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Interferon-inducible phospholipids govern IFITM3-dependent endosomal antiviral immunity

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Thank you for submitting your manuscript to The EMBO Journal. We have now received comments from three reviewers, which are included below for your information.

As you will see from the reports, the reviewers find the study interesting, but they also raise multiple substantial concerns with the study, in particular regarding the data presentation, inclusion of appropriate controls, especially for validation of the IFITM3 3KR and 4KR mutant proteins, and discrepancies between the presented findings and published literature, e.g., for Amphotericin B effect on viral entry. They find that the analysis has a somewhat preliminary nature, and they further indicate that reorganisation of the study would be needed to focus on the role of IFITM3/PIP3 interaction in viral entry restriction and extension of the findings on SARS-CoV-2 Omicron variant.

Based on the overall interest expressed in the referee reports and your willingness to engage in a major revision as expressed in the preliminary revision plan provided during the pre-decision consultation, I would like to invite you to address the comments of all reviewers in a revised version of the manuscript. I should add that it is The EMBO Journal policy to allow only a single major round of revision and that it is therefore important to resolve the main concerns at this stage.

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, please contact me as soon as possible upon publication of any related work to discuss the appropriate course of action. Should you foresee a problem in meeting this three-month deadline, please let us know in advance to arrange an extension.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess. Please also see the attached instructions for further guidelines on preparation of the revised manuscript.

Please feel free to contact me if have any further questions regarding the revision. Thank you for the opportunity to consider your work for publication, and I look forward to receiving the revised manuscript.

Referee #1:

The authors have performed an extensive study to understand how IFITM3 restricts the entry via the endosomal route. They find that conserved lysine residues in IFITM3 are essential for endosomal viral restriction but dispensable for surface entry restriction when IFITM3 is retained on the surface by a Y20A mutation. They use the lysine residue mutant to identify a host of putative cofactors including a number of PIP3 binders. As the authors and others have previously shown that manipulation of MTOR signalling drives IFITM3 to degradation, the authors make the case with mutants and inhibitors that PIP3 interaction with IFITM3 is a key regulator of its endosomal function.

While there is huge amount of interesting data here, and I don't dispute a necessity for PIP3 in IFITM3 function in endosomes

that explains the AKT/MTOR relationship, it seems to me that the key to their interpretation that IFITM3/PIP3 interactions are essential in the endosome is the experimentation that the authors use to conclude that the 4KR mutation (despite phenocopying the Y20A mutant) is not affected in its subcellular localization. The authors' experiments as far as I can tell are not controlled sufficiently for this interpretation, and it could simply be that the 4K mutant is similarly restricted more prominently to the cell surface, and thus not enriched in PIP3+ compartments. Given the extensive literature on the role of ubiquitination in receptor trafficking as well as turnover, and that a K-to-R phenotype is often highly indicative of a Ub-dependent process, this needs to be rule in or ruled out properly.

Specifically, the authors rely on steady-state PLA-based or Ab-based localization with LAMP or PIP3 to conclude that 3KR is not mis-localized to the PM. This experiment is not controlled properly. LAMP (and the majority of endosomal markers) traffic between compartments, and at some point most membrane proteins will end up in endosomes for degradation. Therefore, the experiment needs a robust specificity control. If the authors are correct, then IFITM1 and crucially, IFITM3 Y20A, should display significantly reduced co-localization. Secondly, 3KR, but not Y20A, should not accumulate on the plasma membrane by flow/IF. Without these controls the localization of 3KR is not interpretable, and all subsequent experiments (be they manipulation of PIP3 that will affect endosomal traffic, interaction with cofactors, or cell-type dependant effects in the presence of IFN) might simply be a reflection of 3KR not getting into the right compartment in the first place. I note that surface fusing measles LVs have not been tested against the 3KR mutant alone, but against the double Y20A/3KR that would mask any effect of 3KR on trafficking.

Other points:

• The Ks are conserved in IFITM1 - what happens to surface fusers if they a mutated?
• Given the complexities of SARS CoV-2 entry, site of fusion and cell tropism, in my opinion the SARS2 data doesn't really add much to the conclusion of this paper, and may be better used in a different MS.
• I am really surprised that authors have managed to get such robust infection of THP1 cells with SARS-CoV-2. In many people these are refractory to infection even at high MOI. Do the authors' THP-1 cells express ACE2 endogenously or have been engineered to express it?

Referee #2:

The manuscript „IFN-inducible phospholipid levels govern endosomal antiviral immunity" by Unali et al. characterizes the anti- and proviral roles of distinct members of the IFITM protein family (IFITM1, 2, 3) in the context of different virus infection assays (lentivirus-based pseudotypes, authentic viruses) in different immortalized and primary cell systems. Adding another layer of complexity, the authors compare overexpression of IFITM genes with their knock-down and/or knock-out. They identify four lysine residues (one in the N-terminal domain, three in the CIL) to be essential for antiviral activity of IFITM3, but not IFITM1, against viruses which use the endosomal entry route, as opposed to viruses that fuse at the plasma membrane. Mutation of lysines to arginines results in the loss of higher molecular IFITM3 complexes, potentially dimers, that are readily formed by WT IFITM3, and these lysine residues seem to be responsible for recruitment of PIP3, an essential cofactor for IFITM3 restriction. Interestingly, IFITM3-mediated restriction of virus entry was absent in one cell line, K562, and could be restored by exogenous addition of PIP3. The overall conclusion of the manuscript is that IFITM3 requires PIP3, an IFN-inducible phospholipid, as a cofactor for its antiviral activity. While this message is novel and very interesting, large parts of the paper unfortunately distract from the main message. The in silico analysis of interactors appears a bit immature, and figures 6-7, for unclear reasons, largely deal with viruses (pre-omicron SARS-CoV-2 variants) which actually are not susceptible to IFITM3/PIP3-mediated restriction. It would have been much more informative to place the main finding (PIP3 recruitment by the lysines, differential abundance of PIP3 in cell lines) in the center and focus on its deeper characterization, and maybe use omicron for an extensive analysis of this new phenotype. Identifying the enzyme which is responsible for PIP3 formation in the phospholipid biosynthesis pathway and which may be expressed in a cell line-dependent manner, quantifying PIP3 turnover/stability, widening the relationship of PIP3 abundance and IFITM3 restriction to more cell lines/titration of PIP3/IFITM3 expression, and provide some understanding regarding how PIP3 assists IFITM3 at the molecular/physical level would have been very exciting.
Major points:
1. Figure 1 is a recapitulation of known properties of IFITM proteins: IFITM1 and IFITM3 mutants that are redirected to cell surface inhibit plasma membrane-fusing viruses, IFITM2-3 inhibit those entering via endosomes. While it is informative and required to introduce the system and to demonstrate that it recapitulates established findings, I advise to move it to the supplement.
2. Figure 1, The bar diagrams indicate "TD efficiency (%)" but no information is provided about the normalization (what is 100%?). Also, the concrete read-out is not mentioned. Based on the transfer vector used, it seems to be GFP expression. Are the bar diagrams based on the percentage of GFP-positive cells, or the mean fluorescence intensity (MFI), or both? Adding a few FACS dot plots to illustrate the gating strategy and the overall sensitivity of the assay would be very informative.
3. Figure 2: the literature contains several reports of Amphotericin B-antagonized IFITM3 restriction (e.g. Influenza A virus, PMID: 24268777; PMID: 33112230). What is the reason for the absent impact of Amphotericin B on VSV-G-mediated entry, then?
4. The 4KR and 3KR mutants seem to have lost a large proportion of their antiviral activity against VSV-G lentiviral vectors in THP-1, but it seems that the loss is incomplete; yet the authors phrase "both the quadruple and triple lysine to arginine mutants

of IFITM3 lost antiviral activity against endosomal viral entry of VSV-g pseudotyped LV" (lines 162-163) - that interpretation should be softened to "partially reduced". Is there still a statistically significant difference between the transductions in the context of KR mutants versus empty vector?
5. The finding of VSV-G pseudotypes being unaffected by IFITM3 overexpression in K562 cells, while IFITM1-mediated restriction of Measels gp entry was preserved, is very interesting and should take center stage of the manuscript. However, the entire lack of IFN-mediated inhibition of transduction in these cells is puzzling, since IFN is expected to inhibit also the post-entry steps of the lentiviral transduction (e.g. reverse transcription), and deserves further characterization. Are these cells at all sensitive to IFN? The induction of IRF7 and IFITM3 mRNA around at best 10-fold seems to indicate that these cells respond very poorly to IFN treatment under these experimental conditions. Is the inability of IFITM3 to impose an antiviral effect reproduced in other myeloid cell lines (with low Amphotericin B levels)?
6. Figure 4. This is mostly an in silico analysis of cellular interactors identified (via mass-spec) to be specifically binding WT IFITM3 (intact lysines) but not 4KR-IFITM3. The authors state that "146 [proteins] were binding IFITM3 independently of the lysine residues", however the Venn diagram seems to show that this applies to only 22 proteins specifically, and 31 interactors were specifically binding to the wild-type IFITM3. Therefore, the rationale to investigate a number of 146 proteins is unclear, and it may have been more informative to compare the 22 to the 31 proteins. The strategy applied by the authors needs some correction or clarification. In B, it remains unclear what is shown exactly. The legend states "significance of overlap between IFITM3 interacting proteins and interactors of IFITM3-sensitive viruses". Overlap in which regard? What is meant by numbers of interactors: IFITM3 interactors and/or viral proteins? Which mass spec candidates were imputed here exactly? Those specifically binding wt IFITM3 but not 4KR-IFITM3? The analysis shown in B is not very informative since it would probably come up with similar numbers using any kind of input. In C, the authors state to identify "74 interactors with higher affinity for IFITM3 WT" compared to what? What do the individual columns represent? What is the fold change difference in "higher affinity" and how is "higher affinity" determined and quantified? Red-labelled proteins are reported to represent interactors linked or localizing to endolysosomes, but this applies to some non-labelled proteins as well (Rab14, e.g.). Why would endolysosomes being the only interesting subcellular compartment (excluding endosomes e.g.?) Do A and C base on the identical data set? In D and E, the venn diagram, the interactor protein map and functionally enriched terms are, unfortunately, quite uninformative because basically everything is interconnected with everything and no specific pattern is visible. The reviewer is unable to deduce information that would inform about differences between IFITM3 wt interactors and 4KR-IFITM3 although it seems that the authors aim to show them here.
7. Figure 5: while the authors showed that overexpression of IFITM3 does not restrict VSV-G pseudotypes in K562 cells, it seems that knock-out of IFITM3 has a (facilitating) effect on transduction. The reviewer is not sure if he/she interprets figure 5O correctly because no values for parental cells are shown. Furthermore, even within the IFITM3 knock-out situation, why does PIP3 addition induce a restriction? The authors state that "this rescue [of endosomal IFITM3 restriction] was lower in cells knock out for IFITM3" (lines 304-305) but it looks like a 2-fold rescue in both situations (Oe-IFITM3 and KO-IFITM3). This would be puzzling because the authors seem to favor the hypothesis that IFITM3 and PIP3 need to be co-present to exert the restriction, while either one on its own should be insufficient. However, this is not supported by these data.
8. Figure 6 and 7 adds to the current complexity of published findings about the pro- or antiviral context of IFITMs, depending on the context of IFITM expression (heterologous versus endogenous), the context of spike presentation (authentic virus or pseudotyped). While the data are carefully acquired and worth reporting, they distract a lot from the main massage of the paper, which is about PIP3 representing a cofactor of IFITM3-mediated restriction of endosomal viral entry. Furthermore, since they show that all pre-omicron variants are sensitive to restriction merely by plasma membrane-redirected IFITMs, it remains a bit unclear why the authors devoted two entire figures to a virus which is expected (and shown by the authors) to not be susceptible to modification of the PIP3-recruiting lysines in IFITM3. It may have a better strategy to use omicron as a virus potentially susceptible to the PIP3-recruiting function of the IFITM3 lysines (this is only hesitantly addressed in panel 8I and J and could be extended largely by using the panel of existing plasma membrane-redirected IFITM3 mutants, with intact or mutated lysines, in the context of pseudotypes and authentic omicron infection, in different cell lines, importantly also in the K562 cells).
Minor points:
1. The authors mention that"IFITM3 delta 21 isoform that mimics an IFITM3 variant predicted to be encoded by the human SNP rs-1225-C" - this statement is highly debated in the literature (PMID: 28531322; PMID: 29202190; PMID: 34078739) and should be toned down.
2. The labels in the figures are somewhat imprecise, switching between indicating the glycoprotein used for pseudotyping versus the virus whose glycoproteins were used for pseudotyping. It is the glycoprotein that should be indicated (e.g. VSV-G LV, Measles envelope gp LV etc.).
3. Throughout the manuscript, the language tends to be a bit imprecise, e.g. "BaEV, RD114 and Measles pseudotyped LV" (lines 132-133) should be changed to "BaEV envelope-, RD114 envelope-, and Measles envelope glycoprotein-pseudotyped LV"
4. The glycoprotein of VSV is sometimes VSV-G, or VSV-g. Please make consistent.
5. Figure 1, why does IFITM3 delta 21 not present the expected lower molecular weight (as shown in several publications, e.g. PMID: 23055554; PMID: 22446628)?
6. Figure 8H, the label of the Y axis says "fold inhibition of infection, Ctrl vs PIP3"; I believe it should be "PIP3 vs Ctrl", because PIP3 addition is supposed to enhance inhibition?

