nature portfolio

Peer Review File

Rab27a regulates the transport of influenza virus membrane proteins to the plasma membrane

Corresponding Author: Professor Hongbo Zhou

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In the present study, the authors provide molecular detail on how Rab27a and its effectors (mainly SYTL1 and SYTL4) promote the transport of IAV envelope proteins to the cell surface. Overall, the study provides mechanistic insights on the role of Rab27a in IAV egress out of the host cells. The conclusions drawn from the experiments are appropriate; however, there are certain points that need attention and further experimentation, as described below.

1. What happens to IAV infection when constitutive active (Q78L) or constitutive inactive (T23N) Rab27a mutants are expressed in the background of a Rab27a knockout? How does the expression of these GTP- and GDP-locked Rab27a mutants affect IAV assembly and budding? What happens to the intracellular localization as well as the surface levels of viral proteins when these Rab27a mutants are expressed?

2. Can the author mention the level of Rab27a-Flag/HA overexpression used in the experiments shown in Fig. 1 (C-E)?

3. Fig. 1F illustrates how Rab27a KO impaired the growth of different subtypes of IAV at 12 and 24 hpi. Later post-infection, these levels further decreased, but the growth of H5N1 appears to have recovered at 36 hpi. Additionally, the graph plots up to 48 hpi for other isotypes, but only up to 36 hpi for H5N1. Is there any particular reason for that?

4. According to confocal images, the Rab27a distribution appears highly cytosolic. Could the location of the tag, which tags the Rab27a construct in these experiments at the C-terminus instead of the N-terminus, explain this?

5. In Fig. 4, it's difficult to comprehend the bands of LC3b-I and LC3b-II. The authors should use arrows to mark the bands. Additionally, all the figures should include the molecular weight of proteins.

6. In Figs. 4A and 4B, what are the levels of p62 in these conditions?

7. The authors should provide sequencing results for SYTL1-KO and SYTL4-KO cell lines.

8. Line 72, "In the this study, we employed siRNAs" please correct this line.

9. There is some writing issue with this line 135 "Notably, vRNA levels of viral proteins were no difference between……."

10. Please rename Bafilomycin A to BafA1, rather than BFA1. Brefeldin A commonly uses the term BFA, which could lead to confusion.

11. The authors should correct the GFP-LC3 and Merge panels in the Rab27a KO images in Fig. 4C.

12. In Figure 4I, the anti-phospho-ATG4B blot shows that levels of phospho-ATG4B decrease in samples co-expressing ATG4B-Flag and Rab27a-Flag as compared to samples expressing ATG4B-Flag alone. But, in Figure 4J, the levels of phospho-ATG4B look similar in both samples expressing ATG4B-Flag alone and samples co-expressing ATG4B-Flag and Rab27a-Flag. It would be helpful if the authors could quantify the blots.

13. In line 239, rewrite “detected” to "detect.".

14. In Fig. 5A, correct the spelling of "GFP.".

15. Line 103: It should be Rab27a instead of Rab27, since there are two isoforms.

16. Line 107: Instead of "impair," it should say "impaired."

17. Fig. 4A: Upon virus infection, levels of LC3BII increased, whereas virus-infected cells treated with BFA1 had reduced levels of LC3BII compared to only virus-infected cells or only BFA1-treated cells. Can the authors clarify this point?

18. Line 224: It should be “golden” instead of "gulden."

19. Fig. 5C, E, and 5L, as well as supplementary Fig. 6D, incorrectly label "HA" as "HAo."

Reviewer #2

(Remarks to the Author)
In this study, Chen and colleagues investigate the potential involvement of some RAB GTPases in the intracellular transport of Influenza A virus (IAV) surface proteins to the plasma membrane of infected cells. They identify RAB27A/B as well as their effector proteins SYTL1 and 4 as regulators of IAV HA, NA and M2 proteins trafficking to the cell surface and show that this host factors are important for IAV life cycle. This work relies on a combination of biochemistry, flow cytometry, electron and fluorescent microscopy experiments performed on depleted or knocked-out A549 cells. The authors also achieve efficient depletion of RAB27A and SYTL4 in the mouse lung tissue in vivo through intranasal delivery of siRNAs to validate their importance. Although the role of the identified host factors in the transport of IAV surface proteins is interesting and important for the general comprehension of how they make their journey within the host cell, the submitted work lacks essential controls that are needed to verify whether this transport pathway is specific to IAV transmembrane proteins or whether it merely constitutes a canonical route that any other secreted viral or cellular protein uses to reach the plasma membrane. Moreover, a thorough identification of the cellular compartments through which IAV surface proteins transit towards the plasma membrane would strengthen the impact of that work, by using specific tools now available and importantly by increasing the quality of the fluorescence microscopy data provided, which do not meet the standards of what is nowadays achievable and usually published in a journal such as Nature Communications.

Main concerns:

- It would be both important and interesting to test whether the host factors identified herein also participate in the trafficking of surface proteins from other viruses or whether this transport pathway is specifically hijacked upon IAV infection. For instance, respiratory syncytial virus (RSV), which interestingly also seems to hijack the RAB11-dependent pathway upon infection, efficiently infects A549 cells, such as vesicular stomatitis virus (VSV), whose G glycoprotein has been very well described to use the secretory pathway en route to the plasma membrane. Is the anterograde trafficking of other viral surface proteins altered upon RAB27 and SYTL4 depletion/KO? Does the over-expression/depletion/KO of these factors impact the formation/budding of other viral particles and other viruses growth?

- An essential question raised by the authors’ findings and that remains to be fully addressed is the identification of the precise transport pathway used by IAV surface proteins en route to the plasma membrane. In which compartment(s) do HA/NA accumulate in the absence of RAB27? Are they stuck in the Golgi apparatus, in the trans-Golgi network, in some endosomal structures? Specific immuno-stainings of such organelles followed by high resolution confocal microscopy in RAB27 KO cells would greatly improve the authors’ study. Ectopic expression of at least HA using a tool such as the RUSH system, which allows for the retention of a secreted cargo of interest in the endoplasmic reticulum followed by its synchronous release and tracking would be ideal to answer that question. Performing such experiment in WT and KO cells and use VSV-G for instance as a control would to me be powerful and greatly enhance our understanding of the transport pathway IAV surface proteins use to reach the plasma membrane, and of how/where RAB27 participates to its regulation.

Major points:

- How the screening of RAB GTPases was designed is to me questionable. RAB6 for instance, which has been clearly identified as a major, if not universal, regulator of post-Golgi transport to the plasma membrane should have been included. What about RAB17 and RAB23 which have been described as participating in IAV HA and NA traffic to the apical membrane of polarized MDCK cells?

- There are inconsistencies about the impact of RAB27A depletion/KO on the total levels of viral proteins. Indeed, while NP levels (why not rather monitoring HA/NA here?) decrease and increase upon RAB27A knock-down and over-expression respectively in figure 1, NP/ surface proteins levels increase upon RAB27 KO. How do the authors explain this difference?

- According to figures 3L, M/S2A and to the method section, GFP has been fused to the C-terminus of RAB27A. This is very surprising to me as it is well known in the field that RAB GTPases must only be tagged to their N-termini since C-terminus

fusion leads to alteration of the hypervariable domain which is necessary to efficient membrane targeting and renders RABs inactive. This must explain why the RAB27 pattern looks diffused within the cytosol rather than on membrane structures in figure S2A, which does not allow for proper co-localization analysis. This also accounts for the other RAB27 fusion constructs (HA, FLAG…) if they happen to have been designed the same way.

To sum up the issues about the identification of the transport pathway that IAV surface proteins take along their way to the plasma membrane, I believe the authors should:
(i) increase the quality of the imaging to provide convincing and quantified co-localization data between HA/NA and RAB27 in infected cells. This could easily be solved by generating a GFP-RAB27 A549 stable cell line. (ii) Identify precisely the cellular organelles that are involved in HA/NA transport and in which of them they remain stuck in the absence of RAB27 (see main concerns).

- The role of RAB27 in M2 transport remains puzzling to me despite the efforts of the authors to connect this part of their story with autophagy. RAB27A KO induces increased levels of surface M2. The authors claim that this is due to autophagy inhibition that leads to an increase in M2 total levels and that RAB27A does participate in M2 secretion. But then, how does M2 makes it to the plasma membrane in the absence of RAB27A? By diffusion? Via an alternative transport pathway? If not clarified I believe the manuscript would gain in clarity by focusing on HA/NA transport.

Minor points:

- In figure 3 (L, M) as well as in figure S2 (A, B, C), the authors first transfected A549 cells for 24h and then infected the cells. This is quite surprising to me as to my knowledge, not only A549 cells are not very easy to efficiently transfect but it is also well known that infection with IAV in previously transfected cells is difficult. If the authors decide to stick with this protocol rather than with the establishment of stable cell lines (see major points), I suggest that they provide representative images allowing for the visualization of the amount of transfected as well as transfected+infected cells. What does n=5 mean for instance in the case of figure S2? How many cells were analyzed?

- To what time post-infection do the quantifications relate? The control cell line should be used as a reference for normalizing the intensity of the signal, not the other way around.

- in figure S6D, RAB27A KO leads to a much stronger increase in HA total levels at 6hpi when compared with the same experiment showed in figure 3A. How do the authors explain such heterogeneity?

- Authors must provide molecular weights with the western-blot data

- Unusual and not very fluid building of the manuscript with supplemented figures only mentioned in the discussion section. These figures should be mentioned within the results section together with the main figures they relate to. Figure S6, which describes the involvement of RAB27B, is related to the first part of the manuscript that describes a role for RAB27A. Figure S5 relates to figure 4.

Reviewer #3

(Remarks to the Author)
The paper submitted to Nature Communications by Chen et al. investigates the transport mechanism of influenza A virus (IAV) envelope proteins to the cell surface following Golgi processing. Using siRNA screening, the authors identify Rab27a as a critical host factor that regulates the trafficking of viral envelope proteins: HA and NA glycoproteins and the M2 ion channel. Interestingly, the mechanism of the transport of HA/NA was different from that of M2. Rab27a is a small GTPase of the ras-associated family and plays an important role in the trafficking and secretion of intracellular proteins and has been reported to be involved in the replication cycle of other clinically relevant viruses, such as human parainfluenza virus, human cytomegalovirus, etc. In addition, the authors show that Rab27a exerts its regulatory function during viral infection through its effectors SYTL1 and SYTL4. Knocking out Rab27a and SYTL4 does not block the early stages of the IAV life cycle but restricts IAV assembly and budding by reducing the distribution of viral envelope proteins on the cell surface. Importantly, SYTL4 knockdown provides significant protection in a mouse model of IAV infection. Moreover, Rab27b was also seen to cooperate with Rab27a for the transport of IAV envelope proteins.
Understanding how IAV envelope proteins are transported to the cell surface of infected cells is crucial for the identification of novel targets and the development of innovative antiviral strategies against IAVs. Overall, this is a well-designed systematic study. The information derived from this study is relevant to the field of influenza and may guide research on other viruses. Nevertheless, there are several minor suggestions that may lead to some improvements and clarifications.

1) The EM of Supplementary Figure 1 shows cell surface extensions at 2 and 3 hpi that resemble filopodia. It may be worth stating in the figure legend what these extensions are so as not to confuse them with actual budding viruses.

2) The description for the Figure 2 (B to D) seems to be for B to E.

3) The timepoint for the WB in the Figure 3B is missing.

4) There appears to be an error in Figure 4L as it shows a band for Rab27a flag (-) and no band for Rab27a flag (+).

5) GAPDH is misspelled as GADPH in several figures.

6) Scale bars are not visible or clear enough on IFA and EM images.

7) All main and supplementary figures should include the molecular weight (MW) of the proteins identified by WB.

