nature portfolio

Peer Review File

Structures of African swine fever virus topoisomerase complex and their implications

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In “Complex structures and implications of African swine fever virus topoisomerase”, by Yang et al., the authors deepen the knowledge on pP1192R, a unique type II DNA topoisomerase coded by the African Swine Fever virus, by gaining new structural and mechanistic insights on how this enzyme works. It is a very welcome addition to the recent publication of pP1192R’s cryo-EM structures (doi: 10.1128/mbio.01228-23), which it complements and is already acknowledged by the authors. The structures obtained in both cases are similar, which strengthens both findings and respective methodologies.

The manuscript is clear and most of the conclusions are sound and justified by the results shown.

Still, while I have no doubt about the merits of the work and that it deserves to be published, I do have some questions about the work and think it needs a revision before acceptance. Furthermore, minor improvements and changes should be made and some information added.

Doubts and suggestions concerning the main text:

- “Dimerization is mainly mediated the GHKL subdomain. -> it seems that a “by” is missing between “mediated” and “the”

- “With respected to the central region…” -> “respect”

- “Structural analysis showed that, at the tilting point, the side chain of one Pro residue (Pro852) deeply inserts into the minor groove, which may play critical role in the bending of DNA1.” -> indication of Fig. 2C should be added at the end of the sentence

- section “T-DNA is bound by AsfvTopII in an asymmetric manner” -> in my opinion, it is difficult to understand, with only what is shown and described by the authors, why the interaction of AsfvTopII and the T-DNA is assymetric, i.e., why there are only 3 interacting regions and not 4, with the coiled-coil arm of the partner molecule also interacting the the T-DNA (as suggested to happen in Fig. 4A); a panel showing the interaction from a different angle, and a more detailed explanation should be added to clarify this point and justify the authors’ claim

- “However, carefully analysis…” -> “careful”

- all claims related to Fig. S4 are impossible to judge given that there is no Fig. S4 (see below my point concerning this)

- “One Mg2+ ion is also captured at the catalytic site of the partner molecule (Fig. S8A-B), coordinating with the side chains of Glu438, Asp539 and Asp541, the main chain of Lys615, and the phosphate group of nucleotide C12.” -> why is Glu438 indicated as participating only in one of the AsfvTopII molecules and not both?

- “WT AsfvTop Ⅱ was used as the temple, the primers” -> correct temple to template

Concerning the Figures included in the manuscript:

- why do the authors present their structures reversed (upside-down) when compared to the vast majority of TopoII structural representations? This makes figure side-by-side comparison harder.

- Figures S4 and S6 are identical; looking at other sections of the manuscript, namely the M&Ms, I can conclude that Figure S4 is missing (for ex., in the current Fig. S4A the shown DNA sequence represents DNA2 and not DNA1 as stated in the main text or figure legend)

- a Figure of an SDS-PAGE showing the several purified proteins, for quality assessment, should be included as supplementary material

- details are missing in the legends of several Figures and Supplementary Figures; for example, the authors never specify what the red, grey or black spheres, or the dashed lines between residues, are supposed to represent

- in Figure 6C, the authors show a plasmid relaxation assay that can be used to assess activity of either type I (ATP-independent) or type II (ATP-dependent) DNA topoisomerases; therefore, a lane with AsfvTopII but without ATP should be shown, to demonstrate that the activity observed is typeII-specific

Overall, the methodology used is indicated with sufficient detail. I do, however, have some doubts and suggestions:

- Given that ASFV is a double-stranded DNA virus, and that it has no introns, why do authors use the designation of “codon-optimized cDNA sequence”? Coding sequence

would suffice and be more adequate. Furthermore, the nucleotides changed for the optimization should be highlighted in Table S3 and, ideally, the original and codon-optimized sequences should be presented together and aligned.

- "…recombined into the pET-His6-MBP-TEV-LIC cloning vector…" -> which version of the vector?

- "The proteins were treated with TEV protease at 4℃ overnight" -> for the non-specialist the purpose of this step may not be clear; please clarify

Some improvements or corrections can be done in terms of references:

- "AsfvTopII is encoded by the p1192r gene that is highly conserved in all ASFV isolates." -> a reference for this statement should be indicated

- "Transcription of the p1192r gene begins as early as 2 hours after infection and is active throughout the entire ASFV infection course." -> a reference for this statement should be indicated (for example, reference 15)

- "Like all other replicative proteins, previous study confirmed that AsfvTopII is critical for the replication of the viral DNA13,14" -> the references indicated here do now show what the authors claim; better references should be indicated or the sentence changed

- "…fully abrogates the replication of ASFV in vitro15-17." -> reference 16 refers to inhibition by genistein, which is not a fluoroquinolone derivative; this sentence should, therefore, be changed or the indicated reference removed

Reviewer #2 (Remarks to the Author):

In "Complex structures and implications of African swine fever virus topoisomerase", by Yang et al., the authors deepen the knowledge on ASFV pP1192R, a unique type II DNA topoisomerase. Using the X-ray crystallography the authors determined four protein's complex structure (ATPase domain with ATP; DNA-bound domain with G-DNA and T-DNA), and further characterized its activity in vitro by using biochemical assays. However, the

recent publication of pP1192R's cryo-EM and crystal complex structures (doi: 10.1128/mbio.01228-23; doi: 10.1128/mbio.03086-23), hinders the novelty of this study.

Issues with the main text:

Now, several research groups have reported the structure of AsfvTopII; related experiments should use recombinant virus technology to return to the biological problems of the virus itself.

- lines 28-29, "the unique Pro852 and T-DNA-binding 28 residues are important for the function of AsfvTopII", The authors believe that the key amino acids confirmed by biochemistry in vitro are important for the function of AsfvTopII and can be used as targets for the development of inhibitors. Related virus experiments should be added.

- lines 192-194, The main innovation of this paper seems to be the complex structure of pP1192R, G-DNA and T-DNA. Apart from in vitro biochemical experiments, the authors did not return to the virus itself; what is ASFV's preference for G-DNA and T-DNA, and how it regulates virus replication?

- lines 249, How ASFV chooses to use its own coding TopIIs to complete the replication process instead of using host-related proteins.

Reviewer #3 (Remarks to the Author):

Jie Yang and coauthors present crystal structures of fragments of the African Swine Fever Virus (ASFV) type II topoisomerase (AsfvTopoII). The isolated ATPase domain, and an N-terminus truncation of AsfvTopoII were crystalized in the presence of ATP and its analogs, and with two different double-stranded DNA sequences. The structure of the isolated ATPase domain with ATP or AMPPNP are similar to other type II topoisomerases and are largely confirmatory. The structure of the N-terminus truncation with a gate-segment bound double-stranded DNA sequence reveals the structural details of gate segment binding, which is conserved among topo II topoisomerases, but the structural details of gate segment binding and bending have not previously been determined for AsfvTopoII. The most interesting aspect of this work is the observation of a second double-stranded DNA bound asymmetrically across the C-terminal gate in addition to the gate segment. The authors identified key resides that make contact with the DNA segment bound in the C-

terminal gate, and show that mutating these residues degrades topoisomerase relaxation of DNA supercoils to varying degrees of severity.

