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Maturation of flaviviruses starts from one or more icosahedrally independent nucleation centers

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1st Referee Reports

REFEREE REPORTS

Referee #2 (Remarks to the Author):

Plevka et al. present important and novel data on the icosahedral envelope structures of dengue virus particles during their different pathways of maturation which can result in the secretion of fully mature, partially mature and completely immature virions from infected cells. The relative proportions of these particles can vary, depending on the virus/virus strain, as a consequence of differences in the extent of proteolytic cleavage of one of the viral glycoproteins (prM) in the TGN during exocytosis. Even in the complete absence of proteolytic cleavage, the acidic pH in the TGN induces a dramatic rearrangement of the glycoproteins prM and E in the virion envelope to a 'mature-like' state that is reversed when the particles again encounter neutral pH upon release from the cells. In their present study the authors build on previous work in which they provided a number of structural details of this flavivirus maturation pathway. They show - using cryo-electron tomography - that a) in partially immature virions the mature and immature glycoprotein complexes are not distributed evenly but segregate in two separate regions of the virion and b) in completely uncleaved particles the reversal of the acid pH-induced glycoprotein rearrangement at neutral pH is not synchronized over the whole virion but is initiated from one or more nucleation centers. As a consequence, the icosahedral symmetry of these immature particles is broken and not homogeneously distributed over the particle surface, in contrast to that of the immature particles initially assembled in the ER.
The demonstration of a breakdown in the icosahedral symmetry under certain in vivo conditions is of general interest to structural biologists dealing with crystallographic or cryo-EM analyses of

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icosahedral particles. In addition to that, the work presented provides new structural input to investigations on the pathogenesis of dengue virus infections. Specifically, it was shown recently that completely immature and non-infectious virions (such as those described in this manuscript) can become infectious upon uptake into cells by antibody-mediated endocytosis followed by post-entry maturation cleavage in the endosome. Antibodies to prM can thus potentially contribute to the development of severe forms of dengue, such as dengue hemorrhagic fever and dengue shock syndrome in secondary dengue infections.
For people not well acquainted with the biology of flaviviruses and the structural details of their assembly and maturation processes, some parts of the paper may be difficult to comprehend (see specific comments below).

Specific comments:
1. Summary: The first two sentences give the impression that there is simply a reorganization of a set of 60 glycoprotein trimers in immature, fusion-incompetent viruses into 90 dimers of the same glycoprotein in infectious viruses. This is not the case since the trimers are composed of heterodimers between E and prM . Rewording is suggested.

2. Introduction, p.4 line 2: '....viruses that infect mammals'. Except for some remnants of non-human primate reservoirs, dengue virus is maintained exclusively in a cycle between humans and mosquitoes. Rewording is suggested.

3. Introduction, p.4 line 12: '.... can be cleaved by furin .....only after the conformational change'. The reader should be informed already before this notion that the generation of infectious virus requires the cleavage of prM by furin.

4. Introduction, p.5 line 4: '....they can become infectious upon interaction with some anti-prM antibodies'. For non-insiders this is a misleading statement because it suggests that the interaction with the antibody makes the particles infectious. This is not the case; the antibody - which can be directed to prM but also to E - mediates particle uptake into Fc-receptor bearing cells and only the maturation cleavage of prM in the endosome makes the particles infectious. Rewording is suggested.

5. There appears to be a discrepancy between the statements on p.4 line 6 from bottom 'Fully and partially immature particles together constitute ~40% of all particles in wild-type dengue virus type 2 .....' and on p.6 line 2 from bottom 'Preparations of DENV 2 contain only ~3% of immature-looking particles'. This should be clarified.

6. I wonder whether Fig. 5 could be already referred to in the Introduction to familiarize the reader with the problem addressed and structural transitions of flaviviruses during exocytosis and maturation. E and prM could be marked in the figure.