8) In the discussion, the authors discuss the presence of Rab27a in leukocytes and the absence of the effector SYTL4 in these cells. The authors suggest that this may affect the humoral response of the animals and be responsible for the differences in body weight loss and survival. This could be investigated, for example, by measuring influenza-specific endpoint serum titers of Ab isotypes by ELISA.

9) The model for Rab27a-mediated IAV envelope proteins in Figure 7 shows the envelope proteins on the wrong side of the transport vesicle and the cell membrane. The budding is in the wrong direction.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed the comments/suggestions, and the manuscript can be accepted for publication.

Reviewer #2

(Remarks to the Author)
In the reviewed version of their study entitled “Molecular mechanism of influenza virus envelope proteins transport”, describing a role for RAB27 and its effectors SYTL1/4 as regulators of Influenza A virus HA, NA and M2 proteins trafficking to the cell surface, Chen and colleagues strengthened their investigation and notably tested the importance of the described transport pathway for the trafficking of another unrelated virus (VSV), and used the RUSH system in an attempt to precisely identify the organelles through which they transit en route to the plasma membrane. Even though the absence of effect of RAB27 depletion on VSV replication suggests some kind of specificity of the described molecular pathway for Influenza virus surface proteins, the data provided here do not fully support the author’s claim according to which RAB27 participates in the traffic of viral enveloppe proteins from the trans-Golgi network to the plasma membrane.

Major concerns:
• Role of different RAB GTPases on viral envelope transport towards the cell surface:
siRNAs specific for other relevant RAB GTPases were added. I have to admit that I find the absence of effect on HA transport to the surface upon RAB6 depletion very surprising, knowing how key this cellular factor is for anterograde post-Golgi transport and I might not be the only one. Having said that, the flow cytometry data are clear. Maybe showing immunofluorescence images of HA levels at the plasma membrane to illustrate the difference would help making these results more convincing?
The authors should at least discuss the fact that contrary to what Sato et al. (PFID 31456775) described, depletion of RAB17 and RAB23 depletion does not affect trafficking of surface proteins in their hands.

• Identification of the cellular compartments involved in Influenza virus surface proteins transport

In general, the data provided by the authors do not fully support their conclusions according to which RAB27 (and its effectors) regulates the transport of viral envelope proteins from the trans-Golgi network to the plasma membrane of infected cells. Despite nicely designed RUSH-based assay which tend to show that, the results obtained in an actual infectious context do not :

- According to Fig 5b, KO of RAB27 does not lead to any accumulation of HA in the TGN, as it should do if RAB27 was essential for a “TGN-to-plasma membrane” transport route.

- According to Fig 5k, most GFP-RAB27 seems to localize on ER membranes (with a pretty clear nuclear envelope signal) where most HA is found as expected. However, when looking at the most peripheral part of the cell, where post-Golgi vesicles are found, there is almost no colocalization between HA and RAB27, again not supporting a “TGN-to-plasma membrane” transport route.

- According to Supp Fig 4, GFP-RAB27 is slightly relocalized to LC3+ structures upon infection but not to the TGN. This is again in total contradiction with RAB27 being involved in HA trafficking within a “TGN-to-plasma membrane” transport route.

- In Fig 5C, CD63 stainings (which allows for the visualization of multivesicular bodies, not late endosomes), show an endosomal-like pattern while while HA accumulates in the ER, as expected. Thus, there is very low co-localization between the 2 signals but the quantification provided with the images indicates ~70% of co-localization! This makes the way co-

localization analysis were performed very questionable.

The authors themselves, and I quote here the point-by-point response to reviewers, write that “Rab27a functions after infection is not in the Golgi or late endosomes, but in the autophagosome” (sic), which adds to the overall confusion.

Altogether, even though the authors made a strong effort in an attempt to identify the subcellular transport pathway through which influenza virus surface proteins transit with nicely designed RUSH assay-based experiments, their results do not fully support their conclusions, yet they are sometimes contradictory.

• The title of the study is too general. This work does not elucidate the “Molecular mechanism of influenza virus envelope proteins transport” but rather identify RAB27 and effectors as potential regulators of their traffic.

Minor comments:

- Line 27 : Introduction not instruction
- Line 33 : M2 is not an envelope protein
- Line 36 : RAB11 was not identified as driving vRNP transport from “vacuolar structures”.
- Line 39 : Membrane proteins are not “attached” to membrane vesicles. “transported through” maybe?
- Line 53 : RAB GTPases are not involved in Golgi “post-processing”. “Post-Golgi transport” maybe?
- Line 61/62 : this work does not fully “elucidate the molecular mechanism of IAV proteins transport”
- Lines 136 to 167 + line 245 : A549 cells cultured in 2D are not polarized and hence do not exhibit apical versus basolateral membrane domains. My point regarding the use of VSV-G was to make sure that the RAB27-dependent transport pathway identified here was specific of IAV envelope proteins in infected cells and not merely a canonical route to the plasma membrane. The authors should thus avoid mentioning apical vs basolateral membranes as their work was not performed in polarized cells.
- Line 173 : Why would impairment of autophagy lead to accumulation of M2 to the cells surface? I keep thinking that the part of M2 transport makes the message very confusing and I do not understand how RAB27 could transport M2 from the Golgi apparatus to the plasma membrane if M2 accumulates at the surface in RAB27 KO cells.
- Fig 5D “release” : The transport of a cargo from the ER to the Golgi apparatus occurs through vesicles which should be drawn of the cartoon.

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
In the last reviewed version of their study now entitled “RAB27A regulates the transport of influenza virus membrane proteins to the plasma membrane” (which better reflects their findings), Chen and colleagues notably put some efforts at better identifying the organelles hijacked by Influenza A virus (IAV) to ensure the transport of the viral surface proteins, and propose a model in which RAB27A would regulate the transport of post-Golgi vesicles carrying viral proteins to the plasma membrane.

As this remains to me the weakest part of the proposed study, I will mostly comment on that and on what seems to me being an over-interpretation of the data:

The conclusion that RAB27+ vesicles containing IAV membrane proteins are post-Golgi vesicles en route to the plasma membrane is mostly, if not only, based on the fact that they are “observed in areas far from the nuclear periphery” (sic, Point-by-point response to the reviewer’s comments and concerns), which is not a solid argument for such a claim. These vesicles may also be involved in ER-to-Golgi transport and represent ER exit sites (ERES) or vesicles generated from the ER-to-Golgi Intermediate Compartment (ERGIC). Such structures are not necessarily observed in the perinuclear area and can be found at more distal locations. This alternative proposition actually fits with the fact that GFP-RAB27 exhibits both a ER and a Golgi distribution. Only a 20°C block in the Golgi apparatus followed by a switch at 37°C to synchronise the release of vesicles from the Golgi towards the cell periphery would formally show that the identified vesicles actually are post-Golgi vesicles. In other words, the current set of data rather convincingly show that RAB27A regulates the transport of IAV envelope proteins to the plasma membrane (so says the new title) but the exact nature of the vesicles involved remains unclear. This should to the least lead the authors to revisit and tone down their current conclusion.

Minor: To my opinion, including the data that show that VSV-G transport relies on RAB6A (figure R1B-C) and not on RAB27A (while it’s the exact opposite in the case of IAV surface proteins) would strengthen the idea that the RAB27A-mediated pathway is somehow specific to IAV membrane proteins. For instance, in a supplementary figure that would relate to Figure 3.

Point-by-point response to the reviewers' comments and concerns

Comments from Reviewer 1:

In the present study, the authors provide molecular detail on how Rab27a and its effectors (mainly SYTL1 and SYTL4) promote the transport of IAV envelope proteins to the cell surface. Overall, the study provides mechanistic insights on the role of Rab27a in IAV egress out of the host cells. The conclusions drawn from the experiments are appropriate; however, there are certain points that need attention and further experimentation, as described below.

Response: We feel great thanks for your professional review work on our article. As you are concerned, there are several problems that need to be addressed. According to your valuable suggestions, we have made extensive corrections to our previous draft, the detailed corrections are listed below.

1. What happens to IAV infection when constitutive active (Q78L) or constitutive inactive (T23N) Rab27a mutants are expressed in the background of a Rab27a knockout? How does the expression of these GTP- and GDP-locked Rab27a mutants affect IAV assembly and budding? What happens to the intracellular localization as well as the surface levels of viral proteins when these Rab27a mutants are expressed?

Response: We wholeheartedly appreciate this professional suggestion. We stably expressed wild-type Rab27a (Rab27a-WT), a constitutively active mutant of Rab27a (Rab27a-Q78L), or a dominant-negative mutant of Rab27a (Rab27a-T23N) in Rab27a KO cells. The virus replication levels (Revised Fig. 1i), the number of viral budding (Revised Fig. 2f), intracellular localizations (Revised Supplementary Fig. 2a) as well as the surface levels of viral envelop proteins (Revised Fig. 3l, m) were compared in these cells. The effects of expressing the above proteins in Rab27a-KO cells on above levels are as follows:

1) The virus replication levels in those cells followed the order in Rab27a-Q78L-expressing cells > Rab27a-WT-expressing cells > Rab27a-T23N-expressing cells > Rab27a-KO. While there was a statistically significant difference in the virus titers between Rab27a-T23N and Rab27a-KO cells,

the absolute levels were relatively close (Revised Fig. 1i).

2) The number of viral buddings in those cells followed the order in Rab27a-Q78L-expressing cells > Rab27a-WT-expressing cells > Rab27a-T23N-expressing cells = Rab27a-KO (Revised Fig. 2f).

3) Intracellular localizations (Revised Supplementary Fig. 2a) as well as the surface levels of viral envelop proteins (Revised Fig. 3l, m): Rab27a-Q78L-expressing cells > Rab27a-WT-expressing cells > Rab27a-T23N-expressing cells > Rab27a-KO. Similarly, the surface levels of HA between Rab27a-T23N and Rab27a-KO were relatively close.

Overall, these levels in Rab27a-T23N-expressing cells were comparable to those in Rab27a-KO cells, and both were significantly lower than those in Rab27a-WT -expressing cells. These levels in Rab27a-Q78L- expressing cells were higher than those in Rab27a-WT-expressing cells. These results suggested that the GTP-bound form of Rab27a plays a dominant role in transport of viral envelop proteins.

Moreover, you may have noticed that the expression levels of these three mutants are inconsistent, which might be due to the impact of site mutations on protein expression efficiency. We recognize this as a minor shortcoming of our study, but we think it does not detract from the validity of our findings. A series of results indicate that although the Rab27a-Q78L mutant exhibited the lowest expression level, it had the strongest promotive effect on influenza virus. In contrast, while the Rab27a-T23N protein was indeed expressed in Rab27a-KO cells, it did not significantly impact the budding or replication of the influenza virus. Therefore, we conclude that our results highlight the critical role of Rab27a in the viral life cycle, which depends on its GTP-bound form.

We observed another phenomenon that might raise your concerns. We used the anti-Rab27a antibody to detect the expression of these proteins in Rab27a-KO cells, with A549-Cas9 cells serving as a control. The results showed that although the protein levels of Rab27a-WT/Q78L in Rab27a-KO cells appeared higher than the endogenous Rab27a in A549-Cas9 cells, the growth of influenza virus, the number of viral buddings, and the distribution of HA on the cell surface were not higher than

those in A549-Cas9 cells. We propose that these stable expression cells were generated based on Rab27a-KO monoclonal cells and represent polyclonal stable expression cells. Consequently, these cells may include subpopulations of cells with low expression levels or uninfected by the lentivirus, which could compromise the overall effectiveness of the cell lines. This phenomenon is relatively common in the construction of stable cell lines.

2. Can the author mention the level of Rab27a-Flag/HA overexpression used in the experiments shown in Fig. 1 (C-E)?

Response: Thank you for pointing out this problem in manuscript. We have clarified the expression levels of Rab27a-Flag/HA-Rab27a overexpression used in the figure legend of Fig. 1f-h in the revised manuscript (lines 808).

