacts with U2 were observed in all models, while the conformational flexibility of RNA in the binding pocket was reflected by the instability of contacts with other bases, including U4. In contrast, we observed numerous non-specific contacts between EndoU and the RNA backbone, which might explain why the enzyme can accommodate a variety of substrates. As detailed in the response to point 1, the catalytic promiscuity of EndoU is proposed to play a role at the surface of the ER. Indeed, Schwarz & Blower observed that XendoU is active on the surface of ER membranes and speculated that RNAs targeted by XendoU are likely determined by their proximity to the nuclease upon calcium release from the ER. Therefore, it is likely that the broad substrate specificity of EndoU results from more non-specific contacts with the RNA backbone and stable base-specific contacts essentially limited to the buried uracil at the catalytic site, while catalytic promiscuity might play a role in the RNA clearance function of EndoU at the ER membrane, as suggested by Schwarz & Blower.

11. The manuscript mentions EndoU's relevance as a biomarker in various cancers. Could the authors expand on the potential clinical applications of these findings, particularly in diagnosing or treating diseases?

As reported in the original manuscript, EndoU was characterized as a biomarker in various cancers. In our work, we focused on the calcium-dependent activation of EndoU, and therefore did not investigate EndoU in the context of diagnosing or treating diseases. However, we can discuss a recent specific example regarding EndoU as a biomarker in Head and Neck Squamous Cell Carcinoma (HNSCC) (Xu et al., Front. Oncol., 2021). In this study, the authors identified that EndoU not only showed significantly lower expression in HNSCC cancer samples, but also a further decrease in expression levels in higher-stage cancer samples. Functioning as a tumor suppressor in this context, the Human Protein Atlas (<https://www.proteinatlas.org/>) consistently reports that EndoU is prognostic in HNSCC, with higher expression being favorable for survival.

Based on these data, and in line with our findings, we can speculate on the calcium-dependent RNase activity in this context. In cancer cells, an important hallmark is resistance to apoptosis, despite pro-apoptotic signaling. Given that higher EndoU levels are considered favorable for

survival in HNSCC, and that EndoU functions as a tumor suppressor in this context, we can speculate that EndoU could promote the apoptosis of HNSCC cells by sensing pro-apoptotic signals mediated by calcium, thereby activating the enzyme's broad RNase activity and helping to restore apoptosis in these cells. Although not activated by calcium, the role of broad RNase activity in mediating apoptosis has already been characterized, as in the case of RNase L. Therefore, our speculative answer to the reviewer's question is that EndoU might act as a tumor suppressor in HNSCC by promoting apoptosis through its broad RNase activity in response to pro-apoptotic calcium signaling.

Reviewer #4 (Remarks to the Author):

Please note we participated in the Early Career Researchers initiative and this report represents a summary of our joint review.

In this manuscript the authors used structural and biophysical approaches to investigate the calcium-dependent activation of the human EndoU nuclease. This nuclease specifically cleaves RNA in polyU sequences and has been implicated in several cellular processes but the role that calcium plays in activating this nuclease has remained unclear. First the authors investigated mouseEndoU activity in VL3-3M2 cells. Analysis of cytoplasmic RNA from WT and mouseEndoU knock out cells revealed a strong calcium activation of cleavage. Next the authors investigated how calcium influences the structure of hEndoU. Using ¹⁵N NMR the authors were able to show that saturating calcium as well as RNA + sub-saturating calcium restore well-dispersed NMR peaks. These results suggest that binding of calcium and RNA lock the enzyme in a more fixed position. Next the authors solved a crystal structure of hEndoU with excess calcium, revealing four bound calcium ions, and a putative mechanism of allosteric activation leading to a disorder-to-order transition that stabilizes the active site. The authors confirmed the significance of the calcium binding sites by generating a series of mutants and determining the effect on hEndoU cleavage in vitro. Both in vitro assays and molecular dynamics suggest that sites 1 and 3 are responsible for allosteric activation. Finally, the authors used NMR, alphafold modeling, and molecular dynamics to probe the RNA-binding interface in hEndoU. Overall, the authors work reveals an intriguing mechanism of calcium mediated allosteric activation of hEndoU. While the allosteric mechanism is well supported by structures, in vitro assays and molecular dynamics, some additional work is needed to support the modeled RNA binding interface.

We thank reviewer #4 for taking the time to carefully review our manuscript. We note that reviewer #4 could accurately summarize the essence of our work. As discussed below, we have considered every remark made by reviewer #4 and we implemented relevant suggestions when feasible, while providing consistent explanations otherwise.

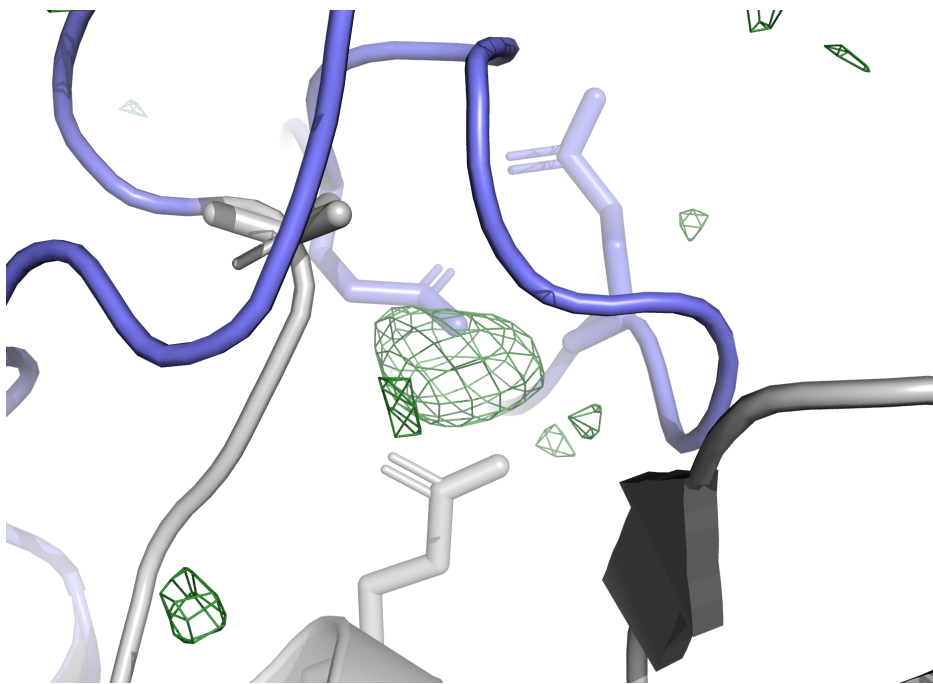
1. Figure S2 – Why do the authors not observe endonuclease cleavage products on the gels shown in panel A? Are the products too small to detect on an 8% denaturing gel? How many different cleavage products are expected for the different substrates? Will hEndoU cleave the RNA surrounding every U or only in UU regions?

The experiments shown in Fig. S2A indeed refer to RNA degradation assays performed with immunoprecipitated mouse EndoU. The sequences corresponding to the substrates are listed in Table S2. These sequences all contain several uracils, which are reported to be the favored sites for RNA cleavage; thus, we expect numerous byproducts upon RNA cleavage. However, EndoU cleavage specificity of U is not absolute, and it is known to be active on a broad variety of substrates. The strict specificity of mammalian EndoU is limited to single-stranded RNA. Establishing the relative specificity of EndoU by visualizing byproducts of RNA degradation assays, as done by others (e.g., Laneve et al., J. Biol. Chem., 2003, 2008), was performed in the presence of Mn^{2+} and not Ca^{2+} (where EndoU activity is significantly lower), and included shorter incubation times and carefully selected protein/RNA concentrations. In Fig. S2A, and more specifically in the two left panels, byproducts can be distinguished from the background, although the corresponding bands remain very weak. Thus, in our hands, in the presence of Ca^{2+} and at the conditions tested, EndoU cleaves at many sites in the RNA, leaving little observable cleavage products.

