THE EMBO JOURNAL

Manuscript EMBO-2016-94565

Structural intermediates in the fusion associated transition of vesiculovirus glycoprotein

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Review timeline:

Submission date:	15 April 2016
Editorial Decision:	20 May 2016
Revision received:	10 November 2016
Editorial Decision:	28 November 2016
Revision received:	02 December 2016
Accepted:	06 December 2016

Editor: Anne Nielsen

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 20 May 2016

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below.

As you will see from the reports, the referees all find that the new crystal structures would be important to the field but at the same time they are not convinced about the functional analysis conducted and the resulting model for membrane fusion. In particular, you will see that none of the referees find the current data to conclusively support functional/physiological relevance for dimer and tetramer formation. Given these somewhat mixed assessments of the overall content of your study (and the open nature of the resulting model) I conducted a round of cross-referee commenting and received the following feedback:

Ref #3:
Given that the crystallized tetrameric complex appears to be a crystallization artifact and given the large number of intersubunit contacts within the tetramer, I am concerned that the exact conformations of the EI and LI proposed intermediates may not be as reflective of true fusion intermediates (which are likely to be monomeric) as is claimed. For these reasons, it is not completely clear to me that the paper provides the expected level of advance (without the dimer/tetramer aspect).

Ref #1:
The structures of the purported G intermediates are very interesting yet alone may not represent a

significant enough advance expected from an EMBO Journal paper, particularly because only the spikes resembling the proposed late intermediate but not the early intermediate were observed by EM on the virions.

Ref #2:
The new intermediate conformations presented by the authors are important advances in the field and provide a first glimpse of the structural transitions. I agree with the other two reviewers that the physiological role of the dimer is not convincing and should be toned down. Nevertheless, the mutagenesis data identifies residues important for fusion and imply that a simple monomer to post fusion trimer does not explain its effect on fusion. Overall, I feel that the manuscript is suitable for EMBO if the authors are able to address all points and better explain the role of the mutation and of its suppressor mutant - other than dimerization.

It is clear from the reports and additional comments that the structural work in itself is interesting but that an extensive amount of further experimentation would be required to address all issues raised and to bring the study to the level of insight and significance required for publication in The EMBO Journal. However, if you were to undertake the efforts to extensively revise the study to include further functional data and a deeper understanding of the fusion process, we would be happy to consider a revised manuscript. I do want to emphasise that this revised version would have to go beyond simply toning down the present conclusions.

Given the referees' overall interest and support for your study, I would therefore 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. I realize that addressing all the referees' criticisms will require a lot of additional time and effort and be technically challenging. I would therefore understand if you wish to publish the manuscript rapidly and without any significant changes elsewhere, in which case please let us know so we can withdraw it from our system.

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

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

REFEREE REPORTS

Referee #1:

This manuscript focuses on the viral membrane fusogen G from related rhabdoviruses VSV and CHAV and reports two new crystal structures of CHAV G ectodomain. By comparing the two new structures to the available structures of the trimeric prefusion form of VSV G (VSV PRE) and trimeric postfusion forms of CHAV G and VSV G (CHAV POST and VSV POST), authors conclude that these represent two distinct intermediates along the fusogenic refolding pathway, namely, an early intermediate and a late intermediate (EI and LI). Previous work from the same lab reported that in-solution refolding of the trimeric VSV PRE to the trimeric VSV POST proceeds through a monomeric intermediate. Here, the authors obtained further evidence in support of this idea by visualizing monomeric G spikes (resembling the LI structure) on the surface of VSV. The crystal structures and the EM data significantly advances our understanding of the fusogenic refolding pathway in G, a representative of the least understood class of viral fusogens, class III, as well as our knowledge of the fusogenic refolding pathway in general.
This part of the manuscript is solid, does not raise any major concerns, and is appropriate for the EMBO Journal. My main criticisms have to do with the remainder of the manuscript, which focuses on the unexpected ability of G to dimerize. Specifically, in the crystal structure, two EI and two LI

molecules pack into a flat dimer of dimers, with extensive interfaces. Dimeric G ectodomain was also observed in solution under certain conditions and found to resemble either EI/LI heterodimer or LI homodimer by EM. While G may dimerize in crystals and in solution, whether this happens in the virus is unclear because only monomers but no dimers were observed on the virions under the "intermediate" triggering conditions. Several mutations that affect fusion map to one of the dimeric interfaces, but in the absence of a more rigorous experimental validation, it cannot be concluded that that such dimers form during fusion and that their formation is necessary for fusion. Such conclusion, stated several times in the manuscript, is premature because it is not sufficiently supported by the available data and detracts from the central message of the manuscript.

