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## Endocytic tubules regulated by Rab GTPases 5 and 11 are used for envelopment of herpes simplex virus

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### Review timeline:

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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

14 March 2012

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Thank you for the submission of your manuscript to The EMBO Journal. We have now received the full set of reports from three referees that were asked to evaluate it, which I copy below. Given their encouraging opinions on your study, I would like to invite you to submit a revised version of your manuscript.

As you will see from their reports, all three referees consider your study interesting and suitable for publication in The EMBO Journal after some modifications are performed. In essence, the main concern posed by the referees relates to the nature/origin of the membranes used during viral envelopment. Referee #1 believes that it is unclear whether they originate directly from tubular invaginations at the plasma membrane or come from recycling endosomes. Along the same lines, referee #2 would like to see the contribution of early/late endosomes assessed and proposes some experiments to address this issue.

I would also like to briefly comment on referee #3 report. While we agree that further insight into the mechanisms of envelopment is the next step and would undoubtedly increase the impact of your study, we believe that further analyses in that direction are out of the scope of this study. Naturally, any addition that would add to our understanding of the interactions between the virus and the endocytic machinery is welcome, but this is not, in our view, essential for the acceptance of your manuscript.

Please be aware that your revised manuscript must address the additional concerns of the referees

and their suggestions should be taken on board. Acceptance of the manuscript will depend on a positive outcome of a second round of review and it is 'The EMBO Journal' policy to allow a single round of revision only. Therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: <http://www.nature.com/emboj/about/process.html>

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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

Yours sincerely,

Editor  
The EMBO Journal

## REFEREE REPORTS

### Referee #1

Hollinshead et al. report a detailed morphological study of Herpes Simplex Virus 1 (HSV) final envelopment in tissue culture cell lines. The cellular sites at which HSV and other herpes viruses undergo the final steps of particle assembly (secondary envelopment) have long been controversial, with the current weight of opinion for HSV favouring the use of membranes associated with the TGN. Using immunofluorescence and electron microscopy, as well as RNA interference, Hollinshead et al. provide compelling evidence that the membranes used for HSV envelopment are derived from the endocytic pathway. Although, in some respects these data disagree with some current views, the data are for the most part compelling and, pending attention to a number of minor points, the paper would be appropriate for publication in the EMBO J.

Specific points.

1. The model proposed by the authors is confused. In parts (e.g. pages 7 and 11), they suggest HSV uses membrane derived from tubules involved in recycling from endosomes (sorting and/or recycling endosomes), but they also suggest (and this is perhaps best illustrated by the cartoon in Fig. 9, but also on pages 6, 12) that the virus can use membranes derived from tubular invaginations of the plasma membrane. Although such tubular invaginations have been described, these are not generally thought to be associated with a clathrin/dynamin/Rab5-dependent endocytic mechanism. Moreover, the analysis using HRP, which shows that the highest proportion of HSV capsids associated with HRP-containing membrane structures is seen after 30 mins HRP uptake, is entirely consistent with the membrane tubules being derived from endosomes. In addition, the fact that dynasore reduced the number of HRP wrapped capsids (page 10) would support this argument. The authors should attempt to provide a clearer explanation of what these tubules are.
2. Although evidence for HSV using endocytic membranes for assembly is novel, there is evidence for the related HCMV using endocytic vesicles and tubules/cisternae (Tooze et al. 1993; Fraile-Ramos et al. 2002). This should be discussed.
3. Some discussion of the literature is in my view incorrect. For example, page 3 - there is little evidence for HIV capsids being targeted to the viral glycoproteins through matrix protein interactions. It is clear that HIV Gag can target the plasma membrane and form VLPs without the HIV Env expression. Is the tegument a 'compartment' (page 3), perhaps complex would be more

appropriate.

4. Page 4, TGN 46 is 'a' major marker, not 'the' major marker.

5. Page 5. The EM imaging of HSV infected cells illustrated in Fig S1 is taken at 12h PI. This is at a time point when virus production is nearing its peak. Are similar morphologies seen at earlier times?

6. The authors should be cautious in their claim that HRP does not traffic to the TGN. As TGN 46 cycles over the cell surface, there must be vesicular trafficking to the TGN and therefore some fluid phase transport. It might be more appropriate to state that HRP trafficking to the TGN is undetectable.

7. Page 7, the polarity of the Golgi cannot be seen in these images, so the claim that TGN 46 remained a marker for the trans side of the Golgi in infected cells is inappropriate.

8. Page 9, as 'morphogenesis' refers to all steps in virus assembly, the notion that Rab knockdown 'was truly at the level of morphogenesis' is a bit vague.

9. Page 10. The authors measure the effects of Rab knockdown on infectious virus release. Have they also looked at the levels of capsid or tegument protein release? If knocking down the Rabs is affecting the trafficking of viral glycoproteins essential for infection, release of non-infectious particles from knockdown cells may still occur?

10. Page 11. It's not clear to me that the distribution of CD63 is significantly different. Moreover, the criteria for describing the tubular recycling membranes as 'abundant' are unclear.