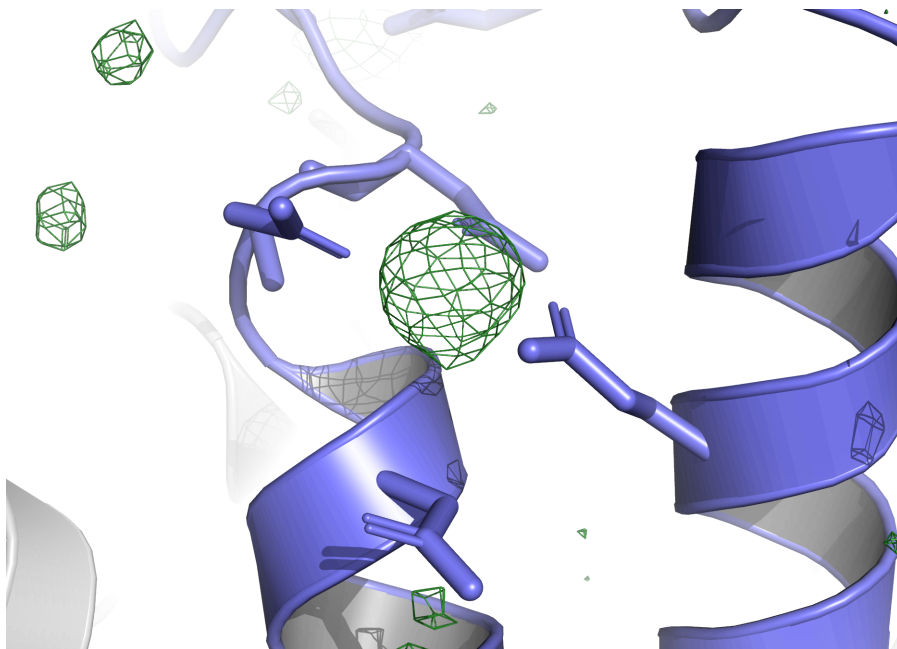
2. Figure 6S – What type of density map is shown here? The authors must show unbiased experimental density, esp for the calcium ions.

For clarity an unbiased map has been calculated and is now shown where the calcium ions have been omitted from the model prior to a novel round of refinements. A Fo-Fc map is now shown contoured at 3 sigmas.

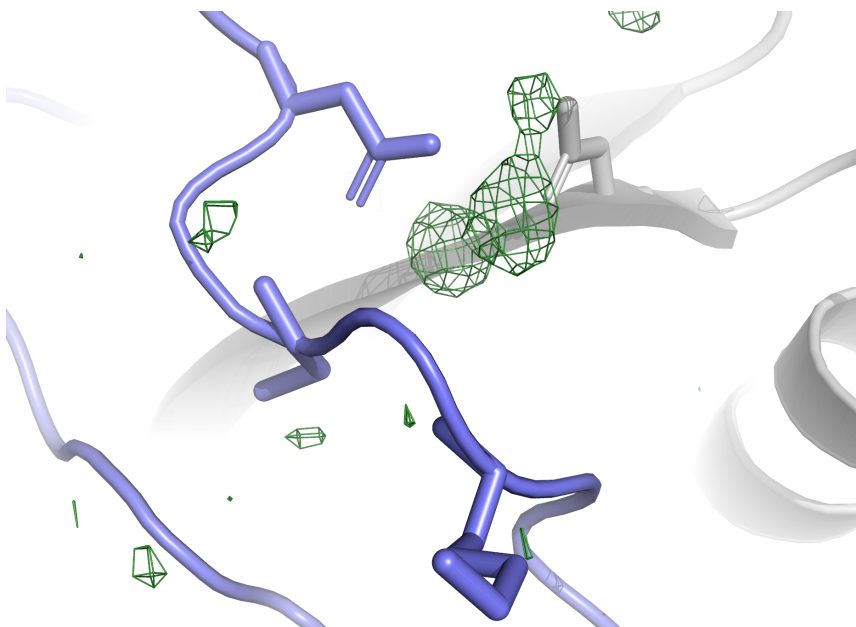
Site1



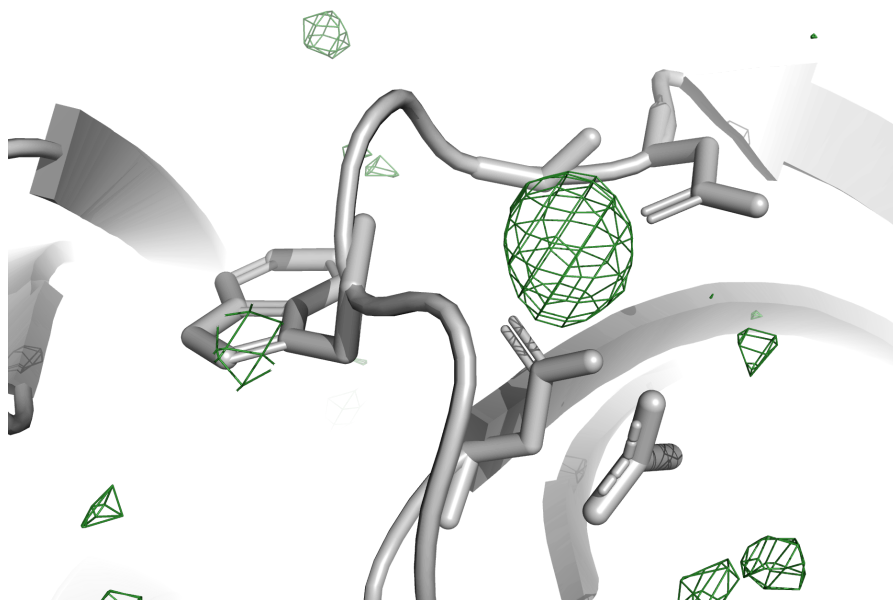
Site2



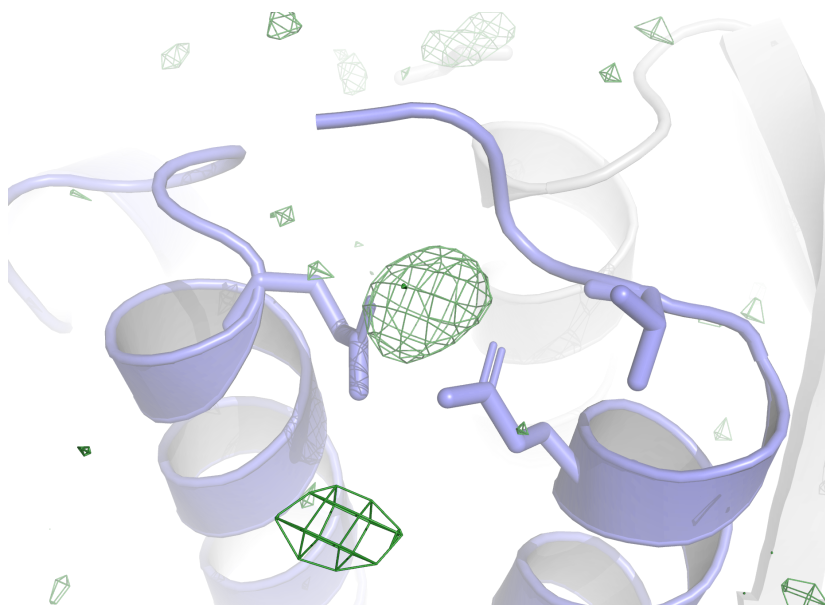
Site3



Site 4



Site 5

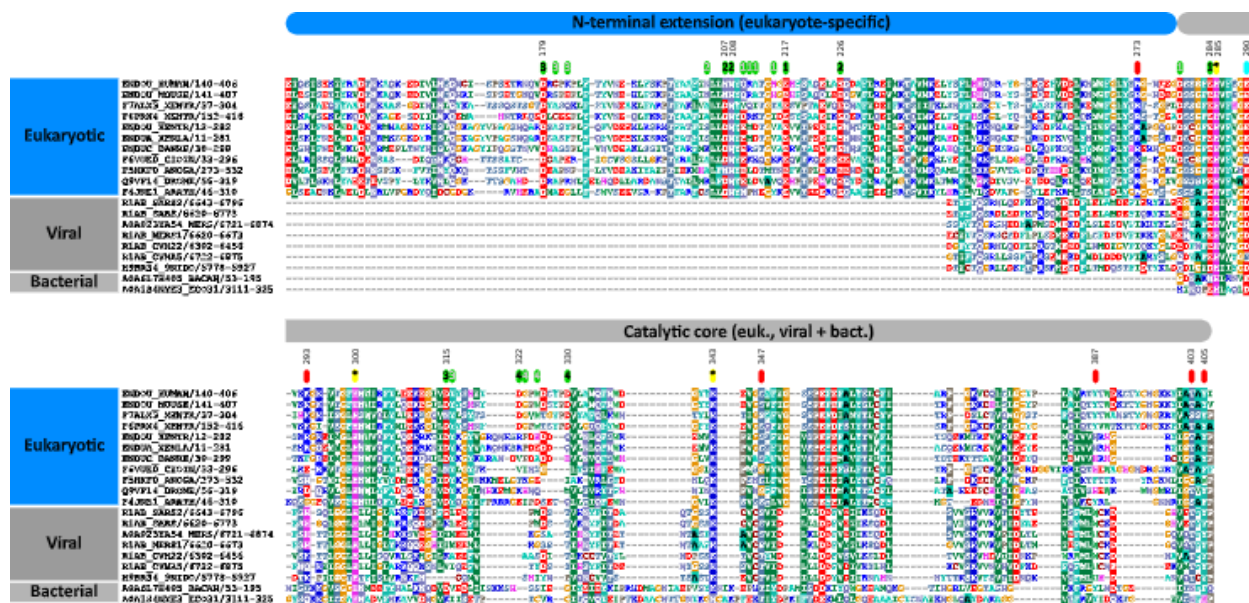


3. It would be very useful for the authors to include a supplemental figure with a comprehensive sequence alignment of different eukaryotic EndoUs, in particular how well conserved are the calcium binding sites, residue E290, putative RNA binding interfaces, etc?

