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IFITM3 requires an amphipathic helix for antiviral activity

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 13 March 2017

Thank you for the submission of your research manuscript to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, all three referees acknowledge the potential interest of the findings. However, they all raise a number of concerns and suggestions to improve the manuscript, or to strengthen the data and the conclusions drawn. All the points of the referees should be addressed in a revised version. Further, both referee #1 and #3 point out that the mechanistic model proposed remains unclear and needs to be better described and discussed in much more detail. Referee #3 also suggests a different title based on this assessment.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be fully addressed in the revised manuscript and in a complete point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

REFEREE REPORTS

Referee #1:

This study examines the mechanism of antiviral activity of interferon-inducible transmembrane proteins (IFITMs) and presents compelling evidence that a conserved amphipathic helix within the

first hydrophobic (HD1) region of IFITM3 is essential for its function. Several experimental approaches employed in this study support the role of a small amphipathic helix for the ability of both IFITM3 and IFITM1 proteins to antagonize virus entry. Although the mechanistic details remain unclear, this is a novel and significant finding that implicates membrane curvature changes in antiviral activity of IFITMs. The results of this study should be of interest to the virology community.

Secondary structure analysis predicted the existence of a small amphipathic helix (residues 59-68) in the IFITM3's HD1 domain, and site-directed mutagenesis revealed the importance of the amphipathic nature of this region for antiviral activity. By contrast, deletion of the transmembrane HD2 domain of IFITM3, although reduced the expression level of this protein, did not abrogate its ability to inhibit the influenza virus fusion. This result suggests that, contrary to the model proposed in the previous study [1], the transmembrane domain, which interferes with the functions of cholesterol trafficking proteins, is dispensable for antiviral activity. These results led to the model that the amphipathic helix of IFITM proteins inhibits viral fusion by inserting into the cytoplasmic face of the endosomal membrane and counteracting the influenza HA-induced membrane curvature changes in the course of fusion.

The paper is well-written, the experiments are clearly described and, for the most part, adequately controlled. The experiments support the main conclusions of this study. Aside from a few experimental concerns discussed below, the main caveat is the undeveloped/unclear model for IFITM-mediated inhibition of viral fusion. The proposed "amphipathicity-based model" is rather vague. It falls short of offering a molecular mechanism for antiviral activity. What is the exact effect of the IFITM3's helix on membrane curvature? What drives the alleged accumulation of IFITM3 at the fusion site? Based on the amphipathic nature of this helix and the proposed shallow insertion into membranes (wedging mechanism, Fig. 7), this region would likely induce positive curvature in the cytoplasmic face of a membrane, which should promote, not inhibit viral fusion (e.g. [2, 3]). It would help the reader to more clearly describe the model and its assumptions, trying to reconcile this with the published work on membrane fusion and the effects of amphipathic peptides. Whereas amphipathic peptides are known to disrupt lipid bilayers, this reviewer is not aware of instances when such peptides/proteins inhibit membrane fusion.

Other essential points:

1. Based upon the inhibition of influenza fusion by the IFITM3 mutant lacking the transmembrane HD2 domain (IFITM3- 88-133), it is concluded that HD2 is dispensable for antiviral activity. However, another study has shown the lack of activity for the IFITM3 chimera in which the HD2 domain has been substituted with that of CD4 or TrfR1 [1]. Can the authors reconcile these discrepant findings? Given the controversial nature of the result suggesting that the IFITM3- 88-133 mutant maintains antiviral activity, in spite of its lower expression, additional controls are warranted. For instance, it would be helpful to show that expression of this mutant does not raise the endosomal pH or interfere with virus endocytosis or trafficking.

2. The structural model of IFITM3 proposed based upon secondary structure prediction is quite similar to the recent NMR structure published by Ling and co-authors [4]. The distinction between the intramembrane model of HD1, presumably proposed in [4], and the structural predictions made in the current manuscript is unclear. The NMR structure does not seem to support an intramembrane loop topology (i.e., loop that inserts deep into the lipid bilayer) for HD1. As expected for amphipathic helices, HD1 is envisioned to shallowly insert into the lipid membrane, at the level of phospholipid head groups. This is unlike the cartoon depiction of the alternative model in Fig. 2B. It thus seems unfair to refer to the published structural results as confirmatory to the secondary structure predictions made in the current study. I'd suggest acknowledging the published results upfront and shortening the section of Results describing the structural predictions.