3. Fig. 1F illustrates how Rab27a KO impaired the growth of different subtypes of IAV at 12 and 24 hpi. Later post-infection, these levels further decreased, but the growth of H5N1 appears to have recovered at 36 hpi. Additionally, the graph plots up to 48 hpi for other isotypes, but only up to 36 hpi for H5N1. Is there any particular reason for that?

Response: We fully understand your concerns and apologize for the inconsistencies in time points used for assessing the replication of different influenza virus subtypes. There was no specific reason for not evaluating the replication levels of the HM/H5N1 subtype at 48 hpi, and the viral titers in Rab27a KO cells consistently remained lower than those in A549-Cas9 cells. To avoid any misunderstanding, we re-assessed the effect of Rab27a KO on HM/H5N1 replication at 12, 24, 36, and 48 hpi, and the results were consistent with the trends observed for other influenza virus subtypes (Revised Fig.1c).

4. According to confocal images, the Rab27a distribution appears highly cytosolic. Could the location of the tag, which tags the Rab27a construct in these experiments at the C-terminus instead of the N-terminus, explain this?

Response: We fully understand your concerns and sincerely appreciate your suggestion, which aligns with the opinion of another reviewer. As your professional insight points out, tagging Rab27a at its C-terminus impairs its targeting to the cell

membrane. Consequently, when Rab27a-GFP is expressed, it predominantly localizes to the cytoplasm (as shown in the previous version of the manuscript, Fig. 3L, M, S2A), whereas HA-Rab27a (incorrectly labeled as Rab27a-HA in the original manuscript) is distributed in both the cytoplasm and the cell membrane (as shown in the previous version of the manuscript, Fig. S2B, C, S2D). We deeply apologize for this oversight. We realize that using the C-terminally tagged Rab27a-GFP does not accurately reflect the relevant localization.

Therefore, we generated A549 monoclonal cells stably expressing GFP-Rab27a, in which Rab27a was properly localized to both the cytoplasm and the cell membrane. We retested the co-localization of GFP-Rab27a with viral envelop proteins under infection conditions and provided representative high-resolution microscopy images. The results showed that GFP-Rab27a co-localized with viral envelop proteins (Revised Fig. 5k, l). In addition, we retested the co-localizations of GFP-Rab27a with different membrane-bound organelles before and after infection, the results showed that there were no significant differences in the co-localizations of Rab27a with Golgi network or late endosomes before and after infection (Revised Supplementary Fig. 4b-d), but the co-localizations with autophagosome was increased (Revised Supplementary Fig. 4e), indicating that the site where Rab27a functions after infection is not in the Golgi or late endosomes, but in the autophagosome or its own positive vesicles. These results provided representative high-resolution microscopy, and statistical analysis was performed on 20-25 cells per group to ensure data reliability.

Additionally, we inadvertently referred to HA-Rab27a as Rab27a-HA in the previous version of the manuscript. We sincerely apologize for this oversight and have corrected it in the revised version.

5. In Fig. 4, it's difficult to comprehend the bands of LC3b-I and LC3b-II. The authors should use arrows to mark the bands. Additionally, all the figures should include the molecular weight of proteins.

Response: Thank you for pointing out this problem in manuscript. We have added indicator arrows next to the bands of LC3BI and LC3BII, and labeled the molecular

weights for proteins in all the figures.

6. In Figs. 4A and 4B, what are the levels of p62 in these conditions?

Response: Thank you for your suggestion. We repeated these experiments and additionally measured the protein levels of P62 (Revised Fig. 4a, b). The results showed that P62 levels significantly increased upon treatment with the inhibitor BafA1, confirming the functionality of the inhibitor. Furthermore, under viral infection, overexpression of Rab27a elevated P62 protein levels, while knockout of Rab27a reduced P62 levels, indicating that Rab27a promotes autophagy. These findings are consistent with our previous conclusions.

We observed that the changes in P62 levels were less pronounced when the inhibitor BafA1was added during infection, although the changes in LC3BII remained quite evident. We speculate that this discrepancy may be related to the complex alterations in mitophagy following viral infection, in which the viral protein M2 promotes incomplete autophagy, while the viral protein PB1-F2 promotes complete mitophagy[1]. Therefore, considering the changes in LC3BII, we maintain that Rab27a promotes the initiation process of autophagy.

Reference：

[1]Wang, R. F. et al. Influenza A virus protein PB1-F2 impairs innate immunity by inducing mitophagy. Autophagy, doi:10.1080/15548627.2020.1725375 (2020).

7. The authors should provide sequencing results for SYTL1-KO and SYTL4-KO cell lines.

Response: Thank you for your suggestion. In the revised manuscript, we have added the sequence information of all knockout cell lines, including Rab27b-KO, Rab27-DK, SYTL4-KO, and SYTL1-KO cells (Revised Supplementary Fig. 1n and Supplementary Fig. 5e).

8. Line 72, “In the this study, we employed siRNAs” please correct this line.

Response: We sincerely appreciate your careful review and apologize for the oversight during the writing process. We have corrected “In the this study” to“In this study” (lines 52)

9. There is some writing issue with this line 135 “Notably, vRNA levels of viral

proteins were no difference between…….”

Response: We sincerely appreciate your careful review and apologize for the oversight during the writing process again. We have corrected “Notably, vRNA levels of viral proteins were no difference between Rab27a-KO and A549-Cas9 cells at 3 hpi, but it was significantly higher in Rab27a-KO cells than that in A549-Cas9 cells at 4 hpi” to “Notably, while the vRNA levels of these viral proteins were similar between Rab27a-KO and A549-Cas9 cells at 3 hpi, they were significantly higher in Rab27a-KO cells at 4 hpi” (lines 120-122). We have thoroughly re-examined the manuscript and have taken measures to avoid similar errors, ensuring the overall quality and accuracy of the paper.

10. Please rename Bafilomycin A to BafA1, rather than BFA1. Brefeldin A commonly uses the term BFA, which could lead to confusion.

Response: Thank you for your professional suggestion. We have corrected “BFA1” to “BafA1” in revised manuscript (lines 175-177) and made corresponding changes in revised Fig. 4a, b.

11. The authors should correct the GFP-LC3 and Merge panels in the Rab27a KO images in Fig. 4C.

Response: We sincerely appreciate your careful review and apologize for the oversight. We have corrected GFP-LC3 and Merge panels in the Rab27a KO images in revised Fig. 4c.

12. In Figure 4I, the anti-phospho-ATG4B blot shows that levels of phospho-ATG4B decrease in samples co-expressing ATG4B-Flag and Rab27a-Flag as compared to samples expressing ATG4B-Flag alone. But, in Figure 4J, the levels of phospho-ATG4B look similar in both samples expressing ATG4B-Flag alone and samples co-expressing ATG4B-Flag and Rab27a-Flag. It would be helpful if the authors could quantify the blots.

Response: Thank you for your professional suggestion. We quantified the proportion of phosphorylated ATG4B relative to total ATG4B protein levels in revised Fig. 4h-j. These results collectively support the conclusion that Rab27a inhibits ATG4B phosphorylation, and that M2 further facilitates this process.

13. In line 239, rewrite “detected” to "detect.”.

Response: We sincerely appreciate your careful review and apologize for the oversight. We have rewritten this sentence and carefully checked the grammar (lines 192-194).

14. In Fig. 5A, correct the spelling of "GFP.".

Response: We sincerely appreciate your careful review and apologize for the oversight. We have corrected the spelling of “GFP” in revised Fig. 6a.

15. Line 103: It should be Rab27a instead of Rab27, since there are two isoforms.

Response: We sincerely appreciate your careful review and apologize for the oversight. We have corrected this word in revised manuscript (lines 86) and reviewed the entire manuscript to avoid similar errors.

16. Line 107: Instead of "impair," it should say "impaired."

Response: Thank you for pointing out this problem in manuscript. We have revised “impair” to “impaired” and improved this sentence in revised manuscript (lines 83).

17. Fig. 4A: Upon virus infection, levels of LC3BII increased, whereas virus-infected cells treated with BFA1 had reduced levels of LC3BII compared to only virus-infected cells or only BFA1-treated cells. Can the authors clarify this point?

Response: We sincerely appreciate your thorough review of our manuscript. We have carefully considered your concerns and realized that the dosage of BafA1 used in our previous experiments (100 nM) might have been suboptimal. This is because the inhibitory effect of BafA1 on LC3 was lower than that induced by viral infection alone, which is unreasonable. Therefore, in the repeated experiments, we used a dosage of 400 nM, which significantly enhanced the inhibitory effect of BafA1 on LC3BII. This adjustment also resulted in LC3BII levels in BafA1-treated infected cells being higher than those in cells infected with the virus alone. We sincerely apologize for any confusion caused by this oversight and are grateful for your keen observation, which has allowed us to ensure the accuracy of these results.

However, we are confident that in multiple repetitions of the experiment, the LC3BII levels in virus-infected cells treated with BafA1 were lower than those in cells treated with BafA1 alone. We believe this discrepancy could be due to the

accumulation of viral proteins during the prolonged infection process, which may influence LC3BII levels. Previous reports have shown that certain viral proteins, such as PB1-F2, can induce complete autophagy, which could potentially explain this observation[1]. Nonetheless, we believe that this issue does not affect the overall conclusion that Rab27a promotes autophagy. We apologize for our oversight again and sincerely appreciate your constructive comments.

Reference:

[1]Wang, R. F. et al. Influenza A virus protein PB1-F2 impairs innate immunity by inducing mitophagy. Autophagy, doi:10.1080/15548627.2020.1725375 (2020).

18. Line 224: It should be “golden” instead of "gulden.”

Response: Thank you for pointing out this problem. We have revised “gulden” to “golden” and improved this sentence in revised manuscript (lines 184).

19. Fig. 5C, E, and 5L, as well as supplementary Fig. 6D, incorrectly label "HA" as "HAo."

Response: Thank you for pointing out this problem in manuscript. We have corrected "HA_0" to "HA" in Fig. 6c, e, l of revised manuscript.

Comments from Reviewer 2:

In this study, Chen and colleagues investigate the potential involvement of some RAB GTPases in the intracellular transport of Influenza A virus (IAV) surface proteins to the plasma membrane of infected cells. They identify RAB27A/B as well as their effector proteins SYTL1 and 4 as regulators of IAV HA, NA and M2 proteins trafficking to the cell surface and show that this host factors are important for IAV life cycle. This work relies on a combination of biochemistry, flow cytometry, electron and fluorescent microscopy experiments performed on depleted or knocked-out A549 cells. The authors also achieve efficient depletion of RAB27A and SYTL4 in the mouse lung tissue in vivo through intranasal delivery of siRNAs to validate their importance. Although the role of the identified host factors in the transport of IAV surface proteins is interesting and important for the general comprehension of how they make their journey within the host cell, the submitted work lacks essential controls that are needed to verify whether this transport pathway is specific to IAV

transmembrane proteins or whether it merely constitutes a canonical route that any other secreted viral or cellular protein uses to reach the plasma membrane. Moreover, a thorough identification of the cellular compartments through which IAV surface proteins transit towards the plasma membrane would strengthen the impact of that work, by using specific tools now available and importantly by increasing the quality of the fluorescence microscopy data provided, which do not meet the standards of what is nowadays achievable and usually published in a journal such as Nature Communications.

Response: We greatly appreciate your professional and constructive comments, which have been extremely helpful in improving the quality of our manuscript. In response to your concern about the specific pathway of Rab27a regulating the transport of influenza virus envelope proteins, we have conducted studies using the two approaches as your suggestion. Our findings demonstrate that Rab27a regulates the transport of secretory vesicles carrying viral envelope proteins along the pathway from the trans-Golgi to the apical plasma membrane. Additionally, following your advices, we generated A549 monoclonal cells stably expressing GFP-Rab27a and retested the co-localizations of GFP-Rab27a with viral envelop proteins and different membrane-bound organelles. Above results provided representative high-resolution microscopy, and 15-28 cells were used for statistical analysis in each group to ensure the reliability of the data.