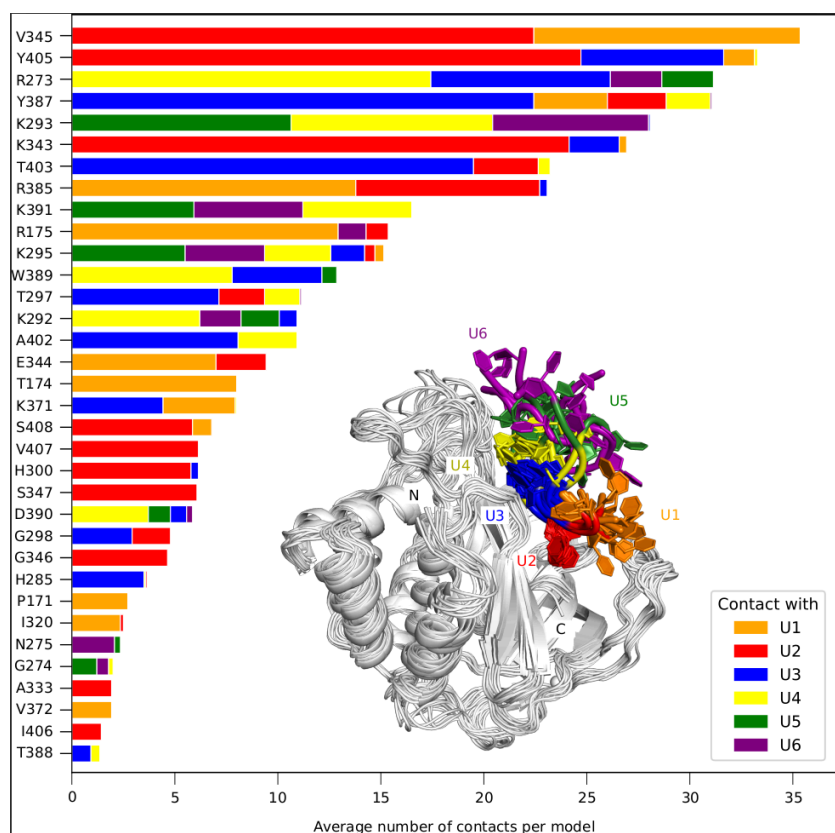
We have added a supplemental figure that includes a comprehensive sequence alignment of various eukaryotic EndoUs, as well as bacterial and viral EndoUs (Fig. S8). In this alignment, we highlighted residues associated with calcium binding sites and the allosteric network identified upon enzyme activation by calcium. Additionally, we included a supplementary figure to

separately map residues that are putatively involved in RNA binding, based on our model ensemble of calcium-activated, RNA-bound EndoU (Fig. S15).

Fig. S8 (see high resolution figure in the manuscript):



A structure-based sequence alignment of eukaryotic, viral and bacterial EndoU homologs. The human EndoU structure from this study, together with the following existing structures of eukaryotic, viral, and bacterial EndoU homologs (2c1wC, 4s1tF, 2gthA, 5yvdB, 2gtiA, 2ozkD, 6vwwA, 5hkqA), were structurally aligned (all-against-all) using the DALI server. The eukaryote-specific N-terminal extensions of the EndoU catalytic domain were aligned separately from the shorter viral N-terminal extensions. The resulting sequence alignment was manually adjusted and annotated to highlight calcium-coordinating residues (green), catalytic residues (yellow, asterisk), and selected RNA-binding residues (red). Residue E290 in hEndoU, which mediates communication between calcium-binding site (1) and the catalytic residues, is also highlighted (cyan). For the calcium-coordinating residues, the numbers 1–4 refer to the calcium-binding site numbering used in this study. Residues that coordinate calcium via their side chains (black numbers) or via their backbone (grey numbers) are distinctly highlighted. Residue numbers refer to the hEndoU sequence.

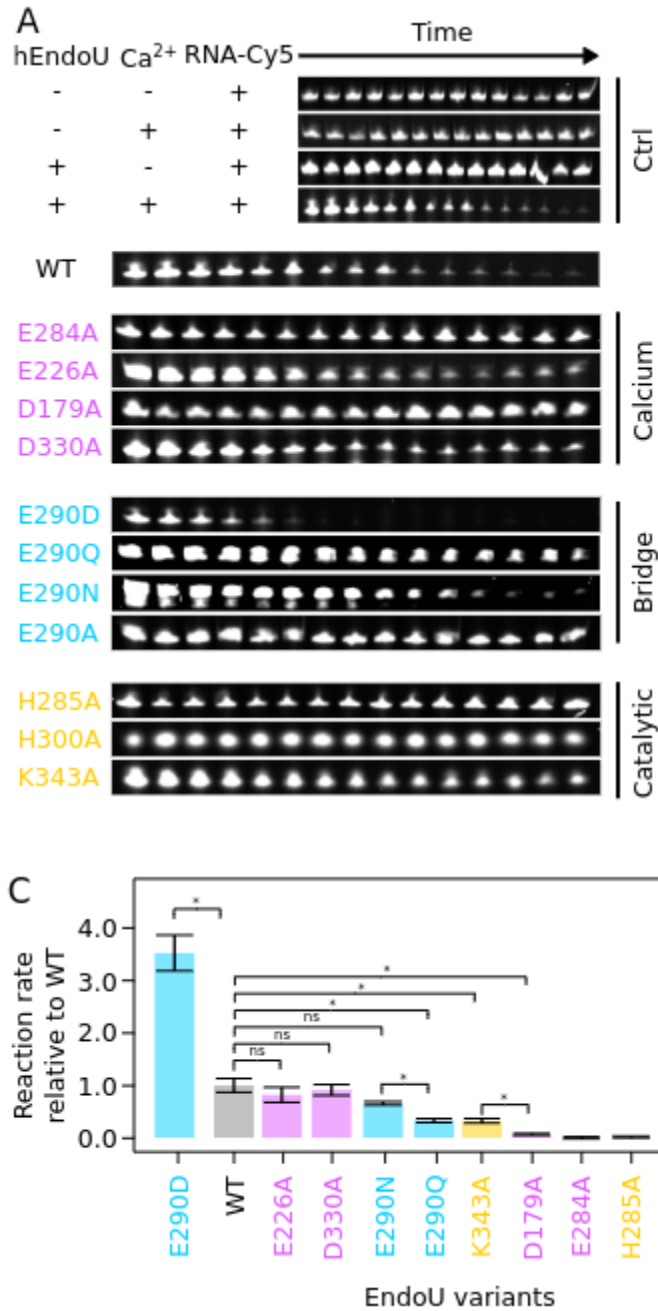


Analysis of intermolecular contacts within the ensemble of calcium-activated hEndoU in complex with RNA. For each model of calcium-activated hEndoU in complex with (U)6 RNA, we measured the distance between each hydrogen atom of the protein and each hydrogen atom of the RNA, defining intermolecular contact as a distance less than 5.5~Å. We averaged the number of contacts per model (N=14) and represented the value for each protein residue toward each base of the RNA. Residues showing fewer than one contact per model with the RNA are not shown. For ease of analysis, we display the ensemble of models for calcium-activated hEndoU in complex with RNA, highlighting bases U1-6, consistent with the plot legend.

4. Figure 3 – The top cleavage gels should be re-sized and aligned with the mutants to allow readers to better compare WT-hEndoU to the mutants. Please indicate in the figure legend how many replicates were done and what RNA substrate was used for these experiments.

