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Structural basis of membrane budding by the nuclear egress complex of herpesviruses

Janna M. Bigalke and Ekaterina E. Heldwein

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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 17 August 2015

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by three referees who also reviewed the related Lye et al. manuscript.

As you can see below, the referees appreciate your analysis and find that it provides a major advance in our understanding of virus nuclear egress. I would therefore like to invite you to submit a suitable revised manuscript. The referees raise a number of specific concerns that I anticipate you should be able to sort out. You can use the link below to upload the revised version. Let me know if we need to discuss the point in more details.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://emboj.embopress.org/about#Transparent_Process

A similar decision was reached for the Lye et al submission. Could you please coordinate with James Hogle and colleagues so that we get the revised versions back around the same time - thanks!

REFEREE REPORTS

Referee #1:

Bigalke and Heldwen describe crystal structures of the nuclear egress complexes from the herpesviruses, pseudorabies virus (PRV) and herpes simplex virus-1 (HSV-1). The structure reveal the folds of the UL31 and UL34 subunits, and the structure of the UL31-34 dimer. Together with the structure of MCMV M50 from Leigh et al., and the companion HCMV paper, we now have multiple

views of herpes viral NEC subunits and complexes. The overall similarities of these structures (together with the high technical quality of the work described here), leaves little doubt that the structural biology being reported here is fundamentally correct. Uniquely this work also reveals how the HSV-1 NEC complex can assemble into hexagonal coats. This is a major advance, and it is made more interesting (but also more complicated) by the fact that two crystallographically distinct hexagonal lattices are observed within the crystal, which comprise similar hexamers, but distinct interhexamer packing interactions. The functional relevance of the two distinct lattices is not resolved in this study, but one possible explanation is that plasticity in NEC assembly may be required to accommodate inequivalent regions of membrane curvature in budding HSV-1 nuclear egress coats. Despite this conformational complexity, there seems to be little doubt that at least one of the crystallographic hexagonal lattices are fundamentally similar to authentic, membrane-associated HSV-1 NEC coats owing to the nice match to previously reported cryo-EM images of HSV-1 NEC coats, and because mutational analyses are consistent with the functional importance of the hexagonal lattice contacts in a GUV budding assay (though such analyses are always challenging given the density of interactions in such a complex lattice). The functional assays have not been extended to studies of authentic viruses, but I do not view this as a significant shortcoming (nor do I think the authors should be required to do this).

Overall, this is a high quality study that represents a major advance in our understanding of herpesvirus nuclear egress.

Minor issues for the author's consideration:
1) Suggest noting in the captions to Fig. 4 and 5 that these are HSV-1 NEC complexes.
2) Given that the authors predict that E37(34) alone is respolsible for the loss of in vitro budding from the D35A/E37A (lines 335-341), I found it surprising that the authors didn't actually test this hypothesis. It's rather rare when single point mutations can functionally disrupt such a lattice, and this experiment would have been a compelling test of the importance of the hexamer.
3) I think there is a typo in line 414 - Should it be "This would implicate helix alpha4 AS an important regulatory..."
4) The authors spend quite a bit of time talking about possible autoinhibitory mechanisms that prevent NEC assembly until the proper time, which is fine (albeit rather speculative). However, they may want to point out that simply binding to a spherical mature capsid, with multiple binding sites that would create avidity effects, could provide the major driving forces for formation of an enveloping vesicle composed of a coat comprising extended patches of NEC hexamers.

Referee #2:

