THE EMBO JOURNAL

Manuscript EMBO-2011-79590

Ebola virus entry requires the host-programmed recognition of an intracellular receptor

Emily Happy Miller, Gregor Obernosterer, Matthijs Raaben, Andrew S.Herbert, Maika S. Deffieu, Anuja Krishnan, Esther Ndungo, Rohini G.Sandesara, Jan E. Carette, Ana I. Kuehne, Gordon Ruthel, Suzanne R. Pfeffer, John M. Dye, Sean P. Whelan, Thijn R. Brummelkamp and Kartik Chandran

Corresponding author: Kartik Chandran, Albert Einstein College of Medicine

Review timeline:

Submission date:	21 September 2011
Editorial Decision:	31 October 2011
Revision received:	03 January 2012
Editorial Decision:	19 January 2012
Revision received:	02 February 2012
Accepted:	03 February 2012

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 31 October 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees whose comments are enclosed. As you will see, all three reviewers express interest in your detailed analysis of NPC1 as a filovirus receptor, and all are broadly in favour of publication - pending satisfactory revision. Their criticisms refer mainly to technical concerns with the immunofluorescence and Western blot data. Regarding the comments of referee 2 re. nomenclature (points 6 and 7), I agree that you do not definitively show NPC1 to have a role in promoting fusion, and would therefore ask that you tone down these particular sections.

Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, 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 COMMENTS

Referee #1

This group, along with that of Jim Cunningham, recently reported that NPC1 is required for filovirus entry. Cunningham presented evidence suggesting that cathepsin cleaved ebolavirus (EBOV) glycoprotein (GP) binds to NPC1. The major new claims of this paper are that (1) detergent solubilized (cathepsin cleaved) GP and NPC interact, (2) that the interaction requires residues in GP1 previously proposed to constitute the receptor binding site (RBS), (3) that the interaction requires the C-loop of NPC, and (4) that the interaction between the RBS in GP and the C-loop in NPC is critical for virus entry. While claims 1 and 2 are well-justified, additional work is needed to bolster claims 3 and 4.

Major Comments

1. Re: claims 3 and 4: The mutant NPC proteins employed are large deletions (of complete loops A, C, or I) or constructs expressing the isolated loops. All deletion mutants show loss of cholesterol transport activity. Information is therefore needed to assess if the mutant NPC proteins are folded correctly. Are there antibodies to assess native NPC structure? Minimally the authors must do a better job assessing the subcellular localization of these constructs (see below).

2. RE: Fig. 2:
a. At the top of p6 you say that the delta C-loop mutant shows similar localization (implied late endosomes) as the other mutants. Higher resolution images should be provided for the supporting figure (S3b). Quantitative data should also be provided (multiple cells, multiple coverslips): how many Lamp positive endosomes stain for each of the NPC constructs?
b. Based on the images shown, it appears that WT NPC co-localizes with Lamp, but that the deletion constructs do not. Are the latter inside the endosomes? If so, comment on how do you think the delta A and delta I constructs work from within the endosomes.

3. RE: Fig. 4: Some features of the C-loop construct used need to be clarified:
a. From the text it appears that the C-loop construct used in Fig. 4c has the WT NPC cytoplasmic tail, which should target it to late endosomes. If so, how did this C-loop construct get onto VSV particles (which assemble at the cell surface)? A later figure aims to show that the C-loop construct with the WT tail is not present at the cell surface.
b. Figures 4d and e show that the C-loop construct (presumably with the tail) supports infection when transfected into NPC null cells. To do this, the construct would presumably have to localize to (the limiting membrane of) late endosomes, but this was not assessed. A quantitative analysis of co-localization of each of the loop constructs used in Fig. 4 with Lamp should be provided.

4. RE: Fig. 5:
a. Comment on why overexpression of NPC should rescue the infection defects of the 3Ala and F88A and F88H mutations (Fig. 5e, f). The binding studies (Fig. 5a, b) were done with the same NPC overexpressing cells and the mutants didn't bind to (overexpressed) NPC.
b. (minor): Should the first bar for the WT GP set be black?

5. RE: Fig. 6.
a. Better images should be provided (and quantitated) for the supplemental Figure (S7a) supporting Fig. 6. Why is there so much intracellular (perhaps Golgi) staining with WGA (done before permeabilization)? Also, it's hard to discern what's going on with the C-loop construct with the WT tail; the image appears to show two small and rounded (possibly recently divided) cells. Provide an image of a more spread cell(s).
b. As per comment 2, provide an analysis of how much of the C-loop constructs (with and without the tail) are in late endosomes.
c. Are the same cells shown in Fig. 6a as in S7a? If so, provide images from different cells.

6. At this stage a term other than "intracellular entry receptor" (used in title, Discussion and elsewhere) should be chosen for NPC. Calling it an entry receptor implies it has an active role in promoting fusion (or internalization if it were at the cell surface). But WT NPC is not a cell surface protein, and is not sufficient for fusion/entry (stated on p13). Until there is evidence that NPC plays an active role in fusion, it is premature to call it an entry receptor and, likewise, to include "NPC" under "fusion triggering" in the model in Fig. 7.