To not alter the layout of Fig. 3A, we answered the need for a better comparison between WT-hEndoU and the mutants by duplicating and re-sizing the gel corresponding to WT-hEndoU. We mentioned the RNA sequence used in the caption of Fig. 3A. We also mentioned the number of replicates in the caption of Fig. 3C, since the figure corresponds to the statistical analysis of Fig. 3A.

Updated Fig. 3A, C with corresponding caption:



(A) RNA degradation assays. Comparison of mutants for calcium-binding sites (magenta), the bridging residue E290 (cyan), and catalytic residues (yellow) with wild-type hEndoU over a 2~hrs degradation assay. The RNA was labeled with Cy5 at the 3' end, with the sequence 5'-CAGGUUCCCCAACGAAAAAAAA-3'.

(C) Relative reaction rates of hEndoU variants compared to wild-type. Experiments were done in triplicate. The fitted reaction rate is expressed relative to the wild-type (plotted value), and the error in the fitted reaction rate was propagated to be expressed consistently (error bars). Statistical significance was assessed by computing a z-score for the difference between each

pair of reaction rates, adjusting for their respective errors, and then these z-score were converted into two-tailed p-values adjusted for multiple comparisons using Bonferroni correction. Statistical significance (*, $p < 0.05$) and non-significance (ns) are highlighted for a representative set of pairwise comparisons.

5. Is calcium required for RNA binding? The inclusion of some type of RNA binding experiment (with and without calcium) would add to the proposed model of allosteric activation. From the NMR experiments shown in Fig. S5 it is unclear if RNA can bind in the absence of calcium.

In Fig. S5, we demonstrate the binding of a 2'F RNA to wild-type EndoU in the presence of 2 mM calcium (S5C) and in its absence (S5D). RNA binding was detected in both scenarios, although the impact on the 1h-15N spectrum of calcium-bound EndoU was significantly stronger compared to apo-EndoU, suggesting that calcium enhances the affinity of the enzyme for RNA. Acknowledging that these simple NMR experiments may not provide the clearest evidence for this claim, we conducted EMSA experiments to demonstrate the effect of calcium on complex formation between a catalytic EndoU mutant (H285A) and a phosphodiester RNA at an equimolar ratio (Fig. S11). In the absence of calcium, no complex was detectable on the gel. Increasing the calcium concentration led to the appearance of an upper band in a dose-dependent manner, corresponding to a 1:1 complex between EndoU and the RNA substrate. The absence of complex formation by EMSA without calcium does not contradict the NMR data, as EMSA is more suitable for detecting stronger interactions, while NMR is a method of choice for detecting biomolecular interactions ranging from very weak to very strong affinities. Altogether, these results indicate that while RNA can bind to EndoU without calcium, the presence of calcium significantly promotes this interaction in a dose-dependent manner.

Of note, the enhancing effect of calcium on RNA binding is not itself responsible for the activation of the enzyme. To support this claim, we repeated the EMSA experiments with the hEndoU mutant E284A, which includes an intact catalytic triad. RNA binding to this mutant could still be easily detected by EMSA in the presence of calcium, in a dose-dependent manner. However, no RNA degradation was observed (Fig. S11B, Fig. 3A, C). This indicates that although calcium positively impacts the binding of EndoU to RNA, this binding alone is not sufficient to enhance catalytic activity, consistent with our model of calcium-dependent activation of EndoU.

Regarding the impact of calcium on EndoU binding to RNA, we added a paragraph in the result section, calcium-activated hEndoU in complex with RNA subsection:

“We established that calcium activates hEndoU by triggering an allosteric network that remotely activates the catalytic triad. Without calcium, binding of 2'F RNA showed limited impact on the 15N SOFAST-HMQC spectrum of hEndoU (Fig. S5D). Repeating the experiment with phosphodiester RNA yielded similar results, indicating weak interaction in the absence of calcium (Fig. S10A). Consistent with our calcium-dependent activation model, this weak binding did not lead to RNA degradation (Fig. 2, 3). To assess the impact of calcium on RNA binding, we used EMSA with the catalytically dead hEndoU variant H285A, which still binds calcium, and

a phosphodiester RNA containing a single uridylate (Fig. S9B, S11A). Without calcium, EMSA did not detect the weak interaction observed by NMR, likely due to its inability to detect complexes with fast dissociation rates. However, increased calcium concentrations strongly populated a low molecular weight (LMW) complex and weakly populated a high molecular weight (HMW) complex (Fig. S11A). A control experiment, in which the single uridylate was replaced by another nucleotide, resulted in a significant increase in free RNA and a weakly populated LMW complex (Fig. S11B). Interestingly, we observed calcium-dependent RNA binding to the hEndoU E284A mutant, despite its lack of catalytic activity (Fig. S11C, 3A,C). This indicates that enzyme activation does not result from the calcium-dependent enhancement of hEndoU interaction with RNA, consistent with our model (Fig. 2C). Altogether, we conclude that calcium promotes the formation of an uridylate-specific complex, but also of higher order complexes due to non-specific secondary binding to other nucleotides.”

Fig. S11:

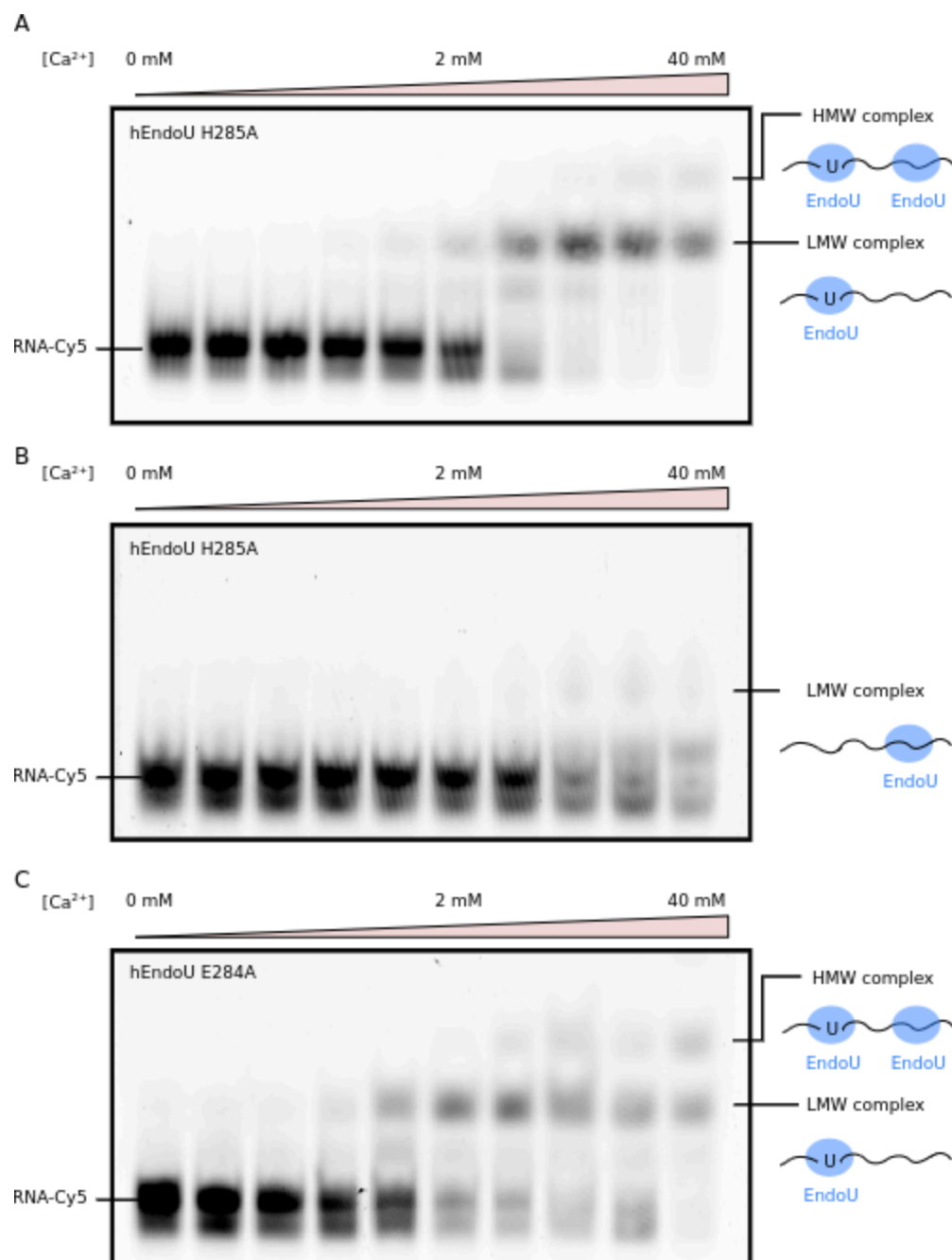


Figure S11: Impact of calcium on RNA binding monitored by EMSA. (A) Binding of a catalytically dead hEndoU mutant (H285A) to a 3'-labeled Cy5 RNA containing a single uridylate with the sequence 5'-CACGAGUACCAGAAAACCAAAA-3'. (B) Control experiment to monitor the binding of hEndoU H285A to a 3'-labeled Cy5 RNA that does not contain an uridylate in the sequence 5'-CACGAAAACCAGAAAACCAAAA-3'. (C) Binding of a calcium-binding hEndoU mutant (E284A) to a 3'-labeled Cy5 RNA containing a single uridylate with the sequence 5'-CACGAGUACCAGAAAACCAAAA-3'. The samples were incubated for 2~hours at room temperature before being loaded onto the gel. For (A, C), the low molecular weight (LMW) complex refers to specific hEndoU binding to uridylate, while the high molecular weight (HMW) complex refers to non-specific secondary binding of hEndoU to a non-uridylate base. For (B),