3. It appears strange to co-express IFITM3 and HA in the same cell for cell-cell fusion experiments. This does not reflect the actual topology of HA and IFITM3, which are normally in different membranes.

4. Figure 6: Needs an additional control of HA not exposed to acidic pH (say at time = 0, before the virus is exposed to acidic pH) to show selectivity of Y8-10C2 antibody binding.

5. Most figures show the results of "representative of {greater than or equal to}3 independent experiments each performed in triplicate". A better practice is to average all experiments.

Minor points:

1. Where VSV and VSV-GFP came from?

2. Colocalization of IAV with IFITM3 (Supp. Fig. 3), is not convincing and does not really carry much information, since IFITM3 covers large perinuclear areas where the virus particles accumulate by default.

3. Page 14, middle: "HA was most prevalent in intracellular compartments..." should probably read "IFITM3 was most prevalent..."

4. Why not use * and ** etc. to differentiate between p values?

References:

1 Amini-Bavil-Olyaee, S., Choi, Y. J., Lee, J. H., Shi, M., Huang, I. C., Farzan, M. and Jung, J. U. (2013) The antiviral effector IFITM3 disrupts intracellular cholesterol homeostasis to block viral entry. Cell host & microbe. 13, 452-464
2 Chernomordik, L. V. and Kozlov, M. M. (2003) Protein-lipid interplay in fusion and fission of biological membranes. Annu Rev Biochem. 72, 175-207
3 Kozlov, M. M., McMahon, H. T. and Chernomordik, L. V. (2010) Protein-driven membrane stresses in fusion and fission. Trends Biochem Sci
4 Ling, S., Zhang, C., Wang, W., Cai, X., Yu, L., Wu, F., Zhang, L. and Tian, C. (2016) Combined approaches of EPR and NMR illustrate only one transmembrane helix in the human IFITM3. Scientific reports. 6, 24029

Referee #2:

In this paper, Chesarino et al. perform a structure-function analysis of IFITM3 and IFITM1 and identify an amphipathic helix that is critical for restriction. First, they show that residues 58 to 78 of IFITM3 are conserved in Homo sapiens and Mus musculus. Secondary structure prediction programs indicated that this domain appears to form two distinct alpha helices separated by a short spacer, with residues 59-68 forming an amphipathic helix. This helix is predicted exposed to the cytoplasmic side of the plasma membrane. Mutations that disturb the amphipathic nature of this domain cause loss of restriction of HIV-1, influenza, Zika and Ebola viruses. Notably, these mutations abolish the anti-fusogenic activity of IFITM3 yet do not appear to affect the well known structural changes of influenza HA triggered by low pH, according to its recognition by conformation-specific antibodies. The authors conclude that the amphipathic helix of IFITM3 is important to allow viral-cell membrane fusion and pore formation in the endosome.

This is a nice and focused paper that sheds light on a poorly understood aspect of IFITM3 restriction. The model proposed is plausible and supported by the data. Although in the paper there is no direct confirmation of the amphipathic helix prediction, this prediction is consistent with recent NMR data (Ling et al. 2016) and is supported by the results based on mutagenesis presented in Figure 3.

While most of the experiments are well performed and controlled, Figure 5B shows rather weak data due to the limited number of multinucleated cells that were counted by eye. This experiment should be analysed by flow cytometry to count a larger number of multinucleated syncitia and the statistical analysis re-run with the newly acquired data.

Referee #3:

This paper explores mechanisms by which interferon-induced transmembrane (IFITM) proteins

suppress diverse viral infections. The paper presents convincing evidence for the important role of newly identified short amphipathic helix in the protein. This is an important finding in a hot research field. My main concern with this paper and especially with its title is that it overemphasizes the less developed aspect of the study: the specific mechanisms by which the alpha-helix inhibits viral fusion. While the authors do suggest a mechanism, it is not proven and, even more importantly, I do not think this hypothetical mechanism is the take home message of the work. In my opinion, the title like 'Virus restriction by IFITM3 depends on amphipathic helix within IFITM1' would better convey the content of the paper than 'An amphipathicity-based mechanism for virus restriction by IFITM3'.

