

MxB N-Terminus Adopts a Stable α -Helix to Engage the HIV-1 Capsid Trimer Interface

Corresponding Author: Professor Peijun Zhang

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The manuscript is very well written and easy to read. It has a clear structure and a precise methodology. The combination of tomography, template matching, and molecular dynamics is a particular strength of the study, allowing the authors to provide new insight into arginine-rich motif-mediated recognition by MxB and other host factors, while also establishing a structural framework for the development of capsid-targeting antiviral therapeutics.

I only noted a few minor typographical issues:

Page 3, line 44: hexametric should be hexameric

Page 3, line 61: "interferon-stimulated genes (IFS)" should be "interferon-stimulated genes (ISG)"

Page 3, line 64: targetting should be targeting

Page 3, line 65: exhibits, did the authors mean restricts?

Page 10: couples of cyoET instead of cryoET.

Page 12, line 344: "(https://github.com/ffyr2w/cet_toolbox)" should read github

Page 15, line 429: "in in the NPT" should be corrected

Page 15, line 438: "forcefield" should be "force field"

Page 21, Figure 3 legend: duplicate label "(d)", one of which should likely be "(g)"

The authors also shared their Python script for processing the tomograms and described their subtomogram averaging approach. It would be valuable if, in the same spirit of transparency, they could also share more details on how emClarity was used in their workflow. It is still relatively uncommon for authors to share full tomograms together with the particle coordinates obtained from template matching in emClarity. Did the authors consider depositing these data in the CryoET Data Portal or otherwise making them available through their GitHub repository?

The same applies to the molecular dynamics simulations. Sharing the optimized RosettaCM structure used as input for the MD simulations, as well as a few representative snapshots from the trajectory, would further improve reproducibility and accessibility.

Overall, this is a strong and carefully executed study that provides important structural and mechanistic insight. My comments are minor and are mainly intended to improve clarity, reproducibility, and data accessibility. I believe the manuscript is suitable for publication after minor revision.

Reviewer #2

(Remarks to the Author)

In this study, Zhu et al. investigate interaction mode of MxB with HIV-1 CA. Zhu et al. employ a combination of CryoET/STA, SPA and MD simulations. Zhu et al. engineer a dimeric protein that includes N-terminal MxB residues 1-35. Zhu et al. relay evidence that MxB engages the CA trimer interface in the curved CA lattice. Zhu et al. further relay evidence that MxB engages this trimeric interface through interaction with its conserved 11-RRR-13 motif. Overall, the manuscript is well-written and the research conducted is sound. Certain parts of the manuscript suffers from hyperbole and Reviewers have requested changes to those parts as reflected in the comments below. The findings and resulting model for MxB/CA interaction is of somewhat incremental value but should be of interest to readers of this journal.

Line 44: Typo, "hexametric". Change to "hexameric".

Line 46: We prefer replication cycle for viruses, however this is up to the Authors.

Line 64: Typo, "targetting". Change to "targeting".

Line 99: Following X-ray crystallographic definitions, "structure" refers to an ensemble of density and atomic model.

Line 92: This is low-resolution. Not high-resolution. This is not a structure. This is a model generated based on a low-resolution density map. Rephrase.

Line 104: "lattice generated". Change to "lattice was generated".

Line 120: Indicate example additional densities with arrow or similar for the average reader.

Line 124-125: Low-pass filtered to what resolution?

Line 136: Change "structure" to "model". Also indicate that this RMSD is a Ca RMSD.

Line 137-138: This is rather confusing. In this paragraph, an SPA structure at 2.8Å resolution is presented. But this RMSD seems to be referring back to a Ca RMSD of the two models generated based on the low-resolution CryoET/STA density maps? If we understand this section accurately, rephrase to make this clear to the reader. A Ca RMSD of 1.94Å is not minor! It is quite substantial and the main reason is likely the limited CryoET/STA resolution resulting in substantial uncertainty in the density-guided atomic models.

Line 142: This cannot be concluded based on the low-quality CryoET/STA models. To make this conclusion, Authors should create an SPA density map of CA in the absence of MxB1-35 of similar quality to the density map they created in the presence of MxB1-35, build the atomic model and redo the RMSD analysis.

Line 149: delete "direct structural".

Line 151: Typo, "re-centred". Change to "re-centered".

Line 159: This cannot be concluded based on the results presented.

Line 166: "coordinates" indicates a high-resolution structure. Rephrase and use "model" instead.

Line 174: Typo, "centred". Change to "centered".