11. Page 27. Legend Fig 5. How long were the cells fed anti-gD antibody?

12. Fig. 6. The Y axes on panels A and D should be labelled.

Referee #2

For a long time the source of the membrane that envelopes herpes virus particles prior to their exit from the cell has been highly controversial. The key issue was whether it is derived from the biosynthetic pathway (most prominently the TGN) or from the endocytic pathway. I would have bet my money on the TGN but this paper provided compelling data arguing in favor of the early endosomes (but see below). The evidence relies on rapid access of a fluid phase marker HRP into the tubular-cisternal membranes that are seen by EM to enwrap the HSV capsids, and on some co-localization by immunofluorescence (IF) using the early endosome marker EEA1 (see below). In contrast by IF there was little overlap between viral capsids and TGN 46, also not when the Golg/TGN was dispersed after nododazole treatment. Most impressive were the data showing that siRNA knockdown of the endosome Rab associated proteins Rab 5, and 11, and both of these together, led to a significant inhibition in the formation of infectious viruses. As controls they showed that knockdown of Rab 24 (ER associated) had no effect, neither did Rab 9, an endosome-associated Rab, while siRNA of the ER-Golgi-associated Rab, Rab1, that was expected to block the early assembly forms of the virus also effectively blocked the infection.

They also analyzed the HSV glycoprotein gD. This part of page 8 is a little confusing in that they introduce gB, gE, that have signals for endocytosis, and gM, that apparently does not. Then they start experiments with gD, without any rationale!

Here they use uptake experiments involving binding of anti gD on ice followed by uptake into cells and after fixation and permeabilization they identify the primary antibody and find that the internalized antibody co-localized with capsids and with EEA1, but not with a Golgi marker giantin. This experiment is then supported by an EM assay of uptake of anti gD followed by an HRP-secondary antibody construct. In these experiments I missed the description of the times used. Curiously, in the EM experiment the gD antibody labeled the cell surface at 40C but apparently not coated pits, even though these structures are by definition open to the outside (at least some should be under their conditions). The HRP could be detected after warming up in coated pits, endocytic tubules and the virus wrapping membranes (times not specified). These data are at odds with their model in Fig 9C in which they show a cell surface invagination enriched in viral membrane proteins. These structures should be accessible to the antibody /HRP on ice, or (if not forming under these conditions) accessible within, say very short periods of antibody accessibility, or even free HRP.

They argue convincingly that EM gives much higher resolution than LM. In agreement with this argument I maintain that the paper would be enormously strengthened by immunogold labeling for gD, for example on cryo sections. This could provide support for the model in Fig 9C, for example. I would also ask that they show ultrastructural localization data for EEA1- where is this marker

really localized with respect to the wrapping membranes? Are the EEA1-positive membranes in continuity with the wrapping membranes-if so this would provide strong support for the wrapping occurring in the bona-fide early endosome, as opposed to the notion that perhaps a specialized viral compartment has been 'induced' by the infection.

I would also ask for one more key experiment. In their experiments with fluid phase HRP they access a few of the viral wrapping membranes within 2 mins of uptake. However, they require 30 mins for 90% of the capsids to have their enveloping membranes labeled. By 30 mins some of the HRP will surely have reached late endosomes. So is the need for longer incubation an indication that both early and late endosomes are involved in providing membranes for wrapping? Alternatively, it may only be the early endosomes but it takes 30 min for the whole system to be accessed by the marker? A more precise identification of the endocytic compartment(s) involved in wrapping would be an important addition. For this, I suggest:

1. Labeling for EEA1, and ideally double labeling of EEA1 with gD.
2. Labeling for a late endosome marker such as Lamp 1 or 2, again with gD.
3. Knock down of Rab 7. I am curious why they selected Rab 9 rather than Rab 7 for si RNA experiments since the role of Rab9 in the endocytic system is vague. In contrast Rab 7 is known to be important for the 'main' endocytic pathway. It is known from the Zerial group's work to come on the endosome precisely when Rab 5 comes off the membrane.

In summary, this paper is a very interesting study that with the extra experiments I suggest would provide significant new information for the field of herpes viruses.

Referee #3

Understanding how viruses infect and replicate in their hosts is of great importance to human health as well as of economic interest. Enveloped viruses, including herpes simplex virus are encapsulated by membrane that for different viruses is acquired from distinct cellular organelles. Wrapping of the herpes simplex virus genome is an incompletely understood process and the intracellular localization where this event is organized has been controversial.

Hollinshead and colleagues use light microscopy and EM methods in combination with perturbation of intracellular transport and kinetic transport assays to address the question where HSV-1 becomes enveloped.

In contrast to what is commonly thought, the new data suggest that wrapping does not occur on TGN membrane. Their results show that this event proceeds on endocytic tubules just below the cell surface and can be inhibited by siRNA of rab5 and inhibition of dynamin activity.