7. Several claims in the Abstract and/or elsewhere should be reworded:
a. "synthetic membrane protein----has viral receptor activity": As above it is premature to term this as viral receptor activity.
b. "promotes viral membrane fusion": Nobody has shown that the GP-NPC interaction promotes fusion.
c. "long-sought filovirus receptor" (end Intro): the long sought after filovirus receptor is a cell surface protein.

Other Comments

1. More information is needed on the reptilian cells. Do they bind and internalize GP particles? Do they have enough cathepsin and endosome acidifying activity? Do they express NPC protein? If so, how does the sequence of its C loop (or the C loop from any reptile or amphibian) compare with that of human/mammalian NPC?
1. Double check Takada. 1997. Did they test a reptilian or amphibian cell?
2. Several minor points re: Fig. 3:
a. 3a legend: Wasn't the control (Ctrl) extract used in 3a, not 3b?
b. Should it read: Fig. 3b on p6, 7 lines from bottom?
c. At what pH were the co-IPs done in Fig. 3b (p6, 3 lines from bottom and in legend)?
d. Were descriptions of 3g and h swapped in the legend?

Referee #2

The manuscript by Miller et al represents a well-constructed set of experiments to examine the role of NPC1 in Ebola virus infection. It fills many gaps in our knowledge of the exact role of NPC1 and its function in infection by this virus and the authors are commended for being thorough in their work. Overall the work is compelling and is very important for the filovirus field. Some issues are still present and it would help if they were corrected.

Figure 1 should be quantitated to give increase in titer after transfection of NPC1 plasmid. Ideally, the cells should have been stained for NPC1-flag to get an idea on transfection efficiency. Otherwise put one of the blots of NPC1-flag expression from the supplemental data into this figure (if same cells).

Do not describe wild type virus as "authentic". This is a non-standard nomenclature. It is "wild type" virus.

The expression levels of the NPC1 deletion mutants are in the supplemental data. They should be in Figure 2. Filipin staining is nice but expression data on the mutants is more important. Please see next comment on these blots. Staining the cells before infection would have been the best approach.

Generally speaking, as well as in fig Supp3A, all the Western blots are grossly overexposed. This is very disappointing in what is otherwise a very well-performed set of experiments. Fig Supp3A has the blots that should be in figure 2. However, bands on the blot are way too dark to give any quantitation of differences in levels of expression except for the delta A mutant (which is very poorly expressed). Also, the levels of protein expression in the panel B immunofluorescence do not seem to correspond to those seen in panel A. In panel A, delta A is the faintest and the other proteins appear as large smears (doublets? - if so please explain). In panel B (for some only a single cell is shown and so it is impossible to determine if it is representative) delta C is the weakest. Also, the staining pattern between the wild type protein and the deletion mutants is quite different with complete overlap with LAMP-1 obvious for the wild type while the deletion mutants appear luminal even though the expression on the blots are equivalent. Please explain these differences, add this to the paper and provide images with more transfected cells.

Comment on the doublet bands present in Supp 3c. They may indicate some large change in the maturation of the mutant proteins compared to wild type and this could have a major impact on the interpretation of the work. Comment and include in paper.

Please explain the quantitation of figure 3D. It says "%eGFP positive cells". This suggests a yes/no type of analysis with cells binding or not binding virus. If a cell binds a single virus and another binding 100 are considered the same then this skews the interpretation greatly. For this experiment it is more important to have a read out that incorporates information on binding affinity of each GP construct. A simple way to do this would be to show the FACS data. The interpretation of the subsequent experiments is dependent on this analysis.

Please give some quantitative analysis of panel 3E. The images are again saturated and it is unclear what the total cell loading of virus is for the cell types and the mutant.
It is very unclear how the VSV-M release experiment with overexpression of NPC1 in Fig 5E really gives any information on how NPC1 controls membrane fusion as suggested on top of page 10. Please explain/clarify/remove.

Figure 7 does not add much information to the paper or to the field and should probably be removed. Figure 7 suggests that fusion occurs in late endosomes after contact with NPC1. There is no evidence in this paper or other papers that support this conclusion/assumption. A small amount of NPC1 could be present in early endosomes and this may be the site of fusion (keep in mind that delta A is very poorly expressed and yet supports infection indicating little protein is needed). Either remove this figure or modify it so the figure ends at contact with NPC1 in the endosome and question marks follow this step leading to fusion.

Referee #3

This is an excellent manuscript that builds upon earlier work reported in Nature, by now providing the definitive illustration that NPC1 is an essential filovirus receptor. There is thorough analysis demonstrating that: nonpermissive cell lines can be rendered permissive by transfection with NPC1, that NPC1 specifically reacts with endosomally and enzymatically cleaved GP, that it reacts with cleaved GP from different filoviruses, that point mutations previously shown to be important for binding and entry are important for binding NPC1, that only NPC1 and not the 50% identical NPC1L1 is functional for filovirus entry, and that domain C is the key portion of NPC1 as illustrated by binding and infection studies.