Specific comments:

1) The results of the experiment with Nile red labeling are not easy to interpret. Nile red is a fairly non-specific probe labeling both lipids (and especially lipid droplets) and proteins. It is unclear to me why cells transiently transfected and expressing IFITM3, in contrast to cells expressing IFITM3-88-133, showed a slight increase in Nile Red fluorescence as compared to control cells (Figure 1D,E). Perhaps comparing this increase with cell labeling with probes either more specific for cholesterol or known to non-specifically label all membranes (for instance, FM dyes) may help in clarifying the interpretation. Moreover I am not sure on what this data adds to the story.

2) The suggested mechanism in which helix insertion into distal leaflets of fusing membranes inhibits membrane fusion seems to be related to the stalk-pore pathway in which opening of a fusion pore is inhibited by treatments generating positive spontaneous curvature of distal membrane leaflets. Inserted alpha helix of IFITM in Fig. 7 acts as a wedge adding positive spontaneous curvature similarly to a inverted cone shaped lipid such as LPC to the distal leaflet.

3) How do you define multinucleated cells in the analysis in Fig. 5? Are these cells with more than one nuclei? Cells with more than 2 nuclei? This experimental approach can be used to verify that IFITM has to be expressed in the target cells rather than in the cells expressing HA. Otherwise the sentence "These experiments demonstrate that IFITM3 is able to inhibit HA mediated membrane fusion when localized to the target membrane" over-interprets the results.

4) Something is missing in a sentence "In the case of EBOV infection, cells were for 48 h to allow"

5) The paragraph "We propose that the membrane curvature caused by bilayer asymmetry introduced by insertion of the IFITM3 amphipathic helix into the cytoplasmic face of the endosome membrane may counteract or stabilize regions of membrane curvature induced by HA during its conformational change (Figure 7). Overall, our results support a new amphipathic model for IFITM3 activity." In the end of the Results does noes not belong there and would be more appropriate in the Discussion.

1st Revision - authors' response (in italics) 12 June 2017

We are grateful for the positive reviews and helpful critiques of our manuscript. We have now revised the figures and text to address all of the comments from the reviewers. Major changes include the use of a quantitative luciferase-based readout for cell-cell fusion as requested by all three reviewers, and the revision of several figures so that they now show averages of multiple experiments rather than showing a representative experiment. We also added additional IFITM3 mutants that support our conclusion that IFITM3 possesses an amphipathic helix necessary for its antiviral activity. Further, we have made the requested modifications to the text, including changing the manuscript title and better describing our hypothetical model for IFITM3 activity. We look forward to your publication decision.

Referee #1:

This study examines the mechanism of antiviral activity of interferon-inducible transmembrane proteins (IFITMs) and presents compelling evidence that a conserved amphipathic helix within the first hydrophobic (HD1) region of IFITM3 is essential for its function. Several experimental

approaches employed in this study support the role of a small amphipathic helix for the ability of both IFITM3 and IFITM1 proteins to antagonize virus entry. Although the mechanistic details remain unclear, this is a novel and significant finding that implicates membrane curvature changes in antiviral activity of IFITMs. The results of this study should be of interest to the virology community.

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The paper is well-written, the experiments are clearly described and, for the most part, adequately controlled. The experiments support the main conclusions of this study. Aside from a few experimental concerns discussed below, the main caveat is the undeveloped/unclear model for IFITM-mediated inhibition of viral fusion. The proposed "amphipathicity-based model" is rather vague. It falls short of offering a molecular mechanism for antiviral activity. What is the exact effect of the IFITM3's helix on membrane curvature? What drives the alleged accumulation of IFITM3 at the fusion site? Based on the amphipathic nature of this helix and the proposed shallow insertion into membranes (wedging mechanism, Fig. 7), this region would likely induce positive curvature in the cytoplasmic face of a membrane, which should promote, not inhibit viral fusion (e.g. [2, 3]). It would help the reader to more clearly describe the model and its assumptions, trying to reconcile this with the published work on membrane fusion and the effects of amphipathic peptides. Whereas amphipathic peptides are known to disrupt lipid bilayers, this reviewer is not aware of instances when such peptides/proteins inhibit membrane fusion.