Referee #3:

This study contributes a great deal to our understanding of the mechanisms of IFITM-mediated restriction of enveloped viruses. By employing an impressive panel of cutting-edge approaches, the authors provide evidence for specific regulation of the IFITM3's antiviral activity in endosomes by PIP3. The results with K562 cells that do not suppress VSV entry in spite of IFITM overexpression, unless supplemented with exogenous PIP3, are particularly compelling. Furthermore, the authors identified the three highly conserved Lys in the conserved intracellular loop of IFITMs as residues responsible for interactions with PIP3 and for the virus restriction in endosomes. Additional evidence for cellular compartment-specific antiviral activity of IFITMs comes from the experiments with SARS-CoV-2 Spike pseudoviruses and infectious SARS-CoV-2 virus, which has been shown to fuse directly with the plasma membrane or with endosomes, depending on the availability of cognate host proteases, like TMPRSS2. A clear correlation between the preferred SARS-CoV-2 entry route and restriction by distinctly localized IFITM1 and IFITM2/3 proteins is established. IFITM1, but not IFITM2/3, is shown to inhibit SARS-CoV-2 infection in cells that appear to support direct virus fusion with the plasma membrane. The experimental results thus support the notion of distinct requirements for IFITM-host factor interactions for efficient antiviral activity. Overall, the findings of this study provide a strong support for the role of PIP3 in IFITM3-mediated restriction of diverse viruses that enter via endosomal pathways and implicate the PI3K/Akt/mTORC pathway in regulating the antiviral activity.
The paper is very well-written and the experimental approaches are rigorous and, for the most part, are carefully controlled. The findings of this study will be of great interest to a broad virology and cell biology audience. However, there are several major concerns with some experiments that need to be addressed.
1. The lack of significant effect of AmphoB on IFITM3-mediated restriction of VSV LV transduction in THP1 cells (Fig. 2A) is surprising. Although this result is taken to support the distinct mechanisms of IFITM restriction in endosomes and the plasma membrane, essential details regarding the AmphoB treatment are lacking. There is no description of this treatment in Methods and only the pretreatment duration, but not the concentration of AmphoB is stated in some figure legends. Please include pertinent information and consider performing a dose-dependence experiment to assess the effect on VSV and Measles LV transduction in THP1 and other cells used in this study. Also, to generalize the above notion, it might be worth testing the effects of AmphoB on the THP1 cell transduction efficiency by other viral glycoproteins mediating entry from endosomes or from the plasma membrane.
2. The immunofluorescence results intending to show colocalization of the triple-Lys mutant IFITM3 with LAMP1 (Fig. 3A) are not convincing. First, no LAMP1 signal can be seen in this image. Please increase contrast/brightness to make if visible and perhaps chose another color other than blue for LAMP1 pseudocolor. Second,and more importantly, it is impossible visually determine colocalization of these two proteins in undifferentiated THP1 cells that are rounded. A careful 3D colocalization analysis/quantification must be performed to show colocalization or lack thereof.
3. The PLA results (Fig. 3B) are also quite surprising, since even a low expression of HA- and His-tagged IFITM3 should lead to accumulation and dimerization of these proteins in multiple endosomal compartments. Yet, there are only 1-2 PLA foci per cell. To address this issue, it is essential to also show and analyze colocalization based upon regular immunofluorescence images of HA- and His-tagged IFITM3. This would provide a better sense for the expression levels and the number of IFITM3-positive endosomes. Importantly, the PLA experiment in Fig. 3B lacks a negative control (perhaps use one of the dimerization-deficient IFITM3 mutants). A similar concern related to very scares PLA foci applies to IFITM3-PIP3 colocalization results (Fig. 5A). Here too, it would be helpful to show better images of the staining pattern for PIP3 (see Fig. S3E) to confirm its endosomal localization and colocalization with IFITM3.
4. It might be also worth including a dimerization-deficient IFITM3 mutant as a control for the native gel-based assessment of dimerization/oligomerization of the triple-Lys mutant (Fig. 3C).
5. Fig. 3C: based on the BN gel showing vastly different intensities of IFITM3 dimer and oligomer bands, it is unclear whether densitometry of all bands can be reliably performed.
6. PIP3 is incorrectly called "PIP3 protein" in the figures and the text, which causes confusion. Accordingly, please correct the sentence: "PIP3 has been described to phosphorylate and activate Akt..."
7. Please indicate the source of the PIP3 carrier histone H1 and provide a reference(s) or your own data supporting its role as a PIP3 carrier.

Minor:

1. It appears that the Measles virus pseudovirus was made using just F protein. What pseudovirus titers do you get without the H protein?
2. Do THP1 cells express endogenous TMPRSS2? What drives fusion of SARS-CoV-2 variants (except for Omicron) with the plasma membrane of these cells?
3. Line 132: Spell out the virus' names where these abbreviations first appear in the text.
4. Lines 150-151: It is stated that Amphotericin B has the "capacity to alter lipid membrane stability". Please clarify the mechanism of Ampho B action, which is known to sequester cholesterol/ergosterol.
5. Line 507: Please clarify how "mTOR inhibitors could be employed in the containment of Covid-19".
6. Please provide rationale for using VSV-g mutants, D395N, H80A-Q112P, E276Q instead of WT G.
7. Line 915: Please capitalize selleckchem.
8. Please use Greek symbols for uM and other units, as appropriate.
9. Please format the references in the Mass-spec section of Methods.

10. Line 1044: Please clarify what you mean by: "and then stained for 2 till 16 hours with rabbit anti-IFITM3 polyclonal antibody...".
11. Define the Oe abbreviation in the figure legend to Fig. 1.
12. What is IFITM3im in Fig. 3C?
13. The figure legends tend to refer to a PLA assay as immunofluorescence, which is misleading, as you are not just staining for two proteins but detecting their proximity. Please state clearly when PLA vs immunofluorescence assay was used.
14. Fig. 4B: Any reason why EBOV shows a low number of IFITM3 interacting proteins?

We thank the reviewers for their overall enthusiasm regarding our findings (manuscript ID: EMBOJ-2022-112234), for recognizing our work as high quality, novel and of interest for the field and thank them for their constructive criticism and suggestions. We have now modified the manuscript based on the reviewers' feedback and believe that by properly addressing their concerns we have significantly improved the overall quality of this work.

Briefly, our new experiments confirm through better controlled IF and PLA assays that the lysine mutant IFITM3 correctly localizes within the endolysosomal compartment together with PIP3, as opposed to the phosphomutant forms that confine to the plasma membrane. Moreover, we show that the lysine mutant IFITM3 dimerizes to a similar extent as the WT form, as opposed to a dimerization defective IFITM3 mutant that we generated upon reviewer suggestion. In addition, taking advantage of the susceptibility of SARS-CoV2 Omicron to both endosomal and PM-bound IFITM3, we confirmed that the lysines within the CIL domain of IFITM3 are required only for endosomal restriction. Indeed, replicating Omicron remained susceptible to the PM-confined IFITM3 regardless of the presence of these lysines while endosomal IFITM3 restriction of Omicron was completely abrogated upon their mutation. We also generated a lysine-less IFITM1 and observed that it still restricts PM-fusing viral envelopes and confirm that the 3KR-IFITM3 alone is not able to restrict PM-fusing Measles gp pseudotyped LV. Together, these experiments confirm that the lysines within the CIL domain of IFITM3 do not impact its cellular localization or dimerization and are required only for its endosomal antiviral activity. Finally, to further dissect the cell-type specific determinants of endosomal IFITM3 activity, we performed a genome-wide transcriptomic profiling comparing the restriction competent THP1 and HSPC to the K562 cells in which IFITM3 fails to restrict endosomal viral entry. This experiment aligned well with our lipidomic profiling confirming that phospholipid metabolism and in particular expression of several key players of the PI3K pathway were significantly lower in K562 cells as compared to the restriction competent THP1 and primary human HSPC. These results suggest that low PI3K activity contributes to lack of endosomal IFITM3 immunity by impacting on the PIP3 pools within K562. In agreement, we observed that inhibition of PI3K impaired endosomal IFITM3 restriction in THP1 cells. Together, these novel experiments further support our working model in which sufficient levels of both IFITM3 and PIP3 are required for efficient endosomal restriction and identify the levels of PI3K/Akt/mTOR activity as cell line-dependent regulators of this immunity.