Major criticisms:

1. The conclusion that dimers form during fusion and that dimerization drives fusion is not sufficiently supported by the available data and should be toned down. In the abstract and throughout the manuscript, more emphasis should be placed on the novel and very exciting structures of the fusogenic intermediates rather than on the dimer, the nature and the fusogenic role of which are far less certain.

2. The model of fusogenic pathway that involves G dimers is ambiguous and has multiple inconsistencies, leaving the reader with a number of questions. At which point would a dimer form on the flat edge of the rhabdoviral virion? By referring to dimers as intermediates, the authors appear to suggest that prefusion trimers dissociate into monomers that then form dimeric intermediates and, finally, postfusion trimers. It would be helpful if authors described the conformational pathway that they envision in more detail. An EI homodimer, which is not observed in the crystals, is depicted in Fig. 7. Yet, mutations that affect fusion map to the EI/LI interface. The reader has to guess that the proposed sequence is as follows: PRE trimer to EI monomer to EI homodimer to EI/LI heterodimer to LI homodimer to POST, but this is not clearly stated anywhere. How do dimers of dimers fit into this? How would LI homodimers transition to POST? Although the authors do not explicitly say this, they seem to propose that at the flat end of the virion, fusion may be driven by the conformational changes within the dimer, namely, conversion of the EI homodimer to the LI homodimer (see Fig. 7). This provocative idea, although not supported experimentally at this time, is interesting but needs to be outlined more clearly.

3. Substitution of several residues at the EI/LI interface affects fusion, but no mechanism is proposed to explain how these substitutions may lead to the observed phenotypes (reduced fusion vs. a change in pH threshold). Further, no explanation is proposed for how suppressor mutations that arose in viruses containing His80 mutations, especially, H80A/Q112P mutant, restore fusogenicity.

4. The available data suggest that several residues found at one of the EI/LI interfaces are somehow important in fusion. Authors conclude that the dimers observed in crystals form during fusion, but the alternative hypothesis that interactions between FD domains observed in crystals may reflect the ability of trimers to interact is not considered.

5. On multiple occasions, authors speculate that the flat dimer of heterodimers is "ideally suited" to interact with the target membrane. But, fusion loops appear much better exposed in monomers than in the dimer of heterodimers where only two out of four protomers have exposed loops. If dimers form from monomers, as the authors propose, why would dimers and not monomers be better suited to engage the membrane? In what manner would the dimer of heterodimers be an ideal membrane bridging structure when two of its four protomers (LIs) would have already undergone the fusogenic conformational change? Again, the model needs to be clarified so that the reader does not to have to guess.

Minor criticisms:

1. In the abstract, authors state that " no atomic structure of a transitional intermediate is known for any enveloped virus". This is an overstatement. Structures of late intermediates of Dengue E (Klein et al, JVI 2013) and Rift Valley Fever virus Gc (Dessau et al, PNAS 2013) have been reported and should be acknowledged.

2. Throughout the manuscript, it is not always clear which dimer or dimeric interface the authors are

referring to. I suggest that for clarity, they refer to dimers as EI/LI heterodimer, EI homodimer, or LI homodimer and to dimeric interfaces as EI1/LI1, EI1/LI2, etc. Figures showed be labeled accordingly.

3. Figure EV1. It would be helpful to show VSV G POST structure next to CHAV G POST, to highlight their similarities so as to justify subsequent comparisons of the VSV G PRE with CHAV G POST.

4. In figure 1, it is difficult to distinguish the four molecules in panels A and B partly because FDs of EI and LI are shown in the same color. The same is true of figures 2 and EV2. Please, change the color scheme and label each molecule, i.e., EI1, EI2, LI1, LI2. Figure EV5 is much clearer in this regard.