Line 177-178: There is only density to account for about half of the alphafold predicted helix. It is thus uncertain that this docking is in correct register. From the viewing angle presented in Fig S6A, it cannot be seen if there is partial density for the N-terminal loop prior to the predicted MxB helix. If there is, this helps guiding docking of the model and it should be shown in a figure panel.

Line 193-199: Authors have absolutely no experimental evidence (density or otherwise) to support these chemical interactions. Moreover, the initial model for MD simulations relies on a predicted MxB1-35 model that is docked in low-quality density. Unless the chemical interactions indicated ARE COMPLETELY STABLE THROUGHOUT THE COURSE OF MD SIMULATION, this section should be removed from the manuscript.

Line 201: Change "Immediately downstream, the highly conserved" to "Immediately downstream, according to our model, the highly conserved".

Line 207: Change "The α -helix extends for approximately" to "In our model, the α -helix extends for approximately".

Line 232-233: This is not a high-resolution study nor is it a structural characterization. Rephrase to reflect that Authors are deriving a model from their analyses.

Line 235-236: Rephrase "Using an integrative approach combining cryoET STA with EM-guided MD simulation, we demonstrate that the MxB N-terminus" to "Based on an integrative approach combining cryoET STA with EM-guided MD simulation, we propose that the MxB N-terminus".

Line 237-238: Rephrase "These interactions" to "these proposed interactions".

Line 241: Change "show" to "indicate".

Line 242: Change "interface, with the conserved RRR motif forming key salt bridges" to "interface. Our model indicates that the conserved RRR motif forms key salt bridges".

line 376-377: Authors point to figure S4A as cryoET/STA density maps, however figure S4 relays SPA density maps.

line 400: The representative models are shown in figure S6 instead of S4.

Reviewer #3

(Remarks to the Author)

(General comments)

This manuscript Zhu et al. reported a structural study of a restriction factor MxB and its interaction with the HIV-1 capsid using cryoET/STA, single-particle cryoEM, and MD simulations. The authors propose that residues 10-20 of MxB adopt an alpha-helix that binds the CA trimer of hexameric capsomer interface through electrostatic interactions and that MxB dimers may crosslink adjacent interfaces.

This study challenges the important question in the field on HIV-1 CA-MxB interaction originally identified by Malim and Bieniasz groups in 2013. The study is technically strong and advances our understanding of this critical HIV-host interaction but comes with a few methodological and interpretational limitations.

(Specific points)

The major challenge in this study is that the cryoET/STA density representing the MxB is weak and has limited feature. Single particle cryoEM reconstruction of the MxB-bound CA-NC tube also showed no clear density for the MxB. This is likely due to the intrinsic nature of the relatively low occupancy of the MxB per CA trimer interface, and the flexibility of the disordered regions of the MxB N-terminal domain. MD simulation of the CA docked with an AlphaFold model of MxB NTD partly substantiated the complex structure model, but further biochemical functional study is needed to validate the proposed complex model and structural interface. Specifically, I recommend performing mutagenesis on the proposed MxB-binding residues in CA, such as E187, P207, and E213, for the same co-pelleting assay presented in Fig. 1a or more quantitative assay systems. Additionally, the claim on the helix formation relies heavily on MD and AlphaFold model rather than direct experimental observation. Authors may examine helix-disrupting point-mutation(s) and assess their impact on CA-binding. I recommend discussing the above limitations more explicitly in the discussion session.

(Minor points)

1. There is no mention of the use of an AlphaFold model of MxB1-35 for the MD simulation in the main text under "MxB binding is driven by electrostatic interactions centred on the RRR11-13 motif", section and should be mentioned.
2. Indicate that the comparison of the maps with and without MxB in Fig. S2 was done at similar contour levels.
3. Indicate the extra density representing the MxB in Fig. 1e in comparison to 1d.
4. Explain the models shown in Fig. 4b-f with more details in the main text.
5. In Fig. 6a, the density should be shown in semitransparent or mesh for clarity.
6. In line 61, IFS should be ISG.

Reviewer #4

(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 #3

(Remarks to the Author)

I have reviewed the authors' point-by-point rebuttal and the corresponding revision, which reasonably improved the manuscript. Please see my assessment below:

The authors are responsive and transparent in addressing my concerns. On the major methodological issue with independent validation of the proposed MxB-binding sites, they performed the requested mutagenesis experiments (E76A/E79A, P207A/E213A, and the combined quadruple mutant). All mutants substantially impaired CA-NC tube assembly itself, which precluded a clean separation between the residues' role in capsid assembly and their roles in MxB binding. I understand this limitation due to the nature of these residues, but this means the core structural claim remains without functional validation. I recommend authors to explicitly state in the main text (not just in the rebuttal) that the mutagenesis-based validation was inconclusive due to compromised CA-NC assembly, so readers can appropriately calibrate confidence in the proposed interface.