We also investigated the effects of Rab27a and its effector SYTL4 on VSV replication and the transport of VSV glycoprotein (VSV G). Our results confirmed that Rab27a specifically regulates apical transport rather than basolateral transport. We have addressed the other issues you raised and supplemented the manuscript with the corresponding data. We have tried our best to make all the revisions clear, and we hope that the revised manuscript can satisfy the requirements for publication. Below, we provide detailed point-by-point responses.

Main concerns:

- It would be both important and interesting to test whether the host factors identified herein also participate in the trafficking of surface proteins from other viruses or

whether this transport pathway is specifically hijacked upon IAV infection. For instance, respiratory syncytial virus (RSV), which interestingly also seems to hijack the RAB11-dependent pathway upon infection, efficiently infects A549 cells, such as vesicular stomatitis virus (VSV), whose G glycoprotein has been very well described to use the secretory pathway en route to the plasma membrane. Is the anterograde trafficking of other viral surface proteins altered upon RAB27 and SYTL4 depletion/KO? Does the over-expression/depletion/KO of these factors impact the formation/budding of other viral particles and other viruses growth?

Response: We fully understand your concern about whether the growth of VSV and the transport of VSV-G are regulated by Rab27a or SYTL4, as influenza virus HA and VSV G protein are considered as two typical representatives for studying apical and basolateral transport in polar cells. We greatly appreciate your professional suggestions, which have emphasized the importance of our manuscript. To address this, we investigated the effect of silencing Rab27a or SYTL4 on VSV. We found that silencing Rab27a or SYTL4 had no effect on VSV replication or VSV-G protein transport to cell surface (Revised Supplementary Fig. 2 d, e; Fig. 3n, o; Supplementary Fig. 5k; and Fig. 6q, r). Additionally, using RUSH (retention using selective hooks) system, we observed that the proportion of VSV G proteins on the cell surface after synchronous addition of biotin for 90 minutes did not decrease in Rab27a KO cells compared with A549-Cas9 cells, further confirming the preference of Rab27a in regulating apical transport (Revised Fig. 5d; Supplementary Fig. 4a). Above results indicated that Rab27a and SYTL4 do not participate in basolateral transport.

- An essential question raised by the authors' findings and that remains to be fully addressed is the identification of the precise transport pathway used by IAV surface proteins en route to the plasma membrane. In which compartment(s) do HA/NA accumulate in the absence of RAB27? Are they stuck in the Golgi apparatus, in the trans-Golgi network, in some endosomal structures? Specific immuno-stainings of such organelles followed by high resolution confocal microscopy in RAB27 KO cells would greatly improve the authors' study. Ectopic expression of at least HA using a

tool such as the RUSH system, which allows for the retention of a secreted cargo of interest in the endoplasmic reticulum followed by its synchronous release and tracking would be ideal to answer that question. Performing such experiment in WT and KO cells and use VSV-G for instance as a control would to me be powerful and greatly enhance our understanding of the transport pathway IAV surface proteins use to reach the plasma membrane, and of how/where RAB27 participates to its regulation.

Response: We greatly appreciate your professional and constructive comments. To address your concern about the unclear transport pathway by which Rab27a regulates the transport of influenza virus envelope proteins, we employed two approaches based on your suggestions. We first measured the effects of Rab27a KO on co-localizations of HA proteins with the membrane-bound organelles that they might pass through. The results showed that there were no significant differences in the co-localizations of HA with different organelles (including cis-Golgi, trans-Golgi, and late endosomes) between Rab27a KO and A549-Cas9 cells (Revised Fig. 5a-c). Additionally, we chose specific time points to assess the co-localization of HA with these organelles. The co-localization of HA with the cis-Golgi and trans-Golgi was analyzed at 4 hpi (Revised Fig. 5a, b), while the co-localization with late endosomes was examined at 3 hpi (Revised Fig. 5c). This intentional selection accounts for the differences in HA distribution observed in the tests. At 3 hpi, HA was more uniformly distributed throughout the cells, whereas at 4 hpi, it predominantly formed punctate structures. The time points were carefully chosen to minimize confounding effects. At 4 hpi, viral budding had already occurred, with fewer virus particles budding to the cell surface in Rab27a KO cells. To avoid potential confounding from newly budded virus particles undergoing endocytosis and co-localizing with endosomes (e.g., CD63), we assessed co-localization with late endosomes at 3 hpi, prior to significant viral budding. Furthermore, as shown in Revised Fig. 5k, some HA protein had already reached the cell surface by 3 hpi, making this time point suitable for detection.

Using the RUSH (retention using selective hooks) system, as shown in Revised Fig. 5d, we visualized the transport pathway of HA from the ER to the cell surface to

clarify the specific steps involving Rab27a. The results indicated no significant differences in the co-localization of HA with the cis-Golgi or trans-Golgi between Rab27a KO and A549-Cas9 cells. However, the proportion of HA proteins on the cell surface was notably reduced in Rab27a KO cells (Revised Fig. 5e-h). Importantly, we found that at 90 minutes after biotin addition, a large number of punctate vesicles were retained in the Rab27a KO cells (Revised Fig. 5h), indicating that Rab27a-KO prevents the effective transport of these secretory vesicles carrying HA proteins to the plasma membrane. Furthermore, the RUSH system confirmed that Rab27a knockout did not affect the transport of VSV G protein, reinforcing Rab27a's specific role in apical transport (Revised Supplementary Fig. 4a). Above results provided representative high-resolution microscopy, with 15-28 cells replicates were used for analysis in each group to ensure the accuracy of the conclusions. To enhance the significance of our study, we have incorporated these interpretations into the revised manuscript's Discussion section (lines 349-358).

Additionally, within 0–30 minutes after biotin addition in A549-Cas9 cells, no co-localization of HA protein with late endosomes was observed, suggesting that HA primarily follows the ER-Golgi pathway to reach the cell surface. However, at 3 hpi, co-localization of HA protein with late endosomes was detected (Revised Fig. 5c). We believe it may be due to endocytosis of HA protein after reaching the cell membrane. Since viral budding requires the accumulation of a large quantity of viral proteins on the cell surface, HA proteins likely gradually accumulate on the cell membrane within 0-3 hpi. During normal endocytosis, a small portion of HA may be internalized into endosomes, which subsequently mature into late endosomes.

Major points:

- How the screening of RAB GTPases was designed is to me questionable. RAB6 for instance, which has been clearly identified as a major, if not universal, regulator of post-Golgi transport to the plasma membrane should have been included. What about RAB17 and RAB23 which have been described as participating in IAV HA and NA traffic to the apical membrane of polarized MDCK cells?

Response: We sincerely appreciate your professional and constructive comments. Our

initial screening criteria were established based on a review of relevant literature. Due to an oversight on our part, the Rab6a (Rab6b is mainly expressed in nerve cells, therefore it was not included in the screening), Rab17, and Rab23, which you mentioned, were unfortunately not included in our screening scope. We apologize for this omission and have addressed it in the revised manuscript. Based on your suggestion to include Rab6a, Rab17, and Rab23 in the screening scope, we conducted the screening experiments again (Revised Fig. 1a and Supplementary Fig. 1b-j). The results showed that silencing Rab27a still had the strongest inhibitory effect on HA protein level on the cell surface at 4 hpi. In comparison, silencing Rab6a and Rab17 resulted in weaker inhibitory effects, while silencing Rab23 had no significant impact on HA protein transport.

- There are inconsistencies about the impact of RAB27A depletion/KO on the total levels of viral proteins. Indeed, while NP levels (why not rather monitoring HA/NA here?) decrease and increase upon RAB27A knock-down and over-expression respectively in figure 1, NP/ surface proteins levels increase upon RAB27 KO. How do the authors explain this difference?

Response: We fully understand your concerns. In revised Fig. 1, we conducted experiments under conditions of Rab27a overexpression and knockout during multiple rounds of viral infection at 12, 24, and 36 hpi. These time points reflect the cumulative effects of Rab27a on virus assembly and budding, which resulted in a decrease in NP protein levels upon Rab27a knockout and an increase in NP protein levels upon Rab27a overexpression. We specifically measured NP protein levels because, during multiple rounds of infection, NP protein serves as a reliable marker for overall viral protein expression, effectively reflecting the total viral load in infected cells. This approach aligns with previous studies, where NP protein levels have been commonly and acceptably used to indicate the replication efficiency of viruses during multi-round replication cycles[1,2]. In contrast, in the revised Fig. 2a, we selected earlier time points (3, 6, and 9 hpi) when the virus had completed only 1-2 rounds of replication. We quantified viral protein levels at 6 hpi and found that, under the combined effects of autophagy inhibition and disruptions in viral membrane

protein transport, more viral proteins accumulated in Rab27a knockout cells. At 9 hpi, the differences in viral membrane protein levels between the two groups began to diminish, as more viral particles budded from A549-Cas9 cells and newly synthesized proteins during the second round of infection helped to compensate for the initial differences. It is anticipated that these differences will continue to decrease at later time points. As the infection progresses, the levels of all viral proteins in A549-Cas9 cells will eventually surpass those in Rab27a knockout cells.

References:

1 Zhu, Y. et al. Human TRA2A determines influenza A virus host adaptation by regulating viral mRNA splicing. Science advances 6, eaaz5764, doi:10.1126/sciadv.aaz5764 (2020).

2 Xu, S. et al. MicroRNA-200c-targeted contactin 1 facilitates the replication of influenza A virus by accelerating the degradation of MAVS. PLoS Pathog 18, e1010299, doi:10.1371/journal.ppat.1010299 (2022).

- According to figures 3L, M/S2A and to the method section, GFP has been fused to the C-terminus of RAB27A. This is very surprising to me as it is well known in the field that RAB GTPases must only be tagged to their N-termini since C-terminus fusion leads to alteration of the hypervariable domain which is necessary to efficient membrane targeting and renders RABs inactive. This must explain why the RAB27 pattern looks diffused within the cytosol rather than on membrane structures in figure S2A, which does not allow for proper co-localization analysis. This also accounts for the other RAB27 fusion constructs (HA, FLAG…) if they happen to have been designed the same way.

To sum up the issues about the identification of the transport pathway that IAV surface proteins take along their way to the plasma membrane, I believe the authors should:

(i) increase the quality of the imaging to provide convincing and quantified co-localization data between HA/NA and RAB27 in infected cells. This could easily be solved by generating a GFP-RAB27 A549 stable cell line. (ii) Identify precisely the cellular organelles that are involved in HA/NA transport and in which of them they remain stuck in the absence of RAB27 (see main concerns).

Response: We fully understand your concerns and sincerely appreciate your professional and constructive suggestions.

As your professional insight points out, tagging Rab27a at its C-terminus impairs its targeting to the cell membrane. Consequently, when Rab27a-GFP is expressed, it predominantly localizes to the cytoplasm (as shown in the previous version of the manuscript, Fig. 3L, M, S2A), whereas HA-Rab27a (incorrectly labeled as Rab27a-HA in the original manuscript) is distributed in both the cytoplasm and the cell membrane (as shown in the previous version of the manuscript, Fig. S2B, C, S2D). We deeply apologize for this oversight. We realize that using the C-terminally tagged Rab27a-GFP does not accurately reflect the relevant localization.

Therefore, we generated A549 monoclonal cells stably expressing GFP-Rab27a, in which Rab27a was properly localized to both the cytoplasm and the cell membrane. We retested the co-localization of GFP-Rab27a with viral envelop proteins under infection conditions and provided representative high-resolution microscopy images. The results showed that GFP-Rab27a co-localized with viral envelop proteins (Revised Fig. 5k, l). In addition, we retested the co-localization of GFP-Rab27a with different membrane-bound organelles before and after infection, the results showed that there were no significant differences in the co-localizations of Rab27a with Golgi network or late endosomes before and after infection (Revised Supplementary Fig. 4b-d), but the co-localizations with autophagosome was increased (Revised Supplementary Fig. 4e), indicating that the sites where Rab27a functions after infection is not in the Golgi or late endosomes, but in the autophagosome or its own positive vesicles. These results provided representative high-resolution microscopy, and statistical analysis was performed on 20–25 cells per group to ensure data reliability.