Since rab11 knockdown also affects wrapping, it is possible that recycling endosome tubules constitute a second site where HSV-1 is enveloped. The data cannot formally discriminate between these two possibilities because the rab11 dependent step is distal of rab5 action in the endocytic pathway. In this respect it is surprising that combined knockdown of rab5 and rab11 enhances the phenotype of rab5 knockdown.

The paper makes a strong case for a role of the endosomal system in HSV-1 envelopment, it falls short however in defining the underlying mechanism. Since we know quite a lot of the proteins that are required in these transport pathways, it is important to understand how the virus impinges upon this machinery. Without this information the manuscript does not go beyond a descriptive high quality morphological analysis of HSV-1 wrapping.

Other comment:

Authors state that HRP is not transported via a retrograde pathway to the Golgi complex (top of page 6). They then argue that HRP containing structures with a clathrin coat close to the virions do not derive from the TGN but from the endosomal system.

I doubt whether this is a correct assumption. Earlier work of Orci in this journal (EMBO J 5: 2097-1101, 1986) showed that endocytosed HRP can be transported to an intermediate locale of the Golgi stack!

## Response to Referee's Comments

## Referee #1:

1. *The model proposed by the authors is confused. In parts (e.g. pages 7 and 11), they suggest HSV uses membrane derived from tubules involved in recycling from endosomes (sorting and/or recycling endosomes), but they also suggest (and this is perhaps best illustrated by the cartoon in Fig. 9, but also on pages 6, 12) that the virus can use membranes derived from tubular invaginations of the plasma membrane. Although such tubular invaginations have been described, these are not generally thought to be associated with a clathrin/dynamin/Rab5-dependent endocytic mechanism. Moreover, the analysis using HRP, which shows that the highest proportion of HSV capsids associated with HRP-containing membrane structures is seen after 30 mins HRP uptake, is entirely consistent with the membrane tubules being derived from endosomes. In addition, the fact that dynasore reduced the number of HRP wrapped capsids (page 10) would support this argument. The authors should attempt to provide a clearer explanation of what these tubules are.*

Response: We agree that our original model was confused and lacking certain details, and we have now changed the model to incorporate the points made above by this referee (Figure 9C). Glycoproteins are now seen in clathrin-coated pits at the cell surface, not invaginations, followed by retrieval to an early/sorting endosome, where they are shown to then sort into tubules that subsequently enter the recycling endosomal network. It is these recycling tubules that we suggest are the source of wrapping membranes. This has also been clarified in the Discussion (p15).

2. *Although evidence for HSV using endocytic membranes for assembly is novel, there is evidence for the related HCMV using endocytic vesicles and tubules/cisternae (Tooze et al. 1993; Fraile-Ramos et al. 2002). This should be discussed.*

Response: We have now included these two studies in our discussion about other data that supports endocytosis of virus envelope proteins (p 14).

3. *Some discussion of the literature is in my view incorrect. For example, page 3 - there is little evidence for HIV capsids being targeted to the viral glycoproteins through matrix protein interactions. It is clear that HIV Gag can target the plasma membrane and form VLPs without the HIV Env expression. Is the tegument a 'compartment' (page 3), perhaps complex would be more appropriate.*

Response: The statement on HIV capsid targeting has been removed and we now refer only to the matrix proteins interacting with cytoplasmic tails of envelope proteins (p3). We now refer to the tegument as a complex, not a compartment (p3).

4. *Page 4, TGN 46 is 'a' major marker, not 'the' major marker.*

Response: "The" major marker has been changed to "a" major marker (p4).

5. *Page 5. The EM imaging of HSV infected cells illustrated in Fig S1 is taken at 12h PI. This is at a time point when virus production is nearing its peak. Are similar morphologies seen at earlier times?*

Response: The earliest we have looked by EM is 8h, and at this time we see the same profiles as at 12h. Interestingly, many studies in the literature tend to look at morphogenesis later rather than earlier, but we believe that the earlier times (8h to 12h) are more likely to give a true representation of virus envelopment.

6. *The authors should be cautious in their claim that HRP does not traffic to the TGN. As TGN 46 cycles over the cell surface, there must be vesicular trafficking to the TGN and therefore some fluid phase transport. It might be more appropriate to state that HRP trafficking to the TGN is undetectable.*

Response: We agree that our statement may have been too strong so have changed it to “trafficking of HRP to the TGN is generally undetectable” (p6). See also our response to Referee 3.

*7. Page 7, the polarity of the Golgi cannot be seen in these images, so the claim that TGN 46 remained a marker for the trans side of the Golgi in infected cells is inappropriate.*

Response: This claim has been removed and replaced with “the antigen remained a suitable marker for the TGN” (p7).

*8. Page 9, as 'morphogenesis' refers to all steps in virus assembly, the notion that Rab knockdown 'was truly at the level of morphogenesis' is a bit vague.*

Response: This has been changed to read “the block in virus replication was truly at the level of envelopment rather than release from the cell” (p 11).