the LMW complex refers to non-specific hEndoU binding to a non-uridylate base, since the control RNA does not contain an uridylate.

6. How many times were the molecular dynamics simulations run? The simulations should be repeated at least 3 times to ensure consistent results.

All inputs for the MD simulations were prepared using the CHARMM-GUI interface. In the original manuscript, we conducted two simulations to monitor the binding of calcium to the wild-type EndoU or the D179A mutant. Each of these simulations was performed in triplicate by varying the seed from the first NVT equilibration step. We have updated the methods section to explicitly state that these simulations were conducted in triplicate and that calcium binding events were consistently detected across the replicates:

“To monitor the binding of calcium to apo-hEndoU, we removed calcium ions from the crystal structure and used the resulting structure as input. Calcium ions were then reintroduced into the system as salt ions, and simulations were run in triplicate. Calcium binding events to hEndoU were consistently detected across the replicates.”

For the MD simulation used to derive an ensemble of models for the calcium-activated, RNA-bound EndoU, we did not replicate the experiment. The purpose of this experiment was to qualitatively explore the conformational flexibility of the RNA in the binding pocket of EndoU. We were not examining a precise stochastic event, such as calcium binding, but rather the fast conformational sampling of the RNA, aiming to obtain clusters based on more populated RNA conformations. Although we fully agree with Reviewer #4 that replication of MD experiments is essential to obtain reliable results in general, we believe that in this specific case, it is not absolutely necessary since our interest lies in the rapid conformational sampling of the RNA. Additionally, this MD simulation was performed with the goal of refining the AlphaFold3 model, which lacks information on dynamics at the molecular interface, as suggested by the NMR data. Finally, we performed a qualitative analysis of the ensemble of models, with no intention of elaborating on a quantitative description.

7. The authors NMR/modeling/dynamics suggests several putative RNA interfaces around the active site and a basic-beta sheet groove. The authors should confirm the significance of several of these residues through mutagenesis. Are these residues essential for RNA binding and/or cleavage activity? It would also be of interest if the authors could comment on if this work reveals new insight into Uracil specificity. In particular the authors show some base-specific interactions with U2 and U4 in Fig. S11.

Fig. S11 in the original manuscript corresponds to Fig. S14 in the revised version.

In Fig. S14, we illustrate the interaction between EndoU and RNA based on one model from the ensemble we proposed. In this model, we observed base-specific contacts with U4 and U2. Base-specific contacts with U2 are detected in every model from the ensemble, while those with U4 depend on specific models due to the conformational flexibility of the RNA within the binding

pocket. By limiting the base-specific contacts to U2, we have demonstrated that mutating H300 and K343 leads to either complete abrogation or a substantial reduction of catalytic activity, respectively, indicating that these residues are essential for at least the cleavage activity. Next, we used a catalytically dead hEndoU mutant (H285A, see Fig. 3) as background to further mutate residues K343, S347, R385, Y405, and Y387 to assess the impact of these mutations on RNA binding by EMSA (Fig. 4E-F). Correspondingly, we show below the text we added to the manuscript:

In the method section, Electrophoretic Mobility Shift Assay subsection:

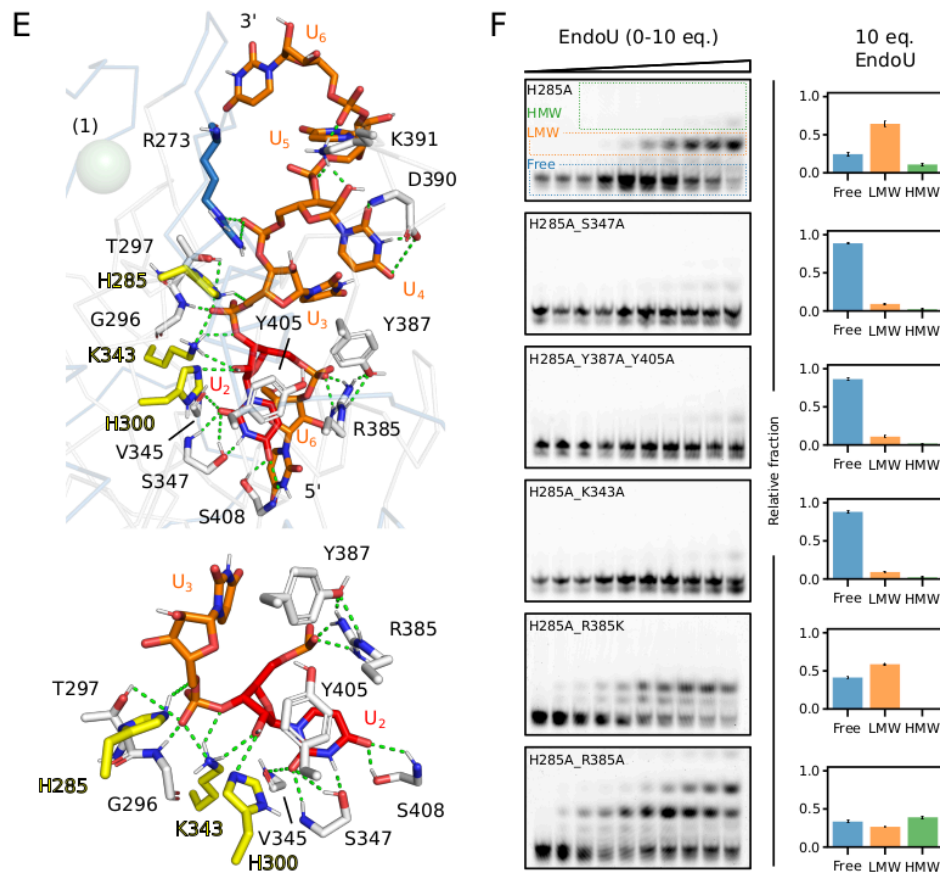
Electrophoretic Mobility Shift Assays (EMSA) were conducted to assess the impact of calcium and mutations on RNA binding to EndoU. We prepared 8% acrylamide/bisacrylamide (19:1) non-denaturing gels in 0.5× TBE (44.5 mM Tris base, 44.5 mM boric acid, 1 mM EDTA) supplemented with 5% (v/v) glycerol. Since calcium was used in our assays, we initially attempted to use an EDTA-free 0.5× TB buffer, but this resulted in inconsistent results, possibly due to trace amounts of heavy metals in the molecular biology-grade glycerol (Sigma-Aldrich G5516) and Tris base (ThermoFisher 17926). Consequently, we used 0.5× TBE. For the EMSA experiments, samples were prepared in a 20 μ L reaction mix with the following buffer composition: 10 mM Tris base (pH 7), 50 mM NaCl, 0.5 mM TCEP. We used a 3'-labeled Cy5 RNA probe containing a single uridylate with the sequence 5'-CACGAGUACCAGAAAACCAAAA-3', at a concentration of 250 nM. To prevent enzymatic degradation of the probe, we used a catalytically inactive hEndoU mutant (H285A), which also served as a background to establish RNA-binding mutants of hEndoU. To assess the impact of calcium on RNA binding, we prepared an equimolar mix of the RNA and hEndoU (H285A), supplemented with increasing concentrations of calcium as follows (mM): 0, 0.1, 0.3, 0.5, 1, 2, 5, 10, 20, 40. To monitor the impact of mutations in hEndoU on RNA-binding activity, we prepared samples containing the RNA probe (250 nM) and calcium ions (2 mM), supplemented with increasing amounts of hEndoU variant as follows (eq.): 0, 0.25, 0.5, 0.75, 1, 2, 3, 5, 10. For all experiments, 20 μ L of loading buffer (50% glycerol v/v, orange G) were added to each sample prior to loading on the gel. All samples were prepared at room temperature and loaded on the gel immediately upon preparation unless specified otherwise. Electrophoresis was conducted at 100~V for 45 minutes at room temperature. Each gel was subsequently scanned with a fluorescence scanner. We used GelAnalyzer software to integrate band intensities to assess the impact of hEndoU mutations on RNA binding. To this end, bands on the lane corresponding to a 1:10 RNA:protein ratio were integrated and expressed as relative fractions of signal for the free RNA, the low molecular weight complex indicating specific binding to uridylate, and the high molecular weight complexes indicating non-specific binding to other bases.

