nature research

Peer Review Information

Journal: Nature Structural and Molecular Biology
Manuscript Title: Structural Basis of CD4 Downregulation by HIV-1 Nef
Corresponding author name(s): Dr. Xiaofei Jia

Reviewer Comments & Decisions:

Decision Letter, initial version:

6th Mar 2020

Dear Xiaofei,

Thank you again for submitting your manuscript "Structural Basis of CD4 Downregulation by HIV-1 Nef". We now have comments (below) from the 3 reviewers who evaluated your paper. In light of those reports, we remain interested in your study and would like to see your response to the comments of the referees, in the form of a revised manuscript.

You will see that the reviewers are positive about the interest of the work, but they all have requests for additional data (pull-downs with Nef mutants; additional mutants), controls and reproducibility information. Both reviewers 1 and 2 are concerned about the lower expression levels of some mutants, which prevent clear interpretation of the cellular results. As commented by reviewer 3, statistics and reproducibility for the experiments should be disclosed. Finally, reviewer 2 has several suggestions to improve clarity and presentation.

Please be sure to address/respond to all concerns of the referees in full in a point-by-point response and highlight all changes in the revised manuscript text file. If you have comments that are intended for editors only, please include those in a separate cover letter.

We are committed to providing a fair and constructive peer-review process. If there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome, do not hesitate to let us know. If you think it would be helpful to discuss the revision, I'd be happy to do so.

We expect to see your revised manuscript within 6 weeks. If you cannot send it within this time, please contact us to discuss an extension; we would still consider your revision, provided that no similar work has been accepted for publication at NSMB or published elsewhere.

As you already know, we put great emphasis on ensuring that the methods and statistics reported in

our papers are correct and accurate. As such, if there are any changes that should be reported, please submit an updated version of the Reporting Summary along with your revision.

Please follow the links below to download these files:

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Please note that the form is a dynamic 'smart pdf' and must therefore be downloaded and completed in Adobe Reader.

Cropped images: Please note that all key data shown in the main figures as cropped gels or blots should be presented in uncropped form, with molecular weight markers. These data can be aggregated into a single supplementary figure item. While these data can be displayed in a relatively informal style, they must refer back to the relevant figures. These data should be submitted with the final revision, as source data, prior to acceptance, but you may want to start putting it together at this point.

SOURCE DATA: we urge authors to provide, in tabular form, the data underlying the graphical representations used in figures (data behind graphs). This is to further increase transparency in data reporting, as detailed in this editorial (http://www.nature.com/nsmb/journal/v22/n10/full/nsmb.3110.html). Spreadsheets can be submitted in excel format. Only one (1) file per figure is permitted; thus, for multi-paneled figures, the source data for each panel should be clearly labeled in the Excel file; alternately the data can be provided as multiple, clearly labeled sheets in an Excel file. When submitting files, the title field should indicate which figure the source data pertains to. We encourage our authors to provide source data at the revision stage, so that they are part of the peer-review process.

Data availability: this journal strongly supports public availability of data. All data used in accepted papers should be available via a public data repository, or alternatively, as Supplementary Information. If data can only be shared on request, please explain why in your Data Availability Statement, and also in the correspondence with your editor. Please note that for some data types, deposition in a public repository is mandatory - more information on our data deposition policies and available repositories can be found below:
https://www.nature.com/nature-research/editorial-policies/reporting-standards#availability-of-data

We require deposition of coordinates (and, in the case of crystal structures, structure factors) into the Protein Data Bank with the designation of immediate release upon publication (HPUB). Electron microscopy-derived density maps and coordinate data must be deposited in EMDB and released upon publication. To avoid delays in publication, dataset accession numbers must be supplied with the final accepted manuscript and appropriate release dates must be indicated at the galley proof stage.

While we encourage the use of color in preparing figures, please note that this will incur a charge to partially defray the cost of printing. Information about color charges can be found at http://www.nature.com/nsmb/authors/submit/index.html#costs

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of our efforts in this direction, we are now requesting that all authors identified as 'corresponding author' on published papers create and link their Open Researcher and Contributor Identifier (ORCID) with their account on the Manuscript Tracking System (MTS), prior to acceptance. This applies to primary research papers only. ORCID helps the scientific community achieve unambiguous attribution of all scholarly contributions. You can create and link your ORCID from the home page of the MTS by clicking on 'Modify my Springer Nature account'. For more information please visit please visit www.springernature.com/orcid.

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We look forward to seeing the revised manuscript and thank you for the opportunity to review your work.

