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Architecture of a nascent viral fusion pore

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(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 21 September 2009

Dear Dr. Lee,

Thank you for submitting your manuscript for consideration by The EMBO Journal. I am very sorry for the undue amount of time it has taken to externally evaluate it; however, we experienced significant initial difficulties in assigning a sufficient number of well-suited referees during the summer vacation period. At this stage, we have now finally received the comments and recommendations of three experts, which you will find copied below. These referees all commend on the images you have been able to obtain and thus consider your results and their implications potentially an important advance; nevertheless they also all raise a number of related issues that would need to be adequately addressed before publication may be warranted. This concerns mainly technical issues, descriptions and controls on the one hand, and interpretation and discussions of the images and results on the other hand.

Should you be able to adequately address these major points, we should be happy to consider a revised manuscript for publication. I would therefore like to invite you to prepare such a revision in the spirit of the reviewers' comments and suggestions. Please be however reminded that it is EMBO Journal policy to allow a single round of major revision only, and that it is therefore essential that you diligently answer to all the points raised at this stage if you wish the manuscript ultimately to be accepted. In any case, please do not hesitate to get back to us should you need feedback on any issue regarding your revision.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

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Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

Lee has collected tomograms of vitrified mixtures of liposomes and influenza virus particles, and describes observed fusion intermediates. In the abstract three observations are highlighted: 1. remodeling takes place in a target-membrane centric fashion. 2. three intermediates can be seen: punctate dimples, an open mouthed liposomal funnel impinging on an unperturbed viral envelope, and a constrained fusion pore. 3. the M1 matrix layer serves as a mesoskeleton for the virus and a foundation for HA during fusion.

The subject of the manuscript is certainly interesting, and the conclusions are potentially exciting. The pictures are striking, and are showing important events. Unfortunately, in the manuscript's current form, I am not convinced that the conclusions are well supported by the data. The most important issues are:

1. I find no discussion of the number of samples/events. Has the author done one fusion experiment or many? How many fusion intermediates has the author observed, 6, 60? How many of each class? Are the tomograms we see representative of many observations, or are they rare events? What fraction of the observed viruses/liposomes are fusing?
2. The results of controls are not clear: What do the liposomes look like after 15 minutes acidification in the absence of virus? What does a mix of liposomes and virus look like 15 minutes after mixing with no pH drop? Some simple statement about controls is needed.
3. In many cases, the features described in the images are difficult to see. I cannot, for example, see any convincing Y shaped features as described in the legend to figure 3; the text suggests that a comparison of Figures 2B,D and 3A makes evident that in some cases the funnel mouth contacts the envelope, while in others a gap is present, but by looking at the figures, I cannot distinguish which panels should represent which conformation. Particular care needs to be taken over interpreting the presence or absence of connections between different features on the 5nm scale in this kind of data. The features they are describing are at the border of what can be interpreted directly from the electron cryo-tomograms, especially considering the noise, the missing wedge, and the defocus.
4. The images are, in my opinion, currently over-interpreted, and there is too much speculation. For example, the coloring and interpretation of the volume rendering in 2C looks close to the level of the noise, but from this image the author counts the number of fingers of density, measures their lengths, and speculates that they may represent HA that has sprung to its extended coiled coil form. The author could equally argue from this figure that the liposome is full of holes. Similarly the black arrow in Figure 5 should show us that the HA particles have migrated up the neck, and suggest to us that HA is still bound to the matrix layer. However, it is not clear to me that the marked density corresponds to migrated HA, rather than density from the other virus which appears in subsequent slices. The authors speculate that the pore is constricted by matrix, but could this not also be membrane? There are other examples of this.

Some other comments:
1. What does a post fusion stage look like? The author suggests that as pH is lowered towards 5, the matrix layer would disintegrate and relax, allowing fusion to go to completion. Has the author tried this? Also along these lines: the author describes fusion intermediates between liposomes and virus lacking matrix. These should then be able to relax and go to completion. Can the final product of this reaction be seen?
2. The authors seem to propose that the small internal vesicles on the interior of liposomes are a by product of a fusion. If this is the case, do they expect them to appear in liposomes which are not in contact with virus? Do they appear?
3. The method simply says that samples were frozen at "various time points following acidification". What time points are represented by the tomograms presented in the paper?

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4. The author has a dye in the liposomes, presumably to follow fusion, but presents no results from this. What lipid mixing is seen under the conditions described in the paper?
5. The use of two different grayscale conventions is unnecessary. If the authors consider white density to be better for clarity, then they should show all figures this way round. Otherwise all figures should be shown with black density.
6. I would recommend that the author separates results from discussion, in order to more clearly separate data from speculation.