In the result section, Calcium-Activated hEndoU in Complex with an RNA Analog subsection, third paragraph:

To further validate our model ensemble, we conducted mutagenesis experiments targeting residues interacting with U2 near the active site (Fig. 4E, S14, S15), which showed consistent

contacts across models with an RMSD of 0.73 $\pm$ 0.35 Å (Fig. 4D, E). We selected K343, S347, R385, Y387, and Y405, and generated mutants in the catalytically dead hEndoU H285A background (Fig. 2A, C), evaluating RNA binding via EMSA in the presence of calcium (Fig. 4F). The hEndoU H285A variant showed consistent RNA binding, with a stoichiometric complex corresponding to uridylyte-specific binding, and higher order complexes corresponding to non-specific secondary binding. Mutations S347A and K343A were expected to disrupt electrostatic interactions with U2, reducing RNA binding. Consistent with our model, double mutants H285A/S347A and H285A/K343A showed dramatically reduced RNA binding. Similarly, the triple mutant H285A/Y387A/Y405A exhibited weak RNA binding, possibly explained by loss of π -stacking between Y405 and U2 and destabilization of R385 conformation due to the absence of the Y387 hydroxyl group (Fig. 4E). The conservative mutation R385K (H285A/R385K) had limited impact on RNA binding. Unexpectedly, the double mutant H285A/R385A, which should reduce RNA binding, showed increased secondary binding at high protein:RNA ratios, suggesting enhanced binding to non-uridylyte bases. Our findings demonstrate that specific residues at the hEndoU–RNA interface are essential for substrate binding, providing further experimental evidence supporting our AI/MD-derived model.

Fig. 4E-F:



(E) Detailed view of a representative model of the ensemble with highlights on protein-RNA interactions. A close-up view of interactions at U2-U3 is also shown.

(F) RNA binding to calcium-activated hEndoU variants monitored by EMSA. The regions corresponding to the free Cy5-labeled RNA, the low molecular weight complex (LMW), and the higher molecular weight complexes (HMW), are highlighted. Experiments were done in triplicate. For each experiment, the relative fraction of Free RNA, LMW, and HMW complexes were computed for each replicate, and the resulting means (plotted values) and standard deviations (error bars) were plotted at 10 eq. hEndoU.

Altogether, these data support the RNA-binding interface we described through NMR, modeling, and dynamics. We also detected specificity for uridylate in Fig. S11A,B. Unfortunately, our work was not designed to give specific insights into the specificity of EndoU for uracil. We believe that a separate study investigating the molecular basis of RNA recognition by calcium-activated EndoU would be necessary to obtain data suitable for explaining or hypothesizing how EndoU could specifically recognize U-containing sequences.

8. The authors should cite and briefly discuss the recent work of Huang et al JBC, 2023 and Wang et al NAR, 2024 which suggests that divalent ion activation by Nsp15 is substrate dependent. This is a different mechanism from the allosteric activation model presented in this manuscript.

As suggested by reviewer #4, we cited the two papers in the discussion section and briefly discussed them in relation to our findings, as follows:

“Consistently, recent in vitro studies demonstrated that Nsp15 retains some activity on single-stranded RNA without divalent metal ions, although manganese increases this activity in a substrate-dependent manner (Huang et al. JBC, 2023; Wang et al. NAR, 2024). Other reports also found enhanced RNA binding by Nsp15 in the presence of manganese (Bhardwaj et al. J. Mol. Biol. 2006) and suggest that manganese may provide charge shielding to promote Nsp15 interactions with the RNA (Frazier et al. NAR, 2021), although it has also been proposed that manganese stabilizes an alternative enzymatic state with a higher cleavage rate (Huang et al. JBC, 2023).”

Minor Issues

1.Introduction, first paragraph – “RNases can be constitutively active (RNase A, angiogenin)”. New work from Korostelev and colleagues (Loveland et al Nature, 2024) suggest that angiogenin is activated by binding to the ribosome.

We removed “angiogenin” from “RNases can be constitutively active (RNase A, angiogenin)”.

2.Introduction, second paragraph – “Xendo U cleaves single-stranded RNA preferentially at 5' of uridylates”. Ref 15 does not show cleavage is 5' of uridylates but rather that it requires two U residues.

We are sorry for the confusion, as the cleavage activity at 5' of uridylates was related to hEndoU and reference Laneve et al. JBC, 2008.

We corrected as follows in the second paragraph of the introduction section:

"In vitro studies demonstrated that **XendoU** is an endonuclease that cleaves single-stranded RNA with **poly(U)-specific** activity (Laneve et al. JBC, 2003)."

"In vitro studies have shown that **hEndoU** cleaves single-stranded RNA preferentially at **5' of uridylates** (Laneve et al. JBC, 2008)."

3.The XendoU versus EndoU nomenclature was a bit hard to follow throughout the text. The authors should consider referring to human endoU as hEndoU (and mouse as mEndoU) throughout the manuscript to make it clear when they are referring to human EndoU and not another EndoU enzyme.

We implemented the hEndoU and mEndoU nomenclature throughout the manuscript to avoid ambiguities, as suggested by reviewer #4.

4.Page 16, first paragraph "mutation of two critical residues" – please specify which active site residues were mutated here.

We explicitly mentioned the residues, as suggested by reviewer #4:

"Based on previous studies (Laneve et al. JBS, 2008), the mutation of two critical residues in mEndoU (**E285A/H286A**) abolished the enzymatic activity (Fig. 1D), confirming the role of mEndoU in RNA cleavage."

5.Figure 1D – CD-HA Please define this in the figure legend including the residues that were mutated.

We defined CD-HA in the legend of Fig. 1D and we included the residues that were mutated:

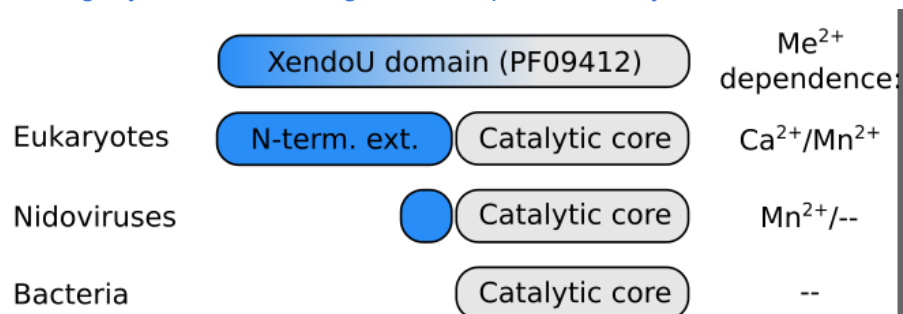
"(D) Cleavage activity of WT mEndoU (WT-HA) and the catalytically dead (CD-HA) mEndoU double mutant E285A/H286A."

6.Figure 1 F. The comparison between different EndoUs is confusing and not to scale. For example, Nsp15 from coronaviruses has two additional domains (N-terminal and Middle domain) with no similarity to hEndoU but the overall length of the protein is almost the same size.

Fig. 1F was intended to present the differences in topology for XendoU domains found in eukaryotic, viral, and bacterial EndoUs. Therefore, the purpose of Fig. 1F is not to highlight the additional domains that can be found in Nsp15 or other specific EndoUs. The XendoU domain

(Pfam 09412) is composed of a C-terminal catalytic core conserved in eukaryotes, Nidoviruses, and Bacteria. In eukaryotes, a long N-terminal extension is also conserved, while a short N-terminal extension is conserved in Nidoviruses.