Overall, the observation of the bound DNA segment to the C-terminal gate and the deidentification of the key residues involved in this interaction along with the impact that mutating these resides have is the most significant aspect of this work, and the only aspects that raises the overall impact and interest to warrant consideration for publication in Nature Communications. That said, there are a number of issues with the work that need to be addressed before I can make a final evaluation of the manuscript for publication in Nature Communications.

Main points:

1. Relaxation gels in figure 6 need to show intermediates in the distribution of DNA topoisomers – or the open circular form needs to be separated from the nicked species – otherwise the relaxation is indistinguishable from nicking. This has been observed in for example figure 6 of ref #18. The authors have to establish that they have a bone fide type II topoisomerase by establishing that the relaxed DNA is intact and not nicked, at a bare minimum. Ideally they would resolve the distribution of DNA topoisomers near the relaxed band that thermodynamically must be present when supercoiled DNA is fully relaxed by a type II topoisomerase. Without these critical demonstrations it is impossible to distinguish type II topoisomerase activity from simply nicking of the DNA.

There are also strange patterns in the background of the gels shown in figure 6C. There are consistent grey bands that appear approximately half way between the relaxed and supercoiled bands. Can the authors comment on these ghost background bands that are noticeably different that the background in other regions of the gel images?

The gels and/or relaxation experiments need to be redone to demonstrate the bine fide type II topoisomerase activity of the enzyme and the various mutants that were tested.

2. Also the relaxation conditions should be adjusted to achieve catalytic rather than stoichiometric, or super stoichiometric, enzyme activity. Specifically, 1.2 ug of Puc19 in a 10 ul reaction, as described in methods on Page 17, corresponds to a concentration of 0.07 uM. This is lower than the lowest concentration of topoisomerase used in the reactions (0.1 um). Type II topoisomerases should be catalytic in that they should be able to relax multiple DNA molecules per enzyme, which is not established in the relaxation assays presented. The authors should redo these relaxation assays with much less topoisomerase

to demonstrate catalytic rather than stoichiometric (equal or greater enzyme to substrate) relaxation conditions.

3. Page 9 “As depicted in Fig. S4B, the phosphate backbones between T11 and C12 (of strand 230 A) and C11 and C12 (of strand B) are all broken in the AsfvTopII_ΔN/DNA1 structure.” The figure is mislabeled since it shows the sequence for DNA2, and shows the structure with DNA – showing both a gate segment and a second putative T segment bound. It appears that Figure S4 was replaced with Figure S6, so the data in S4 are missing and the data in figure S6 incorrectly appear twice.

4. This is a picky point but all of the structures of type II topoisomerases are presented with the G-segment on top and the C-terminal gate below – 180 degrees from how the authors chose to display the structures. This is perhaps just a point of convention, but there are numerous publications that have established the convention that it would be preferable for the authors to follow.

5. In figure 2 or 3 it is imperative to show the cleaved DNA and the 5’ linkage to the catalytic tyrosine. Also in figure the authors should describe in more detail each of the interactions depicted in panels B-D.

6. In figure 4 it would also be helpful to provide more detail describing the key interactions in each panel.

7. Figure 5 is identical to Figure S8. Please indicate the colors for Mg2+ (black?) and water (red?) – please do this for all figures.

8. Figure 6 A-B. What structures are being compared? Yeast, or the two different AsfvTopoII structures? Please specify what is being compared and provide a legend to help guide the reader as to which structure is which.

9. Figure S1. Can the authors include the bound AMPPNP in panel A? this will help in placing panels b and c in context of the overall structure. The PDB or other identifier of the Yeast Topo II structure needs to be specified.

10. Figure S5. If I understand correctly, the Apo AsfvTopoII structures are from the literature. If so the PDB or other identifiers for the published Apo structures need to be included in the figure.

11. Figures S4 and S6 need to be corrected and verified.

12. Figure S9 – the PDB identifier of the yeast structure needs to be included in the figure.

13. In general, the figures would benefit from more detailed captions and proper references to other structures used for comparison purposes.

14. Can the authors speculate as to why only one DNA sequence was bound to the C-terminal Gate?

15. Given the relatively small changes in the conformation of the C-terminal gate DNA bound and free structures, can the authors provide a mechanistic basis for the decrease in activity observed when the key residues interacting with the C-terminal bound DNA are mutated? This is the key finding of the report, which may very well have implications beyond the AsfvTopoII and may provide molecular insights into the activity of type II topoisomerases more generally. A fuller discussion of possible mechanistic or structural explanations for the roles played by the specific dsDNA binding residues in the C-terminal gate domain would significantly enhance the significance and importance of the manuscript.

Once the authors address these concerns, then the paper could be evaluated for possible publication in Nature Communications.

We sincerely thank the reviewers for reading our manuscript with great care, we would also like to thank the reviewers for their very helpful comments, encouragements and criticisms as well. Based on their comments and suggestions, we have carefully revised our manuscript. We believe that the quality of our manuscript has been significantly improved. The following are our point-to-point responses to the reviewers' comments.

Reviewer #1 (Remarks to the Author):

In "Complex structures and implications of African swine fever virus topoisomerase", by Yang et al., the authors deepen the knowledge on pP1192R, a unique type II DNA topoisomerase coded by the African Swine Fever virus, by gaining new structural and mechanistic insights on how this enzyme works. It is a very welcome addition to the recent publication of pP1192R's cryo-EM structures (doi: 10.1128/mbio.01228-23), which it complements and is already acknowledged by the authors. The structures obtained in both cases are similar, which strengthens both findings and respective methodologies. The manuscript is clear and most of the conclusions are sound and justified by the results shown. Still, while I have no doubt about the merits of the work and that it deserves to be published, I do have some questions about the work and think it needs a revision before acceptance. Furthermore, minor improvements and changes should be made and some information added.
Response: We sincerely thank the reviewer for reading our manuscript with great care. We would also like to thank the reviewer for all the nice comments, encouragement and criticism as well.

Doubts and suggestions concerning the main text:
- "Dimerization is mainly mediated the GHKL subdomain. -> it seems that a "by" is missing between "mediated" and "the"
Response: Thanks for pointing out our mistake, which has been fixed.

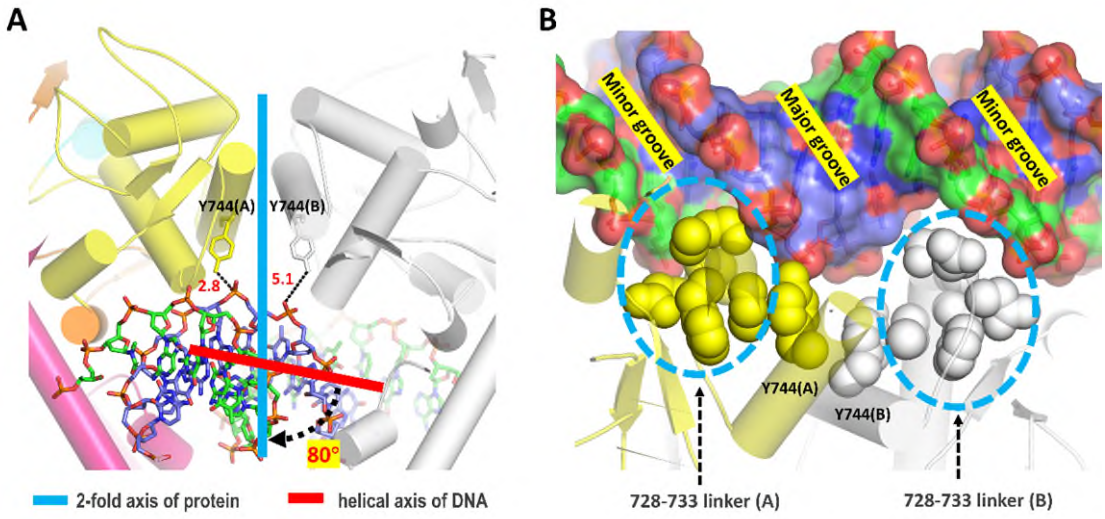
- "With respected to the central region…" -> "respect"
Response: The mistake has been fixed.