We thank the reviewer for the positive critique of our manuscript, and for posing important questions regarding the mechanistic model for IFITM3 that we propose. First, we will note that we have changed the title of the manuscript to more precisely describe its contents ñ ìIFITM3 requires an amphipathic helix for antiviral activity.î We also acknowledge that our proposed mechanistic model is hypothetical and should be more clearly discussed. Our model is built on the work of dozens of other laboratories that have shown that amphipathic helices invariably induce membrane curvature by wedging into only one layer of the lipid bilayer. Amphipathic helices are commonly found in fusogenic ARF, SNAP and SNARE proteins that mediate vesicular membrane fusion in the secretory system and in neuronal synapses. IFITM3 would be expected to induce local curvature through insertion of its amphipathic helix into the cytoplasmic leaflet of the endosome membrane bilayer. Endocytosed viruses, such as influenza virus, induce opposing curvature of the endosome membrane from inside the endosome (pinching the membrane toward the virus). Thus, in our model, IFITM3 induces curvature on one side of the endosome membrane, while the virus is attempting to induce opposing curvature from the other side of the membrane. We have revised the Discussion section in order to increase clarity regarding this proposed mechanism.

Other essential points:

1. Based upon the inhibition of influenza fusion by the IFITM3 mutant lacking the transmembrane HD2 domain (IFITM3- 88-133), it is concluded that HD2 is dispensable for antiviral activity. However, another study has shown the lack of activity for the IFITM3 chimera in which the HD2 domain has been substituted with that of CD4 or TrfR1 [1]. Can the authors reconcile these discrepant findings? Given the controversial nature of the result suggesting that the IFITM3- 88-133 mutant maintains antiviral activity, in spite of its lower expression, additional controls are warranted. For instance, it would be helpful to show that expression of this mutant does not raise the endosomal pH or interfere with virus endocytosis or trafficking.

In response to this comment and comment 1 from Reviewer 3, we removed these data from the

manuscript. Although we are fully confident in the validity of these experiments, we are in agreement with Reviewer 3 that this is not the primary focus of this paper.

2. The structural model of IFITM3 proposed based upon secondary structure prediction is quite similar to the recent NMR structure published by Ling and co-authors [4]. The distinction between the intramembrane model of HD1, presumably proposed in [4], and the structural predictions made in the current manuscript is unclear. The NMR structure does not seem to support an intramembrane loop topology (i.e., loop that inserts deep into the lipid bilayer) for HD1. As expected for amphipathic helices, HD1 is envisioned to shallowly insert into the lipid membrane, at the level of phospholipid head groups. This is unlike the cartoon depiction of the alternative model in Fig. 2B. It thus seems unfair to refer to the published structural results as confirmatory to the secondary structure predictions made in the current study. I'd suggest acknowledging the published results upfront and shortening the section of Results describing the structural predictions.

We have revised the text according to the suggestion of the reviewer.

3. It appears strange to co-express IFITM3 and HA in the same cell for cell-cell fusion experiments. This does not reflect the actual topology of HA and IFITM3, which are normally in different membranes.

As requested here and by the other reviewers, we have replaced this data using an improved cell-cell fusion assay (provided to us by Dr. Mark Peeples of Nationwide Childrenís Hospital). In this assay we express influenza HA in one set of cells and mix those cells with target cells expressing or lacking IFITM3. Cell-cell fusion is compared via measurement of luciferase that is produced only upon fusion of the two cell populations. This affords us a quantitative readout without potential biases of manual counting as requested by Reviewers 2 and 3.

4. Figure 6: Needs an additional control of HA not exposed to acidic pH (say at time = 0, before the virus is exposed to acidic pH) to show selectivity of Y8-10C2 antibody binding.