*9. Page 10. The authors measure the effects of Rab knockdown on infectious virus release. Have they also looked at the levels of capsid or tegument protein release? If knocking down the Rabs is affecting the trafficking of viral glycoproteins essential for infection, release of non-infectious particles from knockdown cells may still occur?*

Response: The only non-infectious HSV1 particles that have really been identified are light (L) particles that comprise envelope and tegument without capsid. As such, we assume (but don't know) they would undergo envelopment by a mechanism similar to full virions, and hence knockdown of Rab5 would be expected to have the same effect on production of L particles as it does on virions. Indeed we do not see any evidence of L particles at the cell surface in Rab5 depleted cells but we have not looked at this biochemically.

*10. Page 11. It's not clear to me that the distribution of CD63 is significantly different. Moreover, the criteria for describing the tubular recycling membranes as 'abundant' are unclear.*

Response: We have replaced the image in the original figure with a new, hopefully more convincing, image and have included a brightfield image to show the extent of the cytoplasm in relation to where the CD63 structures are localized. We agree that the difference is subtle but it is highly reproducible. These images are now discussed on p 10 (Figure S4). We have also included an image of CD63 in Rab7 depleted cells, which was carried out in response to comments from Referee 2. The reason for describing the recycling membranes as abundant is based on Figure 9A & B where we have measured the abundance of transferrin positive membranes relative to TGN46 positive membranes. However, as we are referring to relative abundance not overall abundance, we have clarified this through the manuscript.

*11. Page 27. Legend Fig 5. How long were the cells fed anti-gD antibody? Response: This information has now been included in the figure legend for figure 5 (p31) – 30 min on ice followed by 30 min at 37 °C. 12. Fig. 6. The Y axes on panels A and D should be labelled. Response: These axes have now been labeled.*

Referee #2 :

*They also analyzed the HSV glycoprotein gD. This part of page 8 is a little confusing in that they introduce gB, gE, that have signals for endocytosis, and gM, that apparently does not. Then they start experiments with gD, without any rationale!*

Response: We realize that this introduction on p8 was somewhat confusing and have now expanded it to explain our reasoning behind using gD as a marker. Essentially, we reason that for our model to be correct, all glycoproteins must be endocytosed from the plasma membrane – even if they don't have retrieval signals of their own, they would be retrieved maybe by forming complexes with glycoproteins that do. Hence, antibody-feeding experiments should be possible with any glycoprotein. As we had a monoclonal antibody to the extracellular domain of gD available to us, we used gD for the experiment. We have also done this experiment with antibody to gM with similar results.

*Here they use uptake experiments involving binding of anti gD on ice followed by uptake into cells and after fixation and permeabilization they identify the primary antibody and find that the internalized antibody co-localized with capsids and with EEAI, but not with a Golgi marker giantin. This experiment is then supported by an EM assay of uptake of anti gD followed by an HRP-secondary antibody construct. In these experiments I missed the description of the times used. Curiously, in the EM experiment the gD antibody labeled the cell surface at 40C but apparently not coated pits, even though these structures are by definition open to the outside (at least some should be under their conditions). The HRP could be detected after warming up in coated pits, endocytic tubules and the virus wrapping membranes (times not specified). These data are at odds with their model in Fig 9C in which they show a cell surface invagination enriched in viral membrane proteins. These structures should be accessible to the antibody /HRP on ice, or (if not forming under these conditions) accessible within, say very short periods of antibody accessibility, or even free HRP.*

Response: We apologize for having worded this section very badly, as we did not mean to give the impression that the gD antibody did not label coated pits at 4 °C. In fact as this referee anticipated, the gD antibody clearly labeled pits at 4 °C, and we have now included a panel in Figure 5E showing this result. We have changed the text accordingly (p9).

*They argue convincingly that EM gives much higher resolution than LM. In agreement with this argument I maintain that the paper would be enormously strengthened by immunogold labeling for gD, for example on cryo sections. This could provide support for the model in Fig 9C, for example. I would also ask that they show ultrastructural localization data for EEAI- where is this marker really localized with respect to the wrapping membranes? Are the EEAI-positive membranes in continuity with the wrapping membranes-if so this would provide strong support for the wrapping occurring in the bone-fide early endosome, as opposed to the notion that perhaps a specialized viral compartment has been 'induced' by the infection.*

Response: With regard to the suggestion to carry out immunogold labeling for gD – we feel that our ultrastructural studies on gD retrieval from the plasma membrane, which we have now clarified as described above, are much more informative for our proposed model (Figure 9C) than total immunogold studies. General immunolabelling of infected cells for gD will label most membranes in the cell – the ER, the Golgi, the TGN, the plasma membrane and membranes of the endocytic pathway. In this situation it is impossible to say where the gD that is in HSV wrapping membranes has derived from. By labeling cell surface gD with antibody and allowing the antibody to be endocytosed into the cell, we can identify glycoprotein that has started off at the cell surface, and ask if this population is detectable in the wrapping membranes at the ultrastructural level. The results we present in Figure 5E shows that this is the case.