- "Structural analysis showed that, at the tilting point, the side chain of one Pro residue (Pro852) deeply inserts into the minor groove, which may play critical role in the bending of DNA1." -> indication of Fig. 2C should be added at the end of the sentence
Response: Thanks for the helpful suggestion. Fig. 2C has been cited at the end of the sentence.

- section "T-DNA is bound by AsfvTopII in an asymmetric manner" -> in my opinion, it is difficult to understand, with only what is shown and described by the authors, why

the interaction of AsfvTopII and the T-DNA is asymmetric, i.e., why there are only 3 interacting regions and not 4, with the coiled-coil arm of the partner molecule also interacting the T-DNA (as suggested to happen in Fig. 4A); a panel showing the interaction from a different angle, and a more detailed explanation should be added to clarify this point and justify the authors' claim

Response: We sincerely thank the reviewer for the thoughtful comments. Follow the reviewer's suggestion, we included one new figure showing the asymmetric interactions between AsfvTopII and the T-DNA. As depicted in the figure below and Fig. S9 in the revised manuscript, the two AsfvTopII protomers are related by a 2-fold axis in the structure. However, the two halves of the T-DNA are not related by the 2-fold axis; the angle between the 2-fold axis of the protein and the helical axis of the T-DNA is approximately 80°. The side chain of Tyr744 of protomer A forms stable H-bond interaction (2.8 Å) with the T-DNA backbone. Whereas, no interaction forms between the T-DNA and the side chain of Tyr744 of protomer B, supported by the long distance (5.1 Å). T-DNA interacts with the 728-733 linkers of both protomers A and B. However, the linker of protomer A interacts with the DNA from the minor groove side, whereas the linker of protomer B interacts with the DNA from the major groove side. Due to the asymmetric binding, the T-DNA cannot interact with the coiled-coil arm of the protomer B; the shortest distance between them is more than 4.7 Å.

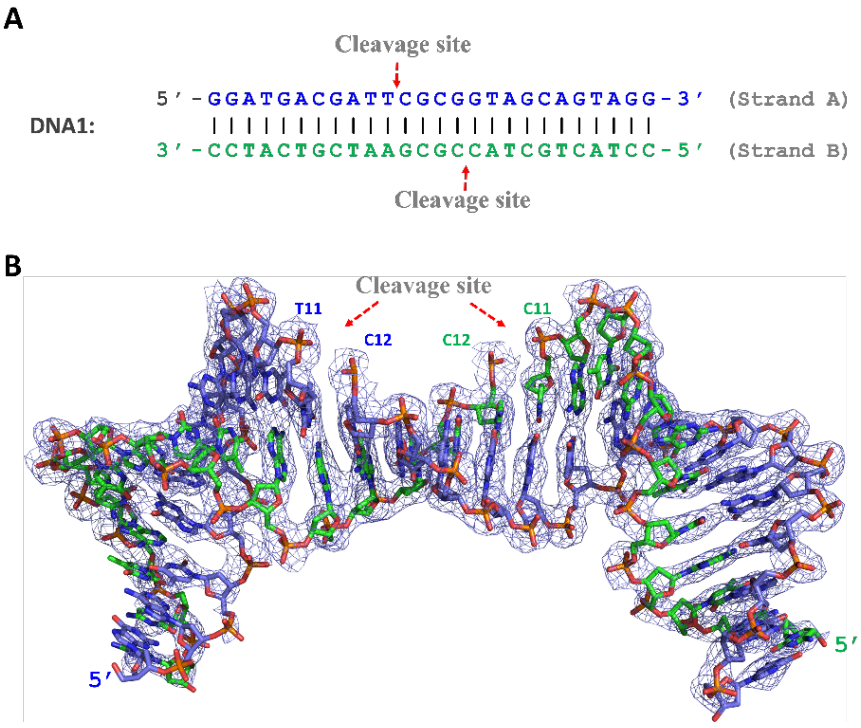


- "However, carefully analysis…" -> "careful"

Response: The mistake has been fixed.

- all claims related to Fig. S4 are impossible to judge given that there is no Fig. S4 (see below my point concerning this).

Response: We are terribly sorry that we misplaced a wrong Fig. S4 in the original manuscript. Please see below for the correct figure, which has also been included and renumbered as Fig. S6 in the revised manuscript.



- "One Mg^{2+} ion is also captured at the catalytic site of the partner molecule (Fig. S8A-B), coordinating with the side chains of Glu438, Asp539 and Asp541, the main chain of Lys615, and the phosphate group of nucleotide C12." -> why is Glu438 indicated as participating only in one of the AsfvTopII molecules and not both?

Response: We sincerely thank the reviewer for the thoughtful comments. To verify the detailed coordination of the Mg^{2+} ion, we carefully checked our structures. As depicted in Fig. 5A-B, Glu438 of AsfvTopII protomer A does not coordinate with Mg^{2+} ion in the AsfvTopII_ΔN/DNA_1 structure. The side chain of Glu438 of the protomer B points toward the Mg^{2+} ion. However, as indicated by the long distance between them (3.15Å), Glu438 does not form stable coordination with the Mg^{2+} ion. We are so sorry for the mistake in our previous manuscript. To avoid possible confusion, we have updated the figures, which has been renumbered as Fig. S11A-B in the revised manuscript. The related sentence has been replaced with "One Mg^{2+} ion is also captured at the catalytic site of the protomer B of AsfvTopII (Fig. S11A-B), coordinating with the side chains of Asp539 and Asp541, the main chain of Lys615, and the phosphate group of nucleotide C12".