Referee #3 (Remarks to the Author):

This manuscript examines the structure of dengue virus 2 particles using cryo-electron tomography. The pathway for dengue virus maturation and the accompanying structural changes that take place in the virus particle have been well-characterized previously, particularly in seminal work from this group. The authors here compare mature virus in which prM is cleaved, partially mature virus (termed "mosaic"), and "immature-like" virus. The latter is virus that exits through the acidic environment of the exocytic pathway but without furin cleavage of the prM protein. The results indicate that the mosaic virus has regions of icosahedral symmetry reflecting trimeric E-prM spikes and regions of E-E dimers, each region resembling the features of the immature and mature virus particles, respectively. While this structural characterization is interesting it is not, at least to this reviewer, so surprising (and perhaps the novelty here could be made clearer). The immature-like virus has two regions each resembling immature virus but with mismatched icosahedral symmetry. This is a novel finding that suggests that the switch back to immature structure upon exit of the virus into a neutral pH environment occurs at two sites. The authors interpret the data as indicating that the conformational changes on the virus particle are not synchronized but propagate from distinct nucleation sites. This is a significant new finding that is important to our understanding of the mechanism of icosahedral virus assembly and function.

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1. As summarized above, an important new finding is the mismatched icosahedral symmetry observed in the prR201A mutant. However, this mutation does not completely block furin cleavage. The authors should address the possibility that some cleavage is occurring on the immature-like particles, resulting in the "space" between the observed regions of symmetry and perhaps causing their mismatch.

2. Why are only two regions of mismatched icosahedral symmetry observed in the prR201A virus particles? It would seem that the model the authors propose (the domino effect) could just as likely occur with 3 nucleation sites, for example.

3. A number of parts of the text are confusing and clarification would greatly help readers who are not familiar with the intricacies of the virus structure and maturation. Thus, it would help to explain/define the terms used to refer to the various types of virus particles, right at the outset: for example, immature, immature-like, immature-looking, mosaic, partially mature, etc. Please standardize the nomenclature and define.

4. On p.4 the text states that fully/partial immature wildtype virus is ~40%, while on p.6 the authors say that only 3% of the wildtype virus is immature-looking. This should be more clearly explained.

5. Emphasize that the low pH-triggered conformational rearrangement of the virus particle is reversible until prM cleavage.

6. Figure 2 should be made more accessible to a virology audience. Tough going as it reads now.

7. On Fig. 5 legend, explain the pr release from the mature particle, as is nicely summarized in the introduction. Label the mosaic particle (clarify nomenclature).

8. The middle paragraph on p7 is confusing.

9. The title is rather general and perhaps could better differentiate what is new compared to previous work.

Referee #4 (Remarks to the Author):

The authors present a cryo-ET study of partially-mature Dengue virus particles, and of a mutant with low cleavage efficiency.

Particles containing both mature and immature regions have been described previously in the cited reference Junjhon 2010, and the description of these particles presented here does not add significant new information.

The authors also conclude that 50% of the low cleavage efficiency mutant prR201A contain two independent orientations of icosahedral symmetry. It is assumed that this results from the reversion of uncleaved glycoproteins from a mature to an immature organization, beginning from more than one nucleation center. This is a more interesting observation, but I am not convinced that it is of sufficient interest to warrant publication in EMBOJ.

The study would benefit from increased clarity and detail in the results and methods sections. Examples:

1. "the sub-tomogram" is introduced in the sixth line of the results, but it is not clear what is within this volume. Later the authors refer to "the sub-tomogram for each volume covering an icosahedral asymmetric unit". In the methods there is no use of "sub-tomogram", only an indication that 50 x 50 x 50 boxes were extracted containing the image of a virus. I believe that initially a sub-volume from the tomogram containing the whole virus is aligned against an icosahedral reconstruction of the virus, in this way the orientation is calculated, and subsequently, based on this alignment, cross correlations are calculated between one unit cell of the icosahedral reconstruction, and the equivalent region in the tomogram. If so, this can be made much clearer.

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2. There is no indication of the size of the dataset. Did the authors analyze 2, 20 or 200 mixed particles? The authors need to show data for multiple particles, and describe in more detail the variability.

3. Figure 3b gives the impression that the two different immature orientations have different centers, but only rotational searches are described in the methods. Are the centers defined independently for the two orientations?

Some comments/questions on the results:

1. If one of the icosahedral orientations covers a much smaller area than the other, then the peaks for the smaller are presumably difficult to detect in the cross correlation searches. Does the method only find particles where the two orientations both cover a large area of the particle? Did the authors ever identify three centers. Would the signals be strong enough/easy enough to interpret if there are three or more centers?

