

ADAM17 Is a Broad HIV-1 Restriction Factor Targeted by Nef to Restore Env Maturation

Corresponding Author: Professor Yong-Hui Zheng

This manuscript has been previously reviewed at another journal that is not operating a transparent peer review scheme. The manuscript was considered suitable for publication without further review at Nature Communications.

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)

Review of NCOMMS-26-014054-T

Review of the manuscript entitled "ADAM17 Is a Broad HIV-1 Restriction Factor Targeted by Nef to Restore Env Maturation" by Professor Zheng and colleagues.

The authors claim that the protein ADAM17 acts as a host restriction factor against HIV-1 — however the results are not always convincing and overinterpreted, especially because statistics are missing in all the presented results. Main findings are that ADAM17 is trapping the gp160 Env of HIV-1 in the Endoplasmic reticulum. Consequently, gp160 cannot mature, cannot traffic properly and be incorporated in the virions > virions are not infectious. This effect is enhanced in the absence of Nef. Nef would counteract ADAM17 restriction via its PxxP motif. This antiviral activity is due to the signal peptide of ADAM17 independently of its metalloprotease activity.

This work is of significance in the field of HIV.

However, this manuscript requires major revisions. There are serious issues with transparency, and several results are overinterpreted.

- Overall, throughout the entire manuscript, no statistical tests have been performed. Authors should calculate p-values and add them to the graphs, both in the main and supplementary figures. It is also never specified what the error bars represent (SD? SEM?). What I find most concerning is that in the "Reporting Summary," Authors checked "Confirmed" for several categories, particularly regarding statistical tests and error bars, which is not true. This is misleading. Similarly, in the "Editorial Policy Checklist," they checked "Confirmed" for "Clearly defined error bars are present and what they represent (SD, SE, CI) is noted," which is false. I found no mention—neither in the text nor in the figure legends—of what the error bars represent.
- Regarding the BiFC experiments: there is very little in the Materials and Methods section describing the protocol. Occasionally, some details appear in figure legends, but there should clearly be a dedicated paragraph in Materials and Methods explaining how the BiFC experiments were performed. In addition, in the figure legends for this type of experiment, they should indicate the number of images acquired per condition (or the number of cells analyzed).
- Figure 1E: there are 4 data points per condition, yet the legend states that the experiments were repeated 3 times. This needs clarification.
- Figure 2A: they performed a western blot to assess whether Ser5 and A17 are more abundant in Δ Nef virions than in WT. Authors show the blot in Figure 2A and state, "As expected, Ser5 and A17 were more abundant in Δ Nef virions than in WT," but there is no quantification of the western blot. They must quantify their blots (from three independent experiments) and present a graph showing the results. The same applies to other bands. This issue also applies to other western blots in the entire manuscript where they claim "there is more of X in condition Y." but serious quantification is lacking.
- Figure 2D: neither the text nor the legend explains what the graphs on the right represent. Moreover, these graphs are very small, difficult to read, and of poor graphical quality.
- Figure 2E: same comment as for 2D. Additionally, the fact that the intensity profile is drawn directly on the image obscures the underlying pixels. This is particularly evident in the A17 (–) conditions with Env–Env BiFC labeling.

- Overall, the section showing that A17 retains the envelope in the ER is acceptable.
- Figure 4A: it is not stated whether they ensured the same number of virions were used to infect TZM cells. It is therefore unclear whether differences in infectivity are due to changes in virion infectivity (as they claim) or simply differences in the number of virions produced (by the 293T cells with different KO).
- The supplementary figures are cited out of order; for example, Figure S6 is cited before S5, which is itself cited before S4. Figures need to be ordered.
- For the CD4+ T cell KO experiments, authors provide no viability values. Therefore, lower p24 production in some cases might simply be due to fewer live cells in the well. This needs to be correctly normalized.
- Figure 5, the IF/confocal microscopy images need to be zoomed because we cannot see anything on the images : we cannot distinguish any trafficking Lamp1 or CD63 or Rab7 or Rab11 (+) vesicles, so we have to « believe » the colocalization in graph H. how the colocalization graph was obtained (nothing is detailed about the plugin used for colocalization calculation). Is it Comdet or Mander's or else ?
- Confocal microscopy protocol should be detailed (for example, it is written that permeabilization was done with Saponin but nothing is written for the primary antibody incubation buffer that should also contain saponin, otherwise the epitope will not be recognized properly...ect)
- Most of the experiments were done in HEK293T cells ; however the IF/confocal microscopy was done in HeLa cells : this is not relevant because the vesicular trafficking is probably different in HeLa, HEK293T and CD4 T cells (main target for HIV). At least, important results should be verified by IF/confocal microscopy in HIV infected MOLT3 cell lines.
- In Figure 5, graph J, how do we know that this fraction is containing EVs ? and Which EVs ? CD63(+) exosomes or else, like plasma membrane EVs ? This part of the manuscript is weak. The exosomal fraction should be analyzed with different techniques for EV characterization (as in MISEV2023 recommendation).
- The methods and figure legends need to be more detailed/completed.

Reviewer #2

(Remarks to the Author)

The authors discovered a gene that impacts HIV NL4.3 replication in 293T cells and go on to show that this gene, A17, affects Env processing, suggesting this may be one mechanism that reduces HIV infectivity. Their further studies show reproducible effects on infectivity upon expression of A17, and these findings extend to a T cell line. They further map the determinants of A17 activity and also extend these studies to show that expression of A17 in 293T cells impacts infectivity of a range of primary isolates. This is important but the major issue here is whether the levels of expression of A17 in 293T cells is similar to what is found in CD4T cells as this could otherwise be something of an artifact of overexpression - a painfully common issue in the HIV antiviral gene field. This needs to be addressed.

Another key issue is the difference in NL4.3 and a primary virus in terms of Nef dependence on A17 in primary cells. They appear very different (Fig 6G) and yet this is not really explained.

This is potentially an interesting gene that impacts Env processing, but I am not yet convinced it is relevant to HIV replication in primary cells at endogenous levels. Some of the issue is just the paper is so dense, but the data in Fig 6G, which gets to the heart of this as it examines replication effects of A17, do suggest very little impact of A17 on WT virus, which is the situation of natural HIV infection

Specific points:

Line 312-314: They certainly cannot conclude that A17 is solely responsible for HIV restriction in primary cells based on their experiments. Indeed, comparing no gRNA and A17 RNA for WT AD8, which seems the most relevant comparison, the effects of A17 are quite small. (They could do area under the curve to quantify differences or values at a fixed time point) How do they explain other genes that previously have been shown to restrict in primary cells?