Sincerely,
Ines

Ines Chen, Ph.D.
Chief Editor
Nature Structural & Molecular Biology

ORCID 0000-0002-1405-9703

Referee expertise:

Referee #1: trafficking, cellular

Referee #2: trafficking, structural

Referee #3: HIV-1, host factors

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
HIV-1 Nef interaction with AP-2 is essential for CD4 downregulation, and cristal structure data is available explaining how Nef associates with the α−σ2 subunits of AP-2. However, structural data on the interaction of Nef with AP-2 in the presence of the CD4 cytoplasmic domain (CD) was missing. This study by Kwon and colleagues is important in providing the crystal structure of the complete tripartite complex formed by Nef, CD4 CD, and the four AP-2 subunits.

This is a solid and complete study in which functional experiments nicely support the structural data. The study clearly shows how Nef connects CD4 to AP-2, leading to CD4 endocytosis. Moreover, the work provides novel information into the sequence requirements for this Nef activity. In this regard, a significant contribution of the study was to map the interaction site of CD4 in Nef. It is shown that CD4 CD binds to a pocket in Nef that had been previously demonstrated by these authors to be involved in MHC-I recruitment. The authors propose that this common CD4/MHC-I interaction site in Nef could be useful for pharmacological targeting. However, the common Nef residues contacting both CD4 and MHC-I are not readily visible in the structures shown. Also, it was not experimentally demonstrated that specific residues in this pocket are required for the downregulation of both CD4 and MHC-I.

Specific comments

- It seems important to experimentally show the correlation between loss of CD4 downregulation and AP-2 binding capacity for the novel Nef mutants described in this study. In this regard, the authors could expand the GST pulldown assays presented in Figure 1 only for Nef WT.

- Did the authors consider that poor expression displayed by some Nef mutants (e.g., M79D, T138D, L37D, W57A-L58A) might result from instability caused by changes in global protein structure? This would challenge their interpretation that compromised activity is specifically due to loss of CD4/AP-2 interaction. Testing the capacity of these mutants to downregulate MHC-I would help to show if they still are biochemically active.

- It would be helpful to include a panel in Figure 4, highlighting the Nef residues contacting both CD4 and MHC-I. Also, to sustain their hypothesis of a shared interaction site, the authors should test whether Nef mutants predicted to lose interaction with both CD4 and MHC-I are defective in downregulate the two targets. If those common residues were among the ones already tested for CD4 downregulation (Figure 2), they should also be tested for MHC-I. This would sustain their hypothesis of using a small-molecule for Nef inhibition targeting a single interaction site.

- Nef is thought to prevent MHC-I targeting to the cell surface by hijacking AP-1 at the TGN, whereas Nef hijacks AP-2 to induce CD4 endocytosis (Collins and Collins PLoS Pathog. 2014 Jan; 10(1): e1003851). Based on their comparative structural analysis of MHC-I:Nef:AP-1 and CD4:Nef:AP-2 tripartite complexes, can the authors discuss the apparent specificity of AP-1 and AP-2 participation in these two similar but mechanistically distinct Nef functions? In other words, is there any structural basis for a Nef's preference in using AP-1 to downregulate MHC-I and AP-2 to downregulate CD4?

Minor:
- In the Extended data Figure 8, It would be useful also to highlight the residues that are involved in MHC-I downregulation and mark the Nef residues corresponding to CD4/MHC-I binding pocket.

- In Figure 2f, the numbers for residues W57 e L58 are inverted.

Reviewer #2:
Remarks to the Author:
Hijacking of endogenous membrane trafficking systems seems to be a common feature of many viral

infection cycles. In the case of HIV, the viral protein Nef has previously been shown to interact with both the AP1 and AP2 clathrin adaptor complexes. These complexes are central to the formation of clathrin-coated vesicles in the golgi (AP1) and the plasma membrane (AP2).

In this work, the authors determined an x-ray crystal structure of a portion of the AP2 complex bound to the HIV protein Nef and a portion of the CD4 receptor. This structure shows that while Nef engages host cargo (CD4 and MCH-I) using the same binding site, Nef interacts with AP1 and AP2 in distinct manners. To validate the biological importance of the interaction seen in the crystal structure, the authors make several mutations in various Nef binding pockets, which reduce the ability of Nef to downregulate CD4 levels at the plasma membrane. Additionally, the authors take a comprehensive approach to model building and to probing the dynamics of AP2 when engaged in the AP2:Nef:CD4 complex.

This work will be of much interest to the membrane trafficking community and researchers interested in HIV. This data likely explains the mechanism the cells use to internalize CD4 via AP2-mediated endocytosis.

I go into more detail below, but the major issue with this work is that the cell biology data is poorly organized, and the authors use a less-than-convincing approach to justify the significance of mutations in Nef that seem to destabilize the protein and/or reduce expression in cells. If the authors can address the concerns below, considering that the structural component of this work is solid, the manuscript should strongly be considered for publication.

Major issues

• The lack of an introduction section will make this study difficult for non-experts to understand. The only introduction or background information to be found is in the abstract, which nicely sets the stage. However, this should be expanded upon in the main text so that readers are better prepared to contextualize the results.