*With regard to immunogold of EEAI, a protein that is localized specifically to the early endosome - although we never meant to give the impression that we thought the virus envelope was derived from the early endosome, it is clear that our attempt to explain our results was confused and we have now clarified this in the manuscript (see our responses to Referees 1 and 2, and the revised model in Figure 9C). Our model has been refined to show that wrapping occurs in tubules that have been sorted out of the early endosome and are destined to recycle to the plasma membrane. This is why Rab11 depletion has an effect on virus production, because Rab11 is localized to recycling endosomes and is involved in transport from the early endosome to recycling endosomes, and recycling endosomes to the cell surface. Moreover, Rab11 depletion has no effect on the appearance of early endosomes (now presented in Figure S4), and hence the effect of its depletion is downstream of the early endosome. Based on the evidence that Rab11 depletion affects virus production; none of our EM images suggest that the wrapping membranes are contiguous with early endosome structures; the wrapping membranes have the appearance of discrete tubules of varying lengths; and HRP uptake timing suggests wrapping in membranes downstream of the early endosome, we do not anticipate and do not suggest that the early endosome is the site of virus wrapping. Furthermore, unlike gD, capsids do not show obvious colocalisation with EEAI in infected cells by immunofluorescence (now presented in Figure 5D), and we believe that gD is sorted out of the early endosome into the final wrapping membranes of recycling tubules. Finally, the appearance of HRP positive membranes is the same in uninfected compared to infected cells (Figure 2) suggesting that endocytic trafficking pathways are similar in infected cells – hence we do not believe that wrapping occurs in a virus-induced compartment.*

*I would also ask for one more key experiment. In their experiments with fluid phase HRP they access a few of the viral wrapping membranes within 2 mins of uptake. However, they require 30 mins for 90% of the capsids to have their enveloping membranes labeled. By 30 mins some of the HRP will surely have reached late endosomes. So is the need for longer incubation an indication that both early and late endosomes are involved in providing membranes for wrapping? Alternatively, it may only be the early endosomes but it takes 30 min for the whole system to be accessed by the marker? A more precise identification of the endocytic compartment(s) involved in wrapping would be an important addition. For this, I suggest:*

*1. Labeling for EEA1, and ideally double labeling of EEA1 with gD. 2. Labeling for a late endosome marker such as Lamp 1 or 2, again with gD. 3. Knock down of Rab 7. I am curious why they selected Rab 9 rather than Rab 7 for si RNA experiments since the role of Rab9 in the endocytic system is vague. In contrast Rab 7 is known to be important for the 'main' endocytic pathway. It is known from the Zerial group's work to come on the endosome precisely when Rab 5 comes off the membrane.*

Response: We agree that it is vital to determine if late endosomes are involved in HSV1 envelopment, and have now carried out a number of experiments to address the issue of whether wrapping membranes are provided by late endosomal MVBs:

1. In our HRP uptake studies described in Figure 2 and quantitated in Table I we have now included results whereby infected cells were incubated in HRP for 10 or 30 min, and then chased for a further 60 min in media lacking HRP to chase all intracellular HRP out of early cisternae into MVBs (Figure 2A & 2L, and Table I). The data shows that when HRP is present only within MVBs and not in early/recycling endosomes, HSV1 wrapping membranes no longer contain HRP. Hence these wrapping membranes must originate from a source other than the late endosome.
2. In Figure 3, we have now included immunofluorescence of the MVB marker CD63 in cells infected with the HSV1 green capsid virus. As for TGN46 there is little colocalisation of capsids with CD63 positive membranes.
3. In Figure 6, we have now included results for Rab7 depletion in conjunction with depletion of the other endocytic Rabs. Rab7 was efficiently knocked down as measured by Western blot (Figure 7C), but its absence reduced virus yield by just over 2 fold compared to 50 fold for our positive control Rab1, and 20 fold for Rab5. So while we cannot rule out a role for Rab7 in virus envelopment, any contribution it makes would seem to be limited.

In short we would suggest that the reason it takes 30 min for 90% capsids to have HRP positive enveloping membranes is not that the late endosomes are involved but that it takes 30 min for HRP to saturate the early/recycling endocytic network and for those membranes to encounter capsids to enwrap.

Referee #3:

*Since rab11 knockdown also affects wrapping, it is possible that recycling endosome tubules constitute a second site where HSV-1 is enveloped. The data cannot formally discriminate between these two possibilities because the rab11 dependent step is distal of rab5 action in the endocytic pathway. In this respect it is surprising that combined knockdown of rab5 and rab11 enhances the phenotype of rab5 knockdown.*

Response: As in our response to referee 2, although we never meant to give the impression that we thought the virus envelope was derived from the early endosome, it is clear that our attempt to explain our results was confusing. In response to Referee 1 we have clarified our model of HSV1 envelopment to show glycoproteins trafficking from the cell surface to the early endosome where we suggest they are then sorted into tubules destined to recycle through the recycling endosome back to the cell surface. Wrapping of capsids would occur in this population of recycling tubules (many of which cluster around the MTOC). Hence Rab5 and Rab11 would function sequentially along this pathway. Rab5 depletion would block glycoprotein retrieval from the cell surface that would affect all downstream steps in the envelopment pathway, while Rab11 depletion would interfere with the final envelopment stage. Based on the fact that it is unlikely to get 100% depletion



using siRNA, the reason for enhancement by double depletion may be that Rab11 depletion interferes with any residual glycoprotein retrieval that has escaped Rab5 depletion.