Line 354. Brings up the point of overexpression noted above. How does the level of A17 expressed related to endogenous levels in T cells? This is very relevant given the KO effects are much less impressive than the overexpression effects, e.g 6B and 6C. The authors should add a western blot showing A17 levels in producer 293T cells compared to primary CD4 cells. This should ideally be done for the experiment in 6A as well.

Line 104-106, I don't see how Nef is counteracting the A17 effect. In both cases, there is a big drop (maybe this is the 15-35 fold they are describing?) - with A17. If Nef was counteracting A17, shouldn't there be similar levels of infection with A17 to the control in the WT virus, but not delta Nef? Or are they arguing that 15 fold and 35 fold are different? In which case some statistics are needed, but even then the magnitude of the effect is not convincing. Perhaps the issue here is it is unclear what is being compared to what- as presented, the figure implies it is the added gene compared to the control for each virus.

In fact, in 4A it looks like the Nef effect is about 2-fold (~7000 with Nef vs 3000 without Nef in A17 cells). In the double KO there is no effect of Nef. So, I am just convinced about the Nef part of this story as presented.

The double KO aspect of the story is interesting because the effect in 4C is the most convincing in terms of infectivity. Nef reduces all three ADAMs (Fig 1H and 1I) but only A17 restricts. Doesn't that also suggest this is not due to Nef?

I can't really follow how they draw the results from their Westerns, and I think quantitation across replicate Westerns may help, allowing for discussion of fold-differences. Some examples:

For the western in Fig 2A, the explanations seem a bit arbitrary. For example, gp120 in lanes 8 and 11 seem the same, but gp41 is lower in lanes 11 vs 8. So, does this really support the statement for a marked effect of Nef on processing? It would also be helpful to see the gp160 band to see if it is processing specifically and how overall levels of Env play into this.

In 2B, comparing Ser5 and A17, gp41 seems to not be impacted by A17, or perhaps lower, but gp120 is higher. How do they explain this differential impact on the two subunits and what is driving this? Or is the real focus the ratio compared to WT, in which case, again quantification of ratios would help as it is very hard to do this accurately by eye.

The differences in AD8 vs NL4.3 replication in primary cells with Nef deleted are striking. What is the basis for this and is this an example of the issues with using NL4.3?

What is the conclusion of the role of Nef in A17 restriction in primary cells using primary virus as it appears different than all the NL4.3 findings? Is it that absent A17 in primary cells, Nef is not relevant to replication? It seems like this is an important finding of the paper. In fact, on lines 310-311, the authors say the results mirror the cell line findings, but they should clarify that they mirror for NL4.3, but not for the AD8 virus.

It is great to see the primary cell/virus experiment, but it raises more questions than it answers for the A17 KO cells. At least seeing replication of a few more viruses in normal vs A17KO cells could be helpful.

Discussion: The first paragraph says A17 leads to a 100-fold reduction in infectivity for Nef deficient viruses - the authors should clarify what this finding is based on. Again, I am bit confused because of the different findings with NL4.3 and AD8 and at this point, the replication curves suggest the effect is virus dependent. So, that needs to be noted and discussed more fully as the replication curves seems most relevant to infection outcomes.

Related to the above point is that AD8 shows minimal Nef dependence when produced in A17 KO cells. Given the model is A17 effects Env processing, this setting where Env is being produced for virions seems the most relevant place where the role of A17 is tested. How does this support the 100-fold effect noted above?

Regarding the ADAM10 conflict described in the discussion, were the viruses and cells used the same? Here they show that it is not active against NL4.3 in 293T cells (and I suppose in the double KO in Molt4 cells?), but these types of results vary widely by virus and cell type. The authors should discuss this aspect to see if it might contribute to the differences observed. Also, the point made about pools versus clonal KOs. It is also important to acknowledge that clonal KOs are problematic unless multiple clones are tested. Individual clones can be a select variant so most people test 3-5 clonal lines to test a question. Something worth noting in the discussion as well if they used single clonal lines.

Minor;

What are the two bands in Fig 1B and which one is the one being discussed? For example, the two bands for A17 and in the A9 panel, the input is one size and the lp another?

Note, I don't have much experience with the methods used for microscopy so will defer to other reviewers for those aspects.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

1. The authors answer correctly the concern by adding statistical analysis to their figures and the details of the analysis in the caption were added.

2. The detailed description provided in Methods section is now sufficient.

3. Ok

4. The Western blot quantifications and their statistical analysis has been added throughout the manuscript, reinforcing greatly the paper.

5. The new Figure 2D and its caption are way clearer and easy to read now.

6. The modification of the figure are good, and adding of flow cytometry experiment is a very good idea

7. Ok

8. Ok

9. The order is now correct.

10. For the CD4+ T cell KO experiments, authors provide no cell viability datas. It could be a minor remark because no impact for the comprehension of the paper, but if the authors have the data, please add it in the supplementary figures. It's usually required to add cell viability to this type of data when experimenting with PBMCs that undergo proteins knockout.

11. Figure 5G is now clearer. The question was not about what software was used (Fiji) because this was already mentioned in the manuscript. The question was more about the method that was used, because it exists many different way to define and to measure colocalisation. However, the authors added the details of the analysis in the methods section during the revision, and explain clearly that they are talking about percentage of overlapping area after choosing a threshold for each channel, which is satisfactory.

12. The method section for confocal microscopy is now more detailed and sufficient.

13. Most of the experiments were done in HEK293T cells ; however the IF/confocal microscopy was done in HeLa cells : this

not relevant because the vesicular trafficking might be different in HeLa/ HEK293T cell lines and CD4 T cells (main target for HIV). At least, important decisive results should be verified by IF/confocal microscopy in HIV infected MOLT3 cell lines. This point was not addressed by the authors and it is not difficult to achieve; a lot of protocols exist in the literature to perform IF on infected T cells.

14. The answer on EVs is acceptable.

Minor remark : please mention the technique used for the figure S5D in its caption

Reviewer #2

(Remarks to the Author)

The authors have worked hard to address issues raised. For the most part, the A17 effect on viral replication is supported by the studies presented. I do remain puzzled as to how they conclude that A17 is antagonized by Nef and think they spin this too far. A17 significantly reduces infection of WT type NL4.3 virus (a virus that expresses Nef) and deleting Nef does exacerbate this but the effect is relatively modest (~5-fold). Similarly, all the primary viruses are inhibited by A17 despite having an intact Nef. So Nef may only be a part of this antiviral mechanism.