We performed the suggested control and now include these data as Figure EV5. We observed no staining with the Y8-10C2 antibody at time 0 after virus binding to cells, though we observed staining at 1 h post transitioning to warm media to allow virus entry. The main text Figure has also been moved to the Figure EV5B due to the main text figure limitations of the journal and since these data are not essential to the primary conclusion of the manuscript.

5. Most figures show the results of "representative of {greater than or equal to}3 independent experiments each performed in triplicate". A better practice is to average all experiments.

Any of our main text figures that did not previously show averages of at least three experiments have been replaced with averages of at least three identical experiments. Updated figures include Fig 2A, 3D, and 4A. Figure 5 was also replaced completely and is an average of four experiments. This is also true for all Extended View figures with the exception of microscopy experiments, which show average quantifications of numerous cell fields derived from two independent experiments (Fig EV3), and antibody staining experiments where mean fluorescence intensity magnitudes differed between experiments (though patterns of staining were consistent) and we chose to show a representative experiment (Fig EV5).

Minor points:

1. Where did VSV and VSV-GFP come from?

VSV-GFP used in our manuscript and was provided to us by Dr. Dominique Garcin (University of Geneva). We have corrected this oversight, and have added this information the Methods and Acknowledgements section.

2. Colocalization of IAV with IFITM3 (Supp. Fig. 3), is not convincing and does not really carry much information, since IFITM3 covers large perinuclear areas where the virus particles accumulate by default.

We have removed this Supplementary Figure.

3. Page 14, middle: "HA was most prevalent in intracellular compartments..." should probably read "IFITM3 was most prevalent...".

Given the new fusion assay that we have utilized, this section has been rewritten entirely.

4. Why not use * and ** etc. to differentiate between p values?

We have modified the figures as suggested.

References:

1 Amini-Bavil-Olyaee, S., Choi, Y. J., Lee, J. H., Shi, M., Huang, I. C., Farzan, M. and Jung, J. U. (2013) The antiviral effector IFITM3 disrupts intracellular cholesterol homeostasis to block viral entry. Cell host & microbe. 13, 452-464
2 Chernomordik, L. V. and Kozlov, M. M. (2003) Protein-lipid interplay in fusion and fission of biological membranes. Annu Rev Biochem. 72, 175-207
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Referee #2:

In this paper, Chesarino et al. perform a structure-function analysis of IFITM3 and IFITM1 and identify an amphipathic helix that is critical for restriction. First, they show that residues 58 to 78 of IFITM3 are conserved in Homo sapiens and Mus musculus. Secondary structure prediction programs indicated that this domain appears to form two distinct alpha helices separated by a short spacer, with residues 59-68 forming an amphipathic helix. This helix is predicted exposed to the cytoplasmic side of the plasma membrane. Mutations that disturb the amphipathic nature of this domain cause loss of restriction of HIV-1, influenza, Zika and Ebola viruses. Notably, these mutations abolish the anti-fusogenic activity of IFITM3 yet do not appear to affect the well known structural changes of influenza HA triggered by low pH, according to its recognition by conformation-specific antibodies. The authors conclude that the amphipathic helix of IFITM3 is important to allow viral-cell membrane fusion and pore formation in the endosome.

This is a nice and focused paper that sheds light on a poorly understood aspect of IFITM3 restriction. The model proposed is plausible and supported by the data. Although in the paper there is no direct confirmation of the amphipathic helix prediction, this prediction is consistent with recent NMR data (Ling et al. 2016) and is supported by the results based on mutagenesis presented in Figure 3.

While most of the experiments are well performed and controlled, Figure 5B shows rather weak data due to the limited number of multinucleated cells that were counted by eye. This experiment should be analysed by flow cytometry to count a larger number of multinucleated syncitia and the statistical analysis re-run with the newly acquired data.

We thank the reviewer for the positive review. As described for Reviewer 1, we have replaced Figure 5 with an improved cell-cell fusion assay. In this assay we express influenza HA in one set of cells and mix those cells with target cells expressing or lacking IFITM3. Cell-cell fusion is compared via measurement of luciferase that is produced only upon fusion of the two cell populations. This affords us a quantitative readout similar to the suggested flow cytometry assay.