• The cell sorting data in Figure 2 and expanded upon in Extended figure 1 are hard to follow in the text, and the dose compensation assays are unconvincing. The assay requires co-transfection of a GFP-containing plasmid and a Nef-containing plasmid, and then cells are sorted using two colors: GFP signal (a proxy for Nef transfection) and antibody staining of surface-exposed CD4.

The first problem with this data is that it is poorly described in the text. There needs to be at least a single sentence describing the assay in the main text. Just a simple explanation would help readers be able to interpret Figure 2e.

Regarding Figure 2e, there are 8 separate mutants displayed. Four of these mutants are mentioned on page 2 line 32 (F121D, D123R, M79D, and T138D) when describing the CD4 binding pocket on Nef, two are mentioned on page 3 line 6 when describing the N-loop on Nef (W57A/L58A, L112D), and one is mentioned on page 4 line 15 when mentioning the beta binding pocket on Nef (L100A-I109A). This corresponds to seven mutants discussed throughout the text. However, there are 8 mutants in figure 2e. The L164A-L165A mutant is never described in the text, as far as I can tell.

To summarize, the various mutants described in figure 2e are described in a haphazard manner

throughout the text. It makes following the functional assays difficult to the reader. In addition, several of the mutations seem to destabilize Nef, and this makes interpreting the various mutations more difficult as figure 2e is discussing mutations in several binding pockets around Nef.

• The dose-compensation assay shown in Extended Figure 1 and 6 does not seem like a good way to probe whether the defects of the mutation come from a simple destabilization of Nef or from an impaired interaction with CD4.

• For all of the flow-cytometry data, is there any sort of quantitative metric that can be used to say if a cell line has downregulated CD4? The difference between no Nef and WT plots are visually distinctive, but some of the mutants seem to not be so striking. For example, comparing L37D and L76D in Extended Data Fig 1 is difficult. One is suggested to break CD4 interaction (L37D) and the other is suggested to not (L76D).

• Mutant Q104-R-W113A in Ext. Data Fig 6 is never discussed in the text.

• I believe that the authors should re-arrange the figures by putting all of the Nef immunoblots in the supplement, and only put flow cytometry data for mutants that do not affect Nef expression/stability in the main text. A figure showing the multiple binding sites on Nef (CD4 site, Nef Loop, etc.) and highlighting the residues that when mutated abolish the ability to downregulate CD4 would be a powerful combination of the two techniques.

Essentially, I am not convinced that Nef mutants that show reduced Nef stability/expression can be claimed as vital to CD4 downregulation with confidence. If the authors simply stated that those mutations could not be fully analyzed because of their effects on protein stability, and instead focus only on the mutants that do not affect Nef stability, the results would be much clearer.

Minor issues

• In Fig 3d, the grey N-terminal helix of Nef should be labeled in the figure.

• Related to my comment about the introduction above in "Major Issues", I think the manuscript is difficult to read without sub-headings to guide the reader. I'm unaware of the journal guidelines, but overall I found the paper difficult to read as it just blends together as a series of structural description. Breaking the main text up with sub-headings might re-enforce the various findings of the paper.

• Is there anything to gain from comparing the structure with that from the AP1:Arf1:Tetherin:Nef structure (Morris, et al., 2018)? Or the AP2:Tetherin:Nef structure (Buffalo, et al., 2019)?

Reviewer #3:
Remarks to the Author:
Kwon and coworkers report a structural analysis of a complex of a CD4-Nef fusion protein with AP-2. The problem is significant in that the mechanism of CD4 downregulation, a highly conserved activity of HIV-1 Nef, has not been fully described at a structural level. Prior studies had reported the structure

of Nef bound to AP-2 but without CD4. In the current complex, the CD4 cytoplasmic tail, though contiguous with the Nef polypeptide, appears to be occupying its bona fide binding site in Nef, and AP-2 is bound in its open conformation. Interestingly, the CD4 binding site in Nef overlaps the binding site for MHCI, and the core domain of Nef binds differently to AP-2 and AP-1.
The work appears to have been performed rigorously, and the functional data, including analysis of new Nef point mutants, supports the biological relevance of the structure. However, the functional data are devoid of information regarding replicates, and there is no statistical analysis, thereby casting doubt on the reproducibility of those experiments. The text of the paper is clear though somewhat terse. The prior literature is appropriately cited.

The authors must indicate the number of times each of the following experiments was performed, and provide mean values and standard deviations for the data, along with appropriate statistical analysis:
Fig. 2e
Extended Data Fig. 1
Extended Data Fig. 6
Extended Data Fig. 7

Author Rebuttal to Initial comments

Manuscript #: NSMB-A42830-T

Structural Basis of CD4 Downregulation by HIV-1 Nef

We sincerely thank the reviewers for their critical insights and thoughtful suggestions. We found them to be very helpful in identifying weaknesses in our earlier manuscript and pointing us to the right directions for improvement. We have addressed each of these concerns and suggestions as detailed below.