One particularly interesting finding is that GP-NPC1 binding does not occur at the cell surface. This could be assumed because only cleaved GP binds, but the authors have actually demonstrated this using domain C-tailless construct that localizes exclusively to the plasma membrane that increases viral attachment. The second particularly interesting conclusion is that binding of NPC1 to cleaved GP in the presence of acid pH is still not sufficient to cause fusion, leading to the speculation that there may an additional factor (co-receptor?) for triggering.

A few suggestions:

Page 13 It might be appropriate to add in some discussion of the possibility of filoviruses achieving initial entry through macropinocytosis in the absence of any specific attachment factor.

Page 12 I wonder if the remodeling of GP truly is a way of avoiding humoral immune responses. It is a quite acute infection. Of course, what happens in the natural host is likely quite different and the massive, mucin-like domains do lead to production on non-neutralizing antibodies.

point mutants: It would be good to say somewhere that it is still unclear if mutations like F88A are detrimental to binding because they remove a key contact or because the structure is somehow disrupted.

Figure 1. The legend is a bit confusing. Perhaps rewrite as "Human NPC1 confers susceptibility to filovirus entry and infection. Viper VH-2 cells expressing empty vector or human NPC1 were infected with either (a) filoviruses or (b) recombinant VSIV..."

Figure 2. Infection of I-deletions is weaker than A-deletions. Is there any evidence that domain C would interact with domain I, so hat I might be partially involved in infection? Or are NPC1 with deletions of domain I less stable or less highly expressed. Are relative expression levels of full-length NPC1, and domain A-, C-, and I-deletions shown anywhere?

Figure 3. There are quite a few separate ideas in this figure, and I'm wondering if it would be possible to separate them into different figures. I don't think it would take up any more journal page space, but would make working through the legend much easier. (a) and (b) are about NPC1 and GP pulling each other down. (c-f) could be another figure saying LPC1L1 will not suffice. (i-j) seem repetitive with Figure 4 until one realizes that Figure 3 is about binding , while Figure 4 is about infection. The authors could either clarify this a little more immediately in the legend or rearrange the Figure panels so binding and infection were in the same figure.

Figure 3b: What are the asterisks, and the identity of the band to which they correspond?

Figure 3 g-h: are these panels switched?

Figure 5 a-d are about the Lys and Phe mutations and not about functioning of NPC1 late in entry. I wonder if panels a-d should be a separate figure or grouped in with other binding studies.

Figure 7. Perhaps the blue arrowheads in (a) could be colored red so they stand out better. I think also you could label the grey membrane differently in the cartoon so it is clear that domain C with tail is on the endosomal membrane while tail-less C is on the plasma membrane.

1st Revision - authors' response 02 January 2012

Responses to reviewers' comments

Reviewer #1

The major new claims of this paper are that (1) detergent solubilized (cathepsin cleaved) GP and NPC interact, (2) that the interaction requires residues in GP1 previously proposed to constitute the receptor binding site (RBS), (3) that the interaction requires the C-loop of NPC, and (4) that the interaction between the RBS in GP and the C-loop in NPC is critical for virus entry. While claims 1 and 2 are well-justified, additional work is needed to bolster claims 3 and 4.

We thank the reviewer for their detailed and constructive feedback. To bolster claims (3) and (4) above–that domain C is necessary and sufficient for binding to GP, that it binds directly to GP, and that GP-NPC1 interaction is required for entry–we have added three important new experiments.

1. We now show that virus-derived cleaved GP but not uncleaved GP binds to a soluble, secreted, and biologically-active form of domain C (Fig. 5d).

2. We show that cleaved GP derived from a purified GP ectodomain can bind to the purified domain C protein. (Fig. 5e)

3. We show that this soluble domain C protein can inhibit infection by virus bearing cleaved GP but not uncleaved GP (Fig. 5f).

Re: claims 3 and 4: The mutant NPC proteins employed are large deletions (of complete loops A, C, or I) or constructs expressing the isolated loops. All deletion mutants show loss of cholesterol transport activity. Information is therefore needed to assess if the mutant NPC proteins are folded correctly. Are there antibodies to assess native NPC structure? Minimally the authors must do a better job assessing the subcellular localization of these constructs (see below).

Unfortunately, no conformation-specific antibodies against NPC1 are currently available. We have included the results of new microscopy experiments to address the subcellular localization of the domain-deletion and individual-domain mutants (Figs. S3 and S4). These images demonstrate that all of the mutants (except domain I-flag) localize to LAMP1-positive endo/lysosomal compartments. Domain I flag appears to be retained in the ER, indicating a profound folding/maturation defect (Fig. S4b). We describe these findings in the text and have removed binding and infection experiments done with this mutant from the revised manuscript.