Referee #3:

This paper explores mechanisms by which interferon-induced transmembrane (IFITM) proteins

suppress diverse viral infections. The paper presents convincing evidence for the important role of newly identified short amphipathic helix in the protein. This is an important finding in a hot research field. My main concern with this paper and especially with its title is that it overemphasizes the less developed aspect of the study: the specific mechanisms by which the alpha-helix inhibits viral fusion. While the authors do suggest a mechanism, it is not proven and, even more importantly, I do not think this hypothetical mechanism is the take home message of the work. In my opinion, the title like 'Virus restriction by IFITM3 depends on amphipathic helix within IFITM1' would better convey the content of the paper than 'An amphipathicity-based mechanism for virus restriction by IFITM3'.

We have changed the title of the manuscript to "IFITM3 requires an amphipathic helix for antiviral activity."

Specific comments:

1) The results of the experiment with Nile red labeling are not easy to interpret. Nile red is a fairly non-specific probe labeling both lipids (and especially lipid droplets) and proteins. It is unclear to me why cells transiently transfected and expressing IFITM3, in contrast to cells expressing IFITM3-88-133, showed a slight increase in Nile Red fluorescence as compared to control cells (Figure 1D,E). Perhaps comparing this increase with cell labeling with probes either more specific for cholesterol or known to non-specifically label all membranes (for instance, FM dyes) may help in clarifying the interpretation. Moreover I am not sure on what this data adds to the story.

We agree that these data were a side note to the primary focus of the manuscript, and due to the controversial nature of the data as noted by Reviewer 1, we have removed them as suggested.

2) The suggested mechanism in which helix insertion into distal leaflets of fusing membranes inhibits membrane fusion seems to be related to the stalk-pore pathway in which opening of a fusion pore is inhibited by treatments generating positive spontaneous curvature of distal membrane leaflets. Inserted alpha helix of IFITM in Fig. 7 acts as a wedge adding positive spontaneous curvature similarly to a inverted cone shaped lipid such as LPC to the distal leaflet.

We thank the reviewer for bringing experiments using LPC to our attention, as they indeed provide a precedent for alteration of membrane curvature as an effective antiviral strategy. We have added a sentence in the discussion section regarding LPC inhibition of virus fusion.

3) How do you define multinucleated cells in the analysis in Fig. 5? Are these cells with more than one nuclei? Cells with more than 2 nuclei? This experimental approach can be used to verify that IFITM has to be expressed in the target cells rather than in the cells expressing HA. Otherwise the sentence "These experiments demonstrate that IFITM3 is able to inhibit HA mediated membrane fusion when localized to the target membrane" over-interprets the results.

As described for Reviewers 1 and 2, we have replaced Fig 5 with an improved cell-cell fusion assay in which HA and IFITM3 are expressed in distinct cell populations, and which uses a quantitative luciferase readout to report on fusion.

4) Something is missing in a sentence "In the case of EBOV infection, cells were for 48 h to allow"

This is now corrected.

5) The paragraph "We propose that the membrane curvature caused by bilayer asymmetry introduced by insertion of the IFITM3 amphipathic helix into the cytoplasmic face of the endosome membrane may counteract or stabilize regions of membrane curvature induced by HA during its conformational change (Figure 7). Overall, our results support a new amphipathic model for IFITM3 activity." In the end of the Results does noes not belong there and would be more appropriate in the Discussion.

We agree and have deleted this statement from the Results section.

2nd Editorial Decision 05 July 2017

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the report from the referees that were asked to re-evaluate your study (you will find enclosed below). As you will see, all referees support the publication of your manuscript in EMBO reports. Referee #1 has still concerns regarding the proposed model and the related discussion, we would ask you to address in a final revised version of the manuscript.

Further, I have the following editorial requests that need to be addressed:

Please add up to 5 keywords and a running title to the title page.

Please format the references according to EMBO reports style.