On the second major concern, regarding reliance on AlphaFold and MD simulation, the authors have adequately clarified the modeling workflow. The AlphaFold model served only as an initial template for RosettaCM density-guided refinement, and the model used in subsequent MD simulations was selected from a broader conformational ensemble based on fit to the STA density and Rosetta energy scoring, not taken directly from AlphaFold. They have also added an explicit limitations discussion regarding potential bias from initial model selection.

All other minor points have been addressed appropriately.

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Point-by-Point Responses to reviewers' comments

Reviewer #1 (Remarks to the Author):

The manuscript is very well written and easy to read. It has a clear structure and a precise methodology. The combination of tomography, template matching, and molecular dynamics is a particular strength of the study, allowing the authors to provide new insight into arginine-rich motif-mediated recognition by MxB and other host factors, while also establishing a structural framework for the development of capsid-targeting antiviral therapeutics.

Response: We sincerely thank the reviewer for the positive feedback.

I only noted a few minor typographical issues:

Page 3, line 44: hexametric should be hexameric

Response: We thank the reviewer for catching this typo. The word “hexametric” has been corrected to “hexameric” in the revised manuscript.

Page 3, line 61: “interferon-stimulated genes (IFS)” should be “interferon-stimulated genes (ISG)”

Response: We apologize for the typo. The abbreviation “IFS” has been corrected to “ISG” (interferon-stimulated genes) in the revised manuscript.

Page 3: line 64: targetting should be targeting

Response: The spelling “targetting” has been corrected to “targeting” in the revised manuscript.

Page 3: line 65: exhibits, did the authors mean restricts?

Response: We thank the reviewer for the careful reading. We agree that “restricts” more accurately conveys the antiviral effect in this context. Accordingly, we have changed “exhibits” to “restricts” in the revised manuscript.

Page 10: couples of cyoET instead of cryoET.

Response: We thank the reviewer for catching this typo. The misspelling “cyoET” has been corrected to “cryoET” in the revised manuscript.

Page 12, line 344: “(https://gothub.com/ffyr2w/cet_toolbox)” should read github

Response: We thank the reviewer for catching this typo. The URL has been corrected from “gothub” to “github” in the revised manuscript.

Page 15, line 429: “in in the NPT” should be corrected

Response: We have corrected it to “in the NPT” in the revised manuscript.

Page 15, line 438: “forcefield” should be “force field”

Response: We thank the reviewer for the careful wording suggestion. The term “forcefield” has been corrected to “force field” in the revised manuscript.

Page 21, Figure 3 legend: duplicate label “(d)”, one of which should likely be “(g)”

Response: We thank the reviewer and have corrected duplicate label “(d)” to “(g)” in the revised manuscript.

The authors also shared their Python script for processing the tomograms and described their subtomogram averaging approach. It would be valuable if, in the same spirit of transparency, they could also share more details on how emClarity was used in their workflow. It is still relatively uncommon for authors to share full tomograms together with the particle coordinates obtained from template matching in emClarity. Did the authors consider depositing these data in the CryoET Data Portal or otherwise making them available through their GitHub repository?

Response: We thank the reviewer for the suggestion regarding data sharing.

Regarding the emClarity workflow, a detailed step-by-step description of how emClarity was used for subtomogram averaging, including the key parameters and processing stages, has already been published in a dedicated *Nature Protocols* paper (T. Ni *et al.*, *Nat Protoc* **17**, 421-444 2022), which is also cited in Materials and Methods section. Readers can therefore refer directly to that publication for complete implementation details.

Regarding data deposition: We agree that sharing tomograms and particle coordinates enhances transparency and reusability. At present, the cryoET data (tomograms and particle coordinates obtained from template matching in emClarity) are available upon request. We are considering depositing the full dataset in a public repository such as the CryoET Data Portal or a suitable generalist repository (e.g., EMPIAR), which may take some time to sort and upload the data. For the current manuscript, we have included a statement that raw tomograms and particle lists are available upon request.

The same applies to the molecular dynamics simulations. Sharing the optimized RosettaCM structure used as input for the MD simulations, as well as a few representative snapshots from the trajectory, would further improve reproducibility and accessibility.