Lines 107-110. Fig 1D

To the point above, A17 reduces wild type by 17-fold, and Dnef by another 5-fold (90-fold total), so the A17 effect seems to be only moderately impacted by Nef, yet the authors conclude that A17 exerts potent antiviral effect that is efficiently counteracted by Nef. I think 'efficiently' should be 'partially'. Similarly, in 1H, there is a partial Nef effect, with 14-fold reduction of WT in the A17KO

Line 253 says A9 does not affect infectivity but in Fig 4A that is not clear and those KO cell results should be compared to WT. The p values that are there seem to relate to another point in lines 254-255 about Dnef vs WT virus.

Line 336 and 341, Ser failed to restrict AD8 dNef should be reworded to say the effect was modest and only a trend for significance (0.055) or something that acknowledges the trend.

Figure 6H is the key experiment and I am still puzzled by the fact that there are striking differences in A17 cells for NL4.3 vs ADA. Why does Nef have no effect on NL4.3 infection in WT vs A17KO cells? This should be discussed.

One of reasons this paper is so dense and confusing may be that the figure sometimes compare the wrong data. An example is Fig 6F. There they compare WT and dNef within cell pools/types. But isn't the real point comparing dNef in WT versus A17 cells?? I think my problem following this whole story was partly that the Figures often were structured in a way that did not optimally follow the text or point being made.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

I have reviewed the revised manuscript and am satisfied with the modifications made in response to the comments. The authors have adequately addressed my concerns, and I have no further comments. I therefore recommend the manuscript for publication.

Reviewer #2

(Remarks to the Author)

The authors have clarified to some extent, most of the points raised and while still confusing in places to me, at this point it is perhaps up to the readers to determine the key takeaways.

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Reviewer #1:

General comments:

The authors claims that the protein ADAM17 acts as a host restriction factor against HIV-1 — however the results are not always convincing and overinterpreted, especially because statistics are missing in all the presented results. Main findings are that ADAM17 is trapping the gp160 Env of HIV-1 in the Endoplasmic reticulum. Consequently, gp160 cannot mature, cannot traffic properly and be incorporated in the virions > virions are not infectious. This effect is enhanced in the absence of Nef. Nef would counteract ADAM17 restriction via its PxxP motif. This antiviral activity is due to the signal peptide of ADAM17 independently of its metalloprotease activity.

This work is of significance in the field of HIV.

However, this manuscript requires major revisions. There are serious issues with transparency, and several results are overinterpreted.

We sincerely thank the reviewer for the careful evaluation of our manuscript and for recognizing the significance of this work to the HIV-1 field. We agree that the original submission required improved statistical rigor, clearer data presentation, expanded methodological detail, and more restrained interpretation in several sections. In response, we have extensively revised the manuscript by adding full statistical analyses with p-values, quantification of all major western blot and infectivity datasets, expanded Methods and Figure Legends, improved microscopy presentation and quantification, and several new validation experiments.

Specific comments:

1. Overall, throughout the entire manuscript, no statistical tests have been performed. Authors should calculate p-values and add them to the graphs, both in the main and supplementary figures. It is also never specified what the error bars represent (SD? SEM?). What I find most concerning is that in the “Reporting Summary,” Authors checked “Confirmed” for several categories, particularly regarding statistical tests and error bars, which is not true. This is misleading. Similarly, in the “Editorial Policy Checklist,” they checked “Confirmed” for “Clearly defined error bars are present and what they represent (SD, SE, CI) is noted,” which is false. I found no mention—neither in the text nor in the figure legends—of what the error bars represent.

We appreciate the reviewer highlighting this important issue. In the revised manuscript, we have now incorporated statistical analyses throughout the study, including p-values, definitions of error bars, biological replicate numbers, and corresponding updates to the Reporting Summary and Editorial Checklist. Statistical analyses are now included in Fig.1D, Fig.1F, Fig.1G, Fig.1H, Fig.1I, Fig.1J, Fig.2A, Fig.2B, Fig.2F, Fig.2H, Fig.3B, Fig.3C, Fig.3D, Fig.3G, Fig.4A, Fig.4B, Fig.4C, Fig.4D, Fig.4H, Fig.4J, Fig.5A, Fig.5C, Fig.5E, Fig.5G, Fig.5H, Fig.6A, Fig.6B, Fig.6C, Fig.6E, Fig.6F, Fig.6G, and Fig.6H. All figure legends have also been revised to explicitly define error bars and statistical methods.

2. Regarding the BiFC experiments: there is very little in the Materials and Methods section describing the protocol. Occasionally, some details appear in figure legends, but there should clearly be a dedicated paragraph in Materials and Methods explaining how the BiFC experiments were performed. In addition, in the figure legends for this type of experiment, they should indicate the number of images acquired per condition (or the number of cells analyzed).

We thank the reviewer for this suggestion. Detailed descriptions of the BiFC experiments are now provided in the Methods section, including imaging procedures and analysis conditions. We have also revised the corresponding figure legends to indicate the number of images acquired and the number of cells analyzed for each condition. On average, at least 10 images from 3 biologically independent experiments were analyzed, and representative images are shown.

3. Figure 1E: there are 4 data points per condition, yet the legend states that the experiments were repeated 3 times. This needs clarification.

We appreciate the reviewer identifying this inconsistency. The dataset in this figure (now Fig.1F) has been carefully reanalyzed, and the figure legend has been corrected accordingly.

4. Figure 2A: they performed a western blot to assess whether Ser5 and A17 are more abundant in Δ Nef virions than in WT. Authors show the blot in Figure 2A and state, “As expected, Ser5 and A17 were more abundant in Δ Nef virions than in WT,” but there is no quantification of the western blot. They must quantify their blots (from three independent experiments) and present a graph showing the results. The same applies to other bands. This issue also applies to other western blots in the entire manuscript where they claim “there is more of X in condition Y.” but serious quantification is lacking.

We agree with the reviewer that quantitative analyses are important for rigorous interpretation of these results. In the revised manuscript, the levels of SERINC5 and ADAM17 in WT and Δ Nef virions are now quantified from 3 independent experiments. Similarly, all major western blot datasets throughout the manuscript have now been quantified and statistically analyzed.