Referee #2 (Remarks to the Author):

This manuscript describes a cryo-electron tomographic analysis of the interactions of influenza virus particles with artificial liposomes made of di-oleoyl-phosphatidyl choline (DOPC), a type of lipid that is frequently found in natural membranes. The authors demonstrate a striking deformation of the liposomes induced by the contact with the virus particles, after lowering the pH to 5.5. This effect is consistent with the pH sensitivity of the influenza virus hemagglutinin (HA), which undergoes a major conformational change when exposed to low pH. The conformational transition causes the transient exposure of the "fusion peptides", which insert in the target membrane, before acquire the well known "hairpin conformation" that has both fusion loops and viral transmembrane segments juxtaposed at the same side of the molecule. Importantly, the images show that there is an apparent disruption of the liposome membrane at the point of contact, which is likely to be surrounded by a number of HA molecules, perhaps in the pre-hairpin intermediate conformation, with the fusion loops inserted in the liposome. These are striking images, which have not been observed previously, and are therefore extremely valuable to get a better picture of the membrane fusion process.
This work also highlights the role of the matrix protein layer that underlies the viral membrane, in preserving the particle shape and concentrating the deformation on the target membrane. This matrix protein layer appears to make an ultimate barrier to complete fusion, which apparently requires dissociation of the matrix layer to proceed, which the authors propose is an important feature and requires lowering the pH to about 5.

Altogether, this work provides valuable new data. I strongly support its publication in the EMBO Journal. I have a few issues, however, which the author should address in a revised version.

1. The liposomes. As the author states, DOPC is a curvature-neutral lipid, and liposomes composed exclusively of DOPC usually are quite large, having a tendency to make planar lipid bilayers. However, the liposomes imaged in contact with the virus particles are all very small, often smaller than the virions. Such small liposomes have a strong curvature, certainly imposing a lot of stress on the liposomes to begin with. There is no explanation in the Materials and Methods about the way in which the liposomes were prepared.
2. The effect on the liposome is likely to be the result of insertion of the fusion loops from a number of HA protomers into the target membrane. Because the insertion is only in the outer leaflet of the bilayer, this can result in a more pronounced curvature at the site of contact, which would make a sort of dimple, as observed. My question is, concerning the breks (or holes): has the author taken into account the possibility that there is still continuity in the inner leaflet of the liposome in those regions? Can the density become diffuse enough when many fusion loops accumulate in that region of the liposome, such that the contrast is affected, resulting in the impression of a break? This is just a point for the discussion, the images are quite clear, but it would be important to state that there is no other interpretation possible.
3. DOPC liposomes are known for not yielding complete fusion. Has the author monitor fusion by some other means, to see if this system yields full fusion if brought to pH 5, for instance? I understand that it is a very nice way to trap an intermediate state of the process to be able to visualize it, but the questions that comes next is: is this an intermediate in the pathway to full fusion, or is it a dead end.
4. The colored representation given in Figure 3C is confusing. The liposome is expected to have a continuous density. I this Figure, it is difficult to tell what are the proteins, what are membranes.
5. In Fig 5, the author claims that there are HA molecules invading the liposome up to the level of the "neck". Since there are virus particles also underneath, cand those spots belong to this other particles? It is not clear to me from the picture.

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Referee #3 (Remarks to the Author):

The author used cryo electron tomography to analyze the fusion process of Influenza virus particles with the endosome membrane. As model for that process a liposome-based fusion assay was employed. The chosen elegant investigation system offers thin specimens as required for cryo electron tomography of reasonably good resolution and conditions suitable to catch fusion intermediates as achieved by lowering the pH to 5.5. The presence of the long distinctly-shaped HA-spikes on the virus membrane make the virus model very attractive for studying virus-host fusion by cryo electron tomography.