*Authors state that HRP is not transported via a retrograde pathway to the Golgi complex (top of page 6). They then argue that HRP containing structures with a clathrin coat close to the virions do not derive from the TGN but from the endosomal system. I doubt whether this is a correct assumption. Earlier work of Orci in this journal (EMBO J 5: 2097-1101, 1986) showed that endocytosed HRP can be transported to an intermediate locale of the Golgi stack!*

Response: The referee refers to an early paper showing HRP undergoing retrograde transport to the Golgi. However, this study was carried out in specialized secretory cells (insulin secreting cells) that are designed to move large volumes from the Golgi to the cell surface. Other published studies on a range of other cell types such as BHK, for example in the papers we referred to on p6, did not detect HRP transport to the Golgi. Furthermore, in our own studies presented here in 3 different cell-types, we did not detect HRP positive Golgi stacks (eg Figure 2D), even after long periods of HRP incubation. While we are unclear as to why retrograde trafficking may be different in secretory cells, it may be that these cells have a specialized mechanism to replenish the Golgi. We have now referred to the above-mentioned paper by Orci on p6.

2nd Editorial Decision

29 May 2012

Thank you for your patience while your manuscript has been reviewed and please accept my apologies for the delay. Your study has been sent to former referees #2 and #3, who now consider that most of their concerns have been properly addressed, although some minor issues still remain.

As you will see below, both referees still suggest a number of experiments to further improve your manuscript. As you know, it is our policy to allow for a single round of revision, but we occasionally allow for further experimental work if justified by the manuscript, provided that the remaining concerns are addressable in a timely manner. In this case, after further consultation and cross-referee commenting, both referees agree in the importance of the main message of your study, and further agree that double-label immuno-EM studies using combinations of gD-EEA1, gD-LAMP and gD-TGN marker (for negative control) is necessary for definitive support of your main claims. Other points raised will not be determinant in the acceptance of your manuscript.

Do not hesitate to contact me in case you have any further questions.

Thank you again for your patience and the opportunity to consider your work for publication. I look forward to the final version of your manuscript.

Yours sincerely,

Editor  
The EMBO Journal

## REFeree REPORTS

### Referee #2

The revised manuscript is definitely improved, and the final model (Fig 9 C) now makes more sense. I am still not satisfied with their response to my request to carry out double immunogold labeling with anti gD and key cellular markers of the early endosome (e.g EEA1) and late endosomes (e.g LAMP 1). They claim (without evidence) that gD will be found almost everywhere. However, Fig 9C (the figure that most readers will scrutinize most carefully) shows (quite reasonably) the notion that the early endosome shows sorting: some domains are suggested to be enriched in viral glycoproteins, others depleted. This is a general theme in virus assembly. Double labeling at the EM level with these two markers would tell us much more about where gD concentrates, and where it is depleted. In addition they would be able to visualize the budding

capsids in the same images. With these additional data the paper would be a definitive study.

Referee #3

The authors strengthened the idea that wrapping of the HSV-1 capsid occurs from endosomal tubules. They show this convincingly in several cell types and using kinetic experiments in combination with microscopy, they eliminate the relevance of other compartments as source of membrane. From phenotypes arising of siRNA-mediated knock-down of several endosomal rab GTPases they conclude that wrapping depends on rab5 and rab11.

As before I find that the paper makes a convincing case for wrapping from early endosomes instead of the TGN. Nevertheless, the mechanism via which this happens is not clear. Since rab11 function in endocytic transport is downstream of rab5, a combined knock-down should not have a stronger effect than the single rab5 knock-down. An alternative explanation for the synergistic knock-down involves an indirect function of rab11 via directing biosynthetic cargo or other molecules from the TGN to the plasma membrane, that might subsequently be needed for wrapping. In support of this, inspection of Fig. 6F reveals that expression of several HSV-1 proteins including ICP27, gE and gD is reduced as a consequence of combined rab5 and rab11 knock -down. Of different note, knock-down of rab7 and rab9 also produces a moderate but nevertheless 2-fold decrease in virus release (Fig. 6A,B), which is difficult to reconcile with the main message of the paper.

At this point the underlying mechanism responsible for endosomal wrapping of HSV-1 is not sufficiently developed. This descriptive paper elucidates where HSV-1 wrapping occurs, but it does not give us a mechanism or provides new molecular insights in endosome function and is therefore more suited for J. Virol or perhaps PLoS Pathog.