Would it be possible to reduce the number of references? We certainly encourage citing primary papers instead of reviews, but 107 citations for a Scientific Report is quite a lot.

We would like to publish the paper as Scientific Report. For a Scientific Report we require that results and discussion are combined in a single chapter called "Results & Discussion". Please do that for your manuscript.

Please remove the bullet points and the summary from the main manuscript text. I have saved this in a separate file and will export it together with the manuscript to our publisher after acceptance.

Please separate the figure legends for main and EV figures. They should be presented as separate paragraphs with own headlines. The figure legend for Figure EV 5 does not mention that there is EV5A and EV5B. Please fix this.

As the Western blot panels show significantly cropped images, we would like to ask you to provide the original source data for these that will then be published together with the paper (with the aim of making primary data more accessible and transparent to the reader). The source data will be published in a separate source data files online along with the accepted manuscript and will be linked to the relevant figure. Please submit the source data (scans of the entire gels or blots) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

Regarding data quantification and statistics, can you please specify, where applicable, the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable.

Please add scale bars to all microscopic images.

Please also provide a higher resolution image for Figure 2B.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

The revised manuscript by Chesarino and co-authors addressed most of my concerns and is much improved. However, it is still difficult to agree with the proposed model for inhibition of virus fusion by IFITM3 (Fig. 5D). As detailed below, the envisioned effect of IFITM3-induced membrane curvature on membrane fusion is opposite to that reported in many experimental and theoretical

articles. There is a considerable body of work on this subject that is not cited or properly discussed in this manuscript.

According to the stalk-pore model (mentioned by the Reviewer 2 and embraced by the authors), LPC in the contacting/cis leaflets of two membranes inhibits, while oleic acid (a negative curvature lipid) promotes, membrane fusion in diverse experimental systems (for review, see Chernomordik and Kozlov, Annu. Rev. Biochem. 2003). By contrast, increased positive curvature of the distal/trans leaflets, should promote conversion from hemifusion (expanded stalk) to full fusion. In keeping with this nomenclature, positive LPC-like curvature imposed on the cytoplasmic/trans monolayers by IFITM3 is expected to promote virus-endosome fusion, but inhibit SNARE-mediated endosome-endosome fusion - a prediction that is the exact opposite to the experimental evidence reported for these systems, including the results of this study.

Please note that the cited articles are not the original ones that introduced and validated the stalk-pore model. Moreover, the Tamm et al. review referenced in this manuscript incorrectly presented the basic results supporting the stalk-pore model. It is essential to check the original papers that have introduced the stalk-pore model, primarily by M. Kozlov and L. Chernomordik, in order to put the proposed model of IFITM3-mediated restriction of viral fusion in a proper context. If the author's predictions disagree with the stalk-pore model, this should be clearly stated.

Minor points:

The authors demonstrated that IFITM3 expression does not prevent acid-induced conformational changes in HA (Fig. EV5). The demonstration that the functionally inactive IFITM3 mutants still allow conformational changes in HA to occur would add to the value of this work.

Please check the formula for calcium phosphate on page 21.

Referee #2:

The authors have addressed my concern. The new luciferase assay to detect membrane fusion is convincing.

Referee #3:

The Revision has addressed my comments.

2nd Revision - authors' response 18 July 2017

We are pleased to see that all three reviewers of our manuscript are supportive of its publication. We have addressed the concern of reviewer 1 by rewriting the last paragraph of the discussion and devising a new speculative model for IFITM3 activity based on the rich literature on membrane fusion and the insightful comments from the reviewer. In this new paragraph we summarize the stalk-pore model of membrane fusion and, as requested, describe the known effects of the lipid LPC on the hemifusion intermediate of membrane fusion. The reviewer implied that it is perhaps surprising that LPC and IFITM3 have opposite effects on virus fusion when localized to the distal leaflets of the host membrane since they are both predicted to induce similar membrane curvature (LPC promotes opening of the fusion pore while IFITM3 inhibits this). We note in our discussion that while LPC would be free to diffuse into the hemifusion diaphragm, IFITM3 possesses a transmembrane domain that may exclude it from this intermediate structure. Indeed, our new model places IFITM3 at the vertex of the hemifused membranes where its amphipathic helix may stabilize membrane curvature in this region, potentially preventing expansion of the hemifusion diaphragm and transition to a fusion pore. We have replaced Figure 5D accordingly with this new model.