Based on the images shown, it appears that WT NPC co-localizes with Lamp, but that the deletion constructs do not. Are the latter inside the endosomes? If so, comment on how do you think the delta A and delta I constructs work from within the endosomes.

Our improved images (Figs. S3 and S4) now demonstrate clear colocalization of the domain-deletion (and individual-domain mutants) with LAMP1.

RE: Fig. 4: Some features of the C-loop construct used need to be clarified:
a. From the text it appears that the C-loop construct used in Fig. 4c has the WT NPC cytoplasmic tail, which should target it to late endosomes. If so, how did this C-loop construct get onto VSV particles (which assemble at the cell surface)? A later figure aims to show that the C-loop construct with the WT tail is not present at the cell surface.

We now clarify this issue in the text. The domain C construct incorporated into VSV particles in Fig. 5a-c is actually a chimera containing domain C fused at its C-terminus to the VSV G transmembrane domain and cytoplasmic tail. It does not contain the NPC1 cytoplasmic tail.

b. Figures 4d and e show that the C-loop construct (presumably with the tail) supports infection when transfected into NPC null cells. To do this, the construct would presumably have to localize to (the limiting membrane of) late endosomes, but this was not assessed. A quantitative analysis of co-localization of each of the loop constructs used in Fig. 4 with Lamp should be provided.

As discussed above, we include new data demonstrating localization of the synthetic single-pass membrane proteins containing domain A or domain C (but not domain I) to LAMP1-positive endo/lysosomal compartments (Figs. S3 and S4).

Our central conclusion–that domain C is necessary and sufficient for GP-NPC1 binding and filovirus entry– does not require such quantitative localization data. Because NPC1 is absolutely essential for infection (no NPC1 = no detectable infection; e.g., Fig. 2c), we interpret any infection with a mutant construct as evidence that it contain the minimal machinery to act as a viral entry factor. We show that all of the entry-supporting mutants clearly localize to LAMP1-positive compartments. Just as important, two mutants that do not contain domain C (NPC1ΔC and domain A-flag) and cannot support viral entry also clearly colocalize to LAMP1-positive compartments. We fully agree that a detailed understanding of why the mutant proteins are suboptimal receptors will require the assessment of a number of factors, including quantitative differences in protein folding, maturation, localization, and GP binding. However, we feel that those studies are beyond the scope of the current manuscript.

Comment on why overexpression of NPC should rescue the infection defects of the 3Ala and F88A and F88H mutations (Fig. 5e, f). The binding studies (Fig. 5a, b) were done with the same NPC overexpressing cells and the mutants didn't bind to (overexpressed) NPC.

The infectivity assay is several orders of magnitude more sensitive than the binding assay, and the level of rescue obtained by NPC1 overexpression, while dramatic, still brings infection only up to the ~10-20% level relative to WT. Therefore, our current binding assay might not be sensitive enough to detect the reduced binding capacity of these mutants.

Should the first bar for the WT GP set be black?

We thank the reviewer for pointing this out and have changed the color of the bar.

5a. Better images should be provided (and quantitated) for the supplemental Figure (S7a) supporting Fig. 6. Why is there so much intracellular (perhaps Golgi) staining with WGA (done

before permeabilization)? Also, it's hard to discern what's going on with the C-loop construct with the WT tail; the image appears to show two small and rounded (possibly recently divided) cells. Provide an image of a more spread cell(s).

We have added new, improved images to demonstrate the plasma membrane localization of domain C-flag^{tailless} (now in Fig. S10a). We tried a variety of fixation and staining protocols to reduce Golgi localization of WGA in the CHO CT43 cells (presumably through uptake prior to permeabilization); however, we have been unable to eliminate it entirely. We believe it is a cell type-dependent phenomenon, since no such problems were encountered with the viper VH-2 cells (Fig. S2a). Nevertheless, we believe that the WGA staining in Fig. S10a clearly outlines the cell surface, and, together with the surface biotinylation experiment in Fig. S10b, clearly demonstrates that only the tailless domain C construct localizes extensively to the plasma membrane.

5b. As per comment 2, provide an analysis of how much of the C-loop constructs (with and without the tail) are in late endosomes.

We have added new images that assess the localization of domain C-flag and domain C-flag^{tailless} to LAMP1-positive compartments (Fig. S4). While some degree of LAMP1 localization is observed with domain C-flag^{tailless}, it appears reduced relative to domain C-flag (with the NPC1 cytoplasmic tail). Please see our response above regarding quantitative colocalization studies.

5c. Are the same cells shown in Fig. 6a as in S7a? If so, provide images from different cells.

The revised manuscript includes images of different cells in these figures (new Figs. 8 and S10).