Reviewer #1:

Major issues:

"It seems important to experimentally show the correlation between loss of CD4 downregulation and AP-2 binding capacity for the novel Nef mutants described in this study. In this regard, the authors could expand the GST pulldown assays presented in Figure 1 only for Nef WT."

This is a great suggestion. Following it, we have analyzed the effect of these novel Nef mutations using an *in vitro* fluorescence polarization assay (Fig. 2e). Here, binding of a fluorescence tag-labeled CD4 tail

to the Nef/AP2 complex was characterized for both WT and mutant Nefs. This assay goes a step further than the GST pulldown assay in that it allowed a quantitative comparison of CD4-binding abilities. As expected, correlation was observed between the loss of CD4 downregulation (Fig. 2f-h) and *in vitro* CD4-binding abilities for each Nef mutation tested.

"Did the authors consider that poor expression displayed by some Nef mutants (e.g., M79D, T138D, L37D, W57A-L58A) might result from instability caused by changes in global protein structure? This would challenge their interpretation that compromised activity is specifically due to loss of CD4/AP-2 interaction. Testing the capacity of these mutants to downregulate MHC-I would help to show if they still are biochemically active."

We agree that poor expression of these Nef mutants could be due to their reduced stability. Testing the capacity of these mutants to downregulate MHC-I was a great idea. However, in our experience, poorly expressed Nef mutants are uniformly impaired in their ability to downregulate MHC-I. We have instead addressed this concern by following reviewer #2's suggestion: we left these poorly expressed mutants out and focused only on well-expressed ones (Fig. 2f-h). Results from the latter, we believe, sufficiently validate the structural observations.

"It would be helpful to include a panel in Figure 4, highlighting the Nef residues contacting both CD4 and MHC-I. Also, to sustain their hypothesis of a shared interaction site, the authors should test whether Nef mutants predicted to lose interaction with both CD4 and MHC-I are defective in downregulate the two targets. If those common residues were among the ones already tested for CD4 downregulation (Figure 2), they should also be tested for MHC-I. This would sustain their hypothesis of using a small-molecule for Nef inhibition targeting a single interaction site."

Surprisingly, there is only one Nef residue, Asp123, that is critical for both mechanisms. How this residue makes contact with CD4 is illustrated in Fig. 2b. The effect of mutating D123 on CD4 binding and CD4 downregulation is shown in Fig. 2e-h. Such effect has also been observed in the past (references 28, 29). This same Asp123 residue contributes to MHC-I downregulation by participating in a three-way electrostatic interaction, as we have previously reported (reference 35). There, Asp123 forms salt bridges with two Arg residues of Mu1, both of which then form salt bridges with Asp327 of MHC-I (reference 35, Figure 3d). Although Nef Asp123 does not make direct contact with MHC-I, mutation at this site completely abolishes MHC-I downregulation. This effect has been observed repeatedly in the past including in our own publication (references 35-37). We, therefore, did not test this mutation on MHC-I downregulation again here. Due to the different ways that Asp123 contributes to binding in each case, we find it difficult to structurally illustrate how Asp123 contacts both CD4 and MHC-I in the overlaid structures. We therefore did not include such a panel. We did provide more details on the requirement of Asp123 in the main text. We hope the reviewer will find these changes adequate and

acceptable. Importantly, although only Asp123 is dually critical for both mechanisms, residues that form this dual-functional pocket are conserved likely because both mechanisms are highly conserved functions of Nef (Extended Data Fig. 5).

"Nef is thought to prevent MHC-I targeting to the cell surface by hijacking AP-1 at the TGN, whereas Nef hijacks AP-2 to induce CD4 endocytosis (Collins and Collins PLoS Pathog. 2014 Jan; 10(1): e1003851). Based on their comparative structural analysis of MHC-I:Nef:AP-1 and CD4:Nef:AP-2 tripartite complexes, can the authors discuss the apparent specificity of AP-1 and AP-2 participation in these two similar but mechanistically distinct Nef functions? In other words, is there any structural basis for a Nef's preference in using AP-1 to downregulate MHC-I and AP-2 to downregulate CD4?"