Response: We thank the reviewer for their comments. We agree with the reviewer that the inputs used for the MD simulation as well as the resulting structures should be included to improve reproducibility. We have organized the inputs, scripts and example outputs for the RosettaCM protocol as well as the MD simulations into a Zenodo

repository (<https://doi.org/10.5281/zenodo.21252111>) which will be made available in tandem with the manuscript. We have also included Figure S8 showing the RMSD convergence of the MD trajectories and Table S4 summarizing the simulated CA-MxB complex to improve MD reproducibility and reliability.

Overall, this is a strong and carefully executed study that provides important structural and mechanistic insight. My comments are minor and are mainly intended to improve clarity, reproducibility, and data accessibility. I believe the manuscript is suitable for publication after minor revision.

Reviewer #2 (Remarks to the Author):

In this study, Zhu et al. investigate interaction mode of MxB with HIV-1 CA. Zhu et al. employ a combination of CryoET/STA, SPA and MD simulations. Zhu et al. engineer a dimeric protein that includes N-terminal MxB residues 1-35. Zhu et al. relay evidence that MxB engages the CA trimer interface in the curved CA lattice. Zhu et al. further relay evidence that MxB engages this trimeric interface through interaction with its conserved 11-RRR-13 motif. Overall, the manuscript is well-written and the research conducted is sound. Certain parts of the manuscript suffers from hyperbole and Reviewers have requested changes to those parts as reflected in the comments below. The findings and resulting model for MxB/CA interaction is of somewhat incremental value but should be of interest to readers of this journal.

Line 44: Typo, “hexametric”. Change to “hexameric”.

Response: We thank the reviewer for catching this typo. The word has been corrected from “hexametric” to “hexameric” in the updated manuscript.

Line 46: We prefer replication cycle for viruses, however this is up to the Authors.

Response: We thank the reviewer for the suggestion. In the updated manuscript, we have changed the wording to "replication cycle" as recommended.

Line 64: Typo, “targetting”. Change to “targeting”.

Response: The word has been corrected to “targeting” in the updated manuscript .

Line 99: Following X-ray crystallographic definitions, “structure” refers to an ensemble of density and atomic model.

Response: We thank the reviewer for clarifying the crystallographic definition. To avoid ambiguity, we have changed “structure” to “EM map” in the updated manuscript.

Line 92: This is low-resolution. Not high-resolution. This is not a structure. This is a model generated based on a low-resolution density map. Rephrase.

Response: We have rephrased the description to: “CryoET STA study of the mature CA hexamer from CA-NC tubular assemblies”.

Line 104: “lattice generated”. Change to “lattice was generated”.

Response: We thank the reviewer for the correction. The phrase has been changed to “lattice was generated” in the updated manuscript.

Line 120: Indicate example additional densities with arrow or similar for the average reader.

Response: We thank the reviewer for the helpful suggestion. We have added an arrow pointing to an example of these densities on the tube surface in the updated Figure 1c. The figure legend has also been revised accordingly to guide the reader.

Line 124-125: Low-pass filtered to what resolution?

Response: The map used for template search was low-pass filtered to 30 Å. This information has now been added to the revised manuscript.

Line 136: Change “structure” to “model”. Also indicate that this RMSD is a Ca RMSD.

Response: We thank the reviewer for the suggestion. We have changed “structure” to “model” in the revised manuscript. We have also re-calculated the RMSD using only Ca and indicated in the updated text that the reported value is a Ca RMSD.

Line 137-138: This is rather confusing. In this paragraph, an SPA structure at 2.8Å resolution is presented. But this RMSD seems to be referring back to a Ca RMSD of the two models generated based on the low-resolution CryoET/STA density maps? If we understand this section accurately, rephrase to make this clear to the reader. A Ca RMSD of 1.94Å is not minor! It is quite substantial and the main reason is likely the limited CryoET/STA resolution resulting in substantial uncertainty in the density-guided atomic models.

Response: We thank the reviewer for pointing out the ambiguity. To clarify, the 1.94 Å RMSD value mentioned previously corresponded to an all-atom comparison of two models built independently into low-resolution CryoET/STA maps. In the revised manuscript, we have re-calculated the RMSD using only Ca atoms between the hexamer models built in the presence and absence of MxB1-35. The new Ca RMSD is 0.777 Å (based on 215 pruned atom pairs out of 216 total residues). This minor difference is fully consistent with the high structural similarity of the hexamer under both conditions.