Quantification of Western blots is also provided in Fig.1E, Fig.1I, Fig.1J, Fig.2B, Fig.2H, Fig.3G, Fig.4D, Fig.4E, Fig.4F, Fig.4I, Fig.5E, Fig.5F, and also in relevant supplemental figures.

5. Figure 2D: neither the text nor the legend explains what the graphs on the right represent. Moreover, these graphs are very small, difficult to read, and of poor graphical quality.

We apologize for the lack of clarity in the original presentation. These graphs represent fluorescence intensity profiles from the indicated signals, where overlapping peaks indicate colocalization within the cell. This information is now clearly described in both the text and figure legend. In addition, the graphs have been remade at a higher quality and larger size for improved readability.

6. Figure 2E: same comment as for 2D. Additionally, the fact that the intensity profile is drawn directly on the image obscures the underlying pixels. This is particularly evident in the A17 (–) conditions with Env–Env BiFC labeling.

Reply: We thank the reviewer for this helpful suggestion and agree that overlaying the intensity profile on the original image reduced clarity. In the revised manuscript, we removed the intensity profile from these panels. In addition, to further strengthen the rigor of the BiFC analyses, we quantified the BiFC signals by flow cytometry, which provides population-level validation of the imaging results. This new dataset is now presented in Fig.2F.

7. Overall, the section showing that A17 retains the envelope in the ER is acceptable.

We sincerely appreciate the reviewer's positive assessment of this section.

8. Figure 4A: it is not stated whether they ensured the same number of virions were used to infect TZM cells. It is therefore unclear whether differences in infectivity are due to changes in virion infectivity (as they claim) or simply differences in the number of virions produced (by the 293T cells with different KOs).

We apologize for this lack of clarity. Virion inputs in all infectivity assays were quantified and normalized by p24^{Gag} ELISA to ensure equivalent amounts of virus were used for infection. This information is now explicitly stated in the revised manuscript, including Fig.1D, Fig.1F, Fig.1G, Fig.1H, Fig.3B, Fig.3D, Fig.4A, Fig.5A, Fig.6A, and Fig.6B.

9. The supplementary figures are cited out of order; for example, Figure S6 is cited before S5, which is itself cited before S4. Figures need to be ordered.

We apologize for this oversight. The supplementary figures and citations have now been corrected and reordered appropriately.

10. For the CD4⁺ T cell KO experiments, authors provide no viability values. Therefore, lower p24 production in some cases might simply be due to fewer live cells in the well. This needs to be correctly normalized.

We appreciate this concern. Before infection experiments, we confirmed that all knockout cell populations displayed normal viability and growth kinetics. In addition, neither SERINC5 nor ADAM17 is known to be essential for cell survival under these experimental conditions. We now clarify these points in the revised Methods section.

11. Figure 5, the IF/confocal microscopy images need to be zoomed because we cannot see anything on the images : we cannot distinguish any trafficking Lamp1 or CD63 or Rab7 or Rab11 (+) vesicles, so we have to « believe » the colocalization in graph H. how the colocalization graph was obtained (nothing is detailed about the plugin used for colocalization calculation). Is it Comdet or Mander's or else?

We thank the reviewer for this suggestion. The microscopy images in Fig.5G have now been enlarged and improved for better visualization of the indicated vesicular markers. In addition, the colocalization analysis method is now fully described in the revised manuscript.

Colocalization in Fig.5H was quantified using Fiji and is presented as the percentage of ADAM17-positive signal overlapping with the indicated organelle markers or Nef. Fiji is an open-source image processing package to visualize, process, and analyze complex multi-dimensional scientific images, particularly in the biological and life sciences.

12. Confocal microscopy protocol should be detailed (for example, it is written that permeabilization was done with Saponin but nothing is written for the primary antibody incubation buffer that should also contain saponin, otherwise the epitope will not be recognized properly...ect)

We appreciate this suggestion. A detailed confocal microscopy protocol, including permeabilization and antibody incubation conditions, has now been added to the revised Methods section.

13. Most of the experiments were done in HEK293T cells ; however the IF/confocal microscopy was done in HeLa cells: this not relevant because the vesicular trafficking is probably different in HeLa, HEK293T and CD4 T cells (main target for HIV). At least, important results should be verify by IF/confocal microscopy in HIV infected MOLT3 cell lines.

We appreciate this important point. We initially performed the IF/confocal microscopy analyses in HeLa cells because of their superior morphology, high transfection efficiency, and suitability for high-resolution imaging of intracellular trafficking pathways. HEK293T cells are less commonly used for confocal microscopy because they do not attach tightly to glass slides, making high-resolution vesicular imaging more technically challenging. In addition, high-resolution confocal imaging in suspension CD4 T-cell lines such as MOLT-3 is technically difficult because these cells are small, non-adherent, and poorly suited for detailed vesicular trafficking analyses compared with adherent epithelial cells.

Importantly, there is currently no evidence that the intracellular trafficking pathways examined in this study—including ER retention, Golgi trafficking, endosomal sorting, or CD63/Rab7-associated vesicular transport of HIV-1 Env and ADAM17—behave fundamentally differently between HeLa, HEK293T, and CD4 T cells. These membrane trafficking pathways are highly conserved across mammalian cell types and are therefore routinely studied in adherent epithelial cell systems because of their substantial technical advantages for imaging analyses. In addition, we conducted Env-Env BiFC studies in Jurkat CD4 T cells in our previous studies (PMID: 31043528). Here, we repeated this experiment in HeLa cells (**Fig.2E**) and HEK293T cells (**Fig.2F**), and obtained consistent results, confirming these trafficking pathways are functional in these cells.

14. In Figure 5, graph J, how do we know that this fraction is containing EVs? and Which EVs? CD63(+) exosomes or else, like plasma membrane EVs ? This part of the

manuscript is weak. The exosomal fraction should be analyzed with different techniques for EV characterization (as in MISEV2023 recommendation).

We thank the reviewer for raising this important point. To isolate extracellular vesicles (EVs), we used size-exclusion chromatography (SEC) with qEV columns from IZON, which is a widely used purification approach recommended by MISEV guidelines for EV studies. According to the manufacturer's technical specifications, this method efficiently separates EVs from soluble proteins and removes >99% contaminating protein from cell culture supernatants.