For MHC-I downregulation, the structural basis of the preference of Nef for μ1 over μ2 has been addressed in our earlier publication (reference 35). For CD4 downregulation, structurally, there is no clear indication whether Nef prefers AP2 over AP1. We believe the choice of AP2 over AP1 here is more of a biological reason: Nef needs to target CD4 resident at the cell surface (newly synthesized CD4 is targeted by Vpu) and therefore hijacks the AP2- and clathrin-mediated endocytosis. On the other hand, Nef needs to target MHC-I at the TGN to divert complexes newly loaded with viral peptides from ever reaching the cell surface. Regarding how and why AP1 is used for downregulation of MHC-I while AP2 is used for CD4, the structural aspects of this distinction are based on a molecular switch mechanism involving the alternative binding of the Nef N-terminal helix vs. the β2 N-terminal helix to the hydrophobic crevice on the Nef core in the two complexes. We have tried to more clearly explain this mechanism in the revised text.

Minor issues:

"In the Extended data Figure 8, It would be useful also to highlight the residues that are involved in MHC-I downregulation and mark the Nef residues corresponding to CD4/MHC-I binding pocket."

We have included additional labels in this figure (now Extended Data Fig. 5), which specified residues important for MHC-I downregulation (orange texts) as well as residues corresponding to the binding pocket (black texts).

"In Figure 2f, the numbers for residues W57 e L58 are inverted."

This error has been corrected.

Reviewer #2:

Major issues:

"The lack of an introduction section will make this study difficult for non-experts to understand. The only introduction or background information to be found is in the abstract, which nicely sets the stage. However, this should be expanded upon in the main text so that readers are better prepared to contextualize the results."

We have added an introduction section and expanded on the background knowledge of both Nef and clathrin adaptor protein complexes.

"The cell sorting data in Figure 2 and expanded upon in Extended figure 1 are hard to follow in the text, and the dose compensation assays are unconvincing. The assay requires co-transfection of a GFP-containing plasmid and a Nef-containing plasmid, and then cells are sorted using two colors: GFP signal (a proxy for Nef transfection) and antibody staining of surface-exposed CD4.

The first problem with this data is that it is poorly described in the text. There needs to be at least a single sentence describing the assay in the main text. Just a simple explanation would help readers be able to interpret Figure 2e.

Regarding Figure 2e, there are 8 separate mutants displayed. Four of these mutants are mentioned on page 2 line 32 (F121D, D123R, M79D, and T138D) when describing the CD4 binding pocket on Nef, two are mentioned on page 3 line 6 when describing the N-loop on Nef (W57A/L58A, L112D), and one is mentioned on page 4 line 15 when mentioning the beta binding pocket on Nef (L100A-I109A). This corresponds to seven mutants discussed throughout the text. However, there are 8 mutants in figure 2e. The L164A-L165A mutant is never described in the text, as far as I can tell.

To summarize, the various mutants described in figure 2e are described in a haphazard manner throughout the text. It makes following the functional assays difficult to the reader. In addition, several of the mutations seem to destabilize Nef, and this makes interpreting the various mutations more difficult as figure 2e is discussing mutations in several binding pockets around Nef."

This is a great suggestion. We have re-arranged these flow cytometry data and consolidated them into one figure for CD4 (Fig. 2f-h). The corresponding text is also consolidated into one paragraph. We have included a brief description of the CD4 downregulation assay. We opted not to show the data on the L164A:165A mutant here in Figure 2, although we do show it as a negative control in Figure 4. The dose-response assays, which were intended to address the poor expression of certain Nef mutants, have been removed, following reviewer #2's other suggestion (below).

"*The dose-compensation assay shown in Extended Figure 1 and 6 does not seem like a good way to probe whether the defects of the mutation come from a simple destabilization of Nef or from an impaired interaction with CD4."*

Again, we agree that the concern over the stability of these mutants is valid. We have removed these data and focused only on well-expressed Nef mutants for validation of the structural observations.

"*For all of the flow-cytometry data, is there any sort of quantitative metric that can be used to say if a cell line has downregulated CD4? The difference between no Nef and WT plots are visually distinctive, but some of the mutants seem to not be so striking. For example, comparing L37D and L76D in Extended Data Fig 1 is difficult. One is suggested to break CD4 interaction (L37D) and the other is suggested to not (L76D)."*

We have included a statistical representation of the flow-cytometry data (Fig. 2g). The data for both L37D and L76D mutants are removed due to the concern associated with their poor expression.

"Mutant Q104-R-W113A in Ext. Data Fig 6 is never discussed in the text."

Data for this mutant, although supportive of the molecular switch model, has been removed. This change, however, does not affect our interpretation as the remaining mutagenesis data sufficiently support this model (Fig. 4bc).

"I believe that the authors should re-arrange the figures by putting all of the Nef immunoblots in the supplement, and only put flow cytometry data for mutants that do not affect Nef expression/stability in the main text. A figure showing the multiple binding sites on Nef (CD4 site, Nef Loop, etc.) and highlighting the residues that when mutated abolish the ability to downregulate CD4 would be a powerful combination of the two techniques.