Line 142: This cannot be concluded based on the low-quality CryoET/STA models. To make this conclusion, Authors should create an SPA density map of CA in the absence

of MxB1-35 of similar quality to the density map they created in the presence of MxB1-35, build the atomic model and redo the RMSD analysis.

Response: We agree with the reviewer and have deleted the conclusion sentence from the revised manuscript.

Line 149: delete “direct structural”.

Response: We thank the reviewer for the suggestion. The phrase “direct structural” has been deleted.

Line 151: Typo, “re-centred”. Change to “re-centered”.

Response: The spelling has been corrected.

Line 159: This cannot be concluded based on the results presented.

Response: We appreciate the reviewer’s comment, and have re-calculated the RMSD using only Ca atoms. The new Ca RMSD is 0.777 Å (based on 215 pruned atom pairs out of 216 total residues). This value indicates a very close Ca backbone arrangement between the two models.

Line 166: “coordinates” indicates a high-resolution structure. Rephrase and use “model” instead.

Response: We appreciate the reviewer’s careful reading. To avoid any unintended implication of high resolution, we have replaced “coordinates” with “particle positions in the 3D tomogram”. To avoid confusion, the term “model” is not used because the sentence refers specifically to the localisation of individual particles, not to an atomic model.

Line 174: Typo, “centred”. Change to “centered”.

Response: The spelling has been corrected.

Line 177-178: There is only density to account for about half of the alphafold predicted helix. It is thus uncertain that this docking is in correct register. From the viewing angle presented in Fig S6A, it cannot be seen if there is partial density for the N-terminal loop prior to the predicted MxB helix. If there is, this helps guiding docking of the model and it should be shown in a figure panel.

Response: We thank the reviewer for this important observation. We agree with the reviewer that it is uncertain the docking is in correct register. However, the orientation of the MxB₁₋₃₅ model is constrained by the surrounding CA density. Specifically, the density corresponding to the CA chains at the trimer interface precludes fitting the AlphaFold2 model in the opposite orientation, as the C-terminal portion of the helix would sterically clash with neighboring CA residues.

Importantly, the MxB₁₋₃₅ model utilized for the MD simulations was not determined solely by rigid-body docking of the AlphaFold2 prediction. Rather, the AlphaFold2 model served only as an initial template for RosettaCM density-guided modelling. During this procedure, only MxB residues 11-18, which are predicted with high confidence by AlphaFold2 as an alpha-helical structure, were utilized as template coordinates. To further reduce bias arising from the initial AlphaFold2 model, RosettaCM modelling was performed with a residue shift of up to four positions, allowing the sequence register to vary during model building rather than remain fixed to the initial template. This procedure generated a diverse ensemble of 2,500 independent MxB₁₋₃₅ conformations, with backbone RMSD varying between 7.4 to 49.8 Å compared to the initial AlphaFold2 template, and mean RMSD and standard deviation of 14.0 and 6.5 Å within conformations in the ensemble (Figure S6b-c). A starting structure for MD simulations was then selected based on both its agreement with the experimental density, evaluated by model-to-map FSC, and the optimal Rosetta all-atom energy score.

To clarify this point, we have revised the Methods “MxB₁₋₃₅ modelling” section to emphasize that the AlphaFold2 model was used only as an initial template for RosettaCM modelling and was not directly employed for the MD simulations. We have also revised Figure S6A to highlight residues 11-18 in the AlphaFold2 model used as the RosettaCM template. In addition, we have expanded Figure S6 with new panels (C-E) illustrating the conformational diversity of the RosettaCM ensemble and the best-scoring model selected for MD simulations.

Line 193-199: Authors have absolutely no experimental evidence (density or otherwise) to support these chemical interactions. Moreover, the initial model for MD simulations relies on a predicted MxB₁₋₃₅ model that is docked in low-quality density. Unless the chemical interactions indicated ARE COMPLETELY STABLE THROUGHOUT THE COURSE OF MD SIMULATION, this section should be removed from the manuscript.

Response: We thank the reviewer for this comment. As discussed in our response to the previous point, the initial model used for MD simulations does not correspond to the AlphaFold2 MxB₁₋₃₅ prediction docked into the density. Instead, it was selected from a diverse conformational ensemble of 2,500 models generated via RosettaCM density-guided model refinement, based on both agreement with the STA density and a favorable Rosetta all-atom energy score.