We agree with the reviewer that the EV fraction likely contains a heterogeneous vesicle population and cannot be assigned exclusively to a single EV subtype based on SEC purification alone. Therefore, in the revised manuscript, we have referred to this fraction more conservatively as "EVs" rather than specifically "exosomes" where appropriate.

At the same time, several observations support that CD63⁺ small EVs are enriched in this fraction. First, HIV-1 Nef has been extensively reported in EVs isolated from both cell culture supernatants and plasma from people living with HIV (PMIDs: 18646314, 19912576, 22013042, 29437924), and Nef has been visualized on EV membranes by immunoelectron microscopy (PMID: 39016173). Second, previous studies reported that Nef promotes recruitment of ADAM17 into CD63⁺ EVs (PMIDs: 27211553, 29331674). Consistent with these reports, we found that Nef redistributes ADAM17 into CD63⁺ intracellular compartments in our imaging studies (Fig. 5G–I), supporting the conclusion that Nef promotes ADAM17 sorting into the EV pathway.

We agree that additional EV characterization following MISEV2023 recommendations would further strengthen this part of the study. While a comprehensive EV profiling analysis is beyond the scope of the current work, we have revised the text to clarify this limitation and to avoid overinterpreting the identity of the EV population isolated here.

15. The methods and figure legends need to be more detailed/completed.

We agree with the reviewer and have extensively revised the Methods and Figure Legends throughout the manuscript to provide substantially greater experimental detail and clarity.

Reviewer #2:

General Comments:

The authors discovered a gene that impacts HIV NL4.3 replication in 293T cells and go on to show that this gene, A17, affects Env processing, suggesting this may be one mechanism that reduces HIV infectivity. Their further studies show reproducible effects on infectivity upon expression of A17, and these findings extend to a T cell line. They further map the determinants of A17 activity and also extend these studies to show that expression of A17 in 293T cells impact infectivity of a range of primary isolates. This is important but the major issue here is whether the levels of expression of A17 in 293T cells is similar to what is found in CD4T cells as this could otherwise be something of an

artifact of overexpression - a painfully common issue in the HIV antiviral gene field. This needs to be addressed. Another key issue is the difference in NL4.3 and a primary virus in terms of Nef dependence on A17 in primary cells. They appear very different (Fig 6G) and yet this is not really explained. This is potentially interesting gene that impacts Env processing, but I am not yet convinced it is relevant to HIV replication in primary cells at endogenous levels. Some of the issue is just the paper is so dense, but the data in Fig 6G, which gets to the heart of this as it examines replication effects of A17, do suggest very little impact of A17 on WT virus, which is the situation of natural HIV infection.

We sincerely thank the reviewer for the thoughtful and constructive evaluation of our work and for recognizing the potential importance of ADAM17 in regulating HIV-1 Env processing and infectivity. We appreciate the reviewer's concerns regarding endogenous relevance, strain-dependent phenotypes, and the magnitude of Nef antagonism. In response, we have performed several additional experiments, including repeating the ADAM17 restriction in HEK293T cells by single-round replication (**Fig.1D**), comparison of endogenous versus ectopic ADAM17 expression levels (**Fig.1E**, **Fig.S5D**), confirming Env-Env BiFC in HEK293T cells in addition to HeLa cells (**Fig.2F**), validation of two additional MOLT-3 knockout clones (**Fig.4G**), analysis of an additional primary HIV-1 isolate BaL (**Fig.6F**), and comprehensive quantification of western blot and infectivity datasets.

Specific points:

1. Line 312-314: They certainly cannot conclude that A17 is solely responsible for HIV restriction in primary cells based on their experiments. Indeed, comparing no gRNA and A17 RNA for WT AD8, which seems the most relevant comparison, the effects if A17 are quite small. (They could do area under the curve to quantify differences or values at a fixed time point) How do they explain other genes that previously have been shown to restrict in primary cells?

We agree with the reviewer and have revised the text to avoid implying that ADAM17 is the sole restriction factor operating in primary cells.

Our conclusion is that ADAM17 represents one bona fide Nef-sensitive antiviral factor contributing to HIV-1 restriction in primary cells. Importantly, in Fig.6E, 6F, and 6H, deletion of ADAM17 restored Δ Nef primary strains' replication to levels approaching WT virus, supporting the conclusion that ADAM17 is a major contributor to the Nef-sensitive restriction phenotype under these experimental conditions.

2. Line 354. Brings up the point of overexpression noted above. How does the level of A17 expressed related to endogenous levels in T cells? This is very relevant given the KO effects are much less impressive than the overexpression effects, e.g 6B and 6C. The authors should add a western blot showing A17 levels in producer 293T cells compared to primary CD4 cells. This should ideally be done for the experiment in 6A as well.

We appreciate this important point and have now directly addressed it experimentally. In the revised manuscript, we show that endogenous ADAM17 levels in HEK293T cells are comparable

to those detected in MOLT-3 CD4 T cells (**Fig.S5D**). In addition, titration experiments demonstrate that endogenous ADAM17 expression in 293T cells is approximately equivalent to ectopic expression achieved using ~0.6 µg of ADAM17 plasmid DNA (**Fig.1E**). Because our antiviral assays were performed using 1 µg of ADAM17 expression vector, the ectopic expression level was moderately higher than endogenous expression, which likely contributes to the stronger phenotype observed in overexpression systems.

We share the reviewer's concern regarding interpretation of antiviral activity in HEK293T overexpression systems. For this reason, we extensively validated endogenous ADAM17 antiviral activity using CRISPR/Cas9 knockout approaches. Importantly, we further extended these analyses to CD4 T cells and hPBMCs, where we confirmed the antiviral role of endogenous ADAM17 in more physiologically relevant settings. Together, these results support that the antiviral activity of ADAM17 is not an artifact of overexpression in HEK293T cells, although higher ectopic expression likely amplifies the magnitude of restriction observed in that system.

3. Line 104-106, I don't see how Nef is counteracting the A17 effect. In both cases, there is a big drop (maybe this is the 15-35 fold they are describing?) - with A17. If Nef was counteracting A17, shouldn't there be similar levels of infection with A17 to the control in the WT virus, but not delta Nef? Or are they arguing that 15 fold and 35 fold are different? In which case some statistics are needed, but even then the magnitude of the effect is not convincing. Perhaps the issue here is it is unclear what is being compared to what- as presented, the figure implies it is the added gene compared to the control for each virus.
In fact, in 4A it looks like the Nef effect is about 2-fold (~7000 with Nef vs 3000 without Nef in A17 cells).