This manuscript describes the crystal structure of a large fragment of the nuclear export complex (NEC) of the alpha-herpesviruses herpes simplex virus type 1 (HSV-1) and pseudorabies virus (PrV). This complex is formed by proteins UL31 and UL34, which form a heterodimer. Previous studies had shown that these two proteins alone are sufficient to drive vesiculation in vitro, forming a hexagonal array that induces negative curvature on membranes. Importantly, they show that in the crystals of the HSV-1 NEC, they obtain two hexagonal lattices with very similar parameters to those observed underneath the membranes of vesiculated compartments, indicated that they have captured relevant hexameric interactions. Furthermore, the authors go on to test by site directed mutagenesis the residues involved in the interactions observed in the crystals, showing that vesiculation is indeed impaired when the hexameric contacts are affected. The NEC was also shown to form a coat underneath the inner nuclear membrane (INM), and interact also with the capsid protein in order to envelope it and make it bud into the periplasmic space. Subsequent fusion between the resulting vesicle and the outer nuclear membrane (ONM) - in a step that is not yet understood mechanistically - results in release of the capsids into the cytoplasm, to then undergo secondary envelopment to make infectious virions. The current structures shed light into the initial steps, which involve oligomerization of the NEC underneath the INM and concomitantly induce curvature. How a hexagonal lattice results in a highly curved and closed compartment is not understood, but the authors show an important variation in hexamer-hexamer contacts in the two superposed hexagonal lattices making the crystals. This variation in packing of hexamers could hold a clue to understand how they induce curvature (in the crystals, the hexagonal lattices are of course flat). This is a very important paper and provides profound new insight into the organization of these special proteins; it

is totally appropriate for the EMBO Journal. The statistics show that the structures are of good quality and the refinement was correctly done, although the difference between R and Rfree values is a bit too large for my taste. The manuscript is overall well written, but I have a few comments that I think will help make it more accessible to the general reader of the EMBO Journal;

1 - The section entitled "UL31 and UL34 interact extensively through multiple regions" (lines 183 to 260, two pages in double-spaced format) is way too detailed and quite difficult to follow. It is very difficult for the reader to follow in detail the description of all the mutations that have been seen in the past to affect NEC function, where these residues are and how the interactions observed in the crystal may explain the observed effect. I think the manuscript would gain in clarity if there was a supplementary table listing all of those mutations, their effect and what is the location and the interactions of those residues as seen in the structure, and then in the main text refer to this table and just say that the structure explains why they are essential. Otherwise it becomes tedious to read, which is a shame for a paper that actually is quite exciting in its overall content. The whole paragraph could be cut at least in half. In particular, in lines 245-248, in the description of the effect of mutations in E. Coli and in eukaryotic cells, it is very unclear as written. Apparently the authors mean that the membrane anchored UL34 requires a signal peptide to become anchored in the ER membrane, from where it reaches the INM (I'm not sure I understood correctly here, since this would lead to UL34 anchored in the ONM by lateral diffusion, not the INM...). And that UL31 gets made in the cytoplasm and has an NLS to reach the nucleus. It would then associate with UL34 anchored in the INM. All this requires a much fuller explanation to be clear to an average reader of the EMBO journal.

2- In the paragraph "Hexagonal lattice in HSV-1 NEC crystals resembles NEC coats", the authors fail to explain clearly the actual packing in these crystals. Apparently, the two hexameric rings are right on top of each other, but the hexamer is rotated about the 6-fold axis of the crystal by about 10.5{degree sign}, such that the lateral interactions between hexamers are different. But the reader has to guess thist, there is no clear explanation in the text. Also, they do not say if the rings have the UL31/UL34 complex in the same orientation along the crystal c axis, or if they are head to tail. Presumably the hexamers rings do not make favorable packing interactions if they are placed oriented identically on top of each other, hence this rotation. But this means that the lateral packing of the rings is very flexible, and they could interact in more than just the two ways observed in the crystal, which may actually be dictated by the packing along the 3rd dimension. All of this is extremely interesting, and the authors could spend more space describing this if they shorten the previous paragraph as explained above.
Also, in Fig 4A, it is important to mark which side corresponds to the membrane proximal region of the assembly in the micrograph shown in the bottom panel.