We agree that interactions identified from MD simulations should not be interpreted solely from the initial conformation model. Rather than requiring individual residue-residue interactions to remain continuously present throughout the simulation, we evaluated the persistence of each interaction over the entire trajectory and across four simulation replicas. Since proteins undergo thermal fluctuations, which are directly incorporated in the stochastic thermostat and barostat algorithms utilized in MD simulations, residue-residue interactions are expected to form and dissociate

dynamically through the simulation trajectory, and stable binding is often maintained through networks of transient residue contacts rather than permanently occupied residue pairs (PMID: [15572347](#), PMID: [41536061](#), PMID: [15618400](#), <https://doi.org/10.1038/s42004-019-0205-5>).

To better support our conclusions, we have revised the residue interaction claims in the section “MxB binding is driven by electrostatic interactions centered on the RRR11-13 motif” to report the occupancy of each residue-residue contact observed during the MD simulations and we explicitly state that the proposed interactions are supported from their high occupancies over the MD trajectories. In addition, we performed MM/GBSA calculations to estimate the per-residue energetic contributions of both MxB and CA residues to binding. These results are now presented in the new Supplementary Fig. S7, and independently support the importance of positively charged residues in MxB and at the CA trimer interface, as well as the hydrophobic residue contacts identified in the simulations.

Line 201: Change “Immediately downstream, the highly conserved” to “Immediately downstream, according to our model, the highly conserved”.

Response: We thank the reviewer for the suggestion and have revised the phrase accordingly.

Line 207: Change “The α -helix extends for approximately” to “In our model, the α -helix extends for approximately”.

Response: We thank the reviewer for the suggestion and have revised the phrase accordingly.

Line 232-233: This is not a high-resolution study nor is it a structural characterization. Rephrase to reflect that Authors are deriving a model from their analyses.

Response: We thank the reviewer for the correction. In the revised manuscript, we have changed the wording to “structural model” to accurately reflect that we are deriving a model from our analyses rather than performing a high-resolution structural characterization.

Line 235-236: Rephrase “Using an integrative approach combining cryoET STA with EM-guided MD simulation, we demonstrate that the MxB N-terminus” to “Based on an integrative approach combining cryoET STA with EM-guided MD simulation, we propose that the MxB N-terminus”.

Response: We thank the reviewer for the suggestion. As recommended, we have rephrased the sentence in the revised manuscript.

Line 237-238: Rephrase “These interactions” to “these proposed interactions”.

Response: We have rephrased “These interactions” to “these proposed interactions” in the revised manuscript.

Line 241: Change “show” to “indicate”.

Response: We thank the reviewer for the suggestion. The word “show” has been changed to “indicate” in the revised manuscript.

Line 242: Change “interface, with the conserved RRR motif forming key salt bridges” to “interface. Our model indicates that the conserved RRR motif forms key salt bridges”.

Response: We thank the reviewer for the precise wording suggestion. We have changed the phrase accordingly in the revised manuscript.

line 376-377: Authors point to figure S4A as cryoET/STA density maps, however figure S4 relays SPA density maps.

Response: We thank the reviewer for catching this typo. We intended to refer Figure S6a, which correctly shows the cryoET/STA density maps.

line 400: The representative models are shown in figure S6 instead of S4.

Response: We have changed the citation to Figure S6.

Reviewer #3 (Remarks to the Author):

(General comments)

This manuscript Zhu et al. reported a structural study of a restriction factor MxB and its interaction with the HIV-1 capsid using cryoET/STA, single-particle cryoEM, and MD simulations. The authors propose that residues 10-20 of MxB adopt an alpha-helix that binds the CA trimer of hexameric capsomer interface through electrostatic interactions and that MxB dimers may crosslink adjacent interfaces.

This study challenges the important question in the field on HIV-1 CA-MxB interaction originally identified by Malim and Bieniasz groups in 2013. The study is technically strong and advances our understanding of this critical HIV-host interaction but comes with a few methodological and interpretational limitations.

Response: We sincerely thank the reviewer’s positive feedback.

(Specific points)

The major challenge in this study is that the cryoET/STA density representing the MxB is weak and has limited feature. Single particle cryoEM reconstruction of the MxB-bound CA-NC tube also showed no clear density for the MxB. This is likely due to the intrinsic nature of the relatively low occupancy of the MxB per CA trimer interface, and the flexibility of the disordered regions of the MxB N-terminal domain. MD simulation of the

CA docked with an AlphaFold model of MxB NTD partly substantiated the complex structure model, but further biochemical functional study is needed to validate the proposed complex model and structural interface. Specifically, I recommend performing mutagenesis on the proposed MxB-binding residues in CA, such as E187, P207, and E213, for the same co-pelleting assay presented in Fig. 1a or more quantitative assay systems.