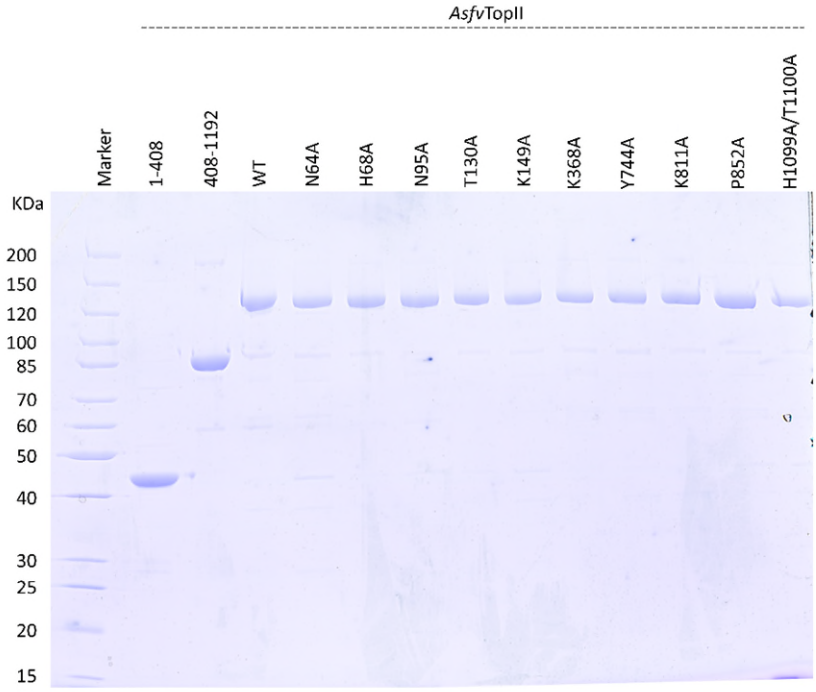
In addition to the AsfvTopII_ΔN/DNA_1 structure, we also checked the coordination of the Mg^{2+} ion in the AsfvTopII_ΔN/DNA_2 complex structure. As depicted in Fig. 5C-D and Fig. S8C-D in our previous manuscript, Glu438 of AsfvTopII does coordinate with Mg^{2+} ion in the AsfvTopII_ΔN/DNA_2 complex structure. Fig. S8C-D has been renumbered as Fig. S11C-D in the revised manuscript. Of note, the two DNA-complexed AsfvTopII structures represent different reaction states, the different conformation of Glu438 and coordination of Mg^{2+} are likely associated with the reaction states.

- “WT AsfvTopII was used as the temple, the primers” -> correct temple to template
Response: The mistake has been fixed.

Concerning the Figures included in the manuscript:
- why do the authors present their structures reversed (upside-down) when compared to the vast majority of TopoII structural representations? This makes figure side-by-side comparison harder.
Response: We sincerely thank the reviewer for the thoughtful comments. Follow the reviewer’s suggestion, we have updated the figures with all structures presented in the conventional upright orientation.

- Figures S4 and S6 are identical; looking at other sections of the manuscript, namely the M&Ms, I can conclude that Figure S4 is missing (for ex., in the current Fig. S4A the shown DNA sequence represents DNA2 and not DNA1 as stated in the main text or figure legend)
Response: We sincerely thank the reviewer for pointing out our mistake on Fig. S4. The correct figure has been included and renumbered as Fig. S6 in the revised manuscript.

- a Figure of an SDS-PAGE showing the several purified proteins, for quality assessment, should be included as supplementary material
Response: Follow the reviewer’s suggestion, we included one figure showing the qualities of the purified proteins (please see the figure below and Fig. S1 in the revised manuscript).

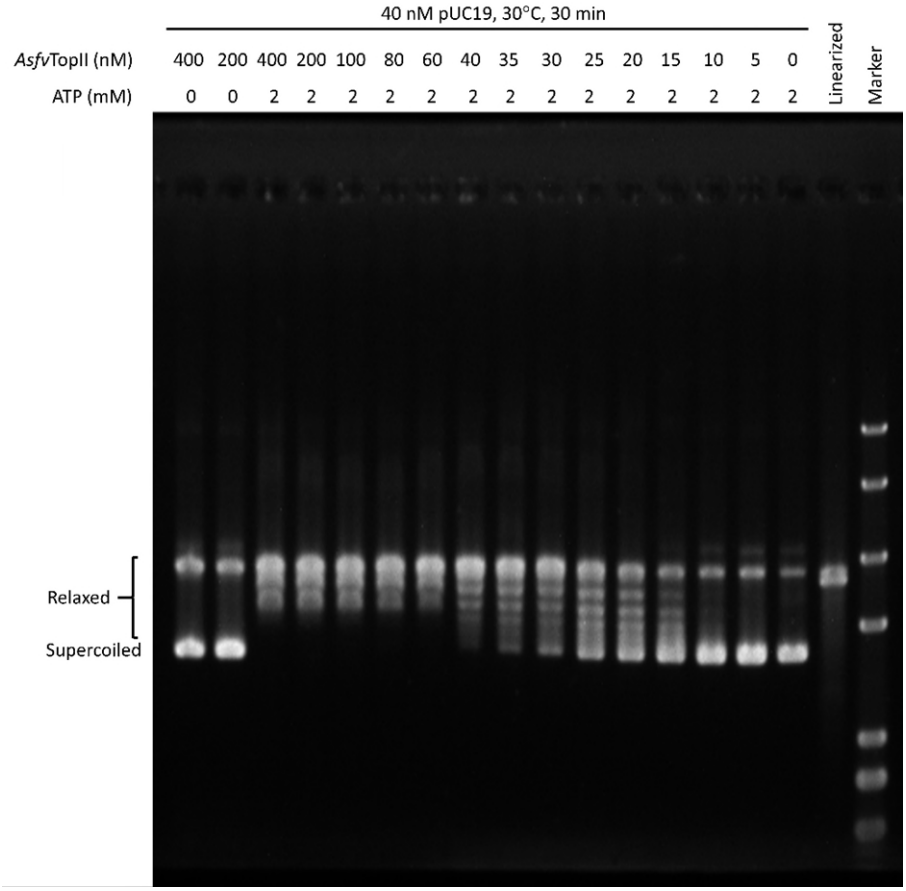


- details are missing in the legends of several Figures and Supplementary Figures; for example, the authors never specify what the red, grey or black spheres, or the dashed lines between residues, are supposed to represent

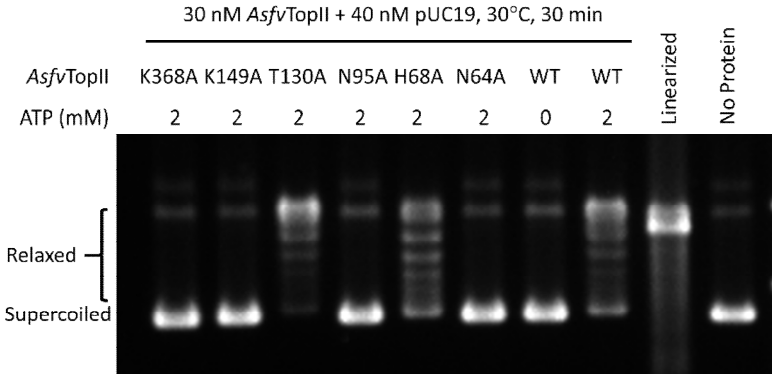
Response: We sincerely thank the reviewer for the criticisms. The detailed information has been included in the legends of the figures.