The presented tomograms are impressive and reveal a number of states in the fusion process. Such detail in resolution and intermediates has not been achieved before. However, I cannot agree with some of the interpretations of the data and conclusion drawn. In particular, one should be very cautious in electron tomography when interpreting density continuity. The absence of continuity might be caused by the imaging conditions. The effect of the contrast transfer function (CTF) might cause bright fringes around objects and can such impair neighboring densities. Given the defocus values used (3-5 micrometers at 120KeV accelerating voltage) this will have an effect. The effect is visible e.g. as white fringe inside and around the liposome membranes and around the HA-spikes in the lower part of the slices in Fig. 2A. Subsequently, it cannot be excluded that the appearance of the liposome membrane as an open funnel that is not connected to the viral membrane in Fig. 2A, slice 1 and 2B might as well be caused by the fringe of the HA-spikes and the viral membrane respectively. This has major consequences in terms of the interpretation of the data in the suggested model.
The steps up to and including the dimpling are well justified and the unilateral dimpling of the liposome membrane caused by the higher rigidity of the viral membrane being reinforced by the underlying matrix layer are well supported by the data and discussion.
The following 'scission' step is an interpretation and the CTF effects might have caused this appearance as outlined above. Even though a similar model is discussed in the literature before, I do not see sufficient evidence for this step presented here. Optimizing the imaging conditions (working closer to focus, using a smaller pixel size to resolve the two bilayers of the liposome membrane unequivocally) might improve the situation and will allow analyzing this aspect of the process in the future. Furthermore, CTF correction as recently introduced (e.g. Briggs et al., 2009, PNAS; Zanetti, et al., 2009, JSB) would allow getting a clearer picture of the events and membrane continuities. The events described as scission and apposition might in fact all present hemifusion states.
The final steps involving pore expansion enabled by disassembly of the matrix layer triggered by the further shift in pH to pH 5 seems likely.
If the author encountered direct evidence for post fusion states at pH 5 when setting up the experimental system and optimizing the pH conditions for finding intermediates, this might support that latter step and should be mentioned.

Nevertheless, when taking out the disputable scission and apposition events the resulting refined model still presents a substantial step forward in understanding the mechanism of HA-mediated fusion.

1st Revision - authors' response 10 December 2009

Referee #1 (Remarks to the Author):

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1. I find no discussion of the number of samples/events. Has the author done one fusion experiment or many? How many fusion intermediates has the author observed, 6, 60? How many of each class? Are the tomograms we see representative of many observations, or are they rare events? What fraction of the observed viruses/liposomes are fusing?

The experiments have been repeated in several independent sessions using freshly prepared materials and grids each time, and the types of complexes observed are consistent from one session to the next. In the study, a total of 53 complexes between virus and liposome were analyzed; the breakdown of the population is included in new text on page 10 (revised version). The majority of viral particles (>100) were not in fact involved in complexes with liposome, this was a result of the experimental conditions that were selected in an attempt to disfavor liposome disruption by multiple viral particles and make virion:liposome pairings more 1:1.

2. The results of controls are not clear: What do the liposomes look like after 15 minutes acidification in the absence of virus? What does a mix of liposomes and virus look like 15 minutes after mixing with no pH drop? Some simple statement about controls is needed.

Dynamic light scattering and fluorescence spectroscopy have also been performed to further characterize liposomes and their stability under acidic conditions. In summary, the liposomes are stable at acidic pH and show no significant change in dimension by DLS and no significant leakage of liposome-encapsulated dye by fluorescence. Additional text and figures regarding controls have been added. A low-dose image of the liposome starting material in the absence of virus has been to the supplementary materials. Lastly, a tomogram of a virus-liposome pair that had been acidified for only 2.5min is now included in Fig 3B.

3. In many cases, the features described in the images are difficult to see. I cannot, for example, see any convincing Y shaped features as described in the legend to figure 3; the text suggests that a comparison of Figures 2B,D and 3A makes evident that in some cases the funnel mouth contacts the envelope, while in others a gap is present, but by looking at the figures, I cannot distinguish which panels should represent which conformation. Particular care needs to be taken over interpreting the presence or absence of connections between different features on the 5nm scale in this kind of data. The features they are describing are at the border of what can be interpreted directly from the electron cryo-tomograms, especially considering the noise, the missing wedge, and the defocus.

The speculative text that suggested the liposomal funnels shown in the old versionís Fig 2B,D, and 3A (revised version Fig 5B, 6A) may be making contact with the viral envelope in one case and separate in another has been removed. I agree it was confusing, especially without the slices numbered. In the revised serial section figures, each slice is numbered in sequence for easier reference. The overall question of whether a gap is present or not is relates to the possibility that CTF artifacts may lead to the appearance of a gap where none necessarily exists or where there is significantly weaker density. This issue is covered now on Page 12 of the revised version.

As for the figures involving the "Y-shaped" features, the image grey-scale for "figure 3" (now Figure 6 in the new version) has been un-inverted (now black/grey density on white background), possibly more suitable for print (the fine features tended to be over-saturated in the inverted image). A schematic has been added to clarify the density and its interpretation. Another case of a Y-shaped feature is also shown in figure 4D (new version numbering). The images shown in this report are comparable to and possibly clearer than similar features shown in an ECT study of herpes simplex virus-1 entry (Fig. 5 in Maurer et al., PNAS v105:10559, 2008), in which fine density features are interpreted as fusion glycoproteins. The figure from Maurer et al, 2008 is provided below (see panels B, E, F, G):

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4. The images are, in my opinion, currently over-interpreted, and there is too much speculation. For example, the coloring and interpretation of the volume rendering in 2C looks close to the level of the noise, but from this image the author counts the number of fingers of density, measures their lengths, and speculates that they may represent HA that has sprung to its extended coiled coil form. The author could equally argue from this figure that the liposome is full of holes. Similarly the black arrow in Figure 5 should show us that the HA particles have migrated up the neck, and suggest to us that HA is still bound to the matrix layer. However, it is not clear to me that the marked density corresponds to migrated HA, rather than density from the other virus which appears in subsequent slices. The authors speculate that the pore is constricted by matrix, but could this not also be membrane? There are other examples of this.