Overall, our findings uncover a novel mechanism governing endosomal antiviral IFITM immunity as we identify PIP3 as a key host factor required for this restriction through binding to the lysine residues of the CIL domain of IFITM3. These findings inform the development of broadly acting antiviral strategies against IFITM–sensitive viruses, including the current and future pandemic-causing SARS-CoV2 variants, and may have broader implications also for the development of anticancer therapies and strategies that exploit endosomal cargo delivery given the emerging role of IFITM proteins in these processes.

Please find below the specific point-by-point reply in response to each Reviewer remark together with access to deposited datasets as follows:

For the proteomic dataset:
Project Name: Wild-type IFITM3 and mut 4KR-IFITM3 comparative interactome analysis in THP1
Project accession: PXD038896
Reviewer account details:
Username: reviewer_pxd038896@ebi.ac.uk
Password: 0eYdVYpy

For the RNA-Seq dataset:

https://www.ebi.ac.uk/biostudies/arrayexpress/studies/E-MTAB-6598?key=bf307083-ddc0-4317-b710-b5cb5af58f36

For the lipidomic dataset:
http://dx.doi.org/10.21228/M8ZB01

Referee #1 (Report for Author)

The authors have performed an extensive study to understand how IFITM3 restricts the entry via the endosomal route. They find that conserved lysine residues in IFITM3 are essential for endosomal viral restriction but dispensable for surface entry restriction when IFITM3 is retained on the surface by a Y20A mutation. They use the lysine residue mutant to identify a host of putative cofactors including a number of PIP3 binders. As the authors and others have previously shown that manipulation of MTOR signalling drives IFITM3 to degradation, the authors make the case with mutants and inhibitors that PIP3 interaction with IFITM3 is a key regulator of its endosomal function.

While there is huge amount of interesting data here, and I don't dispute a necessity for PIP3 in IFITM3 function in endosomes that explains the AKT/MTOR relationship, it seems to me that the key to their interpretation that IFITM3/PIP3 interactions are essential in the endosome is the experimentation that the authors use to conclude that the 4KR mutation (despite phenocopying the Y20A mutant) is not affected in its subcellular localization. The authors' experiments as far as I can tell are not controlled sufficiently for this interpretation, and it could simply be that the 4K mutant is similarly restricted more prominently to the cell surface, and thus not enriched in PIP3+ compartments. Given the extensive literature on the role of ubiquitination in receptor trafficking as well as turnover, and that a K-to-R phenotype is often highly indicative of a Ub-dependent process, this needs to be rule in or ruled out properly. Specifically, the authors rely on steady-state PLA-based or Ab-based localization with LAMP or PIP3 to conclude that 3KR is not mis-localized to the PM. This experiment is not controlled properly. LAMP (and the majority of endosomal markers) traffic between compartments, and at some point most membrane proteins will end up in endosomes for degradation. Therefore, the experiment needs a robust specificity control. If the authors are correct, then IFITM1 and crucially, IFITM3 Y20A, should display significantly reduced co-localization. Secondly, 3KR, but not Y20A, should not accumulate on the plasma membrane by flow/IF. Without these controls the localization of 3KR is not interpretable, and all subsequent experiments (be they manipulation of PIP3 that will affect endosomal traffic, interaction with cofactors, or cell-type dependant effects in the presence of IFN) might simply be a reflection of 3KR not getting into the right compartment in the first place. I note that surface fusing measles LVs have not been tested against the 3KR mutant alone, but against the double Y20A/3KR that would mask any effect of 3KR on trafficking.

We thank the Reviewer for his/her comments and suggestions. To address the reviewers' concerns regarding IFITM localization and specificity of co-localization with PIP3 we have performed IF experiments that evidence how the IFITM3 Y20A mutant clearly remains confined to the plasma membrane in THP1 cells (**Figure 2A and lines 183-185, page 8 of the revised manuscript**), in agreement with our initial observations reported in Petrillo et al., Cell Stem Cell 2018 (Petrillo et al., 2018) and Unali et al., bioRxiv 2021 (Unali et al., 2021). This staining is very different for the 3KR and 4KR mutants form that distributes more evenly within the cell, similarly to the WT form of IFITM3, as reported in **Figure 2A**. In agreement, our immunofluorescence analysis shows that Y20 IFITM3 mutants but not the KR mutants display significantly reduced co-localization with LAMP1 and are confined at the PM (**Figure 2A** and (Petrillo et al., 2018)). Moreover, we have performed co-localization studies between LAMP-1 and WT, 3KR or 4KR mutants of IFITM3 in monocyte-derived macrophages (MDM) in which the flatted cellular morphology facilitates visualization of the different cell compartments. These results confirm substantial and similar co-localization of all three forms of

IFITM3 with LAMP-1 (**Figure 2B and lines 186-189, page 8 of the revised manuscript**). Together, these novel results further support the interpretation that IFITM3/PIP3 interactions are essential in the endolysosomes as both 4KR and 3KR mutants retain the capacity to traffic therein similarly to IFITM3 wild-type and do not accumulate at the plasma membrane as the Y20 mutants.

Moreover, following the Reviewers suggestion, we have tested susceptibility of the surface fusing Measles glycoprotein pseudotyped LV to restriction by the 3KR mutant alone (**Figure EV2A and lines 185-186, page 8 of the revised manuscript**). This data further confirms that the 3KR mutant IFITM3 is not retained at the plasma membrane, given that it is not able to inhibit the PM-fusing Measles gp mediated vector entry, as opposed to the PM-redirected Y20F form.

Additionally, colocalization between IFITM3 and 3KR with LAMP1 was measured in THP1 cells knock-out (KO) for endogenous IFITM3 and transduced with the two lentiviral vectors encoding for HA or His-tagged WT or 3KR IFITM3 at limiting doses to achieve a single copy/vector per cell. No difference in colocalization were detected as Pearson's correlation coefficient, a common measure of colocalization, was similar for IFITM3/LAMP1 and IFITM3-3KR/LAMP1 (**Figure EV2F and lines 202-204, page 9 of the revised manuscript**).

Other points:

• The Ks are conserved in IFITM1 - what happens to surface fusers if they a mutated?

Following the Reviewer's suggestion, we generated the lysine-less version of IFITM1 and tested its capacity to restrict transduction by PM-fusing enveloped LV such as the Measles-gp pseudotyped LV. In agreement with our hypothesis that the lysine residues are not required for plasma-membrane restriction of viral entry, the lysin mutant version of IFITM1 retained full antiviral activity (**Figure 1G, Figure 3F and lines 168-170, page 7 and 220-223, page 9-10 of the revised manuscript**).

• Given the complexities of SARS CoV-2 entry, site of fusion and cell tropism, in my opinion the SARS2 data doesn't really add much to the conclusion of this paper, and may be better used in a different MS.

We agree with the Reviewer that the molecular mechanisms of SARS-CoV2 entry are quite complex and for the benefit of the paper we trimmed as much as possible the related figure panels (**Figure 7 and EV5 of the revised manuscript**). However, given that our data using the different SARS-CoV2 variants agree with our working model regarding the roles of IFITM3 and PIP3 in restricting endosomal but not plasma membrane viral entry and considering the current pandemic situation that remains unresolved, we feel it would be preferable to disclose all possible results regarding SARS-CoV2 within this manuscript.

• I am really surprised that authors have managed to get such robust infection of THP1 cells with SARS-CoV-2. In many people these are refractory to infection even at high MOI. Do the authors' THP-1 cells express ACE2 endogenously or have been engineered to express it?

We agree with the Reviewer and are aware of contrasting results regarding permissiveness of THP1 to SARS-CoV2. Our THP1 cells express quite high levels of ACE2 endogenously, as reported in **Figure EV5E and EV5F and lines 365-369, page 15 of the revised manuscript**), providing us with a rationale for testing SARS-CoV2 replication in these cells. It is possible that distinct clones of the same cell line have diverged over time among laboratories, similarly to what has been reported for the highly used HeLa cell line that may present distinct characteristics and phenotypes across laboratories despite theoretically representing the same cell line (Liu et al., 2019). We have confirmed productive

replication of SARS-CoV2 in all our THP1 cell lines across multiple experiments and different viral variants supporting the robustness of our findings.

Referee #2 (Report for Author)

The manuscript „IFN-inducible phospholipid levels govern endosomal antiviral immunity" by Unali et al. characterizes the anti- and proviral roles of distinct members of the IFITM protein family (IFITM1, 2, 3) in the context of different virus infection assays (lentivirus-based pseudotypes, authentic viruses) in different immortalized and primary cell systems. Adding another layer of complexity, the authors compare overexpression of IFITM genes with their knock-down and/or knock-out. They identify four lysine residues (one in the N-terminal domain, three in the CIL) to be essential for antiviral activity of IFITM3, but not IFITM1, against viruses which use the endosomal entry route, as opposed to viruses that fuse at the plasma membrane. Mutation of lysines to arginines results in the loss of higher molecular IFITM3 complexes, potentially dimers, that are readily formed by WT IFITM3, and these lysine residues seem to be responsible for recruitment of PIP3, an essential cofactor for IFITM3 restriction. Interestingly, IFITM3-mediated restriction of virus entry was absent in one cell line, K562, and could be restored by exogenous addition of PIP3. The overall conclusion of the manuscript is that IFITM3 requires PIP3, an IFN-inducible phospholipid, as a cofactor for its antiviral activity. While this message is novel and very interesting, large parts of the paper unfortunately distract from the main message. The in silico analysis of interactors appears a bit immature, and figures 6-7, for unclear reasons, largely deal with viruses (pre-omicron SARS-CoV-2 variants) which actually are not susceptible to IFITM3/PIP3-mediated restriction. It would have been much more informative to place the main finding (PIP3 recruitment by the lysines, differential abundance of PIP3 in cell lines) in the center and focus on its deeper characterization, and maybe use omicron for an extensive analysis of this new phenotype. Identifying the enzyme which is responsible for PIP3 formation in the phospholipid biosynthesis pathway and which may be expressed in a cell line-dependent manner, quantifying PIP3 turnover/stability, widening the relationship of PIP3 abundance and IFITM3 restriction to more cell lines/titration of PIP3/IFITM3 expression, and provide some understanding regarding how PIP3 assists IFITM3 at the molecular/physical level would have been very exciting.

We thank the reviewer for the constructive criticism and comments. Regarding the identification of the enzyme which is responsible for PIP3 formation in the phospholipid biosynthesis pathway, and which may be expressed in a cell line-dependent manner, we have performed experiments targeting PI3K, the main enzyme that converts PIP2 into PIP3 (Vanhaesebroeck et al., 2010), to assess its impact on IFITM3 restriction in THP1 cells. In line with our working model, inhibition of PI3K, that should lead to decreased PIP3 levels due to failed conversion of PIP2, leads to lower antiviral activity of IFITM3 (**Figure 5H and lines 293-297, page 12 of the revised manuscript**). This effect is specific of IFITM3-mediated restriction as the inhibitor had no significant impact on the control cells or cells knock-out for IFITM3 (**Figure 5H**). Together, these data further support the role of PIP3 in the endosomal restriction by IFITM3 and suggests that PI3K expression levels could at least in part explain cell-type specific variations in levels of PIP3. In agreement, we have performed a genome-wide transcriptomic analysis comparing the restriction competent THP1 and HSPC to the K562 cells in which IFITM3 fails to restrict endosomal viral entry and observed that K562 express lower levels of several PI3K genes and genes involved in phosphatidylinositols binding in comparison to THP1 and HSPC (**Figure 6A and 6B and lines 310-315, page 13 of the revised manuscript**). Together, these novel experiments further support our working model in which sufficient levels of both IFITM3 and PIP3 are required for efficient endosomal restriction and identify the levels of PI3K/Akt/mTOR activity as cell line-dependent regulators of this immunity.