We appreciate the reviewer's concern and agree that the original presentation did not sufficiently clarify the relationship between ADAM17 expression level and Nef antagonism. In Fig.1D, ADAM17 antiviral activity was measured after ectopic expression of 1 µg ADAM17 plasmid DNA in 293T cells. Under these conditions, both endogenous and ectopic ADAM17 are present, and the total ADAM17 level may exceed the antagonistic capacity of Nef, analogous to observations previously reported for other restriction factors such as APOBEC3G and SERINC5. We have now repeated this experiment and show that WT and ΔNef viruses are inhibited by approximately 17-fold and 90-fold, respectively (**Fig.1D**). In Fig.4A, endogenous ADAM17 activity was evaluated in a single-round infectivity assay in 293T cells, whereas experiments in MOLT-3 cells and PBMCs measured multiple-round replication, which likely contributes to the different magnitude of the observed effects. Statistical analyses are now included throughout the revised manuscript.

4. In the double KO there is no effect of Nef. So, I am just convinced about the Nef part of this story as presented. The double KO aspect of the story is interesting because the effect in 4C is the most convincing in terms of infectivity.

Reply: We sincerely appreciate the reviewer's positive assessment of the double-knockout experiments and agree that these results provide strong support for the functional relationship between Nef and ADAM17.

5. Nef reduces all three ADAMs (Fig 1H ad 1I) but only A17 restricts. Doesn't that also suggest this is not due to Nef?

We appreciate this point. At present, we speculate that Nef may employ a related mechanism to reduce ADAM9 and ADAM15 expression, although only ADAM17 demonstrated clear antiviral activity in our assays.

6. I can't really follow how they draw the results from their Westerns, and I think quantitation across replicate Westerns may help, allowing for discussion of fold-differences. Some examples: For the western in Fig 2A, the explanations seem a bit arbitrary. For example, gp120 in lanes 8 and 11 seem the same, but gp41 is lower in lanes 11 vs 8. So, does this really support the statement for a marked effect of Nef on processing? It would also be helpful to see the gp160 band to see if it is processing specifically and how overall levels of Env play into this. In 2B, comparing Ser5 and A17, gp41 seems to not be impacted by A17, or perhaps lower, but gp120 is higher. How do they explain this differential impact on the two subunits and what is driving this? Or is the real focus the ratio compared to WT, in which case, again quantification of ratios would help as it is very hard to do this accurately by eye.

We appreciate the reviewer's careful evaluation of these Western blot data. As suggested, we have now quantified the relevant immunoblots throughout the manuscript.

In Fig.2A, we agree that the gp120 signals were difficult to interpret because the anti-gp120 antibody displayed substantially lower sensitivity compared to the anti-gp41 antibody. We therefore revised the presentation and interpretation accordingly. The gp160 band could not be detected in virion preparations because these samples contain mature virions rather than intracellular Env precursors.

In Fig. 2B, ADAM17 exerted a substantially stronger inhibitory effect on gp41 production than on total gp160 levels. Consistent with this observation, gp160 levels increased much more prominently than gp41 levels in A17-KO cells (Fig. 2H). These findings support a model in which A17 primarily targets and retains gp160 in the ER, thereby limiting its trafficking to the Golgi apparatus, where gp41 processing and maturation occur.

7. The differences in AD8 vs NL4.3 replication in primary cells with Nef deleted are striking. What is the basis for this and is this an example of the issues with using NL4.3? What is the conclusion of the role of Nef in A17 restriction in primary cells using primary virus as it appears different than all the NL4.3 findings? Is it that absent A17 in primary cells, Nef is not relevant to replication? It seems like this is an important finding of the paper. In fact, on lines 310-311, the authors say the results mirror the cell line findings, but they should clarify that they mirror for NL4.3, but not for the AD8 virus.

We appreciate this important point. Differences between laboratory-adapted and primary HIV-1 Env proteins are well established. Laboratory strains such as NL4-3 generally display a more open Env conformation, whereas primary isolates such as AD8 more commonly adopt a closed conformation. This distinction has previously been shown to influence sensitivity to host

restriction factors such as SERINC5 and is consistent with our findings here. Importantly, ADAM17 effectively inhibited all tested primary isolates, supporting its relevance as a broadly active HIV-1 restriction factor. We also corrected the corresponding text (lines 310-311) to avoid any misinterpretation of our results.

8. It is great to see the primary cell/virus experiment, but it raises more questions than it answers for the A17 KO cells. At least seeing replication of a few more viruses in normal vs A17KO cells could be helpful.

We thank the reviewer for this suggestion. In response, we generated an additional Δ Nef proviral clone derived from the primary HIV-1 BaL strain and confirmed its restriction by ADAM17 in MOLT3-CCR5 knockout cells. These new results are now presented in **Fig.6F**.

9. Discussion: The first paragraph says A17 leads to a 100-fold reduction in infectivity for Nef deficient viruses - the authors should clarify what this finding is based on.

We thank the reviewer for pointing out the need for clarification. We now explicitly state that the >100-fold inhibition of Δ Nef HIV-1 infectivity was measured by single-round replication assay under ectopic ADAM17 expression in 293T cells.

10. Again, I am bit confused because of the different findings with NL4.3 and AD8 and at this point, the replication curves suggest the effect is virus dependent. So, that needs to be noted and discussed more fully as the replication curves seems most relevant to infection outcomes.

We agree with the reviewer that these strain-dependent differences merit additional discussion. As noted above, NL4-3 and AD8 Env proteins differ substantially in conformation (“open” versus “closed”), which likely contributes to their distinct sensitivities to ADAM17-mediated restriction.

We have expanded this topic in Discussion and its references are also included.

11. Related to the above point is that AD8 shows minimal Nef dependence when produced in A17 KO cells. Given the model is A17 effects Env processing, this setting where Env is being produced for virions seems the most relevant place where the role of A17 is tested. How does this support the 100-fold effect noted above?

We appreciate the reviewer’s thoughtful question. Our model proposes that ADAM17 retains Env in the ER, thereby reducing its trafficking to the Golgi for processing and subsequent incorporation into virions at the plasma membrane. Because Env is essential for HIV-1 infectivity, inhibition of Env maturation substantially reduces viral infectivity.