The 3-dimensional rendered density in Figure 2C of the old manuscript version has been removed. And the discussion about the HA spikes in Figure 5 has been removed as well. Speculation has been trimmed from the Results text, for example, descriptions of HA lengths and their relation to conformational states has been excised. The highly contoured density was intended to highlight the most robust density, and weaker density such as part of the liposomal "skin" invariably opened up. However this may have introduced more confusion than clarity. The author has shied away from using the common-practice alternative of manual density segmentation due to the subjectivity of the density selection. Instead, where HA spike density is described in the revised manuscript, reference is made to the original tomographic slices.

Some other comments:
1. What does a post fusion stage look like? The author suggests that as pH is lowered towards 5, the matrix layer would disintegrate and relax, allowing fusion to go to completion. Has the author tried this? Also along these lines: the author describes fusion intermediates between liposomes and virus lacking matrix. These should then be able to relax and go to completion. Can the final product of this reaction be seen?

A putative post-fusion complex example has been added to the supplementary materials as Figure S3. They are however rare at the pH 5.5 conditions, which is consistent with the low efficiency of lipid mixing at this elevated pH value (see fluorescence data in Fig. 2 in new version for example). It is not possible at this stage to determine whether the post-fusion complexes that are observed are due to the fusion of matrix-bearing or matrixless particles, due to the noisiness and jumbled density of the observed structures. One type of study that will be pursued in the future is to isolate the matrix-bearing population from the matrixless population (by CsCl density sedimentation perhaps; Fujiyoshi et al. (EMBOJ, (1994) vol 13:318) also proposed that sephacryl-1000 gel filtration could be used to separate various populations of influenza particles), and repeat fusion assays and ECT with those distinct populations.

Efforts have been made to image virus-liposome complexes at pH 5.0, however the sample tends to form large aggregates that are not amenable to ECT (e.g. too thick and it has not been possible to get vitreous ice suitable for ECT). An example low-dose image of X31 virions alone at pH 5.0 has been added to the new version of the paper; the ice conditions are still challenging, however at low-tilts sufficient contrast is available to see some large-scale features. At pH 5.0, it appears that lipid can be stripped from virus particles, and form vesicles. Some of the remaining virions are observed fusing with the vesicles. Fusion in this case appears less constrained and in the image shown for example, two virions are seen "swallowing" a vesicle. The viral envelope also appears to be reverting to a bilayer, however better data is needed to characterize the envelope's structure. The new text is found on page 14 of the revised version.

2. The authors seem to propose that the small internal vesicles on the interior of liposomes are a by product of a fusion. If this is the case, do they expect them to appear in liposomes which are not in contact with virus? Do they appear?

A representative tomogram of an early time point (2.5 min acid) in which a virus and liposome are proximal but not initiating fusion has been added as Fig 3B in the revised version; such colocalized virus-liposome do not exhibit the same abundance of internal vesicles. An image of liposomes alone control has been to the Supplementary Materials Fig. S4. Internal vesicles are observed in only ~15% of the liposomes that have not been exposed to virus. By contrast nearly ever liposome in a

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complex with virus at >5 minute exhibits one or more internal vesicles.

3. The method simply says that samples were frozen at "various time points following acidification". What time points are represented by the tomograms presented in the paper?

Time point annotations (2.5, 5, 6.25, 8, 15 min post-acidification) have been added. Most of the data collection focused on the intermediate range of time-points (5 and 8 min post-acidification) where the fluorescence data suggested interesting changes were taking place.

4. The author has a dye in the liposomes, presumably to follow fusion, but presents no results from this. What lipid mixing is seen under the conditions described in the paper?

This data has been added, and is shown in Figure 2 and Supplementary Figure S1. The data confirm that DOPC liposomes and viruses are capable of merging their membranes, and lipid mixing takes place at pH 5.5, but it is more efficient at pH 5.0. Under both pH conditions, the HA-mediated process is "leaky" and liposome-encapsulated dye leaks out with faster kinetics than lipid mixing. The fluorescence data are consistent with a prefusion target membrane scission model suggested by the electron cryo-tomography data.