Major points:

1. Figure 1 is a recapitulation of known properties of IFITM proteins: IFITM1 and IFITM3 mutants that are redirected to cell surface inhibit plasma membrane-fusing viruses, IFITM2-3 inhibit those entering via endosomes. While it is informative and required to introduce the system and to demonstrate that it recapitulates established findings, I advise to move it to the supplement.

We agree with the Reviewer and have moved this figure to the Expanded View section (**Figure EV1**).

2. Figure 1, The bar diagrams indicate "TD efficiency (%)" but no information is provided about the normalization (what is 100%?). Also, the concrete read-out is not mentioned. Based on the transfer vector used, it seems to be GFP expression. Are the bar diagrams based on the percentage of GFP-positive cells, or the mean fluorescence intensity (MFI), or both? Adding a few FACS dot plots to illustrate the gating strategy and the overall sensitivity of the assay would be very informative.

We thank the Reviewer for this question. The "TD efficiency (%)" refers to the % of BFP+ cells measured by FACS within over-expressing cells (GFP+) or % BFP within viable cells (7AAD-). We have modified all legends to better clarify this, changing the axis title to "TD efficiency (BFP %)" and have added FACS dot plots to illustrate the gating strategy and the overall sensitivity of the assay in supplementary data of the revised manuscript (**Figure EV1A**).

3. Figure 2: the literature contains several reports of Amphotericin B-antagonized IFITM3 restriction (e.g. Influenza A virus, PMID: 24268777; PMID: 33112230). What is the reason for the absent impact of Amphotericin B on VSV-G-mediated entry, then?

We thank the Reviewer for correctly pointing out that Amphotericin B does prevent entry of other viruses. We are aware of previous literature reporting an effect of Amphotericin B on IFITM3 restriction of Influenza (Lin et al., 2013) and HIV (Rahman et al., 2020). However, absence of amphotericin B rescue of IFITM3 restriction on VSV-gp pseudotyped LV is confirmed in the first study (see **Figure C** below).

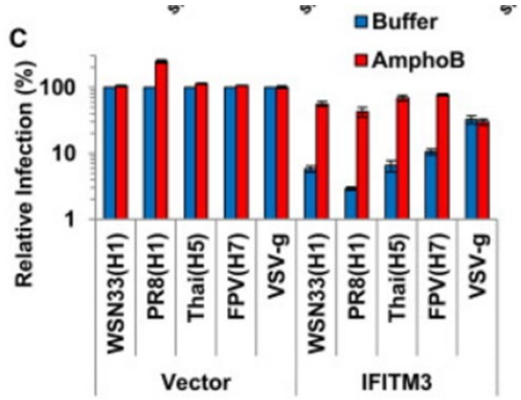

Figure C (Lin et al., 2013). IFITM3's restriction of HA-mediated entry is overcome by AmphoB. A549 cells stably transduced with IFITM3 or with vector alone (Vector) were treated with 1 µM AmphoB (red) or buffer (blue) then challenged with the indicated pseudotyped MLV-GFP particles: WSN/33 (H1), PR8 (H1), Thai (H5), FPV (H7), or the g protein of VSV. Relative infection represents the percentage of GFP-positive cells normalized to that of A549-vector cells as determined by fluorescence-based imaging. Results are the mean of three independent experiments ± SD.

Moreover, current literature on Influenza points toward an antiviral role of endosomal cholesterol accumulation in the cells (Bajimaya et al., 2017). As Amphotericin B sequester cholesterol moieties, we believe that part of the rescue of IFITM3-imposed restriction might be related to this effect. In addition, we have preliminary experimental evidence that cholesterol lowering compounds have no effect on IFITM3 restriction of VSV-gp pseudotyped LVs in our experimental model, as evidenced by the **Figure 1** below, provided here for the Reviewers' consideration.

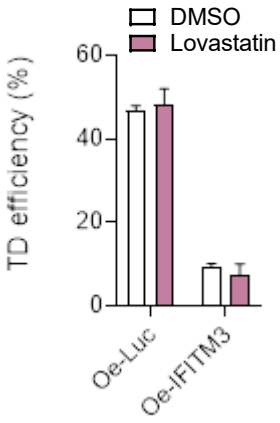

Figure 1. Lovastatin has no effect on IFITM3 restriction of VSV-gp pseudotyped LVs. THP1 over-expressing IFITM3 and control were transduced after 2h treatment with the cholesterol lowering drug Lovastatin (5uM) and then challenged with VSV-gp pseudotyped LV. Transduction was evaluated after 5 days by flow cytometry.

4. The 4KR and 3KR mutants seem to have lost a large proportion of their antiviral activity against VSV-G lentiviral vectors in THP-1, but it seems that the loss is incomplete; yet the authors phrase "both the quadruple and triple lysine to arginine mutants of IFITM3 lost antiviral activity against endosomal viral entry of VSV-g pseudotyped LV" (lines 162-163) - that interpretation should be softened to "partially reduced". Is there still a statistically significant difference between the transductions in the context of KR mutants versus empty vector?

We thank the Reviewer for this observation and have corrected our conclusion to “reduced significantly antiviral activity” and include the statistical comparison between the KR mutants and control (**line 166, page 7 of the revised manuscript**).

5. The finding of VSV-G pseudotypes being unaffected by IFITM3 overexpression in K562 cells, while IFITM1-mediated restriction of Measels gp entry was preserved, is very interesting and should take center stage of the manuscript. However, the entire lack of IFN-mediated inhibition of transduction in these cells is puzzling, since IFN is expected to inhibit also the post-entry steps of the lentiviral transduction (e.g. reverse transcription), and deserves further characterization. Are these cells at all sensitive to IFN? The induction of IRF7 and IFITM3 mRNA around at best 10-fold seems to indicate that these cells respond very poorly to IFN treatment under these experimental conditions. Is the inability of IFITM3 to impose an antiviral effect reproduced in other myeloid cell lines (with low Amphotericin B levels)?

We thank the Reviewer for finding our observations in K562 cells very interesting. Although the ISG upregulation may be lower than in some other cell types, we do think that they do respond to type I IFN stimulation. The fact that no additional effects of IFN are seen despite potentially impacting also other steps of the lentiviral life cycle such as RT may be explained by observations that we have made in the context of THP1 cells. Briefly, in THP1, we have shown that almost all the antiviral activity of type I IFN against VSV-gp pseudotyped LV can be ascribed to the presence of IFITM3 as no inhibition can be observed in THP1 KO for IFITM3 (**Figure 2** below, and reported in (Petrillo et al., 2018)). This data suggests that the most limiting IFN-sensitive step of VSV-gp pseudotyped LV remains the entry step, while later steps such as RT remain less restricted despite efficient responses of the cells to type I IFN. It is possible thus that similarly to THP1, also in the highly proliferating K562, the most rate-limiting restriction that could be induced by type I IFN exposure remains the viral entry step that is non-functional in terms of IFITM3 activity in these cells.

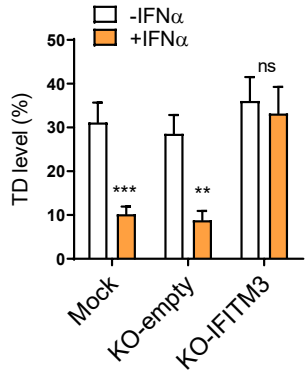

Figure 2. IFITM3 is the main determinant of IFNα-mediated VSV-gp pseudotyped LV restriction in THP1 cells. Adapted from (Petrillo et al., 2018). THP1 KO-IFITM3, control or unmanipulated (mock) were pre-stimulated with IFNα for 24 hours and then transduced with a VSV-gp pseudotyped LV. Transduction was evaluated 5 days post-TD by FACS.

In addition, as illustrated in the **Figure 3D** of the revised manuscript (**lines 217-220, page 9**), we have performed experiments in which type I IFN-treated K562 cells were transduced with the PM-fusing Measles glycoprotein pseudotyped LV and show that in this case the transduction is restricted due to IFITM1 induction that does not require cell-type specific cofactors such as PIP3 for its antiviral activity. We also tested IFNα stimulated K562 with infectious ZIKV observing IFN-mediated inhibition (**Figure 3E and lines 217-220, page 9 of the revised manuscript**).

Together, these data exclude that K562 are unresponsive to IFITM3 restriction due to a more generalized defect in type I IFN-mediated antiviral responses.

6. Figure 4. This is mostly an in-silico analysis of cellular interactors identified (via mass-spec) to be specifically binding WT IFITM3 (intact lysines) but not 4KR-IFITM3. The authors state that "146 [proteins] were binding IFITM3 independently of the lysine residues", however the Venn diagram seems to show that this applies to only 22 proteins specifically, and 31 interactors were specifically binding to the wild-type IFITM3. Therefore, the rationale to investigate a number of 146 proteins is unclear, and it may have been more informative to compare the 22 to the 31 proteins. The strategy applied by the authors needs some correction or clarification. In B, it remains unclear what is shown exactly. The legend states "significance of overlap between IFITM3 interacting proteins and interactors of IFITM3-sensitive viruses". Overlap in which regard? What is meant by numbers of interactors: IFITM3 interactors and/or viral proteins? Which mass spec candidates were imputed here exactly? Those specifically binding wt IFITM3 but not 4KR-IFITM3? The analysis shown in B is not very informative since it would probably come up with similar numbers using any kind of input. In C, the authors state to identify "74 interactors with higher affinity for IFITM3 WT" compared to what? What do the individual columns represent? What is the fold change difference in "higher affinity" and how is "higher affinity" determined and quantified? Red-labelled proteins are reported to represent interactors linked or localizing to endolysosomes, but this applies to some non-labelled proteins as well (Rab14, e.g.). Why would endolysosomes being the only interesting subcellular compartment (excluding endosomes e.g.?) Do A and C base on the identical data set? In D and E, the venn diagram, the interactor protein map and functionally enriched terms are, unfortunately, quite uninformative because basically everything is interconnected with everything and no specific pattern is visible. The reviewer is unable to deduce information that would inform about differences between IFITM3 wt interactors and 4KR-IFITM3 although it seems that the authors aim to show them here.