Essentially, I am not convinced that Nef mutants that show reduced Nef stability/expression can be

claimed as vital to CD4 downregulation with confidence. If the authors simply stated that those mutations could not be fully analyzed because of their effects on protein stability, and instead focus only on the mutants that do not affect Nef stability, the results would be much clearer."

We agree with the reviewer here. Following this suggestion, we have re-arranged the flow cytometry data as well as the corresponding immunoblots. We have now focused only on the well-expressed mutants and presented the mutagenesis data concerning both the CD4-binding and N-loop-binding sites on Nef into one figure (Fig. 2f-h). We have featured these important residues structurally in Fig. 2bd. Correspondingly, we have removed the flow data of poorly expressed Nef mutants. Again, we agree that these data cannot be interpreted with confidence and removing them does not diminish the validation of the structural observations.

Minor issues:

"In Fig 3d, the grey N-terminal helix of Nef should be labeled in the figure."

This label has now been added.

"Related to my comment about the introduction above in "Major Issues", I think the manuscript is difficult to read without sub-headings to guide the reader. I'm unaware of the journal guidelines, but overall I found the paper difficult to read as it just blends together as a series of structural description. Breaking the main text up with sub-headings might re-enforce the various findings of the paper."

This was a weakness of our earlier manuscript. We have now divided the Results into sub-sections with a sub-heading added to each. We agree with the reviewer that the updated format reinforces our findings and we hope make reading easier.

"Is there anything to gain from comparing the structure with that from the AP1:Arf1:Tetherin:Nef structure (Morris, et al., 2018)? Or the AP2:Tetherin:Nef structure (Buffalo, et al., 2019)?"

This is another great suggestion. Comparison between the current structure and the AP2:Tetherin:Nef structure reveals sophisticated ways that Nef proteins manipulate and remodel the β2 N-terminus to target different host proteins. We have now included a paragraph in the Discussion and elaborated on this finding.

Reviewer #3:

"The work appears to have been performed rigorously, and the functional data, including analysis of new Nef point mutants, supports the biological relevance of the structure. However, the functional data are devoid of information regarding replicates, and there is no statistical analysis, thereby casting doubt on the reproducibility of those experiments……..

The authors must indicate the number of times each of the following experiments was performed, and provide mean values and standard deviations for the data, along with appropriate statistical analysis:
Fig. 2e
Extended Data Fig. 1
Extended Data Fig. 6
Extended Data Fig. 7"

We recognize the reviewer's concern on the lack of information on replicates and statistical analysis. Following the reviewer's suggestion, we have added this information as noted above (Fig 2g).

"The text of the paper is clear though somewhat terse."

We have addressed this issue. We added an Introduction and Discussion. We divided the results into sub-sections and added sub-headings for them. We also incorporated more descriptions to facilitate the delivery of the messages and improve the fluency of the text. These additions and modifications will hopefully make the manuscript easier to read and follow.

Decision Letter, first revision:

5th May 2020

Dear Xiaofei,

Thank you again for submitting your manuscript "Structural Basis of CD4 Downregulation by HIV-1 Nef". The reports of one of the original referees are below, and based on these comments, we are happy to accept your paper, in principle, for publication as an Article in Nature Structural & Molecular Biology, on the condition that you revise your manuscript in response to our editorial requirements.

The text and figures require revisions. Note that, within a few days, we will send you detailed instructions for the final revision, along with information on editorial and formatting requirements.

Please send us the main text as a word file, to facilitate our checks. We recommend that you do not start revising the manuscript until you receive this additional information.

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We hope that you will support this initiative and supply the required information. Should you have any query or comments, please do not hesitate to contact me.

If you have any questions, please do not hesitate to contact me directly.

Sincerely,
Ines

Ines Chen, Ph.D.
Chief Editor
Nature Structural & Molecular Biology

ORCID 0000-0002-1405-9703

Reviewer #2 (Remarks to the Author):

I would like to thank the authors for responding fully to all of the concerns of each reviewer. I am satisfied with the changes and I think overall the manuscript is much better. I have no further comments or concerns and would be happy to see this in an upcoming edition of NSMB.

10th May 2020

Dear Xiaofei,

Thank you for your patience as we've prepared the guidelines for final submission of your Nature Structural & Molecular Biology manuscript, "Structural Basis of CD4 Downregulation by HIV-1 Nef" (NSMB-A42830A). Please follow the instructions provided here and in the attached files (sent in separate message), as the formal acceptance of your manuscript will be delayed if these issues are not addressed.

POLICY ISSUES:

1. Data availability: this journal strongly supports public availability of data. Please place the data used in your paper into a public data repository, or alternatively, present the data as supplementary information. If data can only be shared on request, please explain why in your Data Availability Statement, and also in the correspondence with your editor. Please note that for some data types, deposition in a public repository is mandatory.