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Authors' Correspondence

30 May 2012

Thank you for the decision letter concerning our manuscript. Having discussed it with my co-authors I felt it would be best to ask you for some clarification on the requested double immuno-EM experiments, as we actually do not understand how they will provide "definitive support for our main claims".

The only way immuno-EM would provide results as described (critically that the TGN would be a negative control) would be if they were carried out on antibody uptake experiments similar to those used in the manuscript for immunofluorescence and HRP-EM of endocytosed gD antibody. Otherwise, the gD antibody would pick up the membranes of the secretory pathway, including the TGN, through which all glycoproteins are transported to the cell surface - making the TGN a positive not a negative control. However, neither your letter nor the second Referee's report (either first time or second time around) stated anything about doing the immuno-EM on antibody that has been endocytosed from the plasma membrane (our so-called antibody uptake assay).

I would be very grateful for clarification on this important point. Although we have additional further questions about the referees' anticipated results from such experiments, I would like to clarify what we are actually being asked to do before expanding further on these questions.

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Editor's Response

31 May 2012

I apologize for the confusion. Both referees agree in the importance of these co-labeling experiments and both have expressed some doubts regarding your prediction that the experiment would be non-informative. At this point, I don't think we would reach an agreement unless those experiments are actually performed.

In further communication with referee #3 regarding these stainings, s/he literally suggested the following: "Double label immuno EM with antibodies against the viral gD glycoprotein versus markers such as EEA1 or syntaxin 13 (for early endosomes) and LAMP (for late

endosomes/lysosomes) has been done before by others. This should preferably be combined with gD labeling vs a TGN marker (negative control). Antibodies that recognize mentioned markers on mildly fixed ultrathin cryosections prepared from HeLa cells are available".

As I mentioned in my previous letter, other experiments suggested will not be taken into consideration in the final decision. I sincerely hope this helps and please do not hesitate to contact me again in case you need further input.

I am looking forward to seeing the final version of your manuscript.

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Authors' Correspondence

31 May 2012

We are happy to perform these experiments, but absolutely need clarification on the scenario that the referees consider TGN to be a negative control, otherwise we are talking at cross-purposes.

We interpreted the suggested experiments as meaning that they wanted TGN to act as a control for a membrane that had no gD in it - ie for some reason the reviewers seem to think that gD will not be in TGN membranes. But all glycoproteins including gD are in TGN membranes (e.g. see Turcotte et al, 2005) because they are transported through the TGN to the plasma membrane before being re-endocytosed, therefore the TGN would not act as a negative control but rather (as I tried to explain in last email) as a positive control for a gD-containing membrane.

If, on the other hand they want immuno-EM of TGN to act as a negative control for membranes specifically wrapping around capsids, then their suggestion makes absolute sense for TGN. Of course our prediction then would be that all of TGN, EEA1 and LAMP would be negative for HSV1 wrapping membranes, because, as we have made clear throughout our revised manuscript, and in our responses to these 2 referees, we do not believe that wrapping takes place in the limited EEA1 positive sites of the endocytic network, or in late endosomes/MVBs, but in the much more extensive network of tubular recycling endocytic membranes that have been sorted out of the early endosome and are EEA1 negative.

I really apologize for this continued confusion, and as I have said we will of course perform these experiments as requested, but if you could seek further clarification on the use of TGN as a negative control I would be very grateful.

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Editor's Response

01 June 2012

Referee #3 has got back to me with the following answer:

"My apologies for not being clear. The negative control is meant to show by immuno EM that TGN is not the membrane that wraps around the capsids.

There is something to the argument of the authors that gD will be widely distributed in biosynthetic and endocytic compartments. There could be a way around this problem by chasing out gD from biosynthetic compartments using cycloheximide. Such an experiment might be asking too much since it will require fine-tuning incubation conditions with a drug that can affect cell viability.

If the authors are right then immune-EM of EEA1 and LAMP reveals that these are not labeling the membrane that wraps around a capsid. This membrane should however be enriched with syntaxin 12 (also known as syntaxin 12) and rab11 or rab11 effectors!

In summary it boils down to immunolabeling capsid versus:

TGN46

EEA1

LAMP

syntaxin 13 or rab11 (effector)"

I hope this clarifies the experiments that are needed. As the referee acknowledges, further experiments using cycloheximide are out of the scope of this manuscript.

I am looking forward to seeing the revised version of your manuscript.

2nd Revision - authors' response

20 August 2012

Following the response from the referees to our revision, we have had 2 email communications from one referee via the editor that helped clarify the experiments that we were being asked to carry out.