We thank the reviewer for these comments and agree that the Venn diagram could be misleading for the interpretation of the following figures. We have now rearranged figure 4 and included missing

legends. In **figure 4B**, the 74 interactors with higher affinity for IFITM3 WT are compared to IFITM3 4KR, the individual columns represent the biological replicates for the two conditions. We performed a differential analysis between the interactors identified in WT-IFITM3 and the mutant 4KR-IFITM3 using the LFQ intensities from the mass spectrometry data to identify the list of differentially interacting proteins upregulated in WT-IFITM3. We have updated the heatmap with labels that indicate what each individual column represents. The median value of the normalized LFQ intensities for each protein from the mass spectrometry analysis was calculated and the fold change was determined between WT-IFITM3 versus 4KR-IFITM3 (statistical analysis ANOVA Benjamini Hochberg FDR < 0.05) so the higher affinity represents a significantly higher interaction of a protein with the WT-IFITM3 in comparison to the 4KR-IFITM3.

Red labels were meant to indicate the endosomal and lysosomal proteins, we have highlighted RAB15 in the revised **Figure 4B**.

The panel 4B of the previous manuscript version has been removed following the suggestion of the Reviewer.

Regarding panels C and D, The Venn Diagram shows the number of proteins in the overlap between WT-IFITM3 identified in our analysis with ISG interactors, virus interactors and vORF interactors we retrieved from public datasets. This analysis was made to support the novel concept that IFITM3 requires the interaction with multiple proteins to exert its antiviral activity in the endolysosomes. A functional enrichment analysis was performed by clusterProfiler on the the list of differentially interacting proteins (74 interactors) that appeared significantly upregulated in WT-IFITM3 versus 4KR-IFITM3. A network of the resulting functional terms was constructed: Each node represents a specific functional term, the nodes were clustered into functional modules and the connectivity among related terms was constructed using Jaccard similarity coefficients, the Edge thickness is proportional to the Jaccard coefficient between one term and all the other terms. We will better clarify this in the revised manuscript and figure (**lines 1168-1171, page 43 of the revised manuscript**).

7. Figure 5: while the authors showed that overexpression of IFITM3 does not restrict VSV-G pseudotypes in K562 cells, it seems that knock-out of IFITM3 has a (facilitating) effect on transduction. The reviewer is not sure if he/she interprets figure 5O correctly because no values for parental cells are shown. Furthermore, even within the IFITM3 knock-out situation, why does PIP3 addition induce a restriction? The authors state that "this rescue [of endosomal IFITM3 restriction] was lower in cells knock out for IFITM3" (lines 304-305) but it looks like a 2-fold rescue in both situations (Oe-IFITM3 and KO-IFITM3). This would be puzzling because the authors seem to favor the hypothesis that IFITM3 and PIP3 need to be co-present to exert the restriction, while either one on its own should be insufficient. However, this is not supported by these data.

We thank the Reviewer for this observation and agree that there is some effect of PIP3 also in the cell KO for IFITM3 in the context of VSV-gp pseudotyped LV transduction. The experiment of PIP3 addition has given us variable results and we believe this is partly dependent on variable PIP3 intake in each different experiment and partly ascribed to the fact the our K562 have not been clonally selected and might still have heterogenous, although minimal, levels of endogenous IFITM3 that contribute to this intermediate phenotype. We have now performed additional experiments that confirm and statistically enforce our observation that PIP3 addition has a more robust effect in IFITM3 competent cells, as shown in **Figure 6J and lines 331-333, page 14 of the revised manuscript**. To further emphasize this observation, we have included the average fold difference of PIP3 versus control in **Figure 6J** of the revised manuscript to highlight that in KO-IFITM3 cells the fold inhibition of PIP3 is halved. In addition, PIP3 exogenous delivery does not inhibit VSV infection in K562 KO for IFITM3 (**Figure 8H**) or Omicron infection in Calu3 KO for IFITM3 (**Figure 8J**) further supporting the hypothesis

that IFITM3 and PIP3 need to be co-present to exert the restriction (**lines 395-400, page 17 of the revised manuscript**).

8. Figure 6 and 7 adds to the current complexity of published findings about the pro- or antiviral context of IFITMs, depending on the context of IFITM expression (heterologous versus endogenous), the context of spike presentation (authentic virus or pseudotyped). While the data are carefully acquired and worth reporting, they distract a lot from the main massage of the paper, which is about PIP3 representing a cofactor of IFITM3-mediated restriction of endosomal viral entry. Furthermore, since they show that all pre-omicron variants are sensitive to restriction merely by plasma membrane-redirected IFITMs, it remains a bit unclear why the authors devoted two entire figures to a virus which is expected (and shown by the authors) to not be susceptible to modification of the PIP3-recruiting lysines in IFITM3. It may have a better strategy to use omicron as a virus potentially susceptible to the PIP3-recruiting function of the IFITM3 lysines (this is only hesitantly addressed in panel 8I and J and could be extended largely by using the panel of existing plasma membrane-redirected IFITM3 mutants, with intact or mutated lysines, in the context of pseudotypes and authentic omicron infection, in different cell lines, importantly also in the K562 cells).

We agree with the Reviewer and have condensed the figures of panel 6 and 7 of the unrevised manuscript in one figure (**Figure 7 of the revised manuscript**). We included the figures that are overall in line with our working model of the IFITM3-PIP3 axis being relevant for endosomal viral entry, including that exploited by Omicron. This said, given the still ongoing pandemic, we retain useful to report all our findings here including those related to previous variants of concern.

In addition, following the Reviewers suggestion, we have performed experiments with SARS-CoV2 Omicron in our cell lines supporting SARS-CoV2 replication and expressing the different plasma membrane-redirected IFITM3 mutants, with intact or mutated lysines. In agreement with our working model, only mutation of the CIL lysines hampered endosomal IFITM3 restriction of Omicron whereas all PM-mutants IFITM3 maintained antiviral activity (**Figure 8G and lines 390-394, page 16 of the revised manuscript**). Regarding the possibility to test Omicron in K562, unfortunately these cells are not permissive to the SARS-CoV2 viral envelopes and should therefore be engineered to over-express the surface receptor ACE2. We feel that this type of engineering would add an artificial aspect to our experimental system that could potentially skew the results, compared to cell lines such as Calu3 and THP1 that we found to be naturally permissive to the virus.

Minor points:

1. The authors mention that"IFITM3 delta 21 isoform that mimics an IFITM3 variant predicted to be encoded by the human SNP rs-1225-C" - this statement is highly debated in the literature (PMID: 28531322; PMID: 29202190; PMID: 34078739) and should be toned down.

We thank the Reviewer for pointing this out and have rephrased as follows: “that potentially mimics an IFITM3 variant predicted to be encoded by the human SNP rs-1225-C” (**lines 136-137, page 6 of the revised manuscript**), “the PM-bound putative IFITM3-Δ21 isoform that has been predicted to potentially mimic an IFITM3 variant to be encoded by the human SNP rs-1225-C” (**lines 519-520, page 21 of the revised manuscript**) and have sited the debating literature.

2. The labels in the figures are somewhat imprecise, switching between indicating the glycoprotein used for pseudotyping versus the virus whose glycoproteins were used for pseudotyping. It is the glycoprotein that should be indicated (e.g. VSV-G LV, Measles envelope gp LV etc.).

We thank the Reviewer for pointing this out. We have rectified these imprecisions throughout the manuscript.

3. Throughout the manuscript, the language tends to be a bit imprecise, e.g. "BaEV, RD114 and Measles pseudotyped LV" (lines 132-133) should be changed to "BaEV envelope-, RD114 envelope-, and Measles envelope glycoprotein-pseudotyped LV"

We thank the Reviewer for pointing this out. We have rectified these imprecisions throughout the manuscript.

4. The glycoprotein of VSV is sometimes VSV-G, or VSV-g. Please make consistent.

We thank the Reviewer for pointing this out. We have rectified these imprecisions throughout the manuscript.

5. Figure 1, why does IFITM3 delta 21 not present the expected lower molecular weight (as shown in several publications, e.g. PMID: 23055554; PMID: 22446628)?

We agree with the Reviewer that a slight difference in size could be noted between the forms of IFITM3 in other works. It is possible that in our experimental conditions in which samples were run on precast gels these differences are flattened out. Moreover, this construct was generated through synthesis, as now detailed in the materials and methods, and was verified by sequencing.

6. Figure 8H, the label of the Y axis says "fold inhibition of infection, Ctrl vs PIP3"; I believe it should be "PIP3 vs Ctrl", because PIP3 addition is supposed to enhance inhibition?

Yes, the Reviewer is correct, and we have rectified the axis label.

Referee #3 (Report for Author)

This study contributes a great deal to our understanding of the mechanisms of IFITM-mediated restriction of enveloped viruses. By employing an impressive panel of cutting-edge approaches, the authors provide evidence for specific regulation of the IFITM3's antiviral activity in endosomes by PIP3. The results with K562 cells that do not suppress VSV entry in spite of IFITM overexpression, unless supplemented with exogenous PIP3, are particularly compelling. Furthermore, the authors identified the three highly conserved Lys in the conserved intracellular loop of IFITMs as residues responsible for interactions with PIP3 and for the virus restriction in endosomes. Additional evidence for cellular compartment-specific antiviral activity of IFITMs comes from the experiments with SARS-CoV-2 Spike pseudoviruses and infectious SARS-CoV-2 virus, which has been shown to fuse directly with the plasma membrane or with endosomes, depending on the availability of cognate host proteases, like TMPRSS2. A clear correlation between the preferred SARS-CoV-2 entry route and restriction by distinctly localized IFITM1 and IFITM2/3 proteins is established. IFITM1, but not IFITM2/3, is shown to inhibit SARS-CoV-2 infection in cells that appear to support direct virus fusion with the plasma membrane. The experimental results thus support the notion of distinct requirements for IFITM-host factor interactions for efficient antiviral activity. Overall, the findings of this study provide a strong support for the role of PIP3 in IFITM3-mediated restriction of diverse viruses that enter via endosomal pathways and implicate the PI3K/Akt/mTORC pathway in regulating the antiviral activity. The paper is very well-written and the experimental approaches are rigorous and, for the most part, are carefully controlled. The findings of this study will be of great interest to a broad virology and cell biology audience. However, there are several major concerns with some experiments that need to be addressed.

1. The lack of significant effect of AmphoB on IFITM3-mediated restriction of VSV LV transduction in THP1 cells (Fig. 2A) is surprising. Although this result is taken to support the distinct mechanisms of IFITM restriction in endosomes and the plasma membrane, essential details regarding the AmphoB treatment are lacking. There is no description of this treatment in Methods and only the pretreatment

duration, but not the concentration of AmphoB is stated in some figure legends. Please include pertinent information and consider performing a dose-dependence experiment to assess the effect on VSV and Measles LV transduction in THP1 and other cells used in this study. Also, to generalize the above notion, it might be worth testing the effects of AmphoB on the THP1 cell transduction efficiency by other viral glycoproteins mediating entry from endosomes or from the plasma membrane.

We thank the Reviewer for these observations and have included all relevant information regarding the Amphotericin B treatment into the materials and methods section of the revised manuscript (**lines 875-877, page 33**). Although Amphotericin B has been shown to impair entry of several viruses, there are some reports in which VSV has been shown to remain insensitive to this treatment (Lin et al., 2013), in line with our observations, as exemplified by the data below taken (Lin et al., 2013).