2. 50% of the mutant particles look immature. What about the other 50%, are some of them partially mature? I assume that there must be particles which contain only small local regions of the particle which were been cleaved and were mature, and that these particles would therefore be identified as immature. These particles would presumably contain distortions in their icosahedral symmetry. Given that the authors search the initial orientations using a whole icosahedron, can they imagine a situation where such particles might also appear to have multiple symmetry centers?

3. Use of the whole icosahedron to identify the orientations means that local effects, and the boundaries between the different symmetry regions are difficult to interpret. Why not use only a fraction of the icosahedron for the initial orientation search, in a manner comparable to other sub-tomogram averaging studies in the literature, eg the HIV glycoproteins?

1st Revision - authors' response

Referee #2 (Remarks to the Author):
Specific comments:

1. Summary: The first two sentences give the impression that there is simply a reorganization of a set of 60 glycoprotein trimers in immature, fusion-incompetent viruses into 90 dimers of the same glycoprotein in infectious viruses. This is not the case since the trimers are composed of heterodimers between E and prM. Rewording is suggested.
A: The first two sentences were reworded to indicate that the envelope glycoproteins form heterodimers.

2. Introduction, p.4 line 2: '....viruses that infect mammals'. Except for some remnants of non-human primate reservoirs, dengue virus is maintained exclusively in a cycle between humans and mosquitoes. Rewording is suggested.
A: The sentence has been changed to: “Dengue virus is a member of the family Flaviviridae of icosahedral, lipid-enveloped viruses many of which are human pathogens.”

3. Introduction, p.4 line 12: '.... can be cleaved by furin .....only after the conformational change'. The reader should be informed already before this notion that the generation of infectious virus requires the cleavage of prM by furin.
A: We are of the opinion that the sequence of events during maturation is presented correctly.

4. Introduction, p.5 line 4: '....they can become infectious upon interaction with some anti-prM antibodies'. For non-insiders this is a misleading statement because it suggests that the interaction with the antibody makes the particles infectious. This is not the case; the antibody - which can be directed to prM but also to E - mediates particle uptake into Fc-receptor bearing cells and only the maturation cleavage of prM in the endosome makes the particles infectious. Rewording is suggested.
A: The sentence was changed to: “Although purified immature virions are non-infectious they can be endocytosed upon interaction with some anti-prM or E antibodies (Rodenhuis-Zybert et al, 2010).

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Subsequent cleavage of the prM in the endosome may render these particles infectious."

5. There appears to be a discrepancy between the statements on p.4 line 6 from bottom 'Fully and partially immature particles together constitute ~40% of all particles in wildtype dengue virus type 2 .....' and on p.6 line 2 from bottom 'Preparations of DENV 2 contain only ~3% of immature-looking particles'. This should be clarified.
A: There is no discrepancy. The 40% includes both partially immature and completely immature particles. However there are only 3% of completely immature particles. Thus there are 37% partially immature and 3% completely immature particles. Sentence on p6 was changed to state ìcompletely immature."

6. I wonder whether Fig. 5 could be already referred to in the Introduction to familiarize the reader with the problem addressed and structural transitions of flaviviruses during exocytosis and maturation. E and prM could be marked in the figure.
A: Figure 5 has been moved into the introduction. Caption was modified to read: "The pr/prM proteins are shown in red/blue and the E proteins in grey. The red and blue colors of prM indicate mismatched icosahedral symmetries."

Referee #3 (Remarks to the Author):

1. As summarized above, an important new finding is the mismatched icosahedral symmetry observed in the prR201A mutant. However, this mutation does not completely block furin cleavage. The authors should address the possibility that some cleavage is occurring on the immature-like particles, resulting in the "space" between the observed regions of symmetry and perhaps causing their mismatch.
A: Sentences indicating the possibility of partial cleavage were added to the manuscript: "Because the R201A mutation does not prevent the furin cleavage completely, it is possible that the border regions in the double symmetry particles contained cleaved pr proteins and contributed to the observed symmetry mismatch."

2. Why are only two regions of mismatched icosahedral symmetry observed in the prR201A virus particles? It would seem that the model the authors propose (the domino effect) could just as likely occur with 3 nucleation sites, for example.
A: We agree - it is possible that a particle could have three (or more) regions of mismatched symmetry. Nevertheless we never observed such a situation. Explanation indicating possibility of more than two domains was added to the manuscript: "Particles with more than two regions of unrelated icosahedral symmetry were not observed. It is nevertheless possible that small patches of icosahedrally ordered particle envelope were missed because of limited sensitivity of the orientation search."