5. The use of two different grayscale conventions is unnecessary. If the authors consider white density to be better for clarity, then they should show all figures this way round. Otherwise all figures should be shown with black density.

All images are now shown in the standard black density on white background.

6. I would recommend that the author separates results from discussion, in order to more clearly separate data from speculation.

Results and Discussion have been separated in the new version. I thank the reviewer for this excellent suggestion. It has helped to clarify the paper's findings as well as to better place the current results into context with what is known in the field.

Referee #2 (Remarks to the Author):

This manuscript describes a cryo-electron tomographic analysis of the interactions of influenza virus particles with artificial liposomes made of di-oleoyl-phosphatidyl choline (DOPC), a type of lipid that is frequently found in natural membranes. The authors demonstrate a striking deformation of the liposomes induced by the contact with the virus particles, after lowering the pH to 5.5. This effect is consistent with the pH sensitivity of the influenza virus hemagglutinin (HA), which undergoes a major conformational change when exposed to low pH. The conformational transition causes the transient exposure of the "fusion peptides", which insert in the target membrane, before acquire the well known "hairpin conformation" that has both fusion loops and viral transmembrane segments juxtaposed at the same side of the molecule. Importantly, the images show that there is an apparent disruption of the liposome membrane at the point of contact, which is likely to be surrounded by a number of HA molecules, perhaps in the pre-hairpin intermediate conformation, with the fusion loops inserted in the liposome. These are striking images, which have not been observed previously, and are therefore extremely valuable to get a better picture of the membrane fusion process.
This work also highlights the role of the matrix protein layer that underlies the viral membrane, in preserving the particle shape and concentrating the deformation on the target membrane. This matrix protein layer appears to make an ultimate barrier to complete fusion, which apparently requires dissociation of the matrix layer to proceed, which the authors propose is an important feature and requires lowering the pH to about 5.

Altogether, this work provides valuable new data. I strongly support its publication in the EMBO Journal. I have a few issues, however, which the author should address in a revised version.

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bilayers. However, the liposomes imaged in contact with the virus particles are all very small, often smaller than the virions. Such small liposomes have a strong curvature, certainly imposing a lot of stress on the liposomes to begin with. There is no explanation in the Materials and Methods about the way in which the liposomes were prepared.

A more complete description of liposome preparation has been added to the Materials and Methods section. 100 nm liposomes were selected for the study because larger sizes (e.g. 200 or 400 nm diameter) would likely be as thick as or thicker than typical ice thicknesses in the cryo-EM samples. One concern in using the larger liposomes is that they would be squashed in the thin ice layer and subject to significant stresses due to that. The text describing liposome preparation that has been added to the Materials and Methods section reads as follows:

"3-5 mg/ml 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC, Avanti Polar Lipids) liposomes containing 25 mM sulforhodamine-B (SRB) fluorophore (Invitrogen Corp.) were produced by drying a 25 mg/ml DOPC chloroform solution under nitrogen gas. The dried lipid was resuspended in 225 mM NaCl, 10 mM HEPES pH 7.5 buffer containing 25 mM SRB dye; the lower salt concentration was used in order to equalize the osmotic balance on the two sides of the membrane with 250 mM NaCl, 10 mM HEPES pH 7.5 storage buffer. The resuspended lipid was subjected to 5 sequential freeze-thaw cycles in liquid nitrogen, followed by 21 extrusions through polycarbonate filters with 100 nm pore size (Avanti Polar Lipids). 100 nm liposomes were selected for the study because larger sizes (e.g. 200 or 400 nm diameter) would likely be as thick as or thicker than typical ice thicknesses in the cryo-EM samples. One concern in using the larger liposomes is that they would be squashed in the thin ice layer and subject to stresses due to the compression. The dye-encapsulating liposomes eluted as a single band from PD-10 gel filtration columns (GE Healthcare) and were stored in the same pH 7.5 HEPES buffer used for virus storage. Dynamic light scattering (DLS) with a Brookhaven 90+ Nanoparticle Size Analyzer was used to characterize the polydispersity and average dimension of the liposomes at room temperature. Liposome diameters from individual preparations ranged from 125-145 nm by DLS, and polydispersity was typically very low, 0.002-0.020. DLS also confirmed that no significant change in liposome dimensions resulted from incubating the liposomes at pH 5.0-5.5 for up to an hour (longest duration tested). Likewise, when liposomes were acidified to pH 5.0 and 5.5, leakage of sulforhodamine-B was not detected by fluorescence monitoring. It was concluded that the liposomes are stable under the acidic pH conditions used in the fluorescence and ECT studies. Liposomes were used within 24 h of their production."