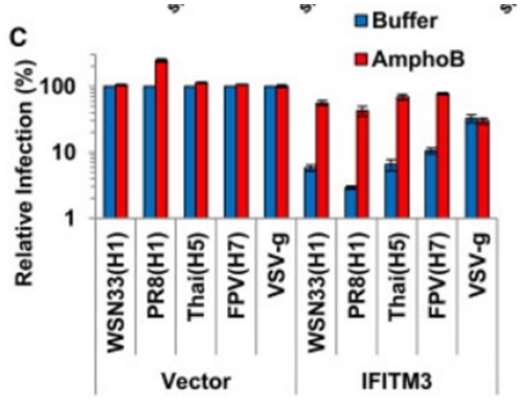

Figure C (Lin et al., 2013). IFITM3's restriction of HA-mediated entry is overcome by AmphoB. A549 cells stably transduced with IFITM3 or with vector alone (Vector) were treated with 1 µM AmphoB (red) or buffer (blue) then challenged with the indicated pseudotyped MLV-GFP particles: WSN/33 (H1), PR8 (H1), Thai (H5), FPV (H7), or the g protein of VSV. Relative infection represents the percentage of GFP-positive cells normalized to that of A549-vector cells as determined by fluorescence-based imaging. Results are the mean of three independent experiments ± SD.

Moreover, all experiments have been performed using 1µM as Amphotericin B dosage. This concentration is the standard dose used to counteract IFITM3 restriction in multiple cell types ((Hensen et al., 2019; Lin et al., 2013; Rahman et al., 2020; Suddala et al., 2019; Wrensch et al., 2015; Zhong et al., 2022) and the dose that also works to counteract PM-IFITM3 restriction in our cellular models THP1 and K562 (**Figures 1B, 1G, 1H and 1I and lines 154-159 and 170-172, page 7 of the revised manuscript**), therefore further increasing the AmphoB dose would unlikely provide any additional information also considering the severe cellular toxicity caused by this compound at higher concentrations.

2. The immunofluorescence results intending to show colocalization of the triple-Lys mutant IFITM3 with LAMP1 (Fig. 3A) are not convincing. First, no LAMP1 signal can be seen in this image. Please increase contrast/brightness to make if visible and perhaps chose another color other than blue for LAMP1 pseudocolor. Second, and more importantly, it is impossible visually determine colocalization of these two proteins in undifferentiated THP1 cells that are rounded. A careful 3D colocalization analysis/quantification must be performed to show colocalization or lack thereof.

We thank the Reviewer for these suggestions and have performed additional experiments to better qualify the localization of the 3KR-IFITM3 mutant within THP1. Firstly, we have included a comparative IF staining performed using the Y20A plasma membrane confined IFITM3 mutant in THP1 (**Figure 2A and lines 183-185, page 8 of the revised manuscript**) to show that despite the small and rounded size of these cells, this form of IFITM3 remains clearly confined to the plasma membrane forming a rim delineating the cell, as opposed to the WT IFITM3 and the 3KR mutant, and confirming our observations in (Petrillo et al., 2018). In addition, we have performed IF staining in monocyte-derived macrophages transduced with lentiviral vectors to over-express IFITM3 WT or 3KR and observe a

similar cytosolic pattern for both forms, further supporting a similar cellular localization between WT and 3KR mutant IFITM3 (**Figure 2B and lines 186-189, page 8 of the revised manuscript**). In addition, we have performed and quantified co-localization of IFITM3-3KR and Lamp1 by IF, as shown in **Figure EV2F and lines 202-204, page 9 of the revised manuscript**. These data confirm that the 3KR mutation does not alter endolysosomal localization of IFITM3.

3. The PLA results (Fig. 3B) are also quite surprising, since even a low expression of HA- and His-tagged IFITM3 should lead to accumulation and dimerization of these proteins in multiple endosomal compartments. Yet, there are only 1-2 PLA foci per cell. To address this issue, it is essential to also show and analyze colocalization based upon regular immunofluorescence images of HA- and His-tagged IFITM3. This would provide a better sense for the expression levels and the number of IFITM3-positive endosomes. Importantly, the PLA experiment in Fig. 3B lacks a negative control (perhaps use one of the dimerization-deficient IFITM3 mutants). A similar concern related to very scares PLA foci applies to IFITM3-PIP3 colocalization results (Fig. 5A). Here too, it would be helpful to show better images of the staining pattern for PIP3 (see Fig. S3E) to confirm its endosomal localization and colocalization with IFITM3.

We thank the Reviewer for these observations and suggestions. Regarding the low numbers of PLA foci, we wish to underscore that the cell lines generated to perform the PLA assays were transduced with very limiting doses of the vectors expressing IFITM3-His or IFITM3-HA (bulk copy number of 0.5) to avoid artificially inducing proximity due to high expression levels of the two proteins. **This experimental detail has been included in the text of the revised manuscript, page 8, lines 192-197.** Following the Reviewers suggestion, we also performed IF-based colocalization studies between His and HA-tagged IFITM3 WT to provide a better sense for the expression levels and the number of IFITM3-positive endosomes in these cells. In addition, we generated and tested a dimerization-defective IFITM3, as described in Rahman et al., eLife 2020, as a negative control and confirmed that it fails to form dimers in the native SDS-PAGE (**Figure EV2G and lines 204-206, page 9 of the revised manuscript**) and leads to significantly lower numbers of PLA foci (**Figure 2C and lines 198-202, page 9 of the revised manuscript**). Regarding IFITM3-PIP3 colocalization results, we have performed additional experiments as requested to confirm PIP3 endosomal localization and colocalization with IFITM3 (**Figure EV3C and lines 265-268, page 11 of the revised manuscript**). Overall, these experiments confirm and support our working model in which the lysines within IFITM3 are not impairing its localization or dimerization capacity but instead affect IFITM3 capacity to interact with PIP3 within the endolysosomal compartment.

4. It might be also worth including a dimerization-deficient IFITM3 mutant as a control for the native gel-based assessment of dimerization/oligomerization of the triple-Lys mutant (Fig. 3C).

We thank the Reviewer for this suggestion. As mentioned in the point above, we generated a dimerization defective IFITM3 as a negative control also for the native gel and confirm that it no longer forms dimers also in this molecular assay (**Figure EV2G and lines 204-206, page 9 of the revised manuscript**).

5. Fig. 3C: based on the BN gel showing vastly different intensities of IFITM3 dimer and oligomer bands, it is unclear whether densitometry of all bands can be reliably performed.

We agree with the Reviewer that these types of quantifications are not absolute and a lot a variability can occur. Therefore, we have performed the native blot several times (n=7) using independent samples to ensure consistency in the observation that the lysine-less IFITM4 presents less intense signal for the high molecular weight complexes compared to WT IFITM3. We hope that the Reviewer can agree that these replicates, together with the evidence of distinct interactomes from the

proteomic analysis in Figure 4, support the claim the IFITM3 lacking the three lysing has altered interactions with other host factors.

6. PIP3 is incorrectly called "PIP3 protein" in the figures and the text, which causes confusion. Accordingly, please correct the sentence: "PIP3 has been described to phosphorylate and activate Akt..."

We thank the Reviewer for pointing this out. We have corrected PIP3 nomenclature throughout the manuscript and figures and have correct the sentence as follows: “Binding with PIP3 has been described to cause phosphorylation and subsequent activation of Akt” (**lines 286-287, page 12 of the revised manuscript**).

7. Please indicate the source of the PIP3 carrier histone H1 and provide a reference(s) or your own data supporting its role as a PIP3 carrier.

We have used PIP3 from Echelon Biosciences 117P-3916 and chose the H1 carrier based on the manufacturers’ recommendations (https://www.echelon-inc.com/product/unlabeled-shuttle-pip-carrier-2/) inserted also in the materials e methods section (**lines 971-974, page 35 of the revised manuscript**).

Minor:

1. It appears that the Measles virus pseudovirus was made using just F protein. What pseudovirus titers do you get without the H protein?

We thank the Reviewer for noticing this. The Measles pseudotype was produced using both the F and the H proteins as previously described (Frecha et al., 2011). We have rectified this in the material and methods section (**lines 827-828, page 29 of the revised manuscript**). The titers of these vectors still remained lower compared to what we obtain with VSV-g (10E6-10E7 TU/ml).

2. Do THP1 cells express endogenous TMPRSS2? What drives fusion of SARS-CoV-2 variants (except for Omicron) with the plasma membrane of these cells?

We have observed that THP1 express quite high levels of endogenous ACE2, as shown in the **Figures EV5E and EV5F of the revised manuscript** and predict that this allows efficient entry of SARS-CoV2 variants into these cells. We have also measured TMPRSS2 levels in these cells and observe that it is also expressed at similar levels in comparison to Calu3 cells (**Figure EV5F and lines 367-369, page 15 of the revised manuscript**).

3. Line 132: Spell out the virus' names where these abbreviations first appear in the text.

We thank the Reviewer for pointing this out. We have rectified these imprecisions throughout the manuscript.

4. Lines 150-151: It is stated that Amphotericin B has the "capacity to alter lipid membrane stability". Please clarify the mechanism of Ampho B action, which is known to sequester cholesterol/ergosterol.

We thank the Reviewer to the suggestion, and we have changed the text in “Amphotericin B has been shown to abrogate IFITM-mediated restriction of several viruses due to its capacity to bind several lipids including cholesterols and ergosterols altering membrane stability” (**lines 151-154, page 7 of the revised manuscript**).

5. Line 507: Please clarify how "mTOR inhibitors could be employed in the containment of Covid-19".

Given that Rapamycin degrades IFITM3 and IFITM3 as well as mTOR activation seem to be important for pre-Omicron SARS-CoV2 variant replication, it is possible that these types of drugs could be useful to inhibit SARS-CoV2. We have reformulated the sentence in "could be employed to reduce SARS-CoV2 replication", (**line 502, page 21 of the revised manuscript**).

6. Please provide rationale for using VSV-g mutants, D395N, H80A-Q112P, E276Q instead of WT G.

We thank the Reviewer for pointing this out, all experiments have been performed with WT VSV-gp in the manuscript. We have rectified this in the materials and methods section of the revised manuscript.

7. Line 915: Please capitalize selleckchem.

We thank the Reviewer for pointing this out and have corrected the spelling to SelleckChem.

8. Please use Greek symbols for uM and other units, as appropriate. We have modified all the units as the Revisor suggested.

We thank the Reviewer for pointing this out and have corrected all unit symbols throughout the manuscript as appropriate.

9. Please format the references in the Mass-spec section of Methods.

We thank the Reviewer for pointing this out and have corrected all references as indicated.

10. Line 1044: Please clarify what you mean by: "and then stained for 2 till 16 hours with rabbit anti-IFITM3 polyclonal antibody...".