6. At this stage a term other than "intracellular entry receptor" (used in title, Discussion and elsewhere) should be chosen for NPC. Calling it an entry receptor implies it has an active role in promoting fusion (or internalization if it were at the cell surface). But WT NPC is not a cell surface protein, and is not sufficient for fusion/entry (stated on p13).
7a. "synthetic membrane protein----has viral receptor activity": As above it is premature to term this as viral receptor activity.
b. "promotes viral membrane fusion": Nobody has shown that the GP-NPC interaction promotes fusion.
c. "long-sought filovirus receptor" (end Intro): the long sought after filovirus receptor is a cell surface protein.

We remain convinced that the term ‘entry receptor’ is appropriate because NPC1 (i) binds directly and specifically to the viral glycoprotein, and (ii) GP-NPC1 binding is absolutely required for entry. Neither of those facts obviate the possibility for an essential cell-surface receptor, nor do they directly show a role for NPC1 in fusion. Accordingly, we no longer refer to NPC1 as the ‘long-sought filovirus receptor’ and have modified the text to apply caveats regarding fusion.

8a. More information is needed on the reptilian cells. Do they bind and internalize GP particles? Do they have enough cathepsin and endosome acidifying activity? Do they express NPC protein? If so, how does the sequence of its C loop (or the C loop from any reptile or amphibian) compare with that of human/mammalian NPC?

We have included new data demonstrating that the entry block in the D. russellii VH-2 viper cells is at a post-internalization step (Fig. S2). We now also show that infection of reptilian cells by rVSV-GPs requires endosomal cysteine cathepsins (and therefore, endosome-acidifying activity) (Fig. 1e). Our filipin staining -/+ U18666A experiment (Fig. S2) provides inferential evidence for a functional NPC1-NPC2 cholesterol transport pathway in these cells. Unfortunately, we do not yet have an antibody that recognizes the reptilian NPC1 (commercially available NPC1 antibodies do not work).

Work to clone the viper NPC1 ortholog from VH-2 by RT-PCR is currently in progress, but the sequence is not yet available. We include below an alignment of NPC1 domain C sequences from humans and other vertebrates. This alignment shows a high degree of amino acid sequence identity for domain C among evolutionarily divergent vertebrates. We anticipate that the D. russellii NPC1 domain C will resemble that of the green anole lizard, Anolis carolinensis, the only reptile for which an NPC1 sequence is currently available. Human and green anole NPC1 domain C amino acid sequences are ~85% identical.

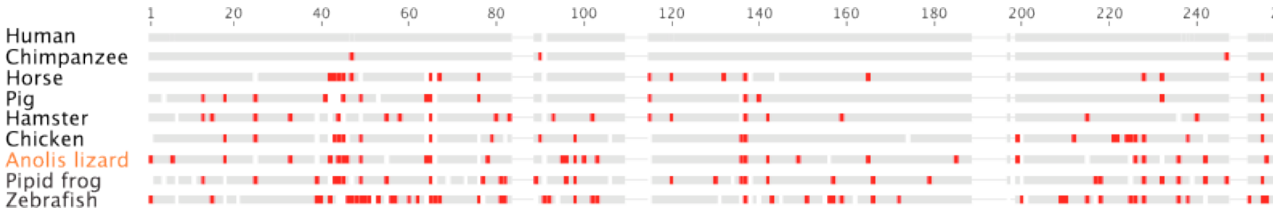

Fig. 1: Alignment of NPC1 domain C amino acid sequences from selected vertebrates. Red lines indicate amino acid changes relative to the human sequence.

b. Double check Takada. 1997. Did they test a reptilian or amphibian cell?

Neither Takada, 1997 nor Wool-Lewis, 1998 tested reptilian nor amphibian cell lines for susceptibility to EBOV GP-dependent entry.

c. Several minor points re: Fig. 3:

1. *3a legend: Wasn't the control (Ctrl) extract used in 3a, not 3b?*
2. *Should it read: Fig. 3b on p6, 7 lines from bottom?*
3. *At what pH were the co-IPs done in Fig. 3b (p6, 3 lines from bottom and in legend)?*
4. *Were descriptions of 3g and h swapped in the legend?*

We have corrected and clarified the Fig. 3 legend.

The co-IPs in Fig. 3b were carried out at neutral pH. This information has been added to the Fig. 3 legened.

The descriptions of Fig. 3g-h were indeed swapped. We have swapped the figure panels to correct this error.

Reviewer #2

The manuscript by Miller et al represents a well-constructed set of experiments to examine the role of NPC1 in Ebola virus infection. It fills many gaps in our knowledge of the exact role of NPC1 and its function in infection by this virus and the authors are commended for being thorough in their work. Overall the work is compelling and is very important for the filovirus field. Some issues are still present and it would help if they were corrected.

We thank the reviewer for their positive and constructive comments.

1. Figure 1 should be quantitated to give increase in titer after transfection of NPC1 plasmid. Ideally, the cells should have been stained for NPC1-flag to get an idea on transfection efficiency. Otherwise put one of the blots of NPC1-flag expression from the supplemental data into this figure (if same cells).