3 - In the paragraph "Analysis of interactions within the honeycomb crystal lattice", apparently there are no UL31/UL31 interactions within the hexamer. But this only indirectly said because the authors write that the contacts are UL34/UL34 and UL34/UL31. The fact that there are no direct UL31/UL31 interactions is interesting since it may also have functional implications. So perhaps the paragraph should be re-written to highlight this observation.
Minor issues: The authors find that UL31 and UL34 have "novel folds", but find DALI scores of about 3.8. In certain cases, a DALI score of 3.8 can be significant (the scores depend also in the size of the compared proteins). To have an idea of how high the scores can be, it would be interesting to provide the DALI scores between the HSV-1 and PRV counterparts, and also between the two independent copies of the NEC proteins within the same crystals (this should be the highest possible DALI score for a protein of this size). This would be a way to normalize. It would also be interesting to see the score obtained when comparing to the CMV NEC proteins, and would put the protein giving a score of 3.8 in a better context (it would be useful to mention what proteins it is, given that the accompanying manuscript did find homology to proteins having a Bergerat fold).
In the various cartoons of the NEC proteins provided in the figures, it would help if the broken loops were connected by dotted lines, to allow the reader to follow the connectivity.
In line 417, when talking about M50, the MCMV homolog of UL34, they use the term "an analogous helix". Since the two proteins are homologs, the most likely is that the helix is homologous not analogous (and these terms have different specific meaning). But the problem would be overcome if the authors used the term "a corresponding helix".
Finally, a very minor point: in my view a virus buds across a membrane and into a different compartment (for instance, across the ER membrane and into the ER lumen). Here, the particles but

across the INM and into the perinuclear space (which is continuous with the ER lumen). So the particles do not bud "into the INM", as the authors explain in the introduction, but across it and into the perinuclear space.

Referee #3:

Overview:

The manuscript by Bigalke and Heldwine looks at the structural basis of herpesvirus capsid assembly, focusing on the nuclear egress complex (NEC) composed of the conserved viral proteins, UL31 and UL34. This group has shown that purified NEC can bud synthetic membranes in vitro, and so determination and analysis of the structures of these two proteins and the complex that they form represent important milestones in understanding of herpesvirus morphogenesis. The authors present structures of NEC from two different viruses, and so the study very nicely demonstrates the extent of conservation of the assembly/budding mechanism, at least in alpha-herpesviruses. A good part of the manuscript is dedicated to exhaustive description of the structures, which appear to be novel folds. The most interesting aspect of the manuscript is that the HSV-1 NEC crystallized in a hexagonal space group. By comparing the crystallographic lattice with coats observed by cryoEM, the authors make a viable argument that the crystal lattice they observe recapitulates the honeycomb lattice formed by the NEC in authentic viruses. While the structural analysis seems solid, the experimental validation of the crystallographically observed lattice contacts needs some strengthening. There is also uncertainty on which of the two observed hexagonal lattices are biologically relevant.

Major points:

My opinion is that the most important aspect of this manuscript is the suggestion that the crystallographic hexagonal lattices formed by HSV-1 NEC recapitulates the viral lattice. However, the analyses performed on the interhexamer and interhexamer mutants are quite limited and could be strengthened. Both the membrane binding and budding assays are key experiments in this paper, so the raw data should be presented as main figures. This is especially important since the P values do not make sense from the apparent differences in bar height in the graphs.

According to Fig. 7, any budding defects that manifest from the mutations could arise from problems in at least 3 prior steps - ideally, the authors should do an assay that directly tests for lattice formation. The authors should seriously consider this as there is disagreement as to the requirements for budding/vesiculation in vitro.

While some of the very detailed structural description is appropriate, it is quite hard to follow descriptions of salt bridges and hydrogen bonds (pages 9-12) in the absence of illustration.

The authors discuss many instances of mutations whose functional phenotypes arise from a misfolding defect; it is important to note that this is a presumption based on the structural models, and not truly experimentally demonstrated. Along these lines, it seems important to do a stability/folding assay on the intrahexamer and interhexamer mutants to rule out this possibility.

The NEC was observed to make two kinds of hexagonal lattices (swapping 2-fold and 3-fold symmetric contact regions), and the authors suggest that this may be relevant to forming curved lattices. This does not really make sense (at least not to this reviewer), since both the A/B and C/D lattices are flat.

Is there any evidence that NEC can form discrete hexamers as depicted in Fig 7? What if the "building blocks" are trimers or dimers?

The section on the relevance of the C-terminal helix present in PRV but not in HSV-1 is better suited for the Discussion section.