2. DATA DEPOSITION: We require deposition of coordinates (and structure factors) into the Protein Data Bank with the designation of immediate release upon publication (HPUB). Accession codes must be provided in your final submission for acceptance, and entries must be accessible or HPUB at the galley proof stage.

3. Nature Research is taking an active approach to improving our transparency standards and increasing the reproducibility of all of our published results. Detailed information on experimental design and reagents is now collected on our Life Sciences Reporting Summary, which will be published alongside your paper. Please provide an updated version of the Reporting Summary (which will be published with the paper) with your final files.
https://www.nature.com/authors/policies/ReportingSummary.pdf

Please also upload a revised Editorial policy checklist.
https://www.nature.com/authors/policies/Policy.pdf

GENERAL FORMATTING:

4. The manuscript is currently 3056 words (main text; Introduction, 422 words; Results: 1890 words; Discussion: 744 words). That's fine.

5. Current title: Structural Basis of CD4 Downregulation by HIV-1 Nef (8 words, 52 characters, spaces included). That's fine too, although a bit vague; please make sure to provide more specific information in abstract.

6. Your abstract is currently 121 words. While it follows our style requirements (below 150 words and should not include citations), I also find that it could be more informative. For example, readers would benefit if abstract would specify the proteins/fragments/fusions contained in the crystallized complex; instead of saying "high resolution", just provide the resolution.

7. The Online methods section is currently 2884 words. That's fine.

8. References: the current manuscript has 42 references in main text and 27 in methods. We usually allow up to 60 references and additional 20 references in online Methods, but it's fine to keep is as is. Please make sure all references are cited in numerical order; Methods-only references are already placed after the Methods section, following the numbering of the main reference list, so that's good.

9. References: the reference list should contain papers that have been published or accepted by a named publication or recognized preprint server. Published conference abstracts, numbered patents and research datasets that have been assigned a digital object identifier may also be included in the reference list.

FIGURES AND TABLES:
10. There are currently 4 Figures and 1 Tables in main article. That's fine, and Table 1 already uses

our template, but please make sure to include the PDB ID.

11. Please make sure all figures and tables, including Extended Data Figures, are cited in the text in numerical order.

12. IMPORTANT: Figures 2 and 4, wWhen cropped gels or blots are shown in figures, all key data should be presented in uncropped form with molecular weight markers (either as Source Data or Supplementary Figure 1, as instructed below). These data can be displayed in a relatively informal style, but must refer back to the relevant figures; figure legend text should refer to the uncropped image and cite the source Data (e.g., Uncropped blot/gel images are shown as xxx").

SUPPLEMENTARY INFORMATION

All Supplementary Information must be submitted in accordance with the instructions in the attached Inventory of Supporting Information, and should fit into one of three categories:

1. EXTENDED DATA FIGURES: these are Extended Data Figures 1-6, please observe formatting requirements below. Legend text should be in inventory.

Extended Data Figures are an integral part of the paper and only data that directly contribute to the main message should be presented. These figures will be integrated into the full-text HTML version of your paper and will be appended to the online PDF. There is a limit of 10 Extended Data Figures, and each must be referred to in the main text. Each Extended Data Figure should be of the same quality as the main figures, and should be supplied at a size that will allow both the figure and legend to be presented on a single legal-sized page. Each figure should be submitted as an individual .jpg, .tif or .eps file with a maximum size of 10 MB each. All Extended Data figure legends must be provided in the attached Inventory of Accessory Information, not in the figure files themselves.
All Extended Data Figure must be called out in order as Extended Data Fig. 1, Extended Data Fig 2, etc.

2. SUPPLEMENTARY INFORMATION: this should be Supplementary Table 1 (not Extended Data Table). If Supplementary Table 1 is submitted as a PDF, it should be listed in section 2A of the inventory.

Supplementary Information is material that is essential background to the study but which is not practical to include in the printed version of the paper (for example, large figures, video files, large data sets and calculations). Each item must be referred to in the main manuscript and detailed in the attached Inventory of Accessory Information. Supplementary Tables containing large data sets should be in Excel format, with the table number and title included within the body of the table. All textual information and any additional Supplementary Figures (which should be presented with the legends directly below each figure) should be provided as a single, combined PDF. Please note that we cannot accept resupplies of Supplementary Information after the paper has been formally accepted unless there has been a critical scientific error.

Supplementary items (such as Supplementary Tables, Videos, Notes, and additional Supplementary Figures if permitted), should be numbered and called out in main article, as Supplementary Figure 1 (not SI1) and so on.

3. SOURCE DATA: these would be uncropped blot images for Fig 2h and 4b (mandatory); and data-behind-graphs for Fig 2e and 2g. Each figure can only have one source data file of each type; you are also welcome to provide a single PDF with all uncropped images, as a "Supplementary Figure 1" (listed in inventory section 2A). If these instructions are confusing, please do contact me.