- in Figure 6C, the authors show a plasmid relaxation assay that can be used to assess activity of either type I (ATP-independent) or type II (ATP-dependent) DNA topoisomerases; therefore, a lane with AsfvTopII but without ATP should be shown, to demonstrate that the activity observed is type II-specific

Response: We sincerely thank the reviewer for the thoughtful suggestions. Follow the reviewer's suggestion, we have redone all the DNA relaxation assays. As depicted in the figure below and Fig. S2 in the revised manuscript, no relaxation activity was observed for AsfvTopII in the absence of ATP. Whereas, in the presence of 2 mM ATP, AsfvTopII can efficiently relax the pUC19 plasmid.



In addition, we also performed plasmid relaxation assays using several AsfvTopII mutants with ATP-binding residues substituted by Ala. In contrast to the wild-type protein, no obvious relaxation activity could be observed for the mutants N64A, N95A, K149A, and K368A (please see the figure below and Fig. 1F in the revised manuscript). All together, these observations confirmed that AsfvTopII is a bone fide type II topoisomerase.



Overall, the methodology used is indicated with sufficient detail. I do, however, have some doubts and suggestions:

- Given that ASFV is a double-stranded DNA virus, and that it has no introns, why do authors use the designation of "codon-optimized cDNA sequence"? Coding sequence would suffice and be more adequate. Furthermore, the nucleotides changed for the optimization should be highlighted in Table S3 and, ideally, the original and codon-optimized sequences should be presented together and aligned.

Response: We totally agree with the reviewer that ASFV is a double-stranded DNA virus and that it has no introns. The cDNA sequence was optimized mainly to facilitate the expression of AsfvTopII in E.coli system. Although it did not change the amino acid sequence of AsfvTopII, the optimized cDNA is more preferred by E.coli. We thank the reviewer for all the thoughtful comments. Follow the reviewer's suggestion, we replaced "codon-optimized cDNA sequence" with "coding sequence" in the revised manuscript. To avoid possible confusion, we also emphasized that the coding sequence has been optimized according to the codon usage bias in E.coli. To highlight the difference between the original and optimized coding sequences of AsfvTopII, we included one figure aligned the two sequences together (please see Fig. S15 in the revised manuscript).

- "…recombined into the pET-His6-MBP-TEV-LIC cloning vector…" -> which version of the vector?

Response: It is the pET-His6-MBP-TEV-LIC cloning vector 1M purchased from Shanghai Hewu Biotechnology Company (http://www.hewusw.com). The detailed information has been included in the revised manuscript.

- "The proteins were treated with TEV protease at 4°C overnight" -> for the non-specialist the purpose of this step may not be clear; please clarify

Response: Thanks for the thoughtful suggestion. TEV protease was used to remove the His6-MBP-TEV fusion tag from the protein. This information has been included in the revised manuscript.

Some improvements or corrections can be done in terms of references:
- “AsfvTopII is encoded by the p1192r gene that is highly conserved in all ASFV isolates.” -> a reference for this statement should be indicated
Response: A related reference (Virology, 2015, 474: 82-93) has been cited in the manuscript.

- “Transcription of the p1192r gene begins as early as 2 hours after infection and is active throughout the entire ASFV infection course.” -> a reference for this statement should be indicated (for example, reference 15)
Response: Thanks for the suggestion. Reference 15 (which has been renumbered as 14 in the revised manuscript) has been cited at the end of the sentence.

- “Like all other replicative proteins, previous study confirmed that AsfvTopII is critical for the replication of the viral DNA13,14” -> the references indicated here do not show what the authors claim; better references should be indicated or the sentence changed
Response: The original reference 14 has been replace by a new reference (Inhibition of African swine fever virus-topoisomerase II disrupts viral replication. Antivir Res, 2016, 134: 34-41).

- “…fully abrogates the replication of ASFV in vitro15-17.” -> reference 16 refers to inhibition by genistein, which is not a fluoroquinolone derivative; this sentence should, therefore, be changed or the indicated reference removed.
Response: Thanks for the suggestion. Genistein related reference has been removed from the sentence.

Reviewer #2 (Remarks to the Author):

In “Complex structures and implications of African swine fever virus topoisomerase”, by Yang et al., the authors deepen the knowledge on ASFV pP1192R, a unique type II DNA topoisomerase. Using the X-ray crystallography the authors determined four protein’s complex structure (ATPase domain with ATP; DNA-bound domain with G-DNA and T-DNA), and further characterized its activity in vitro by using biochemical assays. However, the recent publication of pP1192R’s cryo-EM and crystal complex structures (doi: 10.1128/mbio.01228-23; doi: 10.1128/mbio.03086-23), hinders the novelty of this study.

Response: We sincerely thank the reviewer for reviewing our manuscript and for all the thoughtful comments. We would also like to thank the reviewer for mentioning about the structures recently reported by other groups. In our opinion, the structural similarities further strengthened the findings observed in the reported literature and in our studies. We agree with the reviewer that the overall foldings of the reported structures and our structures are similar, however, they are not exactly identical to each other. The structure reported in the literature (doi: 10.1128/mbio.03086-23) captured a AMPPNP in the ATPase domain of AsfvTopII. In addition to AMPPNP-bound structure, we also performed co-crystallization trial for the ATPase domain and ATP. In the final structure, the ATP has been hydrolyzed, leaving ADP and phosphate group at the catalytic site. This structure directly confirmed the ATP hydrolysis activity of the ATPase domain of AsfvTopII.

The reported AsfvTopII_ΔN structures (doi: 10.1128/mbio.01228-23) are in the apo-form states. In contrast, our structures are in complex with DNAs, which revealed the detailed basis for G-DNA binding by AsfvTopII. Interestingly, the two structures represent two different reaction states, one after the cleavage of the G-DNA, the other prior to G-DNA cleavage or after religation of the G-DNA. These observations directly confirmed that AsfvTopII is functional in DNA relaxation. Most important, our AsfvTopII_ΔN/DNA2 structure captured a T-DNA-mimiking DNA in the C-gate, which represents the only T-DNA bound structure of viral and eukaryotic type II topoisomerase available. Based on all above observations and the functional importance of AsfvTopII, we believed that our study is interested to many researchers working in the related fields.

Issues with the main text:

Now, several research groups have reported the structure of AsfvTopII; related experiments should use recombinant virus technology to return to the biological problems of the virus itself.

Response: We sincerely thank the reviewer for the thoughtful suggestions. We are very sorry that our group mainly focuses on the structural studies of functional important proteins, we do not have the facility and technology to carry out studies with recombinant virus. In fact, African swine fever virus (ASFV) is strictly regulated in our country, only very limited numbers of laboratories are allowed to perform ASFV-related in vivo studies. We thank the reviewer for the kind understanding that

we could not run the in vivo study. In consistent with previous studies, one recent study showed that AsfvTopII is the target of arctiin and pigs administrated arctiin orally showed decreased mortality and tissue damage (J Virol, 2023, 97(11):e0071923. doi: 10.1128/jvi.00719-23). Based on these studies, we are very confident that AsfvTopII is a good target for drug development and our structural studies could provide some clues for the structure-based drug development.

- lines 28-29, “the unique Pro852 and T-DNA-binding residues are important for the function of AsfvTopII”, The authors believe that the key amino acids confirmed by biochemistry in vitro are important for the function of AsfvTopII and can be used as targets for the development of inhibitors. Related virus experiments should be added.
Response: We are very sorry that our group mainly focuses on the structural studies of functional important proteins, we do not have the facility and technology to carry out studies with recombinant virus.