Per reviewer #2ís insights, the following text has been added in the Discussion section (page 17 revised version) in which the choice of DOPC and the relation to the situation in the endosome is discussed:

"Phosphatidylcholine (PC) is the dominant component of the proximal endosomal membrane leaflet. The distal endosomal leaflet is likely to be enriched in phospholipids such as phosphatidylethanolamine and phosphatidylserine that have different curvature and fusion propensities. Understanding the role that intrinsic curvature of these lipids plays in fusion is critical. It is likely for example, that some of the membrane remodeling observed in this study resulted from the curvature-neutral PC bilayer reorganizing under HAís influence to relieve strain present in the curved ~100 nm liposome. Future studies will examine HA-mediated fusion with target membranes that more closely mimic the complex conditions found in endosomes."

2. The effect on the liposome is likely to be the result of insertion of the fusion loops from a number of HA protomers into the target membrane. Because the insertion is only in the outer leaflet of the bilayer, this can result in a more pronounced curvature at the site of contact, which would make a sort of dimple, as observed. My question is, concerning the breks (or holes): has the author taken into account the possibility that there is still continuity in the inner leaflet of the liposome in those regions? Can the density become diffuse enough when many fusion loops accumulate in that region of the liposome, such that the contrast is affected, resulting in the impression of a break? This is just a point for the discussion, the images are quite clear, but it would be important to state that there is no other interpretation possible.

A discussion of the possibility that density may still be in the gaps has been added (page 12, revised version). While it is conceivable that an individual leaflet, such as the inner leaflet persists in the

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gap, and is too weak in density to be imaged by ECT, such a structure with exposed hydrocarbon tails is likely to be highly unstable. A bilayer with still associated leaflets and a hole with a micellar collar and that is lined by fusion peptides seems a more likely structure. However such a structure has not to date been observed, and likely lies beyond the resolution capabilities of the ECT approach.

3. DOPC liposomes are known for not yielding complete fusion. Has the author monitor fusion by some other means, to see if this system yields full fusion if brought to pH 5, for instance? I understand that it is a very nice way to trap an intermediate state of the process to be able to visualize it, but the questions that comes next is: is this an intermediate in the pathway to full fusion, or is it a dead end.

Fluorescence data showing that lipid mixing does take place at pH 5.5 as well as pH 5.0 have been added as Fig 2 and supplementary Fig S1 in the revised version. While lipid mixing takes place at pH 5.5, it is more efficient at pH 5.0. Under both pH conditions, the HA-mediated process is "leaky" and liposome-encapsulated dye leaks out with faster kinetics than lipid mixing. The fluorescence data are consistent with a prefusion target membrane scission model suggested by the electron cryo-tomography data.

4. The colored representation given in Figure 3C is confusing. The liposome is expected to have a continuous density. I this Figure, it is difficult to tell what are the proteins, what are membranes.

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The presented tomograms are impressive and reveal a number of states in the fusion process. Such detail in resolution and intermediates has not been achieved before. However, I cannot agree with some of the interpretations of the data and conclusion drawn. In particular, one should be very cautious in electron tomography when interpreting density continuity. The absence of continuity might be caused by the imaging conditions. The effect of the contrast transfer function (CTF) might cause bright fringes around objects and can such impair neighboring densities. Given the defocus values used (3-5 micrometers at 120KeV accelerating voltage) this will have an effect. The effect is visible e.g. as white fringe inside and around the liposome membranes and around the HA-spikes in the lower part of the slices in Fig. 2A. Subsequently, it cannot be excluded that the appearance of the liposome membrane as an open funnel that is not connected to the viral membrane in Fig. 2A, slice 1 and 2B might as well be caused by the fringe of the HA-spikes and the viral membrane respectively. This has major consequences in terms of the interpretation of the data in the suggested model.

The following 'scission' step is an interpretation and the CTF effects might have caused this appearance as outlined above. Even though a similar model is discussed in the literature before, I do not see sufficient evidence for this step presented here. Optimizing the imaging conditions (working closer to focus, using a smaller pixel size to resolve the two bilayers of the liposome

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membrane unequivocally) might improve the situation and will allow analyzing this aspect of the process in the future. Furthermore, CTF correction as recently introduced (e.g. Briggs et al., 2009, PNAS; Zanetti, et al., 2009, JSB) would allow getting a clearer picture of the events and membrane continuities. The events described as scission and apposition might in fact all present hemifusion states.

The reviewer's point is very well taken. The previous speculative text that suggested the liposomal funnel might be making contact with the viral envelope in one case and separate in another has been removed. I agree that the potential for CTF fringing obscuring fine details is significant. In the revised manuscript, the author has diligently sought to qualify the interpretation with the caveat about CTF effects (see for example pages 12-13, revised version). This new text acknowledges the potential for these artifacts to complicate interpretation of the observed tomographic density. In the revised version, the discussion of scission has been qualified and labeled as putative, subject to the influence of imaging artifacts.