5. Authors discuss in detail the interface forming the antiparallel EI/LI heterodimer but not the rest of the interfaces, some of which bury much larger surface areas. Do these other interfaces have any interesting features?

6. In figure 4, EI, LI, and POST structures should be labeled.

Referee #2:

Gaudin and colleagues present the crystal structures of potential intermediate states ofthe vesiculovirus CHAV G.
The authors describe a new crystal form of CHAV G, which presents new conformations of G different from the prefusion and postfusion. One monomer is similar to the VSV G prefusion protomer with the exception of the position of the R5 region. The second molecule is similar to the CHAV postfusion, but with different orientation of PHD relative to CD. R2 and R3 have the same orientation as in the postfusion conformation, but R1 and R4 are different. The authors claim that they are structural intermediates in the folding pathway from the prefusion to the postfusion conformation. The first conformation, close to the prefusion is being suggested to constitute an early intermediate named E1 and the second one close to the postfusion is a potential late intermediate named L1. Two E1 and two L1 form a tetramer in the asymmetric unit with extensive inter-protomer interactions. The fusion loops of two E1 protomers are exposed at the same side.
Negative staining EM was then used to detect the presence of elongated monomers at pH 6.6, which the authors claim they resemble the L1 structure. This may be true, but I think they cannot exclude, at this resolution, that the structure(s) they observe is the prefusion monomer G or even a postfusion monomer with the fusion peptide pointing towards the viral membrane.
They next probed the solution structure at different pH by native mass spectrometry, which revealed the presence of monomers and dimers at pH7.5. At pH 6 only trimers were observed. The effect of the pH between the two experiments requires better explanation. What is the percentage of particles shown in figure 4D and H compared to all imaged particles ? Based on the MS data it seems to represent a minor species. Thus dimer and tetramer formation as observed in the crystal must have been favored by the crystallization conditions.
Mutagenesis of E1-L1 interface residues was shown to abrogate cell-cell fusion and additional mutations in fusion defective VSV G were isolated that compensate for the original mutation and produce infectious particles.

In summary, the structures nicely present intermediate conformations that can be attributed to the folding pathway from perfusion to postfusion. The detection of such intermediates is an important advancement in the field and will be of broad interest. The interpretation of the physiological relevance of the observed heterodimers in the crystal, however, needs to be toned down.

Although, the strongest evidence that the heterodimer interface is physiologically relevant comes from the mutants that are inactive in fusion. However, because the mutations could affect other steps in the pathway, the authors should consider such possibilities. Secondly, the evidence from negative staining that such heterodimers exist in solution is rather poor. Because negative staining also seems to imply that dimers that associate via the fusion peptide exist as well in vitro, the mass spectrometry analysis might have just detected such dimers.

Further points that need to be addressed:
The authors use the existence of the large interface between E1-L1 as an argument that such dimers constitute functional intermediates. What is the calculated deltaG of this interaction? Shouldn't this be a weak interaction that is only formed transiently before refolding completes into the trimeric postfusion structure? The authors suggest that the heterodimer is positioned parallel between the viral and cellular membrane. How could such a structure proceed to assemble into trimeric post fusion conformations that will bring the membranes into close apposition by positioning the fusion loops and the transmembrane regions at the same end? Is the hexagonal network observed at pH5.5 related to the dimeric arrangement?
Page 17: .. helical network of post fusion trimers such as the one seen in Figure 3F. Figure 3F does not show such a network.
Figure EV3: The quality of the EM images is quite limited with regard to validate the existence of the heterodimers in solution.
A figure with the positions of the mutations in the prefusion structure and the putative heterodimer structure should be included.
Mass spectrometry data on the recombinant mutant that prevents cell-cell fusion in vivo could further indirectly validate the existence of the heterodimer in vitro.