We slightly modified the Fig. 1F for improved clarity:



Of note, the length of the N-terminal extension and catalytic core in XendoU domains found in eukaryotic EndoUs are comparable, hence they are represented with equal length. The N-terminal extension, which consists of an alpha-helical bundle, is short in viral EndoUs compared to eukaryotic EndoUs, hence the corresponding region is represented as short compared to eukaryotic EndoUs.

To avoid confusion and ambiguities, we also modified the figure caption as follows:

“(F) Topology of XendoU domains across phyla. The XendoU domain (PF09412) consists of a C-terminal catalytic core that is conserved across phyla. It features a long N-terminal extension specific to eukaryotes and a short N-terminal extension specific to nidoviruses. In eukaryotes, the presence of the N-terminal extension correlates with a dependence on metal ions..”

7. Page 16, second paragraph “we expressed the XendoU catalytic domain of human....” – This makes it sound like the construct used does not include the N-terminal extension important for calcium binding. Be specific with residue numbers in the results to make it clear this includes the N-terminal extension.

We added the residue numbers as follows:

“We expressed the XendoU catalytic domain (Fig. 1D) of hEndoU (135-410) with [...]”

8. Fig S2B – What substrate was used for these cleavage assays? Please define this in the figure legend.

We updated Fig. S2B caption to:

(B) Cleavage activity in the presence of divalent metal ions. The substrate sequence is as follows:

GATGTrGrArCrArGrCrArUrUrGrCrArArCrCrGrArUrCrCrCrArArCrCrUrGrArArArUrGrUrCrCrUr

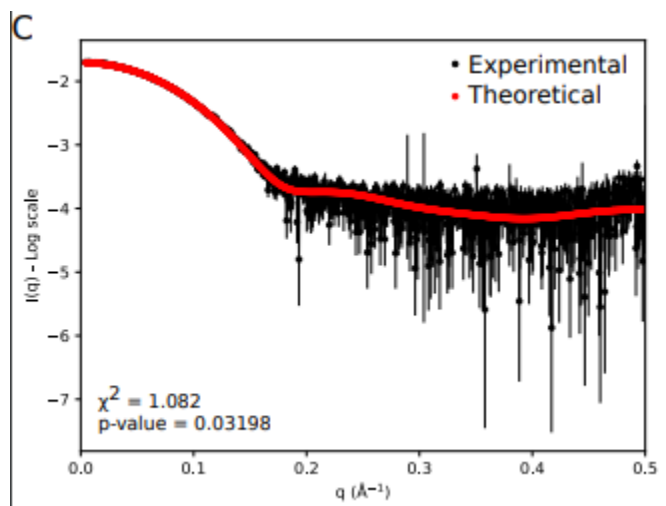
CrGrGTTGGC where G,A,T,C denotes deoxyribonucleotides, and rG, rA, rU, rC denotes ribonucleotides.

9.Fig. S3 – Please include the chi-squared value in panel C to indicate how well the experimental and theoretical curves overlap.

We added Chi-squared and associated p-value in panel C. Values are also reported below:

Chi-square of fit : 1.082
Probability of fit : 3.198E-02

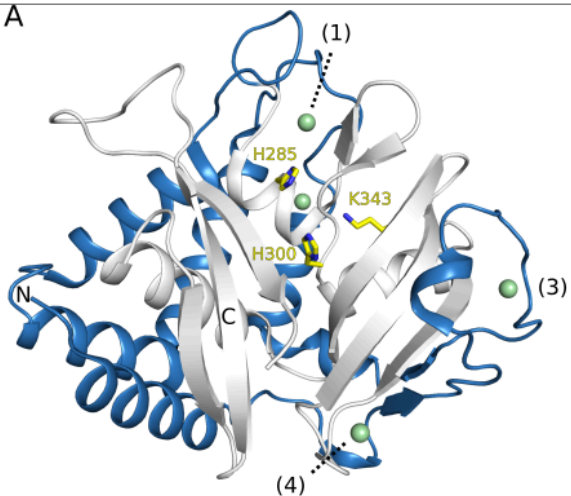
Given the Chi-square of fit and associated p-value (<0.05), we consider that the model fit is statistically significant with regards to the observed data.



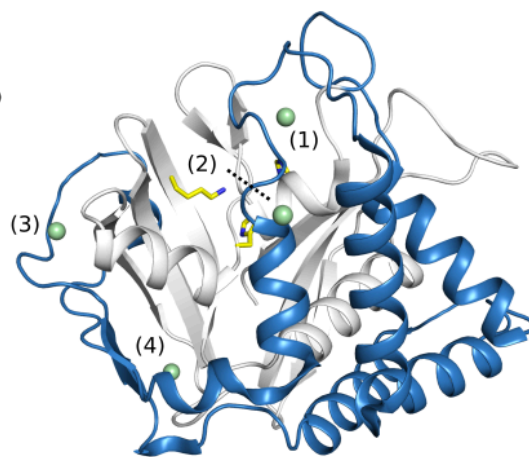
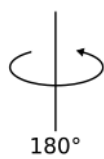
10.Fig 2A – It would be helpful to indicate the position of the active site in the top panel.

We indicated the position of the active site on Fig. 2A by highlighting catalytic residues as follows:

A



● N-terminal extension ● Catalytic core



● Catalytic residues ● Ca^{2+} ● Water

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In their revision, the authors have addressed many of the previous comments. Especially the added biochemical experiments are insightful.

However, some issues remain, were not addressed (e.g. due to experimental difficulties) or were sidelined in the discussion of the previous reviewers' comments. In particular, comparison with previous structures of EndoU homologs are not sufficient, especially structures with substrate (or analogs) were not compared, which might substantiate the proposed active site/RNA binding site placements.

>>> We thank reviewer #1 for taking the time to further review our work. We believe we addressed the remaining remarks in the current version.

Active site of EndoU and substrate binding region:

The proposed active site of human EndoU and the published active site of *Xenopus* EndoU are in different positions. This is not based - as the authors state - on a misunderstanding but rather the statements of a previous publication (PNAS Renzi et al. 2006) and on structural superposition of the deposited human EndoU structure of the authors (PDB 9ftw) and the published *Xenopus* EndoU structure (PDB 2c1w), which has a phosphate (i.e. substrate analog) bound. This means (as already stated in the first review) *Xenopus* EndoU active site = H162, N270, H272, T278 (see PNAS Renzi et al. 2006, where these residues were suggested as active site and some of them were also mutated to confirm importance for catalysis); while human EndoU = p.8 "catalytic triad (H285, H300, K343)". The only residue that is maintained between these two assignments both by location and by type is H162 (X.l.) vs. H285 (H.s.). This means, either the previous assignment in *Xenopus* EndoU is wrong or the current one is wrong.

>>> We thank reviewer #1 for clarifying the initial remark and we now agree on the relevance of the point raised by the reviewer. We apologize for not having understood this point during the first review.

>>> Based on the previous publication mentioned by reviewer #1 (Renzi et al. PNAS, 2006), we agree that a phosphate binding site was proposed, involving « conserved residues: H162 (2.7–3.7 Å), T278 (2.7–3.6 Å), H272 (2.0–2.8 Å), and N270 (3.2–4.5 Å) and one of the conformers of R149 (2.3–3.3 Å) ».

>>> Based on Fig. 2 (Renzi et al. PNAS, 2006) and Fig. S8 (our work), it is clear that H270 and N270 in XendoU are not conserved, in contrast to what authors report. It is therefore extremely unlikely that H270 could act as a catalytic histidine, as the residue is only found in *Xenopus* EndoU (Fig. 2, Renzi et al. PNAS, 2006).

>>> However, residue H162 in XendoU is highly conserved, and corresponds to H285 in hEndoU. Consistently, mutations H162A in XendoU (Gioia et al. JBC, 2005) or H285A in hEndoU (our work) abrogates enzymatic activity, which aligns with the proposed role of the residue in catalysis.

>>> In our assignment, we established that mutation H300A in hEndoU abrogates enzymatic activity. This residue is conserved across phyla, from XendoU (H178) to viral Nsp15 (H250). Moreover, Gioia et al. JBC, 2005 established that H178 was essential for RNA cleavage, which was not recapitulated in the phosphate binding site proposed by Renzi et al.

>>> Overall, we believe that the phosphate binding site proposed by Renzi et al. does not reliably represent the catalytic site of the enzyme. The fact that they present H272 in XendoU as conserved is wrong based on their own data, and this H272 could not be evaluated in the context of hEndoU, simply because it does not exist in the protein.