3. A number of parts of the text are confusing and clarification would greatly help readers who are not familiar with the intricacies of the virus structure and maturation. Thus, it would help to explain/define the terms used to refer to the various types of virus particles, right at the outset: for example, immature, immature-like, immature-looking, mosaic, partially mature, etc. Please standardize the nomenclature and define.
A: The term immature-like was defined in the text "(these are particles that went through complete maturation pathway but their glycoprotein conformation changed into immature because of limited prM cleavage)". The term ìimmature-lookingî was removed. The term mosaic was defined: "(particles that contain both mature and immature regions)". The term "partially mature" is no longer used.

4. On p.4 the text states that fully/partial immature wildtype virus is ~40%, while on p.6 the authors say that only 3% of the wildtype virus is immature-looking. This should be more clearly explained.
A: Please see response to Referee 2, comment 5.

5. Emphasize that the low pH-triggered conformational rearrangement of the virus particle is reversible until prM cleavage.
A: A sentence explaining the reversibility of the conformational change has been added to the manuscript. "If these particles are shifted back to neutral pH their conformation reverts to the immature structure (Yu et al, 2008)."

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6. Figure 2 should be made more accessible to a virology audience. Tough going as it reads now.
A: Brief explanation was added to the caption of figure 2: “Cross-correlation searches to determine the orientation of particles in the sub-tomograms. Model particles in different orientations were compared with particles in the sub-tomograms and the resulting correlation coefficients were plotted onto a stereographic projection (the plots are analogous to the representation of the Earth’s surface onto a flat map). See Materials and methods for details.”

7. On Fig. 5 legend, explain the pr release from the mature particle, as is nicely summarized in the introduction. Label the mosaic particle (clarify nomenclature).
A: This has been done. Sentence “The cleaved pr proteins are released from particles at neutral pH in the extracellular space.” has been added to the figure 5 legend.

8. The middle paragraph on p7 is confusing.
A: We have re-written the paragraph.

9. The title is rather general and perhaps could better differentiate what is new compared to previous work.
A: We propose the more informative title: “Maturation of flaviviruses is initiated from one or more icosahedrally independent nucleation centers”

Referee #4 (Remarks to the Author):

The study would benefit from increased clarity and detail in the results and methods sections. Examples:

1. "the sub-tomogram" is introduced in the sixth line of the results, but it is not clear what is within this volume. Later the authors refer to "the sub-tomogram for each volume covering an icosahedral asymmetric unit". In the methods there is no use of "subtomogram", only an indication that 50 x 50 x 50 boxes were extracted containing the image of a virus. I believe that initially a sub-volume from the tomogram containing the whole virus is aligned against an icosahedral reconstruction of the virus, in this way the orientation is calculated, and subsequently, based on this alignment, cross correlations are calculated between one unit cell of the icosahedral reconstruction, and the equivalent region in the tomogram. If so, this can be made much clearer.
A: A definition of “sub-tomogram” has now been included into materials and methods section. The sentence describing the comparison of a model structure with a particle in a sub-tomogram was changed to make the description clearer. It now reads: "In order to determine which regions of the mosaic particles resemble the mature or the immature structure, correlation coefficients were calculated for each volume covering an icosahedral asymmetric unit between the two known structures and the particle in the sub-tomogram."

2. There is no indication of the size of the dataset. Did the authors analyze 2, 20 or 200 mixed particles? The authors need to show data for multiple particles, and describe in more detail the variability.
A: A total of 14 mosaic particles were analyzed. This information was added to the manuscript. The differences between the particles are in the relative sizes of the mature and immature regions as already described in the manuscript. Since these differences are trivial we do not think that the manuscript would benefit from inclusion of structure analyses of more particles.

3. Figure 3b gives the impression that the two different immature orientations have different centers, but only rotational searches are described in the methods. Are the centers defined independently for the two orientations?
A: Centers of the two immature orientations were indeed refined independently. The following description was added to materials and methods: “The orientation corresponding to the highest correlation coefficient and position of particle centre were then refined with smaller angular and translational increments, respectively.”