Response: We thank the reviewer for this constructive suggestion to validate the proposed MxB binding interface. Following this recommendation, we generated CA mutants at the predicted interaction sites, targeting both the E76A/E79A site (hypothesised MxB K6 interface) and the P207A/E213A site (hypothesised MxB R11–13 interface), alongside a quadruple mutant combining all four mutations.

As shown below (Fig. R1), these residues are critical for CA–NC tube assembly. None of the mutants assembled as efficiently as the A92E variant under the same conditions. Because tube assembly was severely impaired, subsequent co-pelleting assays with the MBP–MxB peptide were not informative.

These findings are consistent with our structure, which places these residues at the trimer interface and indicates that they play an essential role in capsid assembly. Therefore, it may not be possible to separate their structural contribution from their role in MxB binding using a co-pelleting assay. We have included discussions on limitations in experimental validation more explicitly.

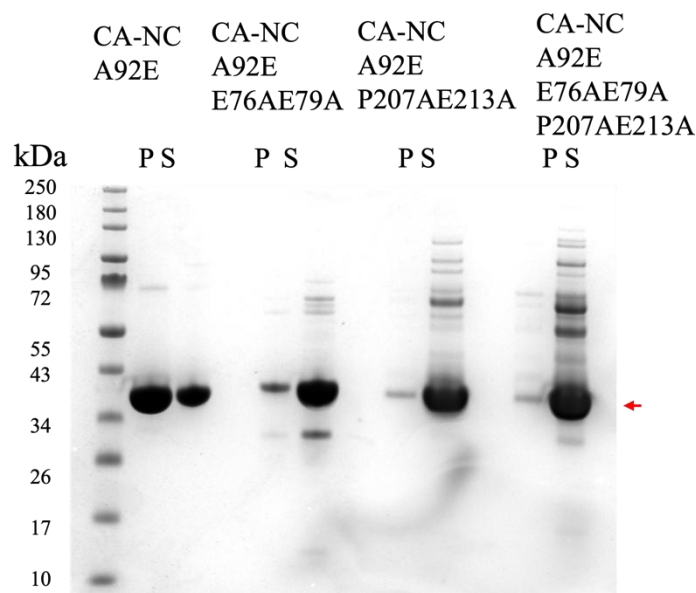


Figure R1 SDS PAGE analysis of pellet/supernatant assembly assays of CA-NC mutants. From left to right: pellet (P) and supernatant (S) fractions of CA-NC A92E, CA-NC A92E E76A/E79A, CA-NC A92E P207A/E213A, and CA-NC A92E E76A/E79A/P207A/E213A following in vitro assembly. The red arrow indicates the CA-NC protein bands.

Additionally, the claim on the helix formation relies heavily on MD and AlphaFold model rather than direct experimental observation. Authors may examine helix-disrupting point-mutation(s) and assess their impact on CA-binding. I recommend discussing the above limitations more explicitly in the discussion session.

Response: We thank the reviewer for this important observation. We have edited the results section “MxB binding is driven by electrostatic interactions centered on the RRR11-13 motif” as well as the methods section “MxB₁₋₃₅ modelling” to clarify that the AlphaFold model of MxB₁₋₃₅ was utilized only as an initial template for the RosettaCM density-guided refinement of the CA-MxB₁₋₃₅ complex and was not used in MD simulations. The initial CA-MxB model used for simulation was selected from a diverse RosettaCM ensemble of 2,500 conformations distinct from the AlphaFold template, based on agreement with the STA density and with an optimal Rosetta all-atom energy score.

We agree with the concern that the extent of helix formation observed in the simulations may be biased by the initial model selection and we have revised the discussion section to discuss this limitation.

(Minor points)

1. There is no mention of the use of an AlphaFold model of MxB₁₋₃₅ for the MD simulation in the main text under “MxB binding is driven by electrostatic interactions centred on the RRR11-13 motif”, section and should be mentioned.

Response: We thank the reviewer for pointing out this omission. In the revised manuscript, we have now explicitly mentioned the use of AlphaFold to generate an initial template model of MxB₁₋₃₅ followed by density-guided model refinement via RosettaCM in the main text section “MxB binding is driven by electrostatic interactions centered on the RRR11-13 motif”.