Referee #3:

Baquero et al report the crystal structure of the envelope protein G from the vesiculovirus Chandipura virus (CHAV) at pH 7.5. The protein forms a flat tetrameric assembly in the crystal. The CHAV G subunits within the tetramer adopt one of two different conformations, with two subunits is each conformation. Each conformation is significantly different from the pre- and postfusion conformations, which were previously determined, but one conformation is most similar to the prefusion form whereas the other is most similar to the postfusion form. The authors conclude that these conformations respectively correspond to early and late refolding intermediates (EI and LI). The crystallographic data are complemented with a negative-stain EM analysis of recombinant VSV at pH 5.5, 6, 6.6 and 8, including tomographic reconstructions of VSV G spikes at pH 6.6, 4˚C or 37˚C and 35 Å resolution. The reconstructions indicated that VSV G formed postfusion trimers at 37˚C but predominantly monomers in the LI conformation at 4˚C. The CHAV G ectodomain was monomeric at pH > 6.5. At lower pHs, clusters of trimers, and "thin dumbbell-like structures" attributed to tetramers similar to those in the crystallographic asymmetric unit were observed. Mass spec analysis indicated that both VSV G and CHAV G were predominantly monomeric in solution at pH > 6.5, although some dimers and trimers were observed at pH 7.5. Lastly, cells with cell-surface VSV G with mutations at His80 were found to lack the cell-cell fusion activity seen in wildtype VSV G, and the activity was partially restored by mutations mapping to the EI/LI protein interface within the CHAV G tetramer.

Comparison of the EI and LI conformations of CHAV G in the new crystal form reported here with the pre- and postfusion G structures reveal enough similarities and differences to make the claim credible that the EI and LI conformations represent structures that are sampled during the fusogenic conformational transition. The EM and mass spec data support the concept of monomeric and possibly dimeric fusion intermediates. These data allow the authors to present an attractive and more complete fusion mechanism (Figure 7) that is consistent with previous literature. There are major concerns, however, with the manuscript in its current form. Certain claims are insufficiently supported by data. Specifically, some of the EM and mass spec data are overinterpreted or do not support the authors' conclusions. In particular, the data presented are not sufficiently convincing to claim that the tetramers observed in the crystal form under physiological conditions. The chronology of the appearance of the observed structural intermediates during membrane fusion is also much less clear than the authors claim in the text.

Major concerns
1. CHAV G crystallizes as a tetramer (a dimer of dimers, with G in two different conformations). However, the evidence that these tetramers form under physiological conditions during membrane fusion is unconvincing. The authors point to mass spec and EM data in Figure 4, but the 2-D average shown in panel 4D at pH 6.25 is not at all sufficient to convince this reviewer that CHAV G

forms tetramers in solution. Moreover, images collected at pH > 6.5 showed entirely monomeric populations and the tetramer crystals were obtained at pH 7.5.

Regarding the mass spec data, it seems that dimers are indeed present (albeit at low abundance), but not tetramers were observed. Trimers are also clearly visible in the VSV G spectrum at pH 7.5 (Fig. 4B), and upon close examination it appears that CHAV G trimers may also at pH 7.5 (Fig. 4A). The presence of trimers, which is not mentioned in the text at all, suggests that the dimer peak may be a trimer assembly intermediate with G in a conformation closer to the postfusion form. Hence there is no convincing direct evidence in Fig. 4 that the crystallized tetramers form in solution, or indeed that G has any other physiological oligomeric state other than trimer and monomer. Indeed, the EM analysis of virions, in which G is membrane-inserted and at high local concentrations, does not reveal any G dimers or tetramers.

The authors also point to the lack of cell-cell fusion activity of VSV G with a mutation within the tetramer interface (H80A) and to "complementing" mutations that recover this activity. However, the mutations that restore partial cell-cell fusion activity are actually either pseudo-revertants (H80K) or compensatory (Q112P) rather than truly complementing. The mutational data is hence also insufficient to support the claim that the crystallized tetramer is physiologically relevant.

Due to the concerns listed above, it is necessary for the authors to remove the claim that the tetramers represent a physiological fusion intermediate (while maintaining the claim that the EI and LI conformations are intermediates), or provide additional data to back it up. Removing the claim would mean moving Fig. 2 to an appendix, removing Fig. 4D and 4H, and modifying the abstract, Results and Discussion throughout to remove claims that the tetramer and its internal protein-protein interfaces are physiologically relevant fusion intermediates. Alternatively, the authors should provide convincing evidence that dimers or tetramers form in solution or in virions (other than by fusion loop clustering, which is a detergent artifact), and/or identify a truly complementing mutation pair (eg. a salt bridge charge reversal) that maintains cell-cell fusion activity.