An additional tomogram has also been added because it provides a useful comparison to assess the influence of CTF effects on density continuity when liposome and virus features are very closely apposed. The complex shown in Fig. 7A may correspond to a state slightly further along the fusion pathway than the putative funnel complexes, in which a funnel-like structure appears to be nestled into the viral envelope. While strong Fresnel fringes are observed around the liposomal density, at the base of the remodeled liposomeís neck, fringes do not obscure the intersection of the neck and virus envelope and density appears continuous without prominent gaps. If CTF artifacts due to the strong liposomal bilayer were always responsible for observed gaps between liposome and virus density, one would expect a similar gap to show up in this case. The defocus in Fig 7A was 5 µm, as it was in the case shown in Fig 5B.

Prefusion target membrane permeability has been documented previously in the literature. Those results as well as their own experimental study led Bonnafous and Stegmann to propose their prefusion target membrane disruption model. Thus the precedent for the putative open-mouthed funnel extends well beyond a single hypothetical model on paper. The fluorescence data that is now included in the manuscript provides insight into the question of whether prefusion target membrane scission takes place under conditions similar to those used in the ECT preparations, and whether an open-mouthed funnel is a reasonable, data-supported interpretation of observed density (Fig. 2, revised version). The data indicates that leakage of liposome-encapsulated dye takes place with faster kinetics than lipid mixing at pH 5.5.

Taking the fluorescence and ECT observations together, it seems that the most consistent explanation for the observed density is that the liposome membrane can be compromised well-before significant lipid mixing and hemifusion is attained. The observation of a break in density at its nexus with a virus particle, is consistent with prefusion leakage. In most cases where liposomal funnels are observed, the virus envelope nearby is seen to be unperturbed, implying that the viral membrane leaflets are not participating in the locus. In fact scission appears to take place at stages before apposition: Figure 5A, slice 1 shows a case where liposome membrane has been disrupted at the site of coordination with a cluster of HA spikes. While this breach is somewhat larger (~10 nm wide) than the funnel-shaped structures that are closely apposed to the membrane, they demonstrate that prefusion membrane scission is observed.

Future efforts will seek to improve the acuity of the reconstructed density in order to clarify membrane leaflet continuity by, as Reviewer #3 suggests, working closer to focus, at higher magnifications, and by applying recently developed CTF-correction algorithms for tomography (Fernandez et al, 2006; Xiong et al, 2009; Zanetti et al, 2009). By transitioning to microscopes operating at 200 or 300 kV, which provide better sample penetration, better quality high angle data will also be obtainable, leading to more complete reconstructions.

The final steps involving pore expansion enabled by disassembly of the matrix layer triggered by the further shift in pH to pH 5 seems likely.
If the author encountered direct evidence for post fusion states at pH 5 when setting up the experimental system and optimizing the pH conditions for finding intermediates, this might support that latter step and should be mentioned.

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An effort has been made to perform the parallel experiments at pH 5.0, however due to significant aggregation, conditions suitable for ECT have yet to be determined. Virus particles alone at pH 5.0, while still prone to some aggregation, have been imaged in the cryo-EM, and the observations, while preliminary, are consistent with the proposed matrix dissolution/weakening at the lower pH condition. A representative image is now included as Fig. 7D in the new version.

Nevertheless, when taking out the disputable scission and apposition events the resulting refined model still presents a substantial step forward in understanding the mechanism of HA-mediated fusion.

2nd Editorial Decision 17 January 2010

We have now received the comments of the two reviewers (1 and 3) who have again evaluated your manuscript, which had been revised in response to their original comments. I am happy to inform you that both of them consider the main issues adequately addressed and the manuscript ready for publication pending a few minor modifications of mostly editorial nature. I am thus returning the study to you once more, kindly inviting you to incorporate these additional changes and to return the final version to us at your earliest convenience. After that, we should be able to swiftly proceed with its formal acceptance and production!

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The majority of points raised by the reviewers have been appropriately addressed. Most importantly, the overly speculative discussion in the previous version has been removed. The following minor points should briefly be considered:

The final subheading of the results section "HA bridges the... and coordinates..." is too bold considering the content of the section which simply shows that some density which may be HA can be found around the site.

The working model is not really summarised in the main text. The authors should perhaps also indicate that the main evidence in the paper is for the dimple step, and for some kind of funnel-like stage which may represent any or a mixture of the subsequent 3 steps.

The "time" axis in fig 2 and fig S1 should be labeled, at least in the legend, as "time after ..." is this time after mixing virus and liposomes, or time after pH change?