We now include quantitative data on viral infection in the viper cell lines (Fig. 1d-e). We have also included a blot of NPC1-flag expression in Fig. 1a. These lines stably express control vector or NPC1-flag (delivered by retroviral transduction, and selected with puromycin). Accordingly, most or all of the cells should express the transgene.

2. Do not describe wild type virus as "authentic". This is a non-standard nomenclature. It is "wild type" virus.

Altered as requested.

3. The expression levels of the NPC1 deletion mutants are in the supplemental data. They should be in Figure 2. Filipin staining is nice but expression data on the mutants is more important. Please see next comment on these blots. Staining the cells before infection would have been the best approach.

We have re-done the blots at lower exposures and included them in Fig. 2a, as requested by the reviewer.

4. Generally speaking, as well as in fig Supp3A, all the Western blots are grossly overexposed. This is very disappointing in what is otherwise a very well-performed set of experiments. Fig Supp3A has the blots that should be in figure 2. However, bands on the blot are way too dark to give any quantitation of differences in levels of expression except for the delta A mutant (which is very poorly expressed). Also, the levels of protein expression in the panel B immunofluorescence do not seem to correspond to those seen in panel A. In panel A, delta A is the faintest and the other proteins appear

as large smears (doublets? - if so please explain). In panel B (for some only a single cell is shown and so it is impossible to determine if it is representative) delta C is the weakest. Also, the staining pattern between the wild type protein and the deletion mutants is quite different with complete overlap with LAMP-1 obvious for the wild type while the deletion mutants appear luminal even though the expression on the blots are equivalent. Please explain these differences, add this to the paper and provide images with more transfected cells.

We thank the reviewer for pointing out the over-exposure issue and have re-done the blots as described in 3. above (Fig. 2a). We believe that they are now much improved and facilitate comparison of expression levels. Only one band is now observed for each protein at the lower exposure level. Thus, the additional bands represent minority species that were visualized out of proportion to their actual abundance as a result of blot over-exposure. They are likely glycoforms and/or break-down products.

The levels of protein expression in the immunofluorescence images do not seem to correspond to those seen in the western blots, because the individual cells chosen for microscopy had lower (or higher, for delta A) levels of protein than average. This was done to facilitate visualization of NPC1's subcellular localization, which was the sole purpose of the microscopy experiments.. The western blots reflect the abundance of NPC1 in the population of cells.

We have added new, improved images for NPC1-LAMP1 colocalization to the revised manuscript, which we believe demonstrate clear endo/lysosomal colocalization of each of the NPC1 domain-deletion constructs (Fig. S3).

5. Comment on the doublet bands present in Supp 3c. They may indicate some large change in the maturation of the mutant proteins compared to wild type and this could have a major impact on the interpretation of the work. Comment and include in paper.

Deglycosylation experiments indicate that the doublet bands observed for domain A-flag and domain C-flag represent alternate NPC1 glycoforms. However, new western blots for the individual-domain mutants (Fig. S4a) now reveal only one predominant band for each protein. For domain C-flag, this predominant species is the one that binds to GP (Fig. 4c-d). Thus, as described above the domain-deletion mutants, the additional forms constitute only a small minority of the total protein for each construct and were visualized out of proportion to their actual abundance as a result of blot over-exposure.

We do not believe that these minor heterogeneities in NPC1 glycosylation alter the fundamental conclusions of the paper regarding the central role of domain C, which are bolstered by inclusion of new data with a soluble domain C protein (Fig. 5d-f).

6. Please explain the quantitation of figure 3D. It says "%eGFP positive cells". This suggests a yes/no type of analysis with cells binding or not binding virus. If a cell binds a single virus and another binding 100 are considered the same then this skews the interpretation greatly. For this experiment it is more important to have a read out that incorporates information on binding affinity

of each GP construct. A simple way to do this would be to show the FACS data. The interpretation of the subsequent experiments is dependent on this analysis.

The GP-cell binding data (now in Fig. 7d) were acquired by flow cytometry, and %eGFP-positive cells were obtained with an eGFP analysis gate (i.e., this is not a 'yes/no' analysis). We have included the FACS data and a depiction of the analysis gate in the supplement (Fig. S8), as requested, along with a more detailed description of how the data were analyzed.

7. Please give some quantitative analysis of panel 3E. The images are again saturated and it is unclear what the total cell loading of virus is for the cell types and the mutant.
It is very unclear how the VSV-M release experiment with overexpression of NPC1 in Fig 5E really gives any information on how NPC1 controls membrane fusion as suggested on top of page 10. Please explain/clarify/remove.

We have reprocessed the images from the M-release expt to more clearly show the localization of the M protein in the different cell samples. Unfortunately, there is no ready way to quantitate the results of this assay, which provides a qualitative yes/no type analysis (punctate staining = no cytoplasmic delivery of virus; diffuse staining = cytoplasmic delivery of virus). Each cell type was infected with an MOI = 200 of each virus in this experiment; we have added this information to the Materials and Methods and Figure Legends.