Below I also provide a point-by-point response to each of your editorial concerns, which we have fully addressed.

I look forward to the publication of our manuscript in EMBO Reports.

Please add up to 5 keywords and a running title to the title page.
--Added.

Please format the references according to EMBO reports style.
--Reformatted using Endnote.

Would it be possible to reduce the number of references? We certainly encourage citing primary papers instead of reviews, but 107 citations for a Scientific Report is quite a lot.
--We eliminated multiple references as requested. However, we also added several references in responding to Reviewer 1ís suggestions for the discussion. We are now at 85 references.

We would like to publish the paper as Scientific Report. For a Scientific Report we require that results and discussion are combined in a single chapter called "Results & Discussion". Please do that for your manuscript.
--Done.

Please remove the bullet points and the summary from the main manuscript text. I have saved this in a separate file and will export it together with the manuscript to our publisher after acceptance.
--Done.

Please separate the figure legends for main and EV figures. They should be presented as separate paragraphs with own headlines. The figure legend for Figure EV 5 does not mention that there is EV5A and EV5B. Please fix this.
--Legend for EV5 is fixed.

As the Western blot panels show significantly cropped images, we would like to ask you to provide the original source data for these that will then be published together with the paper (with the aim of making primary data more accessible and transparent to the reader). The source data will be published in a separate source data files online along with the accepted manuscript and will be linked to the relevant figure. Please submit the source data (scans of the entire gels or blots) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.
--Source data provided.

Regarding data quantification and statistics, can you please specify, where applicable, the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable.
--Done.

Please add scale bars to all microscopic images.
--Done.

Please also provide a higher resolution image for Figure 2B.
--Done.

3rd Editorial Decision 25 July 2017

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Jacob S. Yount
EMBO Reports
2017-44100V1

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- 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.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs 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 and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship 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;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com	Antibodypedia
http://1degreebio.org	1DegreeBio
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo	ARRIVE Guidelines
http://grants.nih.gov/grants/olaw/olaw.htm	NIH Guidelines in animal use
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm	MRC Guidelines on animal use
http://ClinicalTrials.gov	Clinical Trial registration
http://www.consort-statement.org	CONSORT Flow Diagram
http://www.consort-statement.org/checklists/view/32-consort/66-title	CONSORT Check List
http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tum	REMARK Reporting Guidelines (marker prognostic studies)
http://datadryad.org	Dryad
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http://biomodels.net/	Biomodels Database
http://biomodels.net/miriam/	MIRIAM Guidelines
http://jji.biochem.sun.ac.za	JWS Online
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html	Biosecurity Documents from NIH
http://www.selectagents.gov/	List of Select Agents

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	n/a
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	n/a
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	n/a
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	n/a
For animal studies, include a statement about randomization even if no randomization was used.	n/a
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	n/a
4.b. For animal studies, include a statement about blinding even if no blinding was done	n/a
5. For every figure, are statistical tests justified as appropriate?	yes, the only statistical test that we used was unpaired Student's t-test to compare average infection percentages of cells that underwent different treatments.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Kolmogorov–Smirnov testing of our datasets showed that they are consistent with a normal distribution.
Is there an estimate of variation within each group of data?	Standard deviation as indicated by error bars is shown in each figure.
Is the variance similar between the groups that are being statistically compared?	Yes, the F statistic was calculated for our comparison groups.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Examples shown on pages 20 and 22.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	We used a limited number of cell lines in this study (HEK293T and HeLa). Each of these was purchased from the ATCC four years ago when I started my laboratory. We are still using aliquots of our initial freeze downs and are thus confident in the identity of the cell lines.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	n/a
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	n/a
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	n/a

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	n/a
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	n/a
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	n/a
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	n/a
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	n/a
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	n/a
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	n/a

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	n/a
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	n/a
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	n/a
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	n/a

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	n/a