>>> We added the following in the first paragraph of the discussion section:

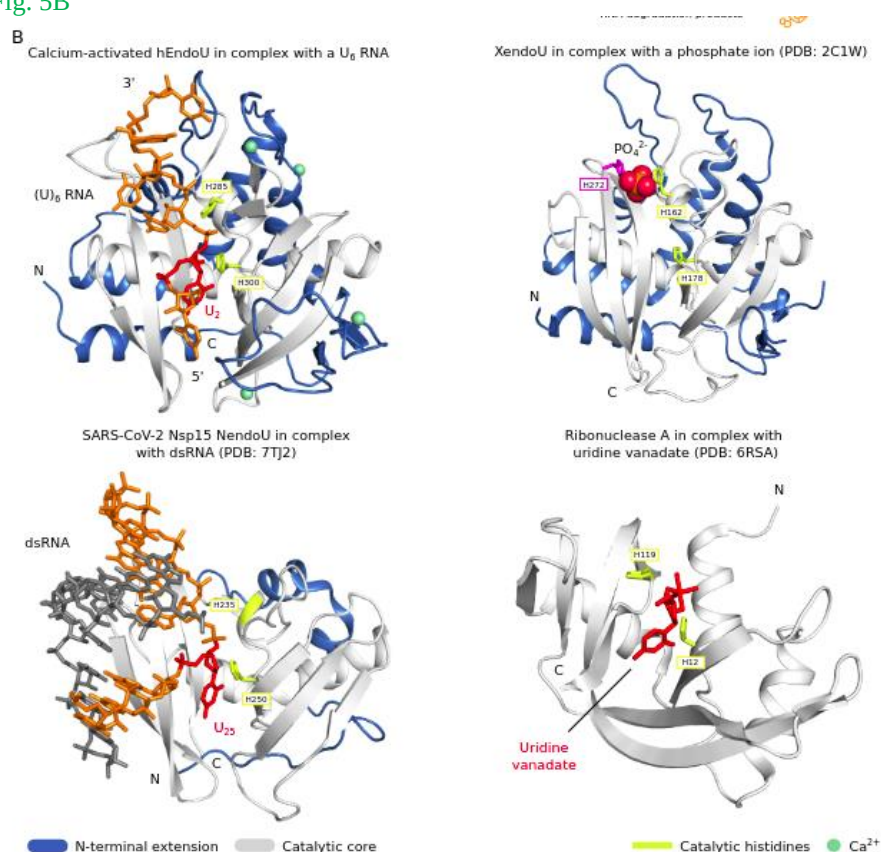
« Of note, the crystal structure of XendoU bound to a phosphate ion suggests that residues H162 and H272 could define the catalytic site (Renzi, PNAS, 2006) (Fig. 5B)). However, H272 in XendoU is not

conserved, and other studies have proposed the universally conserved H162 and H178 instead (Gioia, JBC, 2005), in line with our findings of catalytic H285 and H300 in hEndoU. »

The authors so far omit *Xenopus* EndoU-PO₄ from structural superpositions that show active site comparison with human EndoU (Fig. S16). They also omit structural comparisons of substrate/analog bound EndoU homologs (e.g. Nsp15 with dsRNA PDB 7tj2, *Xenopus* EndoU with phosphate PDB 2c1w, RNaseA with uridine vanadate PDB 6rsa). These comparisons should be included. They would be useful and could support their own data especially since their manuscript relies on modelling and broadly localized NMR peak shifts for identification of substrate binding sites.

>>> We replaced Fig. 5 B with the structure comparison proposed by reviewer #1.

Fig. 5B



(B) A representative model of calcium-activated, RNA-bound hEndoU is shown alongside the crystal structure of apo-XendoU bound to a phosphate ion, the cryo-EM structure of SARS-CoV-2 Nsp15 NendoU in complex with dsRNA, and the liquid-state structure of ribonuclease A in complex with uridine vanadate. For each model or structure, the catalytic histidines are highlighted (yellow). For the crystal structure of apo-XendoU, the residue H272, which was assigned as part of a phosphate binding site in a previous study (Renzi et al, PNAS, 2006), is highlighted (magenta). For hEndoU, XendoU, and Nsp15, the N-terminal extension upstream of the catalytic core is highlighted (skyblue). For the model of calcium-activated, RNA-bound hEndoU, calcium ions are shown (palegreen).

Disorder to order transition:

Counter to what is currently mentioned in the rebuttal letter and text, disorder to order transitions do not mean that a protein region needs to take up an alpha-helical/beta-strand secondary structure. It rather means that it goes from high flexibility (i.e. many transitions between different conformational states) to a more stably populated conformational state. This can also happen in an ordered loop, a long peptide stretch without secondary structure elements or a region that goes from flexible random coil to secondary structure element.

The text in the beginning of p.9 is misleading and should be amended: "In apo-hEndoU, the loop containing E290 does not have a defined secondary structure and is likely flexible in solution. Thus, calcium binding correlates with a disorder-to-order transition in this loop, forming a β -hairpin and stabilizing the catalytic site (Fig. 2 D)." The corresponding E290 residue (X.l. E167) in *Xenopus* EndoU is part of a well structured loop and forms multiple contacts with other residues, it is neither disordered nor flexible. Furthermore, based on which experimental evidence do the authors claim that it is "likely flexible in solution", especially if - as they state in their rebuttal letter - "Specifically, for residues E290 and H285, the corresponding crosspeaks are located in the central crowded region of the 1H-15N spectrum of calcium-bound EndoU, and we could not identify corresponding crosspeaks in the spectrum of the apo-state due to the limitations mentioned above."?

>>> We thank reviewer #2 for emphasizing on this particular point. As the term « disorder-to-order » transition is neither central or critical to the nature of our finding, and although this question could be debated more extensively, we decided to stick to the reviewer comment and we updated the text as follows:

« In apo-hEndoU, the loop containing E290 does not have a defined secondary structure, and calcium binding correlates with the formation of a β -hairpin in the vicinity of the catalytic site (Fig. 2D). »

Figures:

Fig. 2C: orphan white box on blue helix in bottom left

>>> Corrected.

Reviewer #2 (Remarks to the Author):

This reviewer greatly appreciates the revisions made by the authors. In particular, this reviewer agrees with the authors' assertion that "the activation mechanism of EndoU is calcium-dependent and does not involve RNA." However, as noted by this reviewer (and Reviewer #1) during the initial review process, the following critical aspect still requires further clarification.

The authors state that EndoU is specifically activated by calcium, but the molecular mechanism explaining why ONLY calcium triggers activation, while other metal ions do not, has not been demonstrated. This key point is not addressed explicitly in the manuscript at all.

The explanation provided in response to Reviewer #1 regarding the observed electron density in the structural analysis being attributed to calcium is satisfactory. However, this explanation does not shed light on the molecular mechanism underlying EndoU activation by calcium. Although the current manuscript title, "Molecular Basis for the Calcium-Dependent Activation of the Ribonuclease EndoU," implies that the molecular mechanism of calcium-specific activation has been elucidated, the reviewer feels that the manuscript does not provide sufficient molecular evidence to support this claim.

Finally, while the discussion of intracellular calcium concentrations, provided in response to Reviewer #3, is appreciated, this reviewer believes that these details do not offer molecular evidence explaining the specificity of EndoU activation by calcium.

>>> As noted by reviewer #2, the manuscript is entitled "Molecular Basis for the Calcium-Dependent Activation of the Ribonuclease EndoU" and is therefore focused on establishing the mechanism for calcium-dependence, and not specificity. To make this clear, we added the following in the first paragraph of the discussion section:

«Although we unveiled the molecular basis for the calcium-dependent activation of hEndoU, it should be noted that we did not establish the molecular basis explaining the specificity of hEndoU activation by calcium ions.»

Reviewer #3 (Remarks to the Author):

The authors have addressed my initial comments and satisfactorily resolved my concerns. I recommend the revised manuscript for publication.

>>> We thank reviewer #4 for the feedback.

Reviewer #4 (Remarks to the Author):

Please note we participated in the Early Career Researchers initiative and this report represents a summary of our joint review.

The authors have adequately addressed our previous concerns and those of the other reviewers. We appreciate the authors careful and thorough responses in the rebuttal. The additional assays including RNA binding experiments and generating a series of RNA binding mutants help strengthen the proposed model of calcium dependent activation. The additional sequence and structural comparisons also further highlight how this mechanism is distinct from other EndoU enzymes such as Nsp15.

>>> We thank reviewer #4 for the feedback.

Minor Note – There appears to be an issue with black boxes in Fig. 5A but this is probably a file conversion error.

>>> We could not locate this problem on original figure files, but we will pay attention.

Reviewer #5 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.