Additionally, we inadvertently referred to HA-Rab27a as Rab27a-HA in the previous version of the manuscript. We sincerely apologize for this oversight and have corrected it in the revised version.

Regarding suggestion (ii), our response has been addressed in the preceding text.

- The role of RAB27 in M2 transport remains puzzling to me despite the efforts of the

authors to connect this part of their story with autophagy. RAB27A KO induces increased levels of surface M2. The authors claim that this is due to autophagy inhibition that leads to an increase in M2 total levels and that RAB27A does participate in M2 secretion. But then, how does M2 makes it to the plasma membrane in the absence of RAB27A? By diffusion? Via an alternative transport pathway? If not clarified I believe the manuscript would gain in clarity by focusing on HA/NA transport.

Response: Thank you very much for your kind suggestion. With your help, we have recognized that the RUSH system is an excellent tool for analyzing how Rab27a regulates M2 transport. Using the RUSH system, we synchronously released M2 from the Golgi in Rab27a KO and A549-Cas9 cells (Revised Fig. 5i, j). The results showed that the proportion of M2 protein on the cell surface was lower in Rab27a KO cells, indicating that Rab27a regulates the transport of M2 from the Golgi to the cell surface.

Minor points:

- In figure 3 (L, M) as well as in figure S2 (A, B, C), the authors first transfected A549 cells for 24h and then infected the cells. This is quite surprising to me as to my knowledge, not only A549 cells are not very easy to efficiently transfect but it is also well known that infection with IAV in previously transfected cells is difficult. If the authors decide to stick with this protocol rather than with the establishment of stable cell lines (see major points), I suggest that they provide representative images allowing for the visualization of the amount of transfected as well as transfected+infected cells. What does n=5 mean for instance in the case of figure S2? How many cells were analyzed?

Response: We fully understand your concerns and sincerely appreciate your professional suggestions. Following your advice, we generated a stable GFP-Rab27a-expressing cells and re-evaluated the aforementioned experiments. Detailed responses to these experiments have been provided in the preceding text.

Additionally, we attempted to use endogenous LC3 antibodies for staining in the experiments shown in the revised Supplementary Fig. 4e. Unfortunately, despite

testing three different commercial LC3 antibody brands, we were unable to achieve effective and specific staining. To address this issue, we conducted the experiment using a smaller transfection dose and collected data from 20 cells per group to ensure the reliability and accuracy of the results.

- To what time post-infection do the quantifications relate? The control cell line should be used as a reference for normalizing the intensity of the signal, not the other way around.

Response: We greatly appreciate your professional suggestions. Your concern regarding the timing of quantification made us realize that examining Rab27a localization at 24 hpi might be too late, as the cells would have undergone multiple rounds of infection, potentially affecting the accuracy of the results. To address this, we adjusted the detection time to 4 hpi (Revised Supplementary Fig. 4b-e).

- in figure S6D, RAB27A KO leads to a much stronger increase in HA total levels at 6hpi when compared with the same experiment showed in figure 3A. How do the authors explain such heterogeneity?

Response: We fully understand your concerns, we believe the observed differences may be influenced by several factors. Firstly, we note variations in the background signal between the two experiments: the background in Fig. 3A of the original manuscript is relatively high, while the background in Fig. S6D is cleaner. This suggests that differences in antibody binding efficiency between the two experiments may have been a major contributing factor to the discrepancies in the results. Although we use antibodies from the same catalog number, variations in specificity between different batches of commercial antibodies cannot be ruled out. Additionally, differences in cell state between the two experiments may have affected viral infection efficiency, further contributing to the observed differences.

We apologize for any confusion caused by these inconsistencies in the experimental results. Nevertheless, the trends observed in both experiments are consistent, and these trends is also supported by the flow cytometry data (Revised Fig. 3c), which corroborates the accuracy of the findings.

- Authors must provide molecular weights with the western-blot data

Response: We greatly appreciate your professional suggestions. and have labeled the molecular weights for proteins in all the figures.

- Unusual and not very fluid building of the manuscript with supplemented figures only mentioned in the discussion section. These figures should be mentioned within the results section together with the main figures they relate to. Figure S6, which describes the involvement of RAB27B, is related to the first part of the manuscript that describes a role for RAB27A. Figure S5 relates to figure 4.

Response: Thank you very much for your valuable and professional suggestions, which have greatly contributed to the improvement of our manuscript. Following your recommendations, we have combined the section about Rab27b in Fig. S6 of the original manuscript with the first part of the revised manuscript, "Rab27a promotes IAV replication". To ensure the manuscript's logical flow and preserve the overall conclusions, we have removed the data previously presented in Fig. S6C and S6D of the original manuscript. We have retained the findings regarding the involvement of Rab27b in viral replication and the transport of viral HA protein (Revised Supplementary Fig. 1o, p). Additionally, we have combined the Fig. S5 of the previous manuscript with the fourth part of the revised manuscript, "Rab27a promotes autophagy and M2 transport to the plasma membrane" (Revised Supplementary Fig. 3). These adjustments have improved the overall coherence and flow of the manuscript.

Comments from Reviewer 3:

The paper submitted to Nature Communications by Chen et al. investigates the transport mechanism of influenza A virus (IAV) envelope proteins to the cell surface following Golgi processing. Using siRNA screening, the authors identify Rab27a as a critical host factor that regulates the trafficking of viral envelope proteins: HA and NA glycoproteins and the M2 ion channel. Interestingly, the mechanism of the transport of HA/NA was different from that of M2. Rab27a is a small GTPase of the ras-associated family and plays an important role in the trafficking and secretion of intracellular proteins and has been reported to be involved in the replication cycle of other clinically relevant viruses, such as human parainfluenza virus, human

cytomegalovirus, etc. In addition, the authors show that Rab27a exerts its regulatory function during viral infection through its effectors SYTL1 and SYTL4. Knocking out Rab27a and SYTL4 does not block the early stages of the IAV life cycle but restricts IAV assembly and budding by reducing the distribution of viral envelope proteins on the cell surface. Importantly, SYTL4 knockdown provides significant protection in a mouse model of IAV infection. Moreover, Rab27b was also seen to cooperate with Rab27a for the transport of IAV envelope proteins.

Understanding how IAV envelope proteins are transported to the cell surface of infected cells is crucial for the identification of novel targets and the development of innovative antiviral strategies against IAVs. Overall, this is a well-designed systematic study. The information derived from this study is relevant to the field of influenza and may guide research on other viruses. Nevertheless, there are several minor suggestions that may lead to some improvements and clarifications.

Response: We sincerely appreciate your positive feedback and constructive suggestions regarding our manuscript. Your comments are highly valuable and have significantly contributed to the revision and improvement of our paper. We have carefully considered your concerns and provided detailed, point-by-point responses, which are outlined below:

1. The EM of Supplementary Figure 1 shows cell surface extensions at 2 and 3 hpi that resemble filopodia. It may be worth stating in the figure legend what these extensions are so as not to confuse them with actual budding viruses.

Response: We sincerely appreciate your professional suggestions. As you correctly pointed out, the structures resembling filopodia in Supplementary Figure 1 could be mistaken for the virus. To address this issue, we have added black arrows to specifically indicate the filopodia-like structures and red arrows to highlight the virus. Furthermore, we have provided detailed explanations in the figure legend to ensure clarity and avoid any potential confusion (Revised Supplementary Fig. 1a, lines 983-985).

2. The description for the Figure 2 (B to D) seems to be for B to E

Response: We sincerely appreciate your careful review and apologize for the

oversight during the writing process. We have corrected “B to D” to “b to e” (Revised lines 828).

3. The timepoint for the WB in the Figure 3B is missing.

Response: Thank you for pointing out this problem in manuscript. We have added the annotation of “6 hpi” in revised Fig. 3b and provided explanations in the caption (Revised lines 848).

4. There appears to be an error in Figure 4L as it shows a band for Rab27a flag (-) and no band for Rab27a flag (+).

Response: We sincerely appreciate your careful review and apologize for our oversight. We have made corrections in the revised manuscript (Revised Fig. 4l).

5. GAPDH is misspelled as GADPH in several figures.

Response: We sincerely appreciate your careful review and apologize for our oversight. We have corrected these errors in the revised manuscript and carefully checked the entire manuscript to avoid such errors occurring again.

6. Scale bars are not visible or clear enough on IFA and EM images.

Response: Thank you for pointing out this issue in the manuscript. We have re-marked the scales in Fig. 2a, f; Fig. 3i-k; Fig. 7f, g; and Supplementary Fig. 1a in the revised manuscript. Additionally, we have ensured that all scales are clearly labeled and easily identifiable.

7. All main and supplementary figures should include the molecular weight (MW) of the proteins identified by WB.

Response: We greatly appreciate your professional suggestions. and have labeled the molecular weights for proteins in all the figures.

8. In the discussion, the authors discuss the presence of Rab27a in leukocytes and the absence of the effector SYTL4 in these cells. The authors suggest that this may affect the humoral response of the animals and be responsible for the differences in body weight loss and survival. This could be investigated, for example, by measuring influenza-specific endpoint serum titers of Ab isotypes by ELISA.

Response: We greatly appreciate your professional suggestions and fully understand your concern about verifying that silencing SYTL4 can reduce humoral immunity in

mouse infection model. We have carefully considered your proposal to measure influenza-specific endpoint serum titers of Ab isotypes by ELISA. Unfortunately, we unable to supplement these experimental results. We believe that measuring influenza-specific endpoint serum titers of Ab isotypes by ELISA requires detection under viral infection conditions. However, as silencing SYTL4 significantly reduces viral levels in mice, as showed in our results (Revised Fig 7g, h), differences in antibody levels caused by variations in viral load may not accurately reflect the impact of SYTL4 silencing on humoral immunity. Acknowledging this, we realize that including unsupported speculations in the discussion is inappropriate. To avoid misunderstandings, we have removed the relevant discussion in the revised manuscript. Although we did not supplement the relevant results, we emphasize that the potential influence of SYTL4 on humoral immunity is not directly relevant to the molecular mechanism of influenza virus envelope proteins transport. Therefore, it does not affect the overall conclusions of this research. We sincerely hope the reviewers can understand our decision.

9. The model for Rab27a-mediated IAV envelope proteins in Figure 7 shows the envelope proteins on the wrong side of the transport vesicle and the cell membrane. The budding is in the wrong direction.

Response: We sincerely appreciate your careful review and apologize for our oversight. We have corrected the virus budding direction in the pattern diagram (Revised Fig. 8).

Point-by-point response to the reviewer' comments and concerns

Reviewer:

In the reviewed version of their study entitled "Molecular mechanism of influenza virus envelope proteins transport", describing a role for RAB27 and its effectors SYTL1/4 as regulators of Influenza A virus HA, NA and M2 proteins trafficking to the cell surface, Chen and colleagues strengthened their investigation and notably tested the importance of the described transport pathway for the trafficking of another unrelated virus (VSV), and used the RUSH system in an attempt to precisely identify the organelles through which they transit en route to the plasma membrane. Even though the absence of effect of RAB27 depletion on VSV replication suggests some kind of specificity of the described molecular pathway for Influenza virus surface proteins, the data provided here do not fully support the author's claim according to which RAB27 participates in the traffic of viral envelope proteins from the trans-Golgi network to the plasma membrane.