We agree that the M-release experiment does not directly show that NPC1 is acting at the membrane fusion step. Instead, it allows a more limited conclusion that overexpression of NPC1 rescues NPC1-binding viral mutants at a post-binding step in entry. We have modified the text to more accurately reflect this limited interpretation.

8. Figure 7 does not add much information to the paper or to the field and should probably be removed. Figure 7 suggests that fusion occurs in late endosomes after contact with NPC1. There is no evidence in this paper or other papers that support this conclusion/assumption. A small amount of NPC1 could be present in early endosomes and this may be the site of fusion (keep in mind that delta A is very poorly expressed and yet supports infection indicating little protein is needed). Either remove this figure or modify it so the figure ends at contact with NPC1 in the endosome and question marks follow this step leading to fusion.

We agree with the reviewer that there is no direct evidence supporting obligate fusion of virus in late endosomes, or for a direct role of NPC1 in fusion. We have modified the model (Fig. 9) to indicate these ambiguities.

Reviewer #3

This is an excellent manuscript that builds upon earlier work reported in Nature, by now providing the definitive illustration that NPC1 is an essential filovirus receptor. There is thorough analysis

demonstrating that: nonpermissive cell lines can be rendered permissive by transfection with NPC1, that NPC1 specifically reacts with endosomally and enzymatically cleaved GP, that it reacts with cleaved GP from different filoviruses, that point mutations previously shown to be important for binding and entry are important for binding NPC1, that only NPC1 and not the 50% identical NPC1L1 is functional for filovirus entry, and that domain C is the key portion of NPC1 as illustrated by binding and infection studies.

We thank the reviewer for their enthusiasm for this work.

1. Page 13 It might be appropriate to add in some discussion of the possibility of filoviruses achieving initial entry through macropinocytosis in the absence of any specific attachment factor.

We have added a discussion of this possibility to the revised manuscript (Discussion).

2. Page 12 I wonder if the remodeling of GP truly is a way of avoiding humoral immune responses. It is a quite acute infection. Of course, what happens in the natural host is likely quite different and the massive, mucin-like domains do lead to production on non-neutralizing antibodies.

We thank the reviewer for this comment and idea. We have qualified our speculation by restricting to 'natural hosts' (Discussion).

3. point mutants: It would be good to say somewhere that it is still unclear if mutations like F88A are detrimental to binding because they remove a key contact or because the structure is somehow disrupted.

This possibility is now indicated in the Results ('The NPC1-binding site in GP is recessed ... and is unmasked by proteolytic cleavage' section).

4. Figure 1. The legend is a bit confusing. Perhaps rewrite as "Human NPC1 confers susceptibility to filovirus entry and infection. Viper VH-2 cells expressing empty vector or human NPC1 were infected with either (a) filoviruses or (b) recombinant VSIV..."

We have modified the text as the reviewer has suggested, and we hope it now reads more clearly.

5. Figure 2. Infection of I-deletions is weaker than A-deletions. Is there any evidence that domain C would interact with domain I, so hat I might be partially involved in infection? Or are NPC1 with deletions of domain I less stable or less highly expressed. Are relative expression levels of full-length NPC1, and domain A-, C-, and I-deletions shown anywhere?

We have incorporated new expression blots of full-length NPC1 and the domain-deleted proteins directly into Fig 2. Delta I is as well expressed as delta C, and much better expressed than delta A

and WT. Delta I may poorly support infection because loop I makes a (non-essential) direct contribution to NPC1's viral receptor function, and/or because deletion of loop I affects protein folding, maturation, and localization. However, our current findings do not differentiate between these possibilities.

6a. Figure 3. There are quite a few separate ideas in this figure, and I'm wondering if it would be possible to separate them into different figures. I don't think it would take up any more journal page space, but would make working through the legend much easier. (a) and (b) are about NPC1 and GP pulling each other down. (c-f) could be another figure saying LPC1L1 will not suffice. (i-j) seem repetitive with Figure 4 until one realizes that Figure 3 is about binding , while Figure 4 is about infection. The authors could either clarify this a little more immediately in the legend or rearrange the Figure panels so binding and infection were in the same figure.

We thank the reviewer for this suggestion. We have modified the figures to improve their clarity and coherence, as suggested by the reviewer. Specifically, we have split up the old Fig. 3 as recommended.

b. Figure 3b: What are the asterisks, and the identity of the band to which they correspond?

The asterisks indicate protein bands that are nonspecifically detected by the anti-GP1 antiserum used in these experiments. The legend previously omitted a description of these asterisks and has now been corrected.

c. Figure 3 g-h: are these panels switched?

Yes, the panels were accidentally switched. We thank the reviewer for catching the error and have corrected it.

7. Figure 5 a-d are about the Lys and Phe mutations and not about functioning of NPC1 late in entry. I wonder if panels a-d should be a separate figure or grouped in with other binding studies.

We thank the reviewer for this suggestion. Unfortunately, we are already at 9 figures and do not have space to break up this figure.