2. Indicate that the comparison of the maps with and without MxB in Fig. S2 was done at similar contour levels.

Response: We thank the reviewer for this important clarification. The comparison of the maps with and without MxB in Fig. S2 was performed at similar contour levels. We have now explicitly stated this in the updated Figure S2 legend in the revised manuscript.

3. Indicate the extra density representing the MxB in Fig. 1e in comparison to 1d.

Response: We thank the reviewer for the suggestion. Figures 1d and 1e show the cryoET STA maps of CA hexamers from CA-NC tubes without (1d) and with (1e) MxB₁₋₃₅ binding. As noted in the text, MxB₁₋₃₅ is expected to bind at the trimer interface, and its binding was detected using focused 3D classification on the trimer interface. However, in the averaged hexamer map (Fig. 1e), the additional density contributed by MxB₁₋₃₅ is not

resolved at the contour level used. Therefore, we have not indicated an extra density in Figure 1e.

4. Explain the models shown in Fig. 4b-f with more details in the main text.

Response: We thank the reviewer for this constructive suggestion. In the revised manuscript, we have expanded the description of the binding models shown in Fig. 4b–f with additional mechanistic details in the main text (see added text below).

“The first binding configuration (Fig. 4b) corresponds to the shortest MxB pairwise distance peak ($d_1 = 5.5$ nm) observed in the cryoET tomograms (Fig. 2e), in which the flexible N-terminal region enables the two MxB₁₋₃₅ segments to bind adjacent CA trimer interfaces that share the same pair of CA hexamers. The second binding motif (Fig. 4c) corresponds to the most frequent MxB₁₋₃₅ pairwise distance ($d_2 = 9.5$ nm) measured from the cryoET fiducial analysis (Fig. 2e), with the two MxB₁₋₃₅ segments engaging opposite trimer interfaces surrounding the same CA hexamer. The remaining proposed full-length MxB binding configurations (Fig. 4d–f) correspond to combinations of the two predominant MxB₁₋₃₅ pairwise distances: 11 nm ($2d_1$), 16.5 nm ($d_1 + d_2$), and 19 nm ($2d_2$), in which the two MxB₁₋₃₅ segments engage trimer interfaces spanning neighbouring CA hexamers across the lattice. The proposed binding modes suggest that MxB employs a “flexible tethering” strategy, in which the N-terminal region acts as an adjustable arm that enables the dimer to sample and engage multiple CA interfaces across the capsid surface, a feature that may be critical for its lattice-sensing and restriction functions.”

5. In Fig. 6a, the density should be shown in semitransparent or mesh for clarity.

Response: We thank the reviewer for this suggestion. We have made the density semitransparent in Fig. S6a to show the CA fitting and highlighted the density corresponding to MxB with a different color for clarity.

6. In line 61, IFS should be ISG.

Response: We thank the reviewer and correct typo”.

Reviewer #4 (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.

Point-by-Point Response

Reviewer #3 (Remarks to the Author):

I have reviewed the authors' point-by-point rebuttal and the corresponding revision, which reasonably improved the manuscript. Please see my assessment below:

The authors are responsive and transparent in addressing my concerns. On the major methodological issue with independent validation of the proposed MxB-binding sites, they performed the requested mutagenesis experiments (E76A/E79A, P207A/E213A, and the combined quadruple mutant). All mutants substantially impaired CA-NC tube assembly itself, which precluded a clean separation between the residues' role in capsid assembly and their roles in MxB binding. I understand this limitation due to the nature of these residues, but this means the core structural claim remains without functional validation. I recommend authors to explicitly state in the main text (not just in the rebuttal) that the mutagenesis-based validation was inconclusive due to compromised CA-NC assembly, so readers can appropriately calibrate confidence in the proposed interface.

Response: We sincerely thank the reviewer for the positive feedback and constructive recommendation. We have added the mutagenesis experiments to the main text of the revised manuscript, and state that the mutagenesis-based validation was inconclusive due to compromised CA-NC assembly.

On the second major concern, regarding reliance on AlphaFold and MD simulation, the authors have adequately clarified the modeling workflow. The AlphaFold model served only as an initial template for RosettaCM density-guided refinement, and the model used in subsequent MD simulations was selected from a broader conformational ensemble based on fit to the STA density and Rosetta energy scoring, not taken directly from AlphaFold. They have also added an explicit limitations discussion regarding potential bias from initial model selection.

Response: We thank the reviewer for the positive assessment.

All other minor points have been addressed appropriately.

Response: We thank the reviewer for confirming that all remaining minor points have been adequately addressed.