Response:

We sincerely appreciate your professional and constructive comments and fully understand your concerns. As illustrated in the schematic diagram of our manuscript (**Fig. 8**), our study elucidates that Rab27a and its effectors SYTL1 and SYTL4 regulate the transport of post-Golgi vesicles carrying viral envelope proteins to the plasma membrane. However, in the original manuscript, we described this process in a simplified manner as the Rab27a/SYTL1/4-mediated transport of viral envelope proteins from the Golgi apparatus to the plasma membrane. We acknowledge that this description lacks precision and could have led to some confusion. We sincerely apologize for this oversight. In the revised manuscript, we have rewritten the relevant concluding statements to more accurately reflect the molecular mechanism revealed in this study (**lines 8-10, lines 213-214, lines 283-284**).

In addition, we have carefully addressed all the other comments you raised. We hope that the revised manuscript can satisfy the requirements for publication. Below, we provide detailed point-by-point responses.

Major concerns:

- Role of different RAB GTPases on viral envelope transport towards the cell surface:

siRNAs specific for other relevant RAB GTPases were added. I have to admit that I find the absence of effect on HA transport to the surface upon RAB6 depletion very surprising, knowing how key this cellular factor is for anterograde post-Golgi transport and I might not be the only one. Having said that, the flow cytometry data are clear. Maybe showing immunofluorescence images of HA levels at the plasma membrane to illustrate the difference would help making these results more convincing?

The authors should at least discuss the fact that contrary to what Sato et al. (PFID 31456775) described, depletion of RAB17 and RAB23 depletion does not affect trafficking of surface proteins in their hands.

Response:

We sincerely appreciate your professional comments and fully understand your concerns. Following your suggestion, we using indirect immunofluorescence to examine the effects of silencing Rab6a, Rab17, Rab23, and Rab27a on the distribution of HA at the plasma membrane. As shown below in **Fig. R1a**, we confirmed that silencing Rab6a, Rab17, or Rab23 has a much weaker effect on HA levels in the cell surface compared to Rab27a silencing. However, since the flow cytometry data had provided objective and quantitative evidence, and including these immunofluorescence data in the manuscript would significantly increase the volume of results, we have chosen to provide these additional results here in response to your concern. We hope for your understanding. Nevertheless, if you believe these results should be included in manuscript, we are happy to add them in supplementary figure 1.

As you mentioned, Rab6a indeed plays a critical role in post-Golgi anterograde transport. It is involved in the trafficking of cargos such as VSV-G, TNF-α, and herpes simplex virus 1 (HSV-1) to the cell surface. Therefore, to ensure the accuracy of our

results regarding Rab6a, we used the same siRNA as in Fig. 1a of the manuscript to silence Rab6a and examined its effect on the transport of VSV-G to the plasma membrane. The results showed that Rab6a silencing indeed impaired the trafficking of VSV-G to the cell surface (**Fig. R1b, c shown below**), confirming that the biological function of Rab6 was effectively disrupted in our experiments. In addition, we would like to clarify that silencing Rab6a and Rab17 also had a measurable impact on the distribution of HA at the plasma membrane (**Fig. 1a)**. Statistical analyses showed that silencing of Rab6a and Rab17 significantly reduced HA surface levels; however, their effects were notably less pronounced than that of Rab27a. These findings suggest that Rab6a and Rab17 may also be involved, to some extent, in the trafficking of HA to the plasma membrane. Their potential roles in the transport of influenza viral envelope proteins warrant further investigation, and we are indeed interested in exploring this in future studies. Moreover, we have incorporated a discussion of Rab6-related findings into the revised manuscript (**lines 373-379**).

We also sincerely appreciate your suggestion to discuss the study of Sato et al. (PFID 31456775), which are highly relevant to our study. We apologize for not citing their work in the previous version of the manuscript—this was an oversight on our part. Their study showed that the influenza virus HA protein can colocalize with Rab17 and Rab23, and that overexpression of dominant-negative (DN) mutants of Rab17 delays the transport of HA to the plasma membrane, while overexpression of Rab23 DN reduces HA expression on the cell surface. However, our study demonstrated that silencing Rab17 also slightly downregulated the surface distribution of HA, whereas silencing Rab23 even slightly enhanced the localization of HA on the plasma membrane. These discrepancies may be due to differences in cell types, with Sato et al.'s study using polarized MDCK cells and ours using A549 cells, which may exhibit distinct expression patterns of Rab GTPase family gene expression profiles. On the other hand, methodological differences may also contribute to the observed discrepancies, as silencing certain genes could trigger compensatory effects from other members of the same gene family. We have included this content in the

Discussion section in revised manuscript (**lines 362-373**).

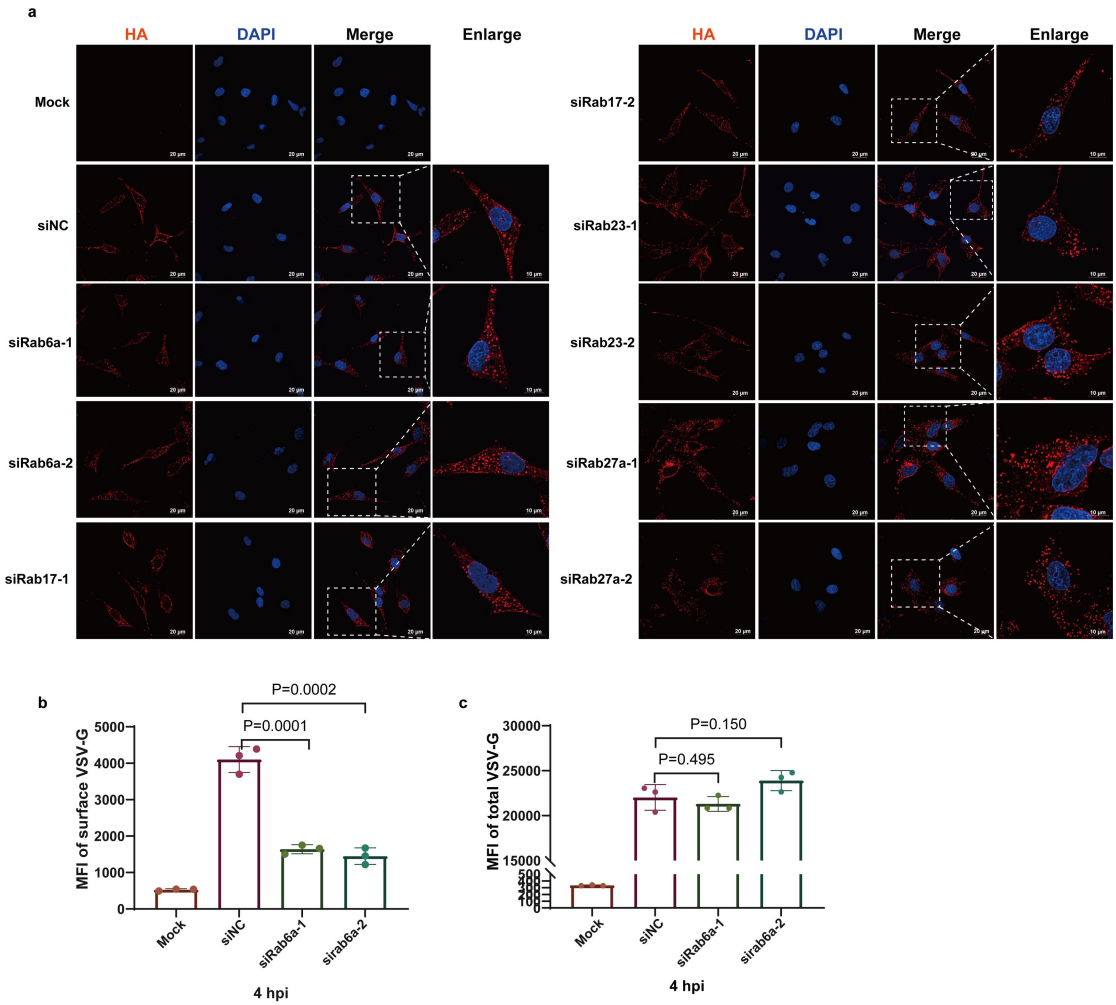

Figure R1

a. A549 cells were transfected with siNC or siRNAs targeting Rab6a (siRab6a-1, siRab6a-2), Rab17 (siRab17-1, siRab17-2), Rab23a (siRab23a-1, siRab23a-2), Rab27a (siRab27a-1, siRab27a-2) for 24 h. The cells were then infected with HM/H5N1 virus (MOI, 10), at 4 hpi, cells were fixed, permeabilized, and stained with antibodies against HA (red) and counterstained with DAPI (blue). Scale bar, 20 and 10 μm.

b-c. A549 cells transfected with siNC or siRab6a (siRab6a-1, siRab6a-2) for 24 h were infected with VSV (MOI, 10). The cell surface (b) and Total (c) levels of VSV-G protein were quantified by flow cytometry. Data are presented as mean ± SD from three independent experiments. Statistical significance was determined using a two-tailed Student's t-test.

• Identification of the cellular compartments involved in Influenza virus surface proteins transport

In general, the data provided by the authors do not fully support their conclusions according to which RAB27 (and its effectors) regulates the transport of viral envelope proteins from the trans-Golgi network to the plasma membrane of infected cells. Despite nicely designed RUSH-based assay which tend to show that, the results obtained in an actual infectious context do not:

- According to Fig 5b, KO of RAB27 does not lead to any accumulation of HA in the TGN, as it should do if RAB27 was essential for a "TGN-to-plasma membrane" transport route.

Response:

We sincerely appreciate your thorough review. We fully agree with your comments, and as previously mentioned, we have revised the imprecise statements in manuscript (**lines 8-10, lines 213-214, lines 283-284**). According to our results, knockout of Rab27a did not lead to the accumulation of viral proteins in the Golgi apparatus (**Fig 5a, b**), indicating that Rab27a does not affect their release from the Golgi. In fact, Rab27a regulates the transport of post-Golgi vesicles carrying viral proteins to the plasma membrane, which is consistent with our overall conclusions.

- According to Fig 5k, most GFP-RAB27 seems to localize on ER membranes (with a pretty clear nuclear envelope signal) where most HA is found as expected. However, when looking at the most peripheral part of the cell, where post-Golgi vesicles are found, there is almost no colocalization between HA and RAB27, again not supporting a "TGN-to-plasma membrane" transport route.

Response:

Thank you for your professional review comments. We fully understand your concerns. We believe that the strong fluorescence signal in the perinuclear region might have limited the visibility of colocalization signals in other areas. To avoid overexposure of perinuclear proteins, the fluorescence intensity was set at a relatively low level, which may have made it difficult to observe the weaker colocalization signals between viral proteins and GFP-Rab27a in regions further from the nucleus.

Therefore, we re-examined our original imaging data and found that, upon simultaneously increasing the intensity of all fluorescence channels, the colocalization between viral proteins and Rab27a could indeed be observed in areas far from the nuclear periphery as shown below in the **Fig. R2a** (yellow arrows indicate the colocalization of HA and Rab27a, and white arrows indicate the colocalization of HA, NA, and Rab27a).

To determine whether the perinuclear colocalization you mentioned corresponds to the endoplasmic reticulum (ER), we performed immunofluorescence using the ER marker Calnexin to examine the colocalization of NA, GFP-Rab27a, and the ER at 3 hpi. As shown below in **Fig. R2b**, we observed clear colocalization between Rab27a, the ER, and viral proteins in the perinuclear region, which consistent with your comments. In addition, in areas distal to the nucleus, Rab27a colocalized with NA without overlapping with the ER (indicated by white arrows).

Additionally, we also examined the colocalization of the Golgi apparatus, HA and GFP-Rab27a. Similarly, we found that in the perinuclear region, HA, Rab27a, and the Golgi could colocalize, while in regions further away from the nucleus, HA colocalized with Rab27a alone, without overlap with the Golgi (**Fig. R2b shown below**).