8. Figure 7. Perhaps the blue arrowheads in (a) could be colored red so they stand out better. I think also you could label the grey membrane differently in the cartoon so it is clear that domain C with tail is on the endosomal membrane while tail-less C is on the plasma membrane.

We have adopted both suggestions in the new Fig. 8.

2nd Editorial Decision 19 January 2012

Thank you for sending us your revised manuscript.

Referee 1 and 2 have now evaluated the manuscript again, and you will be pleased to see that they now support publication of the study here. You may still wish to look into the minor comments put forward by referee 1 and address them by text changes. Please follow the point raised with respect to the term 'entry receptor' at this point.

Furthermore, there are a number of editorial issues that need further attention prior to acceptance:

* Please add statistical details including the number of independent repeats into the legends of all figures with numerical data (Fig.1d/e, 2 c/d, 3 c/e/h, 4 b/d, 5 c/d/f, 6b, 7 b/d/e, 8 c/d, S2b

* Please add scale bars and explanations to figures 6 and S2d

* Please clarify in the legends for the following figure panels whether all lanes come from the same gel: 2a, 3b, 4 a/c, 7a, S5

* We now encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Would you be willing to provide files comprising the original, uncropped and unprocessed scans of all gels used in the figures? We would need 1 file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as a supplementary "Source Data". Please let me know if you have any questions about this policy.

Thank you very much for your kind cooperation.

We are looking forward to receiving your amended manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

This ms. has been clarified and improved. I have only one remaining major comment.

Major Comment

NPC1 is clearly important for Ebola entry and you have provided the strongest evidence that the Ebola glycoprotein (GP) not only binds to NPC1, but also binds to a very specific loop in NPC1. But your evidence (p15, top) suggests that this interaction is not sufficient to trigger the fusion activity of (cathepsin cleaved) GP. Hence, it is premature to call NPC1 an entry receptor. Using the terms viral receptor or filovirus receptor, as done in many places, is appropriate. According to current definitions, if an entry receptor is not used for endocytosis from the cell surface (which NPC1 does not do), then it is a protein that is actively involved in triggering viral penetration, fusion for an enveloped virus (see for example the Introduction to a recent review by Backovic and Rey. 2011. Curr. Opin. Virol.). A few simple text changes will take care of this:

Title: delete 'entry'
Abstract, line 5: delete 'viral entry by'
Discussion, P1, last line, delete 'entry'

Minor Comments

1. For the co-IP experiments (e.g. Figs. 3a,b; 4a,c; 5e): indicate in the Methods sections or legends how much of the supernatants have been loaded on the gels.
2. Fig. 8 legend: Reword the statement on lines 5-7. There's a considerable amount of tail-less C loop inside the cell as well as on the cell surface (e.g., Figs. 8A, S4, and S10).

Referee #2

The authors have addressed all concerns of this reviewer. The paper is much improved and is a valuable contribution to the field.

2nd Revision - authors' response 02 February 2012

Responses to reviewers' comments

Reviewer #1

This ms. has been clarified and improved. I have only one remaining major comment.

We thank the reviewer for their insightful comments. and are pleased that they find our revised manuscript 'clarified and improved'.

NPC1 is clearly important for Ebola entry and you have provided the strongest evidence that the Ebola glycoprotein (GP) not only binds to NPC1, but also binds to a very specific loop in NPC1. But your evidence (p15, top) suggests that this interaction is not sufficient to trigger the fusion activity of (cathepsin cleaved) GP. Hence, it is premature to call NPC1 an entry receptor. Using the terms viral receptor or filovirus receptor, as done in many places, is appropriate. According to current definitions, if an entry receptor is not used for endocytosis from the cell surface (which NPC1 does not do), then it is a protein that is actively involved in triggering viral penetration, fusion for an enveloped virus (see for example the Introduction to a recent review by Backovic and Rey. 2011. Curr. Opin. Virol.). A few simple text changes will take care of this.

Title: delete 'entry'
Abstract, line 5: delete 'viral entry by'
Discussion, P1, last line, delete 'entry'

We have revised the title to remove the juxtaposition of 'entry' and 'receptor'. However, we would prefer to retain the word 'entry' in the title to make clear the phase of the viral life cycle under examination in this study. We have made the other text changes requested by the reviewer.

For the co-IP experiments (e.g. Figs. 3a,b; 4a,c; 5e): indicate in the Methods sections or legends how much of the supernatants have been loaded on the gels.

The requested information is now included in Legends.

2. Fig. 8 legend: Reword the statement on lines 5-7. There's a considerable amount of tail-less C loop inside the cell as well as on the cell surface (e.g., Figs. 8A, S4, and S10).

We have reworded the Fig. 8 legend to emphasize the plasma membrane localization of each protein.

Reviewer #2

The authors have addressed all concerns of this reviewer. The paper is much improved and is a valuable contribution to the field.

We thank the reviewer for helping us improve the manuscript and are gratified that they find it a 'valuable contribution to the field'.