We have corrected with "and then stained for 2h at room temperature or 16 hours at 4˚C with rabbit anti-IFITM3 polyclonal antibody", (**lines 1087-1088, page 40 of the revised manuscript**).

11. Define the Oe abbreviation in the figure legend to Fig. 1.

We thank the Reviewer for pointing this out and have corrected the figure legend as indicated.

12. What is IFITM3im in Fig. 3C?

We thank the Reviewer to point this out. We have corrected the figure with IFITM3 dim (dimer).

13. The figure legends tend to refer to a PLA assay as immunofluorescence, which is misleading, as you are not just staining for two proteins but detecting their proximity. Please state clearly when PLA vs immunofluorescence assay was used.

We thank the Reviewer for pointing this out and have clarified PLA vs IF as necessary through the manuscript.

14. Fig. 4B: Any reason why EBOV shows a low number of IFITM3 interacting proteins?

This analysis was a meta-analysis performed using already published Ebola virus interactome dataset. The low number of common interactors between WT-IFITM3 and the interactome of the Ebola virus could be related to the low number of interactors reported in the Ebola virus dataset we used in this analysis. However, we have followed the suggestion of Reviewer 2 and removed this analysis from Figure 4 of the revised manuscript to help improve the overall data clarity.

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Zhong, L., Song, Y., Marziali, F., Uzbekov, R., Nguyen, X.N., Journo, C., Roingeard, P., and Cimarelli, A. (2022). A novel domain within the CIL regulates egress of IFITM3 from the Golgi and reveals a regulatory role of IFITM3 on the secretory pathway. Life Sci Alliance *5*.

Thank you for submitting a revised version of your manuscript. The study has now been seen by all original referees. While reviewers #1 and #2 are satisfied with the revision, reviewer #3 finds that several of their initial points were not sufficiently addressed. Therefore, I would like to invite you to address the remaining referee comments and the following editorial issues.

Please feel free to contact me if have any questions regarding this final revision. Thank you again for giving us the chance to consider your manuscript for The EMBO Journal, and I look forward to receiving the revised version.

Referee #1:

The authors have addressed my concerns thoroughly. I think the paper is really interesting and should be accepted. I would perhaps suggest that they explicitly state in the text that the THP-1 variant they use unusually expresses ACE2 in contrast to most.

Referee #2:

The authors have carefully adressed all my concerns and have added substantial new data. I am in favor of this revised manuscript being published at EMBO Journal.

Referee #3:

Summary:
The revised manuscript by Unali and co-authors is considerably improved. For the most part, the reviewers' concerns are addressed and new results, including transcriptome and lipidomics, are included. The new results further strengthen the conclusion that antiviral activity of IFITM3 in endosomes is dependent on PIP3 levels. This is a novel and exciting discovery that add a new layer of complexity to regulation of the antiviral activity of IFITM3 in a cellular compartment-specific manner. However, my previous concerns are not fully addressed and some of the results appear to be overinterpreted. Specific comments are as follows.

Specific major comments:

1. My initial concern regarding the effect of Ampho B remains unanswered. Fig. 1A, B and I: The effect of Ampho B on transduction efficiency (or lack thereof) appears to be somewhat overstated. First, Ampho B does enhance VSV-gp LV transduction of IFITM3 overexpressing THP1 cells about 2-fold (panel 1A). If this effect is statistically significant (analysis not shown) , this should be acknowledged. Second, in panels B and I, Ampho B appears to (significantly?) non-specifically increase the control Luc transduction efficiency. This should be considered when interpreting the enhancement of Measles gp-mediated transduction. A fold-increase ratio of IFITM3 over Luc (with proper error propagation) would be a better indicator of the effect of this drug. While the effect of Ampho B on IFITM3-mediated restriction of other viruses has been reported, it is still a good idea to test whether Ampho B also fails to rescue IAV fusion with THP1 cells.
2. Fig. 2A, B: The image quality is improved. However, I am not convinced that all IFITM3 signal that is not plasma membrane-associated is necessarily in endosomes. At least a LAMP1/IFITM3 colocalization analysis would be appropriate here.
3. Fig. 2C: The number of PLA spots per cell (<5 on average) still does not seem to make sense. This number is disproportionately low compared to the expression of HA- and His-tagged IFITM3 proteins and it is only ~2-fold higher than the number of spots for a dimerization-defective mutant. Theoretically, a single protein dimer should generate a visible spot through signal amplification. Are the authors suggesting that there are only a few IFITM3 dimers per cell out of many proteins detected by immunofluorescence? The same issue is with less than 2 IFITM3-PIP3 complexes per cell (Fig. 6H). If, for some reason, PLA detects only a very small subset of dimers (or protein-lipid complexes), this limitation should be acknowledged, and the results should be more carefully interpreted.
4. Fig. 5H: LY294002 caused an almost 2-fold increase in Luc transduction control (although apparently not significant). This calls for an extra caution when interpreting the effect of this inhibitor on IFITm3-mediated virus restriction in the same graph.
5. Line 327 states: "...while IFNα induced both IFITM3 and PIP3 levels in restriction competent THP1 cells, only IFITM3 levels increased in K562 (Figures 6G and EV4C)." However, an increase in IFITM3 level in K562 cells is not nearly as marked as in THP1 cells, which might be partially responsible for the weaker inhibition of infection in the former cells.

6. Fig. 6J: There is a very modest increase in VSV-gp restriction upon exogenous PIP3 addition to IFITM3-expressing vs IFITM3-KO K652 cells. Controls to verify a successful increase in PIP3 content of cells should be shown.

Minor points:

1. Not sure what the authors mean by "defects in IFITM3 localization" (line 226) that were excluded from analysis. What constitutes a defect? If this is a regular occurrence, perhaps it is not a defect? Why is IFITM3 distribution outside "defects" appear to be primarily plasma membrane-localized?
2. Fig. 5A: "Cell periphery (in yellow) is drawn manually" does not appear to be done.

3. Fig 6C, D: How come the PI level ratios are negative in these panels? Same question for Fig. EV4A. Are this log plots of the PI level ratio?

4. Line 981: Cyslosporin H has not been used in this study.

5. Fig. EV2G: The amount of cell lysate is not equal across the three samples (see loading control). The faint band corresponding to IFITM3 dimer might be more apparent if the loading is equalized.

We thank the reviewers 1 and 2 for considering the revised work now acceptable for publication in the EMBO Journal (manuscript ID: EMBOJ-2022-112234). Regarding the last concerns raised by Reviewer 3, we have further modified the manuscript based on the reviewers' feedback, addressing all remaining points as detailed below.

Referee #1:

The authors have addressed my concerns thoroughly. I think the paper is really interesting and should be accepted. I would perhaps suggest that they explicitly state in the text that the THP-1 variant they use unusually expresses ACE2 in contrast to most.

We thank the Reviewer for retaining our work of interest and acceptable for publication in the EMBO Journal. We have better highlighted the unusually high expression levels found in our THP1 cells, as requested, lines 372-373 of the revised manuscript.

Referee #2:

The authors have carefully adressed all my concerns and have added substantial new data. I am in favor of this revised manuscript being published at EMBO Journal.

We thank the Reviewer for appreciating our efforts to address all raised concerns and for retaining the work now suitable for publication in the EMBO Journal.

Referee #3:

Summary:
The revised manuscript by Unali and co-authors is considerably improved. For the most part, the reviewers' concerns are addressed and new results, including transcriptome and lipidomics, are included. The new results further strengthen the conclusion that antiviral activity of IFITM3 in endosomes is dependent on PIP3 levels. This is a novel and exciting discovery that add a new layer of complexity to regulation of the antiviral activity of IFITM3 in a cellular compartment-specific manner.

However, my previous concerns are not fully addressed and some of the results appear to be overinterpreted. Specific comments are as follows.

Specific major comments:

1. My initial concern regarding the effect of Ampho B remains unanswered. Fig. 1A, B and I: The effect of Ampho B on transduction efficiency (or lack thereof) appears to be somewhat overstated. First, Ampho B does enhance VSV-gp LV transduction of IFITM3 overexpressing THP1 cells about 2-fold (panel 1A). If this effect is statistically significant (analysis not shown) , this should be acknowledged. Second, in panels B and I, Ampho B appears to (significantly?) non-specifically increase the control Luc transduction efficiency. This should be considered when interpreting the enhancement of Measles gp-mediated transduction. A fold-increase ratio of IFITM3 over Luc (with proper error propagation) would be a better indicator of the effect of this drug. While the effect of Ampho B on IFITM3-mediated restriction of other viruses has been reported, it is still a good idea to test whether Ampho B also fails to rescue IAV fusion with THP1 cells.

We thank the Reviewer for these observations and have included all the analysis requested. Although Amphotericin B has an approximately two-fold rescue of IFITM3 restriction of VSV-g mediated entry (**Figure 1A**), the effect remains modest in comparison with the full rescue that it exerts on the plasma-membrane confined mutants (**Figure 1B and 1I** of the revised manuscript). The effect of Amphoteric

B observed on the control Luc transduction (**Figure 1B and 1I** of the revised manuscript) may result from an alteration of the membrane lipid composition unlinked to IFITM3. In addition, we cannot exclude some effect due to basal levels of endogenous IFITM3 in these conditions. In any case, this fold rescue of Ampho B on the Luc control appears to be less than 1.5-fold and significantly different from the rescue observed for the over-expressed IFITM3 PM-mutants (**Figure 1B and Figure 1I** of the revised manuscript).

Although we agree with the Reviewer that testing the effect of Ampho B on IAV in THP1 over-expressing IFITM3 would be interesting and could provide additional information on the capacity of the drug to rescue other endocytosis-dependent vectors, we feel that these experiments are out of scope of the present work as the mechanisms of Amphotericin B are not the main focus of our paper. Indeed, experiments with Ampho B were an initial indication of potential distinct mechanisms for plasma membrane vs. endosomal IFITM restriction that we have then extensively elucidated through other experimental approaches throughout the manuscript. In addition, we do not have currently access to IAV and would not be able to perform these experiments in a reasonable timeframe. Therefore, we hope that the Reviewer will agree that these experiments can be part of future investigations beyond the present work.

2. Fig. 2A, B: The image quality is improved. However, I am not convinced that all IFITM3 signal that is not plasma membrane-associated is necessarily in endosomes. At least a LAMP1/IFITM3 colocalization analysis would be appropriate here.

We thank the Reviewer for the suggestion and agree that it is possible that not all non-plasma membrane IFITM3 necessarily localizes to the endosomes. Nevertheless, the main readout of these experiments was to ascertain that similar localization is observed between the WT and the KR mutants. To this end, we have followed the reviewers' suggestion and have performed LAMP1/IFITM3 colocalization analysis. These new results indicated that the Pearson colocalization coefficient is equal for all conditions with the exception of the plasma membrane-confined Y20F mutant, further confirming that WT and KR mutant IFITMs localize to a similar extent with the endosomal compartment. This data has been included it in the manuscript (**Figure 2C** of the revised manuscript). As suggested by the reviewer, it is still possible that other cellular compartments such as the Golgi, as reported by Zhong et al. (Zhong et al., 2022), could also be involved, as cited in the manuscript.