We propose that the observed colocalization of Rab27a and viral proteins within the ER and Golgi apparatus is due to the overlapping locations of protein synthesis and processing. This is supported by the observation that, even prior to infection, Rab27a-GFP was enriched in these organelles, suggesting that Rab27a undergoes synthesis and processing in the ER and Golgi. In peripheral regions, where viral proteins colocalize with GFP-Rab27a independently of these organelles, it suggests that following processing, viral proteins are carrying into Rab27a-positive vesicles for transport toward the plasma membrane.

We sincerely appreciate your thorough review, which made us realize that 3 hpi may not be the most appropriate time point to observe the colocalization between Rab27a and viral proteins. In our study, we found that at 3 hpi, Rab27a had not yet formed prominent punctate accumulations near the plasma membrane (as shown in

Fig. 5c). However, at 4 hpi, more distinct GFP-Rab27a positive vesicle-like structures of could be observed near the plasma membrane (as shown in **Fig. 5a, b** and **Fig S4b, c**), it may colocalize with viral proteins and participate in their intracellular trafficking.

To minimize the interference of budding virions at 4 hpi, we treated the cells with oseltamivir immediately after viral adsorption. Oseltamivir is a neuraminidase inhibitor that blocks both viral budding and re-adsorption, thereby ensuring that the virus undergoes only a single round of infection (**new Fig S4f, g**). We found that under oseltamivir treatment, at 4 hpi, GFP-Rab27a indeed showed distinct punctate colocalization with HA, NA and M2 in regions distal to the perinuclear area (**new Fig. 5k, l**). Interestingly, we observed that the colocalization of HA and NA with Rab27a did not always occur simultaneously. In fact, HA and NA could each independently colocalize with Rab27a (**new Fig. 5k**), indicating that Rab27a regulates the transport of HA and NA in a partially asynchronous manner. To further confirm that the vesicles showing Rab27a–HA/NA colocalization near the plasma membrane had separated from the ER–Golgi network, we examined the localization of these vesicles in relation to the ER and Golgi individually. The results showed that the Rab27a–HA/NA colocalization observed near the plasma membrane did not overlap with either the ER or the Golgi at 4 hpi (**new Fig. S4h**), indicating that Rab27a regulates viral envelope proteins transport to cell surface after they exit from the Golgi apparatus. These results further support our conclusion.

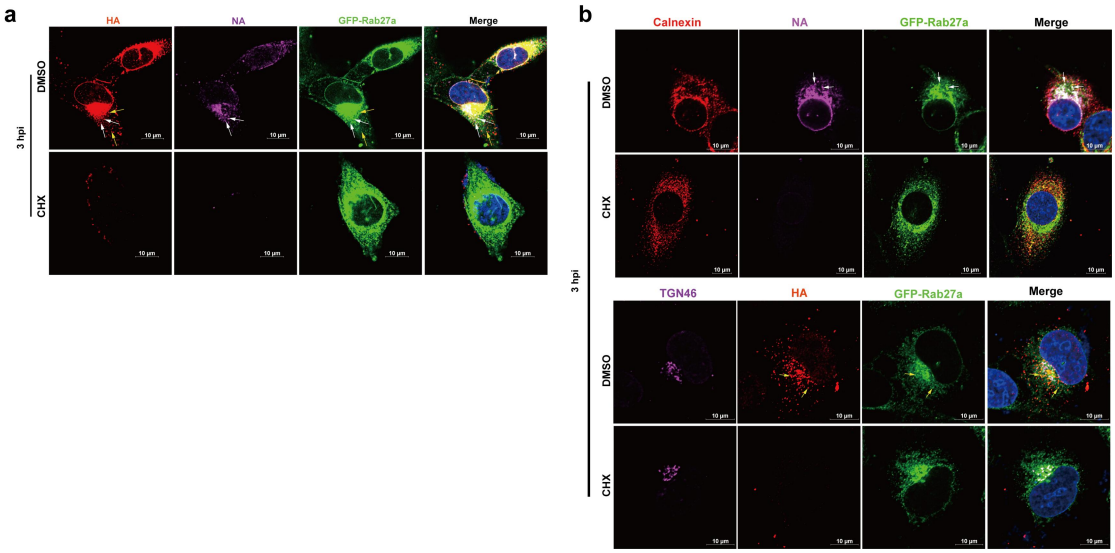

Figure R2

a. Synchronously enhance the fluorescence intensity of Fig. 5k in the original manuscript.

b. A549 cells stably expressing GFP-Rab27a were pretreated with CHX (100 μM) or DMSO for 4 h, and then infected with HM/H5N1 (MOI, 10). Cells were further treated with CHX (350 μM) or DMSO and fixed at 3 hpi. These cells were stained with anti-NA, -Calnexin, -HA, and -TGN46 and DAPI. White arrows indicate colocalization of HA, NA and GFP-Rab27a; yellow arrows indicate colocalization of HA and GFP-Rab27a. Scale bar, 10 μm. Images shown are representative of at least three independent experiments.

- According to Supp Fig 4, GFP-RAB27 is slightly relocalized to LC3+ structures upon infection but not to the TGN. This is again in total contradiction with RAB27 being involved in HA trafficking within a "TGN-to-plasma membrane" transport route.

Response:

Thank you for your professional review comments. In this study, Rab27a primarily functions in regulating autophagy and the transport of post-Golgi vesicles carrying viral membrane proteins to the plasma membrane. Therefore, we believe it is reasonable that Rab27a accumulates on autophagosomes rather than the Golgi

apparatus following infection. We fully understand your concern and have therefore revised the sentence to avoid any potential misunderstanding (**lines 278-279**).

- In Fig 5C, CD63 stainings (which allows for the visualization of multivesicular bodies, not late endosomes), show an endosomal-like pattern while while HA accumulates in the ER, as expected. Thus, there is very low co-localization between the 2 signals but the quantification provided with the images indicates ~70% of co-localization! This makes the way co-localization analysis were performed very questionable.

Response:

We sincerely thank you for your thorough and rigorous review. We fully understand your concerns. As mentioned above, we have demonstrated that the perinuclear accumulation of HA primarily occurs in the ER and the Golgi apparatus. It is known that organelles can engage in partial crosstalk to facilitate material exchange between different subcellular organelle, which may explain the partial colocalization observed between HA and endosomal markers. In addition, the relatively broad distribution of endosomes results in CD63 fluorescence signals being uniform and abundant, which may also contribute to the higher apparent colocalization values with HA. We would also like to clarify that the colocalization value of approximately 0.7 presented in our data does not imply 70% overlap in a literal sense. In fact, it refers to a Pearson correlation coefficient of 0.7, calculated based on the red and green fluorescence channels, as stated in the figure legends in original manuscript. To avoid any misunderstanding, we have revised the y-axis labels in **Fig. 5a–c, f, g** and **Fig. S4b–e** in the revised manuscript to explicitly state “Pearson correlation coefficient”. Our calculation was performed by selecting individual cells as regions of interest (ROIs) and using the Coloc2 plugin in imageJ to calculate the Pearson correlation coefficient between the red and green fluorescence channels. This is a commonly used method in colocalization studies[1].

We completely agree with your point, and we acknowledge that the Pearson correlation coefficient of 0.7 still indicates a relatively high level of colocalization between HA and CD63. Therefore, we carefully re-examined our original data and

identified a potential technical issue that may have led to an overestimation of the PCC values. Specifically, we found that when certain merged images were imported into ImageJ and then split into individual channels, partial signal bleed-through occurred between the green and red fluorescence channels. As shown below in **Fig R3a**, when the merged image was split, some punctate green signals were unexpectedly observed in the red channel. In contrast, when the red channel image was imported separately and converted to 16-bit, these puncta were not observed.

Correspondingly, the PCC value calculated from the split channels of the merged image was 0.65, whereas the PCC value calculated from separately imported images was 0.53. We further examined the specific image highlighted by your comments (**Fig. 5c**), which initially showed a PCC of 0.74. As shown below in **Fig R3b**, after correcting the analysis method, the PCC for this image decreased to 0.58.

In light of this, we reanalyzed all colocalization data in the study using a more accurate method: we directly imported the red and green channel images into ImageJ and converted them to 16-bit format before calculating the PCC. We have updated the corresponding PCC values in the revised manuscript accordingly (**Fig. 5a–c, f, g and Fig. S4b–e**), and these results still support our original conclusions.

We sincerely apologize for this oversight and are deeply grateful for pointing it out, which helped us improve the accuracy and reliability of our data.

References:

1. Dunn, K. W., Kamocka, M. M. & McDonald, J. H. A practical guide to evaluating colocalization in biological microscopy. American journal of physiology. Cell physiology 300, C723-742, doi:10.1152/ajpcell.00462.2010 (2011).

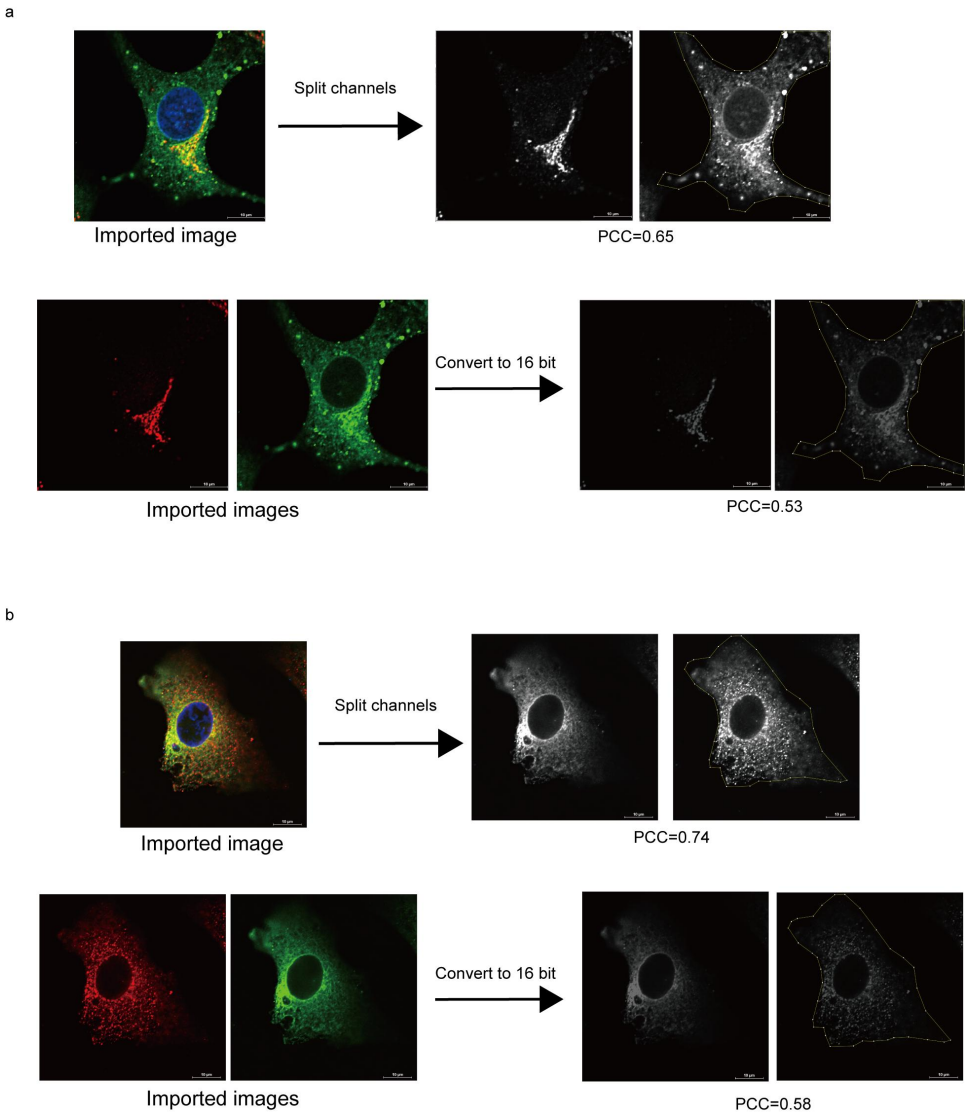

Figure R3

a. Infected group of Fig. S4B

b. Rab27a-KO group of Fig. 5C

The authors themselves, and I quote here the point-by-point response to reviewers, write that "Rab27a functions after infection is not in the Golgi or late endosomes, but in the autophagosome" (sic), which adds to the overall confusion.