#### **Email communication 1.**

*“My apologies for not being clear. The negative control is meant to show by immuno EM that TGN is not the membrane that wraps around the capsids.*

*There is something to the argument of the authors that gD will be widely distributed in biosynthetic and endocytic compartments. There could be a way around this problem by chasing out gD from biosynthetic compartments using cycloheximide. Such an experiment might be asked too much since it will require fine-tuning incubation conditions with a drug that can effect cell viability.*

*If the authors are right than immuno EM of EEA1 and LAMP reveals that these are not labeling the membrane that wraps around a capsid. This membrane should however should be enriched with syntaxin 13 (also known as syntaxin 12) and rab11 or rab11 effectors!*

*In summary it boils down to immunolabeling capsid versus: TGN46 EEA1 LAMP syntaxin 13 or rab11 (effector)”*

**Response:** We have spent several months optimizing immunogold labeling of HSV1 infected HFFF-2 cells for the various markers suggested. Such experiments are made difficult by the fact that HSV1 infected cells express an Fc receptor (the gE/gI complex), which has a strong affinity for many antibodies, particularly those raised in rabbits. Hence we have had to ensure that all our primary and secondary antibodies labeled infected cells specifically, rather than binding non-specifically to gE/gI. Consequently our choice of reagents was somewhat limited and double labeling was not possible in our hands. The results of our immunogold assays were as follows:

**gD** – gD was detected convincingly in many cellular membranes including the Golgi, the plasma membrane, membranes wrapping capsids in the cytoplasm and extracellular virions. This data is presented in Figure S5.

**LAMP** – LAMP2 was detected specifically in late endosomal structures/lysosomes, and was not detected in wrapping or wrapped particles. Capsids were not detected in close proximity to LAMP2 positive structures. These results are presented in Figure S3. This data confirms those already presented in our HRP chase experiments, where HRP was chased into MVBs, with the result that wrapping membranes became HRP negative (Figure 2).

**EEA1** – Labelling of ultrathin sections with EEA1 by both immunofluorescence and immunogold revealed that EEA1 positive structures were extremely infrequent in HFFF-2 cells. Consequently it was very difficult to find EEA1 positive structures by immuno-EM. However, after extensive analysis we found a number of these structures that labeled well with EEA1. Nonetheless, wrapping and wrapped particles did not stain positive for EEA1 and taken together with the rarity of these sites in the cell, and our immunofluorescence data already presented in Figure 5, we conclude that capsids do not wrap at EEA1 positive early endosomes. EEA1 immunogold is presented in Figure S7.

**TGN46** – TGN46 labeled the trans side of Golgi stacks in uninfected cells by immunogold analysis.

However, TGN46 in infected cells was less obvious at this site, and moreover was undetectable at

any other site in the infected cell at the time post-infection that we carried out immunogold labeling. This correlates with previous observations on the behaviour of this cellular marker, and our own analysis presented in Figure 4. Consequently, TGN46 was not detected on wrapping or wrapped particles, as presented in Figure S4.

**Labelling of Recycling Endosomes** – we attempted labeling using 3 different syntaxin 12 antibodies, a Rab11 antibody and a transferrin receptor antibody, but could get none of these to work at the immune-EM level. Hence we have been unable to positively identify recycling endosomes at the ultrastructural level.

In short, our immune-EM data shows that the wrapping membranes do not contain LAMP, EEA1 or TGN46, but we have been unable to provide a positive marker for the compartment involved.

**Email communication 2:**

*“As compromise I would propose a final and 'simple' fluorescence microscopy experiment alike what has already been done in Fig. 9a in HeLa cells. Internalize Cy3-Tf followed by a 37C chase. Cells should then be fixed after different periods of time and labeled for gD. In this manner they can transiently accumulate Cy3Tf in recycling endosomes which could then serve as the recycling endosome marker (compare J Cell Biol. 2000 May 15;149(4):901-14).*

*If it works that would nicely support their conclusion. In case it does not work, or if the authors have a convincing argument that the experimental design is not good, than accept under condition that authors tone down the claims that REs are a principal source of membrane.”*

**Response:** We have previously attempted transferrin-HRP uptake experiments in infected cells to examine recycling endosomes in infected cells by EM, but found that the transferrin remained on the cell-surface (while being successfully internalized into uninfected cells). Nonetheless, we attempted transferrin uptake studies in infected HeLa and HFFF-2 cells, but have found that, as identified in the previous EM studies, at times when capsids are being produced at suitable numbers for analysis, uptake was greatly reduced in comparison to uninfected cells and hence detection was extremely problematic. We do not have an explanation for this, but it may be because the plasma membrane is somehow saturated with virus glycoproteins at this time. As a compromise we stained cells for the transferrin receptor marker of recycling endosomes, and this revealed coincidence between capsids and the receptor containing membranes – this data is now included in Figure 5. Double immunofluorescence could not be carried out as our glycoprotein antibodies are all mouse monoclonals, as is the TFR antibody. Furthermore, as mentioned above, rabbit antibodies are often a problem with infected cells.

In conclusion, we have shown that there is a close coincidence between the TfR and virus capsids, and that virions do not wrap at early or late endosomes, but we are aware that this is not sufficient evidence for pinpointing recycling endosomes as the source of virus membrane. It is, however, noteworthy that a previous proteomic study of extracellular HSV particles identified TfR as one of a number of cellular components in released virions (Loret et al, 2008).

We have now toned down the model in Figure 9C and have removed the mention of recycling endosomes from it.