Other points:

I suggest rephrasing some of the structural description to make them more accessible to non-specialists: e.g., "The crystallized HSV-1 NEC is missing residues M1-K50 ..." (line 118) can be re-written as "The crystallized HCV-1 NEC is missing the first 50 amino acid residues ... which are necessary for membrane interactions."

Figs. 2A and 2B: it would be useful to include in these panels information on the mutagenesis data; e.g., dots or shading can be used to illustrate mutation sites and phenotypes

Figure 2C: it will be useful to indicate the relationship between the main panel and the inset showing Zn-coordination

The discussion in page 16 does not really explain why the D35A/E37A mutant is dominant negative.

The information in Figs. 1 and 2 and Figs. 5 and 6 can be combined into a single figure.

1st Revision - authors' response 05 September 2015

Referee #1:

Minor issues for the author's consideration:

1) Suggest noting in the captions to Fig. 4 and 5 that these are HSV-1 NEC complexes.

We have changed the legends accordingly.

2) Given that the authors predict that E37(34) alone is responsible for the loss of in vitro budding from the D35A/E37A (lines 335-341), I found it surprising that the authors didn't actually test this hypothesis. It's rather rare when single point mutations can functionally disrupt such a lattice, and this experiment would have been a compelling test of the importance of the hexamer.

We agree completely and have tested the NEC containing a single E37A mutation in our budding assay. As expected, this mutation was sufficient to disrupt the NEC budding activity and likely solely accounts for the non-budding phenotype of the double mutant D35A/E37A. We have included this result in Fig. 6C and in the corresponding text on lines 353-355.

3) I think there is a typo in line 414 - Should it be "This would implicate helix alpha4 AS an important regulatory..."

We have corrected the error, see lines 510-511.

4) The authors spend quite a bit of time talking about possible autoinhibitory mechanisms that prevent NEC assembly until the proper time, which is fine (albeit rather speculative). However, they may want to point out that simply binding to a spherical mature capsid, with multiple binding sites that would create avidity effects, could provide the major driving forces for formation of an enveloping vesicle composed of a coat comprising extended patches of NEC hexamers.

We agree with the reviewer that the assembly of the NEC coat in infected cells may be triggered by the capsid. We have included this alternative triggering mechanism in the revised manuscript on lines 543-545: "A mature capsid, with multiple binding sites for the NEC that would create avidity effects, could provide a major driving force for the formation of an enveloping vesicle containing a coat composed of extended patches of NEC hexamers."

Referee #2:

1 - The section entitled "UL31 and UL34 interact extensively through multiple regions" (lines 183 to 260, two pages in double-spaced format) is way too detailed and quite difficult to follow. It is very difficult for the reader to follow in detail the description of all the mutations that have been seen in the past to affect NEC function, where these residues are and how the interactions observed in the crystal may explain the observed effect. I think the manuscript would gain in clarity if there was a supplementary table listing all of those mutations, their effect and what is the location and the interactions of those residues as seen in the structure, and then in the main text refer to this table and just say that the structure explains why they are essential. Otherwise it becomes tedious to read, which is a shame for a paper that actually is quite exciting in its overall content. The whole paragraph could be cut at least in half. In particular, in lines 245-248, in the description of the effect of mutations in E. Coli and in eukaryotic cells, it is very unclear as written. Apparently the authors mean that the membrane anchored UL34 requires a signal peptide to become anchored in the ER membrane, from where it reaches the INM (I'm not sure I understood correctly here, since this would lead to UL34 anchored in the ONM by lateral diffusion, not the INM...). And that UL31 gets made in the cytoplasm and has an NLS to reach the nucleus. It would then associate with UL34 anchored in the INM. All this requires a much fuller explanation to be clear to an average reader of the EMBO journal.

We thank the referee for this helpful suggestion. We have shortened this paragraph (lines 238-244) and have moved the detailed description of previously described mutants, their phenotypes, and the locations of the mutated residues to table S IV.