- lines 192-194, The main innovation of this paper seems to be the complex structure of pP1192R, G-DNA and T-DNA. Apart from in vitro biochemical experiments, the authors did not return to the virus itself; what is ASFV's preference for G-DNA and T-DNA, and how it regulates virus replication?
Response: We sincerely thank the reviewer for the kind understanding that we do not have the facility and technology to run the in vivo study.

- lines 249, How ASFV chooses to use its own coding TopIIs to complete the replication process instead of using host-related proteins.
Response: ASFV is one of the most complex DNA viruses known to date. Its genome encodes more than 170 proteins. In addition to DNA replication pathway, it also expresses proteins involved in other biological pathways, such as entry into host cells, suppression of host immune response, gene transcription, and DNA repair. Like other pathways, using of own coding TopII may allow ASFV to replicate its gene more efficient.

Reviewer #3 (Remarks to the Author):

Jie Yang and coauthors present crystal structures of fragments of the African Swine Fever Virus (ASFV) type II topoisomerase (AsfvTopoII). The isolated ATPase domain, and an N-terminus truncation of AsfvTopoII were crystalized in the presence of ATP and its analogs, and with two different double-stranded DNA sequences. The structure of the isolated ATPase domain with ATP or AMPPNP are similar to other type II topoisomerases and are largely confirmatory. The structure of the N-terminus truncation with a gate-segment bound double-stranded DNA sequence reveals the structural details of gate segment binding, which is conserved among topo II topoisomerases, but the structural details of gate segment binding and bending have not previously been determined for AsfvTopoII. The most interesting aspect of this work is the observation of a second double-stranded DNA bound asymmetrically across the C-terminal gate in addition to the gate segment. The authors identified key resides that make contact with the DNA segment bound in the C-terminal gate, and show that mutating these residues degrades topoisomerase relaxation of DNA supercoils to varying degrees of severity.

Overall, the observation of the bound DNA segment to the C-terminal gate and the deidentification of the key residues involved in this interaction along with the impact that mutating these resides have is the most significant aspect of this work, and the only aspects that raises the overall impact and interest to warrant consideration for publication in Nature Communications. That said, there are a number of issues with the work that need to be addressed before I can make a final evaluation of the manuscript for publication in Nature Communications.

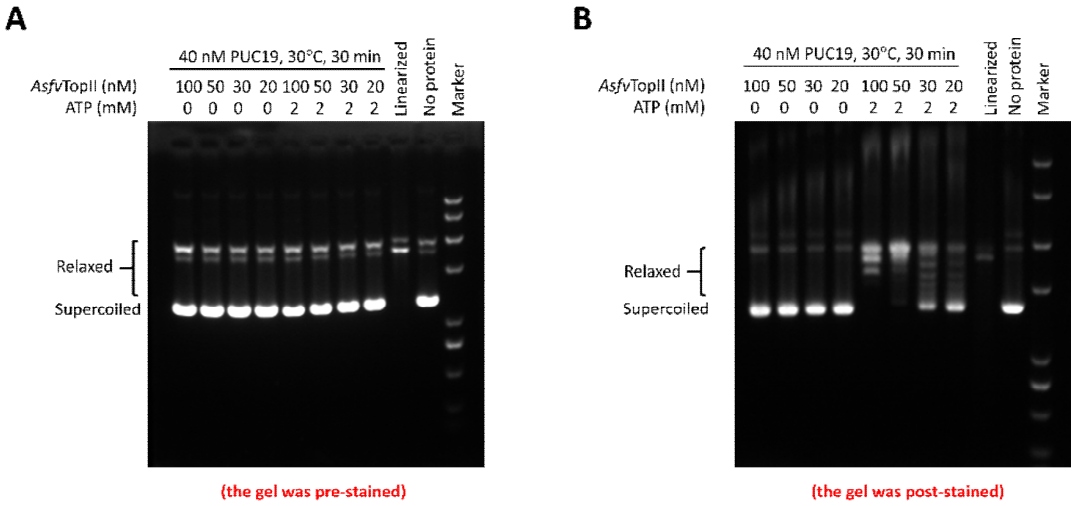
Response: We sincerely thank the reviewer for reading our manuscript with great care. We would also like to thank the reviewer for all the nice comments, encouragement and criticism as well.

Main points:

1. Relaxation gels in figure 6 need to show intermediates in the distribution of DNA topoisomers – or the open circular form needs to be separated from the nicked species – otherwise the relaxation is indistinguishable from nicking. This has been observed in for example figure 6 of ref #18. The authors have to establish that they have a bone fide type II topoisomerase by establishing that the relaxed DNA is intact and not nicked, at a bare minimum. Ideally they would resolve the distribution of DNA topoisomers near the relaxed band that thermodynamically must be present when supercoiled DNA is fully relaxed by a type II topoisomerase. Without these critical demonstrations it is impossible to distinguish type II topoisomerase activity from simply nicking of the DNA.

Response: We sincerely thank the reviewer for raising this important question. To address this question, we have redone all the DNA relaxation assays and found that the assay results are significantly affected by the staining methods of the gel. As depicted in the panel A of the figure below, when the pre-stained gel was utilized, no intermediate could be observed. In contrast, many intermediates of the relaxation

reaction could be observed, when the gel was post-stained by the DNA dye (please see the panel B of the figure). To verify whether AsfvTopII belongs to the type II topoisomerase, we also carried out the reaction in the absence of ATP. No relaxed DNA product was observed on the gel. All together, these results confirmed that AsfvTopII is a bone fide type II topoisomerase and its catalytic activity is ATP-dependent. The missing of the intermediates in our previous DNA relaxation assays are mainly caused by the nucleic acid dye pre-existing in the gel.