We encourage you to provide source data for your figures. Full-length, unprocessed gels and blots must be provided as source data for any relevant figures, and should be provided as individual PDF files for each figure containing all supporting blots and/or gels with the linked figure noted directly in the file. Statistical source data (i.e., data behind graphs) should be provided in Excel format, one file for each relevant figure, with the linked figure noted directly in the file. For imaging source data, we encourage deposition to a relevant repository, such as figshare (https://figshare.com/) or the Image Data Resource (https://idr.openmicroscopy.org).

Source data should be cited in the legend text (e.g., "Uncropped images for panels a-c are available as source data" or "Data for graphs in d-f are available as source data").

STATISTICS and REPRODUCIBILITY

13. GRAPHS: for error bars, please define the type of repeat. E.g., for fig 2e, where those technical replicates, or different samples? For 2g, were those independent experiments conducted with different clones or transfections?

14. Wherever statistical significance has been derived, precise P values should be provided if possible and appropriate. The type of statistical test used needs to be defined in the legend, whether they were one-sided or two-sided or whether adjustments were made for multiple comparisons.

15. When representative experiments are shown, you should state in the legends how many times each experiment was repeated independently with similar results. Please indicate number of times experiments were repeated, number of images collected, etc. If space in the legends is limiting, this information can be included in the "Statistics and Reproducibility" subsection in Methods.

16. Cell lines: the Methods should include a section with cell lines used, origin, whether they were tested for mycoplasma and, where relevant, whether they were authenticated or not.

17. Competing interests statement: Please include a competing interests statement as a separate section after the Author Contributions, under the heading "Competing interests", and enumerate any such circumstances there, or read: The authors declare no competing interests.

18. Reporting Summary statement: This should be placed after Online Methods section and read: Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

19. Data Availability statement: This should be placed after Reporting Summary statement (before Methods-only references). We suggest that you list in this order:
- data deposited in public repositories, with accession codes or DOIs.
- data available as Source Data (e.g. "Source data for figure 3d, 4b and 4c are available with the

paper online.")
- if any data can only be shared upon request, please specify what those data are and explain why.
More information and examples can be found at
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We ask that you aim to return your revised paper within 14 days. If you have any further questions, please feel free to contact me.

Best regards,

Ines Chen, Ph.D.
Chief Editor
Nature Structural & Molecular Biology

ORCID 0000-0002-1405-9703

Reviewer #2:
Remarks to the Author:
I would like to thank the authors for responding fully to all of the concerns of each reviewer. I am satisfied with the changes and I think overall the manuscript is much better. I have no further comments or concerns and would be happy to see this in an upcoming edition of NSMB.

Author Rebuttal, first revision:

Manuscript #: NSMB-A42830A

Structural Basis of CD4 Downregulation by HIV-1 Nef

We sincerely thank the reviewers for approving our resubmission and for the editors for providing the guidance in preparing the final submission. We have addressed each of these concerns and suggestions as detailed below.

We have previously deposited the crystal structure at the PDB database. The coordinates will be released upon the publication of this manuscript. Additionally, we have also deposited the XL-MS dataset at the ProteomeXchange database and the integrative structure model at PDB-Dev. The Data Availability Statement has been updated accordingly.

We have provided the source data and uncropped gels (Supplementary Figure 1). An updated Reporting Summary has been provided.

We have added necessary descriptions in the abstract to make it more informative.

PDB ID has been added to Table 1.

Information about the type of replicates for Figure 2e and 2g has been included in the legend.

Additional information for cell lines has been added in the Methods. We did not create a separate section for this. Rather, we included such information in each of the two assays (CD4 downregulation and MHC-I downregulation) where cell lines were used.

Final Decision Letter:

16th Jun 2020

Dear Xiaofei,

We are now happy to accept your revised paper "Structural Basis of CD4 Downregulation by HIV-1

Nef" for publication as a Article in Nature Structural & Molecular Biology.

Acceptance is conditional on the manuscript's not being published elsewhere and on there being no announcement of this work to the newspapers, magazines, radio or television until the publication date in Nature Structural & Molecular Biology.

Before the manuscript is sent to the printers, we shall make any detailed changes in the text that may be necessary either to make it conform with house style or to make it intelligible to a wider readership. If the changes are extensive, we will ask for your approval before the manuscript is laid out for production. Once your manuscript is typeset you will receive a link to your electronic proof via email within 20 working days, with a request to make any corrections within 48 hours. Please read proofs with great care to make sure that the sense has not been altered. If you have queries at any point during the production process then please contact the production team at rjsproduction@springernature.com. Once your paper has been scheduled for online publication, the Nature press office will be in touch to confirm the details.

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Sincerely,
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Ines Chen, Ph.D.
Chief Editor
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