In ADAM17 knockout cells, this restriction is removed, permitting efficient Env processing and virion incorporation even in the absence of Nef. The >100-fold effect described in the manuscript refers specifically to ectopic ADAM17 overexpression conditions in 293T cells.

12. Regarding the ADAM10 conflict described in the discussion, were the viruses and cells used the same? Here they show that it is not active against NL4.3 in 293T cells (and I suppose in the double KO in Molt4 cells?), but these types of results vary widely by virus and cell type. The authors should discuss this aspect to see if it might contribute to the differences observed.

We appreciate this important point. In our study, we used the same HIV-1 NL4-3 molecular clone and the same MOLT-3 CD4 T-cell line obtained from the NIH AIDS Reagent Program as used in the previous study. Therefore, we do not believe that the discrepancy arises from differences in viral or cellular reagents. Nevertheless, we have now expanded the Discussion as suggested.

We also recently presented our ADAM17 work in Session 7, *Accessory Proteins and Host Factors II*, at the Cold Spring Harbor Retroviruses Meeting, where this topic generated substantial discussion. Notably, Dr. Gottlinger presented his recent ADAM10 study in the same session. Feedback from the meeting further highlighted the importance of clearly distinguishing the roles of ADAM17 and ADAM10 in HIV-1 restriction. In response, we have revised the Discussion to better address these considerations based on the feedback from the meeting and to more constructively place our findings within the context of this emerging area in the field.

13. Also, the point made about pools versus clonal KOs. It is also important to acknowledge that clonal KOs are problematic unless multiple clones are tested. Individual clones can be a select variant so most people test 3-5 clonal lines to test a question. Something worth noting in the discussion as well if they used single clonal lines.

We agree with the reviewer that validation using independent clones is important. To address this concern, we tested two other independent MOLT-3 ADAM17 knockout clones and obtained the same phenotype. These new results are now shown in **Fig.4G**.

Furthermore, our key findings were independently confirmed using CRISPR-edited hPBMC pools (**Fig.6G-H**), minimizing concerns related to clonal artifacts. Finally, reconstitution of ADAM17 expression in MOLT-3 ADAM17-knockout cells restored Δ Nef virus restriction (**Fig.4H-I**), further supporting the specificity and rigor of the observed phenotype.

14. Minor: What are the two bands in Fig.1B and which one is the one being discussed? For example, the two bands for A17 and in the A9 panel, the input is one size and the Ip another?

We appreciate the reviewer's question. ADAM17 is typically detected as two bands by western blot, corresponding to the precursor form and the mature processed form generated after prodomain cleavage. In whole-cell lysates, the precursor form is more abundant, whereas both forms become more readily detectable following immunoprecipitation, making the mature form more visible in the IP samples.

15. Note, I don't have much experience with the methods used for microscopy so will defer to other reviewers for those aspects.

We appreciate the reviewer's consideration and have addressed the microscopy-related concerns raised by Reviewer #1 through expanded methodological detail, revised image presentation, and additional quantitative analyses.

Reviewer #1:

1. The authors answer correctly the concern by adding statistical analysis to their figures and the details of the analysis in the caption were added.
2. The detailed description provided in Methods section is now sufficient.
3. Ok
4. The Western blot quantifications and their statistical analysis has been added throughout the manuscript, reinforcing greatly the paper.
5. The new Figure 2D and its caption are way clearer and easy to read now.
6. The modification of the figure are good, and adding of flow cytometry experiment is a very good idea
7. Ok
8. Ok
9. The order is now correct.
11. Figure 5G is now clearer. The question was not about what software was used (Fiji) because this was already mentioned in the manuscript. The question was more about the method that was used, because it exists many different way to define and to measure colocalisation. However, the authors added the details of the analysis in the methods section during the revision, and explain clearly that they are talking about percentage of overlapping area after choosing a threshold for each channel, which is satisfactory.
12. The method section for confocal microscopy is now more detailed and sufficient.
14. The answer on EVs is acceptable.

Reply: We sincerely thank the reviewer for the careful re-evaluation of our revised manuscript and for recognizing our efforts to address the concerns raised during the initial review. We are pleased that the additional experiments, expanded methodological descriptions, statistical analyses, revised figures, and clarifications have satisfactorily addressed the reviewer's comments and substantially improved the clarity and rigor of the manuscript. We greatly appreciate the reviewer's constructive feedback, which has strengthened the quality of this work.

10. For the CD4⁺ T cell KO experiments, authors provide no cell viability datas. It could be a minor remark because no impact for the comprehension of the paper, but if the authors have the data, please add it in the supplementary figures. It's usually required to add cell viability to this type of data when experimenting with PBMCs that undergo proteins knockout.

Reply: We thank the reviewer for this helpful suggestion. As recommended, we assessed the viability of A17-knockout MOLT-3 CD4 T cells and confirmed that A17 deficiency does not affect cell viability. We also evaluated A17-knockout HEK293T cells and likewise observed no effect on cell viability. These new data have been included in **Fig. S7**. Together, these results indicate that the phenotypes observed in the knockout cells are not attributable to impaired cell viability.

13. Most of the experiments were done in HEK293T cells; however the IF/confocal microscopy was done in HeLa cells: this not relevant because the vesicular trafficking might be different in HeLa/ HEK293T cell lines and CD4 T cells (main target for HIV). At least, important decisive results should be verified by IF/confocal microscopy in HIV

infected MOLT3 cell lines. This point was not addressed by the authors and it is not difficult to achieve; a lot of protocols exist in the literature to perform IF on infected T cells.

Reply: We thank the reviewer for this valuable suggestion and agree that validation in a physiologically relevant HIV-1 target cell is important. Following the reviewer's recommendation, we performed immunofluorescence/confocal microscopy in HIV-1-infected MOLT-3 CD4 T cells. Consistent with our observations in HeLa cells, Nef and ADAM17 exhibited strong colocalization on the cell surface in infected MOLT-3 cells, indicating that the Nef-dependent redistribution of A17 is not unique to HeLa cells as shown in Fig. 5I. These new data confirm our conclusions in a relevant T-cell model and are presented in **Fig. S10**. We have also revised the Results to describe these findings.

Minor remark: please mention the technique used for the figure S5D in its caption.

Reply: Thanks for this suggestion. The technique for Fig. S5D (now Fig. S4D) has been added to the caption.