2- In the paragraph "Hexagonal lattice in HSV-1 NEC crystals resembles NEC coats", the authors fail to explain clearly the actual packing in these crystals. Apparently, the two hexameric rings are right on top of each other, but the hexamer is rotated about the 6-fold axis of the crystal by about 10.5{degree sign}, such that the lateral interactions between hexamers are different. But the reader has to guess this, there is no clear explanation in the text. Also, they do not say if the rings have the UL31/UL34 complex in the same orientation along the crystal c axis, or if they are head to tail. Presumably the hexamers rings do not make favorable packing interactions if they are placed oriented identically on top of each other, hence this rotation. But this means that the lateral packing of the rings is very flexible, and they could interact in more than just the two ways observed in the crystal, which may actually be dictated by the packing along the 3rd dimension. All of this is extremely interesting, and the authors could spend more space describing this if they shorten the previous paragraph as explained above.

Also, in Fig 4A, it is important to mark which side corresponds to the membrane proximal region of the assembly in the micrograph shown in the bottom panel.

We have included a detailed description of the crystal packing on lines 252-265 and 316-321 , which is illustrated in the new Figure EV4. We agree with the referee that the two modes of hexamer packing may be dictated by the hexamer stacking along the c dimension and that this means that the lateral packing of the rings is very flexible, and they could interact in more than the two ways observed in the crystal.

We have also included labels in Fig. 4A for the presumed membrane-proximal and membrane-distal region of the lattice.

3 - In the paragraph "Analysis of interactions within the honeycomb crystal lattice", apparently there are no UL31/UL31 interactions within the hexamer. But this only indirectly said because the authors write that the contacts are UL34/UL34 and UL34/UL31. The fact that there are no direct UL31/UL31 interactions is interesting since it may also have functional implications. So perhaps the paragraph should be re-written to highlight this observation.

We now state that UL31/UL31 interactions are not involved in the hexamer formation, but only mediate contacts between individual hexamers on lines 300-302.

Minor issues: The authors find that UL31 and UL34 have "novel folds", but find DALI scores of about 3.8. In certain cases, a DALI score of 3.8 can be significant (the scores depend also in the size

of the compared proteins). To have an idea of how high the scores can be, it would be interesting to provide the DALI scores between the HSV-1 and PRV counterparts, and also between the two independent copies of the NEC proteins within the same crystals (this should be the highest possible DALI score for a protein of this size). This would be a way to normalize. It would also be interesting to see the score obtained when comparing to the CMV NEC proteins, and would put the protein giving a score of 3.8 in a better context (it would be useful to mention what proteins it is, given that the accompanying manuscript did find homology to proteins having a Bergerat fold).

We have performed Dali pairwise structural alignment of the two NCS mates of HSV-1, HSV-1 vs. PRV, HSV-1 vs. CMV, and HSV-1 and the top hit in the Dali search, the ATP-binding domain of the histidine kinase response regulator DosS, PDB ID 3ZXO. The highest possible Z-score for UL31 and UL34 molecules was, unsurprisingly, for the two HSV-1 NCS mates (35.6 and 30.3, respectively), but the Z-scores were also high for PRV vs. HSV-1 (30.1 for UL31 and 24.5 for UL34) and CMV vs. HSV-1 (24.2 for UL31 and 14.7 for UL34). By contrast, the Z scores for HSV-1 UL31 vs. DosS and UL34 vs. DosS were 3.9 and 3.4, respectively. Notably, the HCMV homologs of UL31 and UL34, UL53 and UL50, have a higher similarity to DosS, with Z scores of 6.0 and 4.0. While HSV-1 UL31 and UL34 incorporate the Bergerat fold characteristic of the GHKL ATPase/kinase superfamily, both proteins contain an additional β-strand between the second strand and the second helix of the classic Bergerat fold. We have included a more thorough discussion of the DALI search results and the non-conventional Bergerat-like fold in the main text on lines 145-158 and 181-189.. We have also changed Figure S2 to compare the Bergerat-like folds of HSV-1 UL31 and UL34 with the classic Bergerat fold of DosS.

In the various cartoons of the NEC proteins provided in the figures, it would help if the broken loops were connected by dotted lines, to allow the reader to follow the connectivity.