Some comments/questions on the results:

1. If one of the icosahedral orientations covers a much smaller area than the other, then the peaks for

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the smaller are presumably difficult to detect in the cross correlation searches. Does the method only find particles where the two orientations both cover a large area of the particle? Did the authors ever identify three centers. Would the signals be strong enough/easy enough to interpret if there are three or more centers?
A: The method can identify icosahedral regions of about 7 icosahedral asymmetric units. Particles with three or more regions were not found; nevertheless small regions conforming to icosahedral symmetry may have been missed. Please see answer to Referee 3, point 2.

2. 50% of the mutant particles look immature. What about the other 50%, are some of them partially mature? I assume that there must be particles which contain only small local regions of the particle which were been cleaved and were mature, and that these particles would therefore be identified as immature. These particles would presumably contain distortions in their icosahedral symmetry. Given that the authors search the initial orientations using a whole icosahedron, can they imagine a situation where such particles might also appear to have multiple symmetry centers?
A: A sentence describing particles produced by the prR201A mutant was added to the manuscript: "(~50% of particles produced by this mutant are immature-like, the remaining ones are mosaic or mature)". The possibility that the double (multiple) symmetry of the particles originated from small fraction of prM being cleaved is possible. Nevertheless there still would have to be two (or more) independent nucleation centers in order to obtain two (or several) regions of local icosahedral symmetry.

3. Use of the whole icosahedron to identify the orientations means that local effects, and the boundaries between the different symmetry regions are difficult to interpret. Why not use only a fraction of the icosahedron for the initial orientation search, in a manner comparable to other sub-tomogram averaging studies in the literature, eg the HIV glycoproteins?
A: We tried searching with fractions of the particle (icosahedral asymmetric unit, three icosahedral asymmetric units forming an icosahedral face, and five icosahedral asymmetric units around an icosahedral vertex). It was more difficult to identify correct solutions, because these searches were more affected by noise since they compared smaller number of voxels than when the whole particle was used. Furthermore, it is difficult to choose suitable fraction of the icosahedron for searching since it is not a priory known what fraction of the mosaic particle would conform to the immature structure. (If the whole particle has a single symmetry then the search with a whole particle would be optimal.)

Editorial Decision

Please accept my apologies for the amount of time it has taken me to return to you with a decision on your manuscript. We have now re received the comments from referee #4. As you will see, s/he essentially supports publication in EMBO reports, although requests a minor inclusion, which would be worth addressing.

Please send the modified text to me by email, and I will incorporate it into your file, as this will be the easiest for you. Once I receive it, I will be able to officially accept your study for publication in EMBO reports.

Thank you very much for submitting your work to EMBO reports.

With best wishes,

Editor
EMBO reports

REFEREE REPORT

The revised manuscript addresses (if rather minimally) all of the requested changes.

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One further minor change should be made, then in my opinion it is OK for publication.
The authors have now indicated how many mosaic particles they have analysed but they have not indicated how many mixed-symmetry particles they have analysed, and only one is shown in the figures. They need to make some comment about the size and variability of the dataset for the mixed-symmetry particles.

2nd Revision – authors' response

The authors have now indicated how many mosaic particles they have analysed but they have not indicated how many mixed-symmetry particles they have analysed, and only one is shown in the figures. They need to make some comment about the size and variability of the dataset for the mixed-symmetry particles.

A: We analyzed 30 immature-looking particles of the prM mutant. Out of these 14 had mixed-symmetry. Sizes of the regions occupied by the two symmetries varied among the particles. We modified the description of the mixed symmetry particles that were analyzed:
"Two independent orientations of icosahedral symmetry were found in 14 of the 30 immature-like particles that were analyzed. The glycoprotein envelope of these particles was organized into two exclusive regions (Fig 3B). Within each of the regions, the glycoprotein arrangement resembled that of the immature particle but the two regions did not fit coherently together in a single icosahedral lattice (Fig 5). As in the mosaic particles described above, the border zone between the two regions was about one icosahedral asymmetric unit wide. The relative size of the two regions varied from particle to particle."

Editorial Decision

Thank you for submitting your revised version, which I consider adequately addresses the referee concerns. Hence, I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future!

With best wishes,

Editor
EMBO Reports

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