3. Fig. 2C: The number of PLA spots per cell (<5 on average) still does not seem to make sense. This number is disproportionately low compared to the expression of HA- and His-tagged IFITM3 proteins and it is only ~2-fold higher than the number of spots for a dimerization-defective mutant. Theoretically, a single protein dimer should generate a visible spot through signal amplification. Are the authors suggesting that there are only a few IFITM3 dimers per cell out of many proteins detected by immunofluorescence? The same issue is with less than 2 IFITM3-PIP3 complexes per cell (Fig. 6H). If, for some reason, PLA detects only a very small subset of dimers (or protein-lipid complexes), this limitation should be acknowledged, and the results should be more carefully interpreted.

We thank the Reviewer for the comments. The limited number of foci can be explained by the very stringent *"find maxima"* used to count the foci. If we decrease the *"find maxima"* threshold for the PLA counts, the average number per cell increases, without however affecting the difference between IFITM3-wt, 3K3 and the dimerization mutant that remains the same as shown in **Figure 1, left panels** below for the Reviewers' consideration. Thanks to the Reviewers comment, we realize that a lower *"find maxima"* might thus better reflect the foci number present in the images and have therefore re-analysed them with an adjusted *"find maxima"* and substituted the graph accordingly (**Figure 2E** of the revised manuscript). The dimerization mutant was previously described to be less competent in forming dimers, however complete loss of dimerization has not been reported and the observed two-fold decrease in foci number reflects what has been described in previous reports (Rahman et al.,

2020). It is also worth to mention that different dimensions of the foci, especially in the cells over-expressing IFITM3-wt and 3KR (**Figure 2D**), possibly indicates a signal overlap originated from different numbers of dimers. This overlap is still counted as one focus by the software, thus likely underestimating the total counts per cell. For this reason, we quantified also the foci mean signal intensity to provide a better idea of the difference between the tested conditions (**Figure 2E**). Also the quantification of foci signal intensity supports a significant difference between IFITM3-wt, 3KR and dimerization mutant, confirming robustness of our analysis. The same applies for IFITM3-PIP3 PLA experiments (**Figure 6F** of the revised manuscript and **Figure 1, right panels** below for the Reviewers' consideration).

Figure 1

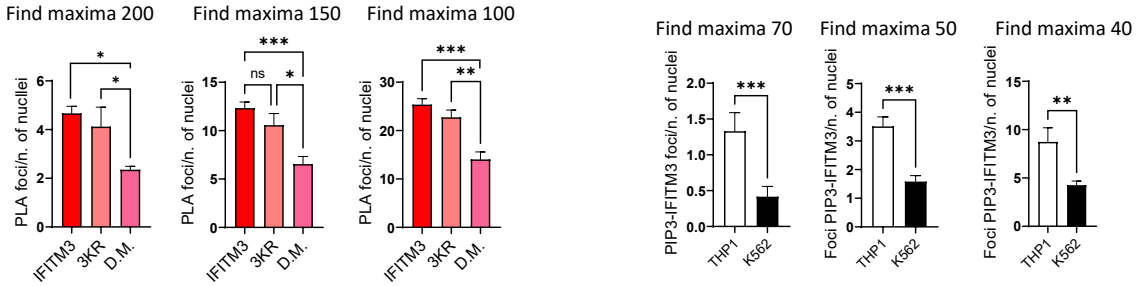


Figure 1. The number of foci were counted on imageJ with different "*find maxima*" and normalized over the number of nuclei. Related to Figure 3C and 6F.

4. Fig. 5H: LY294002 caused an almost 2-fold increase in Luc transduction control (although apparently not significant). This calls for an extra caution when interpreting the effect of this inhibitor on IFITm3-mediated virus restriction in the same graph.

We thank the Reviewer for these observations, and we agree that although non-significant, the small effect of LY294002 on the Luc control should be acknowledged. We modified the text accordingly (line 300-302, page 13 of the revised manuscript). We also calculated the drug fold-rescue of transduction among the different conditions and show that the LY294002 has a significant and stronger effect in IFITM3 over-expression vs. Luc control cells (**Figure 5H** of the revised manuscript). It is also possible that as the Luc control still expresses IFITM3 endogenously the 2-fold increase of transduction could result from blocking some basal IFITM3-PIP3 activity.

5. Line 327 states: "...while IFNα induced both IFITM3 and PIP3 levels in restriction competent THP1 cells, only IFITM3 levels increased in K562 (Figures 6G and EV4C)." However, an increase in IFITM3 level in K562 cells is not nearly as marked as in THP1 cells, which might be partially responsible for the weaker inhibition of infection in the former cells.

We thank the Reviewer for pointing this out. We agree that IFITM3 induction is lower in K562 in comparison to THP1. However, considering the different origin of the two cell lines (monocytic leukemia vs erythroleukemia), it is not unconceivable that intrinsic differences in the fold induction of any given gene, including IFITM3, are observed. Furthermore, we would like to point out that inhibition is not weaker in K562 but completely absent (**Figure 3A and 3B** of the revised manuscript). In addition, similar fold of IFITM3 induction can be detected for HSPC (2.1-fold in K562 vs 2.9-fold in HSPC, **figures EV4C and EV4D** of the revised manuscript) in which a clear and strong inhibition of VSV-g transduction is reported (Petrillo et al., 2018). Moreover, K562 remain unresponsive also upon over-expression of IFITM3 (**Figure 3A** of the revised manuscript) despite similar levels of IFITM3 were achieved between THP1 and K562 (**Figures EV1C and EV2H**, of the revised manuscript). Based on these

considerations, we therefore feel confident in ruling out a potential contribution of weaker IFITM3 expression in lack of IFITM3 antiviral activity in K562.

6. Fig. 6J: There is a very modest increase in VSV-gp restriction upon exogenous PIP3 addition to IFITM3-expressing vs IFITM3-KO K652 cells. Controls to verify a successful increase in PIP3 content of cells should be shown.

We thank the Reviewer for this observation and agree that there is some effect of PIP3 also in the cells KO for IFITM3 in the context of VSV-gp pseudotyped LV transduction. The experiment of PIP3 addition has given us variable results. This can be partly dependent on variable PIP3 intake in each experiment but potentially linked also to the fact that our K562 cell line has not been clonally selected and might still have heterogenous, although minimal, levels of endogenous IFITM3 that contribute to this intermediate phenotype. We have included the requested control (**figure EV4F** of the revised manuscript), confirming successful intake of PIP3 in K562 by immunofluorescence. The two-fold increase of PIP3 levels correlates with the average two-fold inhibition of restriction that we observe in K562 challenged with VSV-gp LV and replicating viruses (**Figure 6J, Figure 8H and Figure 8J** of the revised manuscript). In addition, exogenous PIP3 delivery does not inhibit VSV infection in K562 KO for IFITM3 (**Figure 8H**) or Omicron infection in Calu3 KO for IFITM3 (**Figure 8J**) further supporting the hypothesis that IFITM3 and PIP3 need to be co-present to exert the restriction.

Minor points:

1. Not sure what the authors mean by "defects in IFITM3 localization" (line 226) that were excluded from analysis. What constitutes a defect? If this is a regular occurrence, perhaps it is not a defect? Why is IFITM3 distribution outside "defects" appear to be primarily plasma membrane-localized?

We appreciate the comment of the Reviewer. We have reformulated the sentence into “IFITM3 competence for endo-lysosomal trafficking was confirmed by IF analysis in K562 (Figure 3J) and Pearson’s colocalization coefficients for IFITM3 and LAMP1 were similar in THP1 and K562 (**Figure 2C and 3J**).” (Lines 223-226 of the revised manuscript, page 10).

2. Fig. 5A: "Cell periphery (in yellow) is drawn manually" does not appear to be done.

We thank the Reviewer for pointing this out and have removed this sentence from the figure legend.

3. Fig 6C, D: How come the PI level ratios are negative in these panels? Same question for Fig. EV4A. Are this log plots of the PI level ratio?

We thank the Reviewer for the question. We confirm this is a Log10 representation of the PI level ratio.

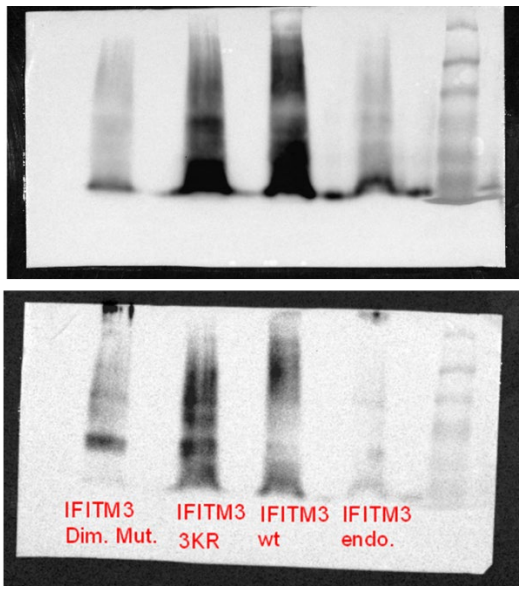
4. Line 981: Cyslosporin H has not been used in this study.

We thank the Reviewer for pointing this out and we have removed Cyclosporin H from the material and methods section of the revised manuscript.

5. Fig. EV2G: The amount of cell lysate is not equal across the three samples (see loading control). The faint band corresponding to IFITM3 dimer might be more apparent if the loading is equalized.

We thank the Reviewer for the comment and agree that the amount of cell lysate loaded seems not to be perfectly equalized for each condition. Upon verification, we noticed an error in the orientation

of the images of the normalizer that were inadvertently flipped during Figure generation. We have corrected this image and have provided original source images with relative markers that will be available as source data for the manuscript. We have included the source images also below for the Revisers' consideration.



α-IFITM3

α-actin

References

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Rahman, K., Coomer, C.A., Majdoul, S., Ding, S.Y., Padilla-Parra, S., and Compton, A.A. (2020). Homology-guided identification of a conserved motif linking the antiviral functions of IFITM3 to its oligomeric state. Elife *9*.

Zhong, L., Song, Y., Marziali, F., Uzbekov, R., Nguyen, X.N., Journo, C., Roingeard, P., and Cimarelli, A. (2022). A novel domain within the CIL regulates egress of IFITM3 from the Golgi and reveals a regulatory role of IFITM3 on the secretory pathway. Life Sci Alliance *5*.

Thank you for addressing these final points. I am now pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

EMBO Press Author Checklist

Corresponding Author Name: Anna KAJASTE-RUDNITSKI
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2022-112234R

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Reporting Checklist for Life Science Articles (updated January 2022)
This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your manuscript.
Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for figures

1. Data
The data shown in figures should satisfy the following conditions:
- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data Presentation.

2. Captions
Each figure caption should contain the following information, for each panel where they are relevant:
- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
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Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	Materials and Methods

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For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Materials and Methods, Figure Legends

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