Reviewer #2:

The authors have worked hard to address issues raised. For the most part, the A17 effect on viral replication is supported by the studies presented. I do remain puzzled as to how they conclude that A17 is antagonized by Nef and think they spin this too far. A17 significantly reduces infection of WT type NL4.3 virus (a virus that expresses Nef) and deleting Nef does exacerbate this but the effect is relatively modest (~5-fold). Similarly, all the primary viruses are inhibited by A17 despite having an intact Nef. So Nef may only be a part of this antiviral mechanism.

Lines 107-110. Fig 1D

To the point above, A17 reduces wild type by 17-fold, and Dnef by another 5-fold (90-fold total), so the A17 effect seems to be only moderately impacted by Nef, yet the authors conclude that A17 exerts potent antiviral effect that is efficiently counteracted by Nef. I think 'efficiently' should be 'partially'. Similarly, in 1H, there is a partial Nef effect, with 14-fold reduction of WT in the A17KO.

Reply: We thank the reviewer for raising this important point. We agree that, in the HEK293T overexpression experiments (**Figs. 1D, 1H, and 6A**), Nef only partially counteracted the antiviral activity of A17. As we acknowledged in our previous response, these experiments were designed to characterize A17 antiviral activity and its mechanism, but the overexpression system is not ideal for quantitatively assessing the physiological antagonism of A17 by Nef.

In our previous response, we further clarified this issue by directly comparing ectopic and endogenous A17 expression levels. As shown in the new **Fig. 1E**, transfection with 1 µg of A17 plasmid, as used in our antiviral assays, resulted in A17 expression levels approximately twofold higher than endogenous A17 in HEK293T cells. Thus, under these overexpression conditions, the amount of A17 exceeded its physiological level, and Nef was unable to completely counteract its antiviral activity. This likely accounts for the substantial reduction in WT virus infectivity observed upon A17 overexpression.

Importantly, we therefore extended our analysis to endogenous A17 in MOLT-3 CD4 T cells and primary PBMCs, which provide more physiologically relevant systems for assessing Nef antagonism. In these cells, depletion of endogenous A17 markedly enhanced the replication of Δ Nef viruses, whereas little or no effect was observed for Nef-expressing WT viruses (**Figs. 4C, 4D, 4G, 5D, 6D, 6G, and 6H**). These results demonstrate that, at endogenous A17 levels, Nef effectively neutralizes A17-mediated restriction. We therefore believe that the apparent partial antagonism observed in HEK293T cells reflects the experimentally elevated level of A17 rather than an intrinsically weak ability of Nef to antagonize A17.

Nevertheless, we agree with the reviewer that this distinction should be made explicit. We have revised the Results associated with **Fig. 1E** to state clearly that Nef only partially antagonizes A17 under HEK293T overexpression conditions and have avoided using “efficiently counteracted” when describing these experiments (**lines 114-120**). We now reserve conclusions regarding effective Nef antagonism for experiments examining endogenous A17 in CD4 T cells and primary cells.

Line 253 says A9 does not affect infectivity but in Fig 4A that is not clear and those KO cell results should be compared to WT. The p values that are there seem to relate to another point in lines 254-255 about Dnef vs WT virus.

Reply: We apologize for the confusion. We agree the original statement that “Knockout of A9, A10, or A15 did not affect viral infectivity” (line 253) is less clear.

Accordingly, lines 253-255 have been revised as below:

“Deletion of A9, A10, or A15 had no detectable effect on the infectivity of either WT or Δ Nef viruses compared with WT HEK293T cells, indicating that these proteins do not measurably restrict HIV-1 under these conditions (**Fig. 4A, left**).”

Line 336 and 341, Ser failed to restrict AD8 dNef should be reworded to say the effect was modest and only a trend for significance (0.055) or something that acknowledges the trend.

Reply: We thank this reviewer for the suggestion. The text has been revised as suggested.

Figure 6H is the key experiment and I am still puzzled by the fact that there are striking differences in A17 cells for NL4.3 vs ADA. Why does Nef have no effect on NL43 infection in WT vs A17KO cells? This should be discussed.

Reply: We apologize for the lack of clarity and appreciate the reviewer for pointing this out. The key difference is that A17 and Ser5 have distinct antiviral specificities. In A17-KO cells, endogenous Ser5 is still expressed and continues to restrict NL4-3 Δ Nef virus. Consequently, deleting A17 alone does not fully relieve the infectivity defect of NL4-3 Δ Nef virus, making the difference between WT and A17-KO cells relatively small. In contrast, Ser5 does not measurably restrict the primary AD8 strain. Therefore, deletion of A17 alone is sufficient to abolish restriction of AD8 Δ Nef virus, resulting in a much more pronounced phenotype.

This interpretation is further supported by our double-knockout experiments in **Fig. 4A**, where simultaneous deletion of *Ser5* and *A17* completely restored NL4-3 Δ Nef infectivity to WT levels, demonstrating that both endogenous restriction factors contribute to NL4-3 restriction, whereas *A17* is the dominant restriction factor for AD8.

As suggested by this reviewer, we have revised the Discussion to clarify this point (**Lines 410-423**).

One of reasons this paper is so dense and confusing may be that the figure sometimes compare the wrong data. An example is Fig 6F. There they compare WT and dNef within cell pools/types. But isn't the real point comparing dNef in WT versus A17 cells?? I think my problem following this whole story was partly that the Figures often were structured in a way that did not optimally follow the text or point being made.

Reply: We thank the reviewer for this thoughtful suggestion and agree that the organization of the figures should clearly highlight the biological conclusion. In this study, we chose to compare WT and Δ Nef viruses produced from the same cell background because this design minimizes variability between independently generated cell lines and provides an internal matched control for assessing the contribution of Nef to viral infectivity. This strategy has been widely used in studies of HIV-1 accessory proteins, including Vif and Vpu, where WT virus serves as the physiological reference for evaluating the infectivity defect of the corresponding mutant virus.

At the same time, we agree that comparison of Δ Nef viruses across different cell backgrounds is also important for illustrating the contribution of individual host restriction factors. For this reason, we have revised the text to more explicitly direct readers to these comparisons and to clarify the conclusions drawn from each experiment. In particular, the key comparison of Δ Nef virus produced from WT versus A17-KO cells is now emphasized in the Results (for example, see the Results section for **Fig. 4C-4D**, **Fig. 4F**, **Fig. 6C-6D**, **Fig. 6F**, **Fig. 6H**), while the WT virus continues to serve as the matched internal control for each genetic background.