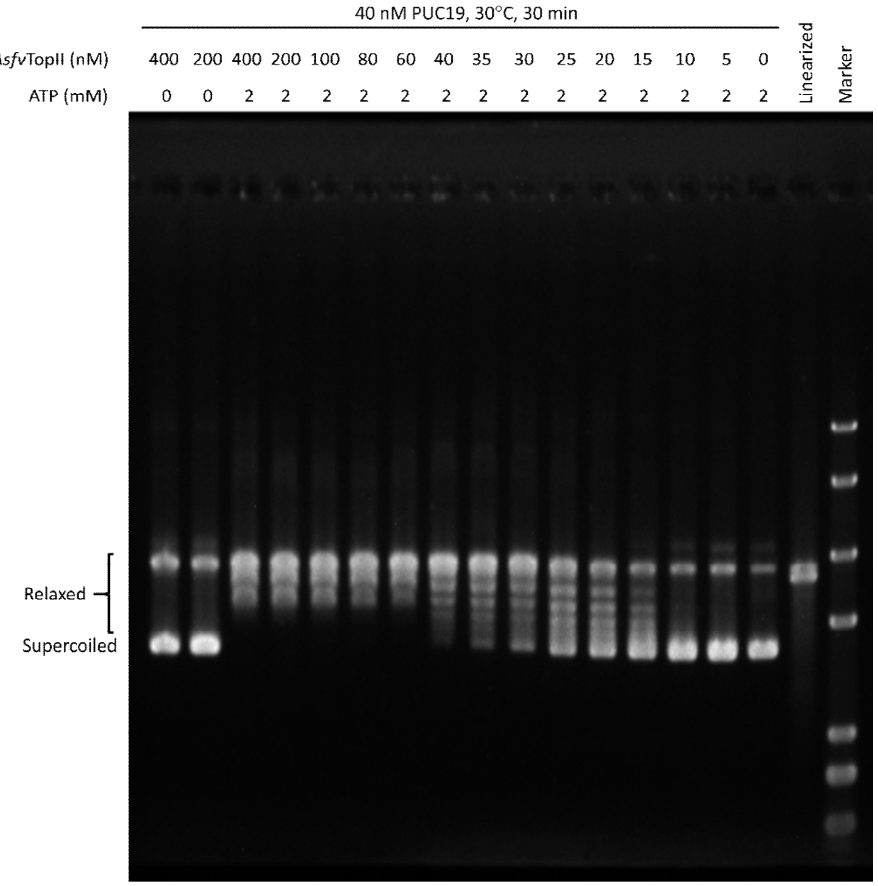
There are also strange patterns in the background of the gels shown in figure 6C. There are consistent grey bands that appear approximately half way between the relaxed and supercoiled bands. Can the authors comment on these ghost background bands that are noticeably different that the background in other regions of the gel images?
Response: Although we are not very sure, the grey bands are likely caused by the dyes present in the loading buffer and/or the gel. We have redone all the DNA relaxation assays using newly prepared loading buffer and different gel staining method. No weird band was observed during our new relaxation experiments.

The gels and/or relaxation experiments need to be redone to demonstrate the bine fide type II topoisomerase activity of the enzyme and the various mutants that were tested.
Response: We have redone all the DNA relaxation assays for the wild-type (WT) and mutant proteins of AsfvTopII. These new assay results confirmed that AsfvTopII is a bone fide type II topoisomerase. The new assay results have been included in the revised manuscript (please see Figs. 1F, 6C-D, and S2).

2. Also the relaxation conditions should be adjusted to achieve catalytic rather than stoichiometric, or super stoichiometric, enzyme activity. Specifically, 1.2 ug of Puc19 in a 10 ul reaction, as described in methods on Page 17, corresponds to a concentration of 0.07 uM. This is lower than the lowest concentration of topoisomerase used in the reactions (0.1 um). Type II topoisomerases should be catalytic in that they should be able to relax multiple DNA molecules per enzyme, which is not established in the relaxation assays presented. The authors should redo these relaxation assays with much

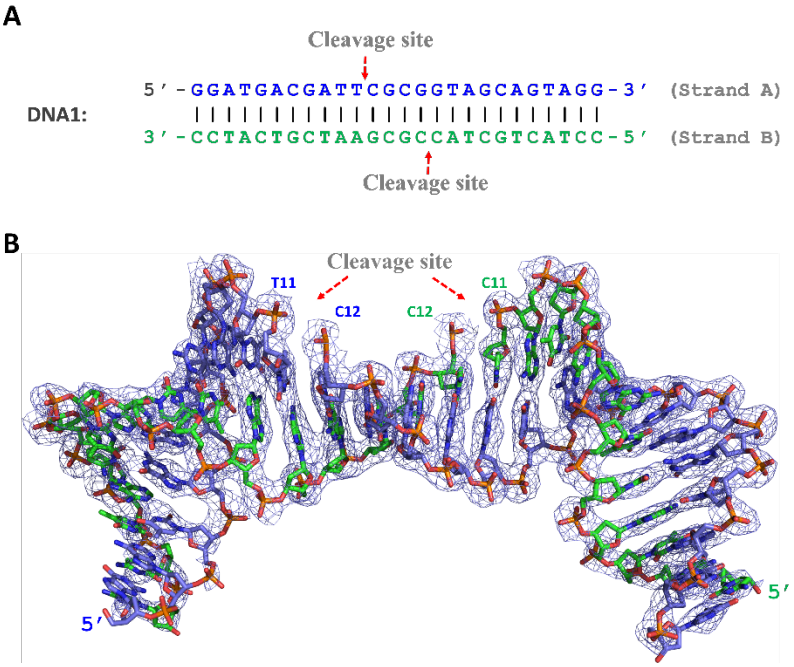
less topoisomerase to demonstrate catalytic rather than stoichiometric (equal or greater enzyme to substrate) relaxation conditions.

Response: We sincerely thank the reviewer for reading our manuscript with great care. We would also like to thank the reviewer for all the thoughtful comments. Follow the reviewer's suggestion, we have redone the relaxation assays using 40 nM pUC19 DNA plasmid as substrates (please see the figure below and Fig. S2 in the revised manuscript). In addition to AsfvTopII with higher concentration (60-400 nM), obvious DNA relaxation activities could also be observed for AsfvTopII under lower concentrations (15-40 nM), .



3. Page 9 "As depicted in Fig. S4B, the phosphate backbones between T11 and C12 (of strand 230 A) and C11 and C12 (of strand B) are all broken in the AsfvTopII_ΔN/DNA1 structure." The figure is mislabeled since it shows the sequence for DNA2, and shows the structure with DNA – showing both a gate segment and a second putative T segment bound. It appears that Figure S4 was replaced with Figure S6, so the data in S4 are missing and the data in figure S6 incorrectly appear twice.

Response: We sincerely thank the reviewer for pointing out our mistakes. The Fig. S4 has been corrected and renumbered as Fig. S6 in the revised manuscript.



4. This is a picky point but all of the structures of type II topoisomerases are presented with the G-segment on top and the C-terminal gate below – 180 degrees from how the authors chose to display the structures. This is perhaps just a point of convention, but there are numerous publications that have established the convention that it would be preferable for the authors to follow.

Response: We sincerely thank the reviewer for the thoughtful comments. Follow the reviewer's suggestion, we updated the figures with all structures presented in the conventional upright orientation.

5. In figure 2 or 3 it is imperative to show the cleaved DNA and the 5' linkage to the catalytic tyrosine.

Response: Thanks for the helpful suggestion. The cleavage sites of DNA1 and the linkage with the catalytic tyrosine residue were shown in Fig. 2B and Fig. 3A in the revised manuscript.

6. In figure 4 it would also be helpful to provide more detail describing the key interactions in each panel.

Response: Thanks for the helpful suggestion. The detailed information has been provided in the legend of Fig. 4. More information can also be found in the Figs. S9-10 in the revised manuscript.

7. Figure 5 is identical to Figure S8. Please indicate the colors for Mg^{2+} (black?) and water (red?) – please do this for all figures.

Response: Like many other TopII proteins, AsfvTopII function as homodimer. Fig. 5A-B only shows the electron density maps and conformations of the nucleotides, residues, and Mg^{2+} at the catalytic site of AsfvTopII protomer A in the DNA1-bound

structure. The electron density maps and conformations of the protomer B in the DNA1-bound structure are shown in Fig. S8A-B (which has been renumbered as Fig. S11A-B in the revised manuscript). For the DNA2-bound structure, the electron density maps and conformations of the nucleotides, residues, and Mg^{2+} at the catalytic site of AsfvTopII protomer A are depicted in Fig. 5C-D, but those observed at the catalytic site of the protomer B are depicted in Fig. S8C-D (which has been renumbered as Fig. S11C-D in the revised manuscript). Mg^{2+} and water molecules are shown as spheres in black and red, respectively. In addition to Fig. 5 and Fig. S11, the color information of water molecules and Mg^{2+} were also included in the legends of many other figures.