The legend to S2 should say what filled and open circles signify.

Referee #3 (Remarks to the Author):

The revised manuscript is substantially improved and I enjoyed very much reading it. I believe it represents a very important paper. Congratulations!
Some minor comments:

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1) in the supplemental figure S1 the legend should provide explanation of the symbols (filled vs. open) despite the fact that it is the same as in figure
2) page 5 line 3: remove "hundred"
3) page 9 end of 1st paragraph: refer to suppl. figure?
4) page 9, 2nd but last line: remove "in"
5) page 9, 2nd last line: consider rephrasing 'the most HA on" to the majority of HA"?
6) page 10, 1st line: consider to insert "post onset of acidification" before the comma
7) page 14, end of 2nd paragraph: interpretation of 3): might as well be the situation that a virus blebs of membrane as discussed as source of the lipid vesicles 2)
8) p.15, end of 2nd paragraph: might as well be some more since 3D ...
9) p. 18 5th line before end of 1st paragraph: 3nm resolution not proven, avoid statement if not calculated
10) p. 19 consider to replace mesoskeleton by endoskeleton
11) Fig 3 legend: in A: black arrows (actuall;y arrowheads) referred here are white in my version of the file; consider to replace "defocus" by "microscope defocus".
12) figure 7 legend, one but last sentence: alternative explanation: lipid blebbing from virus ... (cf. 7)

2nd Revision - authors' response 20 January 2010

Referee #1 (Remarks to the Author):

The majority of points raised by the reviewers have been appropriately addressed. Most importantly, the overly speculative discussion in the previous version has been removed. The following minor points should briefly be considered:

The final subheading of the results section "HA bridges the... and coordinates..." is too bold considering the content of the section which simply shows that some density which may be HA can be found around the site.

The subheading has been changed to "HA is localized around viral and target membrane contact sites"

The working model is not really summarised in the main text. The authors should perhaps also indicate that the main evidence in the paper is for the dimple step, and for some kind of funnel-like stage which may represent any or a mixture of the subsequent 3 steps.

A summary of the working model has been transferred from the Figure 9 legend to the main text in the Discussion section, subsection "Implications for cell invasion and fusion mechanisms". And a statement paraphrasing the reviewerís suggested qualifying statement that the paperís main focus involves the dimpling and funnel-like stages has been added immediately following the working model summary. The statement reads "The primary evidence in this report support the dimpling stage as well as the existence of a funnel-shaped intermediate that may reflect a combination of the subsequent three steps (scission, apposition, and possibly hemifusion)."

The "time" axis in fig 2 and fig S1 should be labeled, at least in the legend, as "time after ..." is this time after mixing virus and liposomes, or time after pH change?

The x-axis in Fig. 2 and S1 have been changed to "Time post-acidification (min)"

The legend to S2 should say what filled and open circles signify.

This has now been done.

Referee #3 (Remarks to the Author):

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The revised manuscript is substantially improved and I enjoyed very much reading it. I believe it represents a very important paper. Congratulations!
Some minor comments:
1) in the supplemental figure S1 the legend should provide explanation of the symbols (filled vs. open) despite the fact that it is the same as in figure

The latest draft incorporates this change.

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7) page 14, end of 2nd paragraph: interpretation of 3): might as well be the situation that a virus blebs of membrane as discussed as source of the lipid vesicles 2)

The latest draft incorporates this addition and reads "(alternatively, this may reflect a virion in the process of shedding its membrane)"

8) p.15, end of 2nd paragraph: might as well be some more since 3D ...

The rough estimate of spike numbers is based upon the 3-D tomographic density, although in the beginning of the paragraph it is acknowledged that limitations due to for example the missing wedge artifact make it challenging to unambiguously count specific numbers of spikes. The rough estimate is consistent with previous reports.

9) p. 18 5th line before end of 1st paragraph: 3nm resolution not proven, avoid statement if not calculated

The latest draft incorporates this change, and no longer specifies "3nm".

10) p. 19 consider to replace mesoskeleton by endoskeleton

The latest draft incorporates this change.

11) Fig 3 legend: in A: black arrows (actuall;y arrowheads) referred here are white in my version of the file; consider to replace "defocus" by "microscope defocus".

Throughout the text and figure legends, "defocus" is now correctly identified as "microscope defocus". Also, "arrows" has been replaced with "arrowheads", and the arrowhead is now correctly identified as white.

12) figure 7 legend, one but last sentence: alternative explanation: lipid blebbing from virus ... (cf. 7)

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The latest draft incorporates this addition and reads "an alternative explanation for this feature is that the virions may be caught in the process of shedding or blebbing their membranes"

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