We have included the connectors in all main figures and EV figures.

In line 417, when talking about M50, the MCMV homolog of UL34, they use the term "an analogous helix". Since the two proteins are homologs, the most likely is that the helix is homologous not analogous (and these terms have different specific meaning). But the problem would be overcome if the authors used the term "a corresponding helix".

We have changed the term to "corresponding" (line 513).

Finally, a very minor point: in my view a virus buds across a membrane and into a different compartment (for instance, across the ER membrane and into the ER lumen). Here, the particles bud across the INM and into the perinuclear space (which is continuous with the ER lumen). So the particles do not bud "into the INM", as the authors explain in the introduction, but across it and into the perinuclear space.

We have changed the text throughout the manuscript to follow the convention: "bud at membrane, into a compartment", which is more common in the relevant literature.

Referee #3:

Major points:

My opinion is that the most important aspect of this manuscript is the suggestion that the crystallographic hexagonal lattices formed by HSV-1 NEC recapitulates the viral lattice. However, the analyses performed on the interhexamer and interhexamer mutants are quite limited and could be strengthened. Both the membrane binding and budding assays are key experiments in this paper, so the raw data should be presented as main figures. This is especially important since the P values do not make sense from the apparent differences in bar height in the graphs.

We have included the raw data in the supplement (table S V). We have also added data for several additional mutants, which strengthen our hypothesis, in our opinion. These mutants are $E37A_{34}$, $V92F_{34}/R229L_{31}$, $R229L_{31}$, $R49A_{34}$, $V247F_{31}$ and $L142E_{31}$. Mutations $E37A_{34}$, $R49A_{34}$, and $V92F_{34}$

presumably interfere with the hexamer formation, while mutations $V247F_{31}$ and $L142E_{31}$ interfere with the hexameric packing, and mutation $R229L_{31}$ strengthens the hexameric packing (lines 402-419).

According to Fig. 7, any budding defects that manifest from the mutations could arise from problems in at least 3 prior steps - ideally, the authors should do an assay that directly tests for lattice formation. The authors should seriously consider this as there is disagreement as to the requirements for budding/vesiculation in vitro.

We thank the referee for raising this point and agree that our budding assay does not directly measure the NEC lattice formation. We have shown, however, that our mutations do not interfere with either the formation of the NEC or with membrane binding. So, the defect is most likely due to a problem in lattice formation. This explanation is supported by our previous work (Bigalke et al., 2014), where using cryoEM, we showed that formation of the honeycomb lattice was disrupted in the DN mutant, which suggested that this is the reason for the non-budding phenotype. While the direct analysis of lattice formation is important, it is technically challenging and beyond the scope of this manuscript. Lattice formation will be analyzed in detail in future work.

While some of the very detailed structural description is appropriate, it is quite hard to follow descriptions of salt bridges and hydrogen bonds (pages 9-12) in the absence of illustration.

We have included an illustration of the salt bridges at the NEC interface as the new Figure S3.

The authors discuss many instances of mutations whose functional phenotypes arise from a misfolding defect; it is important to note that this is a presumption based on the structural models, and not truly experimentally demonstrated. Along these lines, it seems important to do a stability/folding assay on the intrahexamer and interhexamer mutants to rule out this possibility.

All of the mutants generated in our work were tested for correct folding in two ways. First, we tested for NEC formation. This is important, because we know that neither protein (UL31 or UL34) is stable by itself. They cannot be produced separately in our hands and tend to aggregate or get degraded. So, complex formation is a good indicator for correct folding of UL31 and UL34. In addition, we tested all mutants for membrane binding. We doubt that membrane interaction would be possible with misfolded NEC. Last, but not least, all mutations were restricted to solvent exposed residues that should not play a role in structural stability.

Regarding NEC mutations reported in the literature, we agree with the referee that our conclusion that some of the non-functional mutants are misfolded is based on structure analysis alone. Confirming that these mutations indeed cause protein misfolding would require biochemical analysis similar to what was described above. Testing these mutations is beyond the scope of this manuscript, however. Therefore, we limit our conclusions in this case to stating on lines 243-244: "These … mutants may be defective in NEC formation due to protein misfolding".