8. Figure 6 A-B. What structures are being compared? Yeast, or the two different AsfvTopoII structures? Please specify what is being compared and provide a legend to help guide the reader as to which structure is which.
Response: Fig. 6A-B showed the structural comparison of the G-DNA bound AsfvTopII and Yeast TopII structures. The color information of the two structures have been provided in the legend of the figure.

9. Figure S1. Can the authors include the bound AMPPNP in panel A? this will help in placing panels b and c in context of the overall structure. The PDB or other identifier of the Yeast Topo II structure needs to be specified.
Response: Thanks for the thoughtful suggestion. AMPPNP molecule has been depicted in panel A of the updated Fig. S1, which has been renumbered as Fig. S3 in the revised manuscript. As suggested by the reviewer, we also provided the PDB ID of the Yeast Topo II structure in the legends and/or figures.

10. Figure S5. If I understand correctly, the Apo AsfvTopoII structures are from the literature. If so the PDB or other identifiers for the published Apo structures need to be included in the figure.
Response: The PDB_IDs (8J9Y and 8J9Z) of the two reported apo-form AsfvTopoII structures have been included in the updated Fig. S5, which has been renumbers as Fig. S7 in the revised manuscript.

11. Figures S4 and S6 need to be corrected and verified.
Response: We are terribly sorry for the mistake on Fig. S4, which has been fixed. Fig. S4 and S6 have been renumbered as Fig. S6 and S8, respectively, in the revised manuscript.

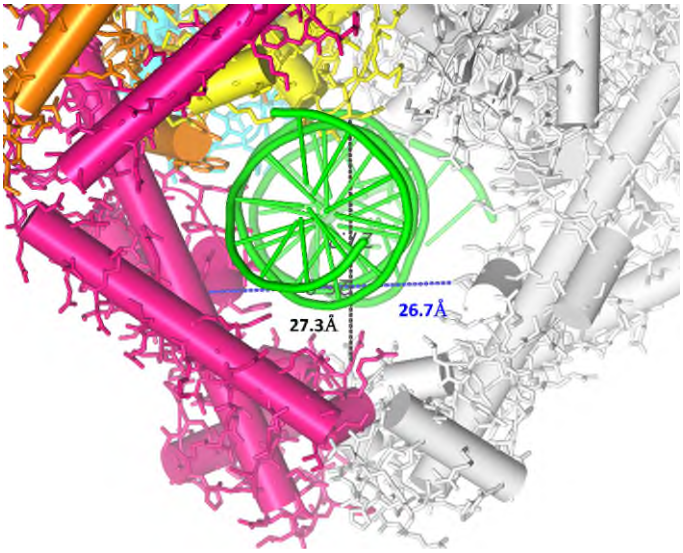
12. Figure S9 – the PDB identifier of the yeast structure needs to be included in the figure.
Response: The PDB_ID of the Yeast TopII structure has been included in the figure and legend in the revised manuscript. Fig. S9 has been renumbers as Fig. S12 in the revised manuscript.

13. In general, the figures would benefit from more detailed captions and proper references to other structures used for comparison purposes.

Response: Thanks for the helpful suggestion. The legends of the figures have been updated and the references related to the compared structures have been cited in the revised manuscript.

14. Can the authors speculate as to why only one DNA sequence was bound to the C-terminal Gate?

Response: As depicted in Fig. 4 in the manuscript, AsfvTopII interacts with both strands of the T-DNA. However, as showed in the figure below, the inner ring of the C-gate is only about 27Å in diameter, which is too small to hold two DNA duplexes simultaneously.



15. Given the relatively small changes in the conformation of the C-terminal gate DNA bound and free structures, can the authors provide a mechanistic basis for the decrease in activity observed when the key residues interacting with the C-terminal bound DNA are mutated? This is the key finding of the report, which may very well have implications beyond the AsfvTopoII and may provide molecular insights into the activity of type II topoisomerases more generally. A fuller discussion of possible mechanistic or structural explanations for the roles played by the specific dsDNA binding residues in the C-terminal gate domain would significantly enhance the significance and importance of the manuscript.

Response: We sincerely thank the reviewer for the helpful suggestion. DNA relaxation by Topoisomerases is a very complicate process, it undergoes multiple conformational changes. Our T-DNA bound structure only captured the reaction at one state, either prior to G-DNA cleavage or after religation of the G-DNA. Although no dramatical conformational changes can be observed between the two AsfvTopII/DNA complexes (please see Fig. S10 in the revised manuscript), our mutagenesis and in vitro assay results suggested that T-DNA interaction with the C-

gate plays a role in DNA relaxation by AsfvTopII (please see Fig. 6C and 6F in the revised manuscript). The key T-DNA interacting residue Tyr744 is not absolutely conserved, but it is shared by AsfvTopII, Yeast TopII, and TopII from T. cruizi, suggesting that it may play similar role in these proteins (please see Fig. S13 in the revised manuscript). Our AsfvTopII/DNA complex is the only T-DNA bound structure of type II topoisomerases. In the future, we will try to determine more DNA-complexed structures of AsfvTopII and TopIIs from other species, which will provide more insight into the functional role of the C-gate of TopIIs.

Once the authors address these concerns, then the paper could be evaluated for possible publication in Nature Communications.

Response: We sincerely thank the reviewer for reading our manuscript with great care. We would also like to thank the reviewer for all the nice comments, encouragement and criticism as well. Based on your suggestions, we have carefully revised manuscript. We hope that our manuscript is now satisfactory to you and is suitable for publication by the journal.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have addressed all of my doubts and concerns.

The quality of the manuscript has increased with the changes now introduced, and I believe it is acceptable for publication.

Reviewer #2 (Remarks to the Author):

The author has answered my concerns and agreed to accept the manuscript.

Reviewer #3 (Remarks to the Author):

The authors have done a nice job in addressing my concerns and criticisms. I would recommend publication after the language is improved through another round of careful editing for grammar and clarity. I appreciate the effort that the authors put into addressing my comments.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have addressed all of my doubts and concerns. The quality of the manuscript has increased with the changes now introduced, and I believe it is acceptable for publication.
Response: We sincerely thank the reviewer for all the nice comments and encouragements.

Reviewer #2 (Remarks to the Author):

The author has answered my concerns and agreed to accept the manuscript.
Response: We sincerely thank the reviewer for the nice comment and supporting of our manuscript.

Reviewer #3 (Remarks to the Author):

The authors have done a nice job in addressing my concerns and criticisms. I would recommend publication after the language is improved through another round of careful editing for grammar and clarity. I appreciate the effort that the authors put into addressing my comments.
Response: We sincerely thank the reviewer for all the nice comments and encouragements. Based on the reviewer's suggestion, we have carefully revised the language of our manuscript.