Response:

We sincerely apologize for the confusion caused during your review process by our unclear description, we have revised the original text to: "indicating that the subcellular compartments where Rab27a functions after infection is in the autophagosomes or its own positive vesicles"(**lines 278-279**).

Altogether, even though the authors made a strong effort in an attempt to identify the

subcellular transport pathway through which influenza virus surface proteins transit with nicely designed RUSH assay-based experiments, their results do not fully support their conclusions, yet they are sometimes contradictory.

Response:

We sincerely appreciate your careful and professional review. We deeply apologize for any confusion caused by our previously imprecise descriptions and the suboptimal choice of time point for analyzing the colocalization between Rab27a and viral proteins. As mentioned above, in the revised manuscript, we have thoroughly corrected the relevant descriptions and selected a more appropriate time point to reassess the colocalization under infection conditions. The updated results show that Rab27a colocalizes with viral proteins in a vesicular pattern in areas distal to the nucleus, which further supports our proposed conclusion.

• The title of the study is too general. This work does not elucidate the "Molecular mechanism of influenza virus envelope proteins transport" but rather identify RAB27 and effectors as potential regulators of their traffic.

Response:

Thank you for your professional suggestions. We fully agree with your opinion, and in the newly submitted manuscript, we have revised the title to:

"Rab27a regulates the transport of influenza virus membrane proteins to the plasma membrane"

Minor comments:

- Line 27: Introduction not instruction

Response:

Thank you very much for your careful review. The manuscript has been revised accordingly (**line 27**).

- Line 33: M2 is not an envelope protein

Response:

We thank the reviewer for this helpful comment. We agree that, although the M2 protein is embedded in the viral envelope and functionally important during virus entry, it is more appropriately described as a membrane protein rather than an

envelope protein[1-3]. The term “envelope protein” is typically reserved for the major glycoproteins such as HA and NA, while M2 is consistently referred to as a membrane protein in the literature.

To improve accuracy and avoid potential confusion in terminology, we have revised the manuscript accordingly by replacing “envelope protein” with “membrane protein” when referring to HA, NA, and M2.

References:

1 Schnell, J. R. & Chou, J. J. Structure and mechanism of the M2 proton channel of influenza A virus. Nature 451, 591-595, doi:10.1038/nature06531 (2008).

2 Wright, A. K., Paulino, J. & Cross, T. A. Emulating Membrane Protein Environments—How Much Lipid Is Required for a Native Structure: Influenza S31N M2. Journal of the American Chemical Society 144, 2137-2148, doi:10.1021/jacs.1c10174 (2022).

3 Taubenberger, J. K. & Kash, J. C. Influenza virus evolution, host adaptation, and pandemic formation. Cell Host Microbe 7, 440-451, doi:10.1016/j.chom.2010.05.009 (2010).

- Line 36: RAB11 was not identified as driving vRNP transport from “vacuolar structures”.

Response:

We sincerely appreciate your valuable feedback. The description of vRNP forming “vacuolar structures” in the infected cell is based on studies by Professor Maria João Amorim[1-2]. Their studies demonstrated that the viral genome forms a type of viral inclusions within the cell, which display characteristics of liquid organelles, segregating from the cytosol without a delimitating membrane, dynamically exchanging material and adapting fast to environmental changes. During IAV infection, the formation of these viral inclusions relies on Rab11-GTP, ATG9A, and vRNP. However, as you correctly pointed out, these studies do not indicate that RAB11 regulates the transport of these vacuolar structures. Therefore, we have

removed the inappropriate description from the manuscript and sincerely apologize for the oversight (**line 35**).

References:

1. de Castro Martin, I. F. et al. Influenza virus genome reaches the plasma membrane via a modified endoplasmic reticulum and Rab11-dependent vesicles. Nat Commun 8, 1396, doi:10.1038/s41467-017-01557-6 (2017).
2. Vale-Costa, S. et al. ATG9A regulates the dissociation of recycling endosomes from microtubules to form liquid influenza A virus inclusions. Plos Biol 21, e3002290, doi:10.1371/journal.pbio.3002290 (2023)

- Line 39: Membrane proteins are not “attached” to membrane vesicles. “transported through” maybe?

Response: Thank you very much for your careful review, and we fully agree with your opinion. The manuscript has been revised accordingly (**line 38**).

- Line 53: RAB GTPases are not involved in Golgi “post-processing”. “Post-Golgi transport” maybe?

Response: Thank you for your professional suggestions. We fully agree with your comments, and the manuscript has been revised accordingly (**line 50**).

- Line 61/62: this work does not fully “elucidate the molecular mechanism of IAV proteins transport”

Response: Thank you for your professional suggestions. The manuscript has been revised accordingly (**lines 60-61**).

- Lines 136 to 167 + line 245 : A549 cells cultured in 2D are not polarized and hence do not exhibit apical versus basolateral membrane domains. My point regarding the use of VSV-G was to make sure that the RAB27-dependent transport pathway identified here was specific of IAV envelope proteins in infected cells and not merely a canonical route to the plasma membrane. The authors should thus avoid mentioning apical vs basolateral membranes as their work was not performed in polarized cells.

Response: Thank you for your professional suggestions. We fully agree with your

opinion. In the revised manuscript, we have completely removed the content related to polarization and apical transport, and revised the text to focus on the specific role of Rab27a in regulating the transport of viral envelope proteins. (**lines 155-157, line 163, lines241-242, line 325**)

- Line 173: Why would impairment of autophagy lead to accumulation of M2 to the cells surface? I keep thinking that the part of M2 transport makes the message very confusing and I do not understand how RAB27 could transport M2 from the Golgi apparatus to the plasma membrane if M2 accumulates at the surface in RAB27 KO cells.

Response:

We fully understand your concerns. In the revised manuscript, we have added direct evidence that M2 can be degraded via autophagy (**new Fig. S3a**), which demonstrates that the knockout of Rab27a, by reducing autophagy, indeed promotes the accumulation of M2 protein. Latest research indicates that the LIR motif of M2 is critical for its localization to the plasma membrane, which promoting its trafficking to cell surface through interaction with LC3[1]. This suggests that M2 can indeed bypass the Golgi apparatus and be transported directly to the plasma membrane via the secretory autophagy pathway. Although Rab27a knockout partially reduces LC3 levels, we propose that the remaining LC3 may still be sufficient to support the transport of accumulated M2 to the cell surface in Rab27a KO cells. In addition, the trafficking of secretory autophagosomes to the plasma membrane is regulated by host factors such as Rab8a[2], Rab37[3], STX3/4[4], and SNAP23/39[4], among others, which may play a role in the elevation of cell surface M2 levels in Rab27A KO cells. However, the underlying mechanisms remain to be elucidated. Therefore, we speculate that M2 reaches the plasma membrane via two distinct pathways: a Rab27a-dependent post-Golgi vesicles-mediated transport pathway and a Rab27a-independent autophagosome-mediated transport pathway. We have added relevant speculations to the discussion section (**lines 416-430**).

References:

1. Figueras-Novoa, C. et al. Caspase cleavage of influenza A virus M2 disrupts M2-LC3 interaction and regulates virion production. EMBO reports 26, 1768-1791, doi:10.1038/s44319-025-00388-7 (2025).
2. Dupont, N. et al. Autophagy-based unconventional secretory pathway for extracellular delivery of IL-1β. Embo J 30, 4701-4711, doi:10.1038/emboj.2011.398 (2011).
3. Wu, S. Y. et al. Secretory autophagy-promoted cargo exocytosis requires active RAB37. Autophagy 20, 933-934, doi:10.1080/15548627.2023.2210446 (2024).
4. Kimura, T. et al. Dedicated SNAREs and specialized TRIM cargo receptors mediate secretory autophagy. Embo J 36, 42-60, doi:10.15252/embj.201695081 (2017).

- Fig 5D “release”: The transport of a cargo from the ER to the Golgi apparatus occurs through vesicles which should be drawn of the cartoon.

Response: Thank you very much for your careful review. We have made the corresponding revisions in **Fig 5d** as your recommendations.

Point-by-point response to the reviewer' comments and concerns

Reviewer (Remarks to the Author):

In the last reviewed version of their study now entitled "RAB27A regulates the transport of influenza virus membrane proteins to the plasma membrane" (which better reflects their findings), Chen and colleagues notably put some efforts at better identifying the organelles hijacked by Influenza A virus (IAV) to ensure the transport of the viral surface proteins, and propose a model in which RAB27A would regulate the transport of post-Golgi vesicles carrying viral proteins to the plasma membrane.

As this remains to me the weakest part of the proposed study, I will mostly comment on that and on what seems to me being an over-interpretation of the data:

The conclusion that RAB27+ vesicles containing IAV membrane proteins are post-Golgi vesicles en route to the plasma membrane is mostly, if not only, based on the fact that they are "observed in areas far from the nuclear periphery" (sic, Point-by-point response to the reviewer's comments and concerns), which is not a solid argument for such a claim. These vesicles may also be involved in ER-to-Golgi transport and represent ER exit sites (ERES) or vesicles generated from the ER-to-Golgi Intermediate Compartment (ERGIC). Such structures are not necessarily observed in the perinuclear area and can be found at more distal locations. This alternative proposition actually fits with the fact that GFP-RAB27 exhibits both a ER and a Golgi distribution. Only a 20°C block in the Golgi apparatus followed by a switch at 37°C to synchronise the release of vesicles from the Golgi towards the cell periphery would formally show that the identified vesicles actually are post-Golgi vesicles. In other words, the current set of data rather convincingly show that RAB27A regulates the transport of IAV envelope proteins to the plasma membrane (so says the new title) but the exact nature of the vesicles involved remains unclear. This should to the least lead the authors to revisit and tone down their current conclusion.

Response:

We are particularly grateful for your constructive comments on our manuscript. Following multiple rounds of revision as suggested, we have gradually improved the

coherence of the evidence chain and further strengthened the overall quality of the study. We fully understand the concerns raised and sincerely appreciate these thoughtful and helpful suggestions.

In response to your comments, we have removed the all description referring to the Rab27a-regulated transport vesicles as "post-Golgi vesicles" and now refer to them simply as "vesicles" or "Rab27a-positive vesicles" in revised manuscript. In addition, we have deleted Figure 8 accordingly. In the Discussion section, we explained why the nature of the vesicles for Rab27a-mediated transport of viral membrane proteins cannot yet be definitively determined and proposed that these vesicles are likely Golgi derived (lines 409-419 in PDF version manuscript). We sincerely thank the your rigorous evaluation, which has enabled us to present a more comprehensive set of conclusions.

Minor: To my opinion, including the data that show that VSV-G transport relies on RAB6A (figure R1B-C) and not on RAB27A (while it's the exact opposite in the case of IAV surface proteins) would strengthen the idea that the RAB27A-mediated pathway is somehow specific to IAV membrane proteins. For instance, in a supplementary figure that would relate to Figure 3.

Response:

Thank you for your professional review comments, we have incorporated the results showing the effect of Rab6a knockdown on VSV G protein transport to the cell surface (originally shown in Figures R1B–C) into Fig S2. Corresponding descriptions have also been added in the "Rab27a regulates transport of HA and NA proteins to plasma membrane" section (lines 185-187 in PDF version manuscript).

Once again, we sincerely thank you for your thorough and constructive review.