The NEC was observed to make two kinds of hexagonal lattices (swapping 2-fold and 3-fold symmetric contact regions), and the authors suggest that this may be relevant to forming curved lattices. This does not really make sense (at least not to this reviewer), since both the A/B and C/D lattices are flat.

We agree with the reviewer that a strictly hexagonal lattice is, indeed, flat. To obtain a curved or spherical lattice there need to be disruptions in the hexagonal lattice. This can be achieved by introducing regular lattice disruptions in the form of pentamers as is seen in icosahedral viral capsids (or soccer balls) or by introducing irregular disruptions. The ability of the NEC hexamers to pack differently in the two lattices observed in the crystals suggests that there is flexibility in how the hexamers can be arranged. And this flexibility could be a way for introducing lattice disruptions, so that a perfect but flat hexagonal lattice becomes an imperfect curved lattice. We have modified our model in Figure 7 to illustrate this hypothesis and have included a more detailed explanation in the manuscript on lines 330-333 and 476-483.

Is there any evidence that NEC can form discrete hexamers as depicted in Fig 7? What if the "building blocks" are trimers or dimers?

The NEC does not oligomerize in solution. We do not know whether the NEC can form discrete hexamers or whether such hexamers immediately pack into an array, but as the hexamers are the same in both crystal lattices, we hypothesize that the hexamers are the building blocks. The dimers and the trimers differ between the two crystal lattices, which argues against them being the building blocks. Furthermore, the interactions within dimers or trimers are much weaker, involve fewer residues and bury a smaller interaction surface than those in hexamers. Thus, we propose that the hexamer should be considered the building block of the lattice. We have commented on this in various sections of the manuscript.

The section on the relevance of the C-terminal helix present in PRV but not in HSV-1 is better suited for the Discussion section.

We have moved this section to the discussion, lines 495-516.

Other points:

I suggest rephrasing some of the structural description to make them more accessible to non-specialists: e.g., "The crystallized HSV-1 NEC is missing residues M1-K50 ..." (line 118) can be re-written as "The crystallized HCV-1 NEC is missing the first 50 amino acid residues ... which are necessary for membrane interactions."

We have rephrased this sentence, see line 120.

Figs. 2A and 2B: it would be useful to include in these panels information on the mutagenesis data; e.g., dots or shading can be used to illustrate mutation sites and phenotypes

We have highlighted mutated residues according to the phenotypes of the mutant NECs (see figure legend 2).

Figure 2C: it will be useful to indicate the relationship between the main panel and the inset showing Zn-coordination.

We have included this in the figure.

The discussion in page 16 does not really explain why the D35A/E37A mutant is dominant negative.

E37 is located at the hexameric interface. We have made a mutant containing only the single mutation E37A and found that this was sufficient to abrogate budding. We have included this in the manuscript (Figure 6C) and changed the text accordingly. We have previously shown that the dominant negative mutant D35A/E37A is unable to form the hexagonal lattice in the presence of membranes (Bigalke et al., 2014). By destabilizing the NEC hexamer formation, the E37A$_{34}$ mutation hinders the correct lattice assembly and, when present in sufficient amounts can "poison" the formation of the NEC coat even in the presence of the WT UL34, which explains the dominant negative effect. We have included this explanation in the manuscript on lines 348-351.

The information in Figs. 1 and 2 and Figs. 5 and 6 can be combined into a single figure.

Given the large amount of visual information in these figures, we think that it will be easier for the reader to follow the manuscript and our conclusions if the figures remain in their current form and prefer not to combine them.

2nd Editorial Decision 30 September 2015

Thanks for submitting your revised manuscript to The EMBO journal. Your study has now been seen by referee #3. As you can see below, the referee appreciates the introduced changes and supports publication here. I am therefore very pleased to accept the manuscript.

REFEREE REPORT

Referee #3:

The authors have thoughtfully addressed all my previous comments. The additional analysis of the new mutants further strengthens the paper.

Additional corrections:
(1) Figure S5 - the labels on the right are not properly aligned with the panels.