2. The authors make specific claims about the CHAV G structural intermediates being "early" or "late" intermediates, but the chronology of the appearance of these structural intermediates during membrane fusion is unclear. In the absence of kinetic studies of membrane fusion, and with experiments performed at 4˚C and with purified, proteolytically cleaved ectodomains, it is not possible to claim with any certainty that structural intermediate occur early or late in the fusion transition. The text should be modified throughout to reflect this, including on p. 5 and p. 7.

3. More emphasis could have been placed on how the EI and LI conformations contribute to our understanding of how the structural fusogenic transition proceeds from (prefusion to LI to EI to post-fusion).

4. Crystallographic data collection, refinement and validation statistics should be provided in the main paper or as an extended view item.

Minor points
1. The last paragraph on p. 4 and the paragraph after that should be deleted or shortened significantly.

2. p. 5 Change "molecular basis of a model" to "molecular basis of a more detailed model"

3. p. 6, 1st paragraph. Specify the pH at which the structure was determined here.

4. p. 16. Points (i) and (ii) seem redundant.

5. p. 16. Delete "(Figure 7)" from the subheading.

6. p. 17. The proposal that some fusogenic conformational transitions go through flat dimers/oligomers and others don't is not supported by data (see also above).

7. p. 19. Remove "carefully".

8. Remove Fig. 4D/H and associated discussion (see above).

9. Some of the extended view figures are unnecessary or not obviously related to main figures, including (EV2, EV3 and EV5).

1st Revision - authors' response 10 November 2016

Ref #3:
Given that the crystallized tetrameric complex appears to be a crystallization artifact and given the large number of intersubunit contacts within the tetramer, I am concerned that the exact conformations of the EI and LI proposed intermediates may not be as reflective of true fusion intermediates (which are likely to be monomeric) as is claimed. For these reasons, it is not completely clear to me that the paper provides the expected level of advance (without the dimer/tetramer aspect).

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The structures of the purported G intermediates are very interesting yet alone may not represent a significant enough advance expected from an EMBO Journal paper, particularly because only the spikes resembling the proposed late intermediate but not the early intermediate were observed by EM on the virions.

Ref #2:
The new intermediate conformations presented by the authors are important advances in the field and provide a first glimpse of the structural transitions. I agree with the other two reviewers that the physiological role of the dimer is not convincing and should be toned down. Nevertheless, the mutagenesis data identifies residues important for fusion and imply that a simple monomer to post fusion trimer does not explain its effect on fusion. Overall, I feel that the manuscript is suitable for EMBO if the authors are able to address all points and better explain the role of the mutation and of its suppressor mutant - other than dimerization.

As the concerns raised here by the referees were very similar, we give a group reply:
1) Concerning the relevance of EI and LI conformations, ***EM data (Figure 2) are consistent with an LI-like conformation present at the surface of VSV****. Previous EM data (Libersou et al. J. Cell Biol, 2010 and Albertini et al. PLoS Pathogens, 2012) are consistent with G being a flexible monomer at the viral surface at high pH. This is in accordance with the fact that, in an EI-like conformation, release of R5 segment from the hydrophobic groove located at the base of CD confers orientational mobility to the rest of the ectodomain.*
2) As explained above, the most parsimonious interpretation of our improved mutational analysis is that the antiparallel interaction between E1 and LI FDs, which is observed in the crystal, is functionally relevant (see our arguments in the last part of the result section and in the second part of the discussion). Therefore, we have kept the notion of a functional role for this interaction in this revised version although we have largely rewritten and toned down the discussion. In particular we no longer suggest that a flat antiparallel oligomer may form the initial bridge between membranes. We do point out that any function of the EI/LI FD interface (now supported by the new mutation data) is at some early stage of fusion since both VSV-G and CHAV-G are found to form dimers above pH 7.
3) We have deleted all the paragraphs suggesting a role for the crystal tetramer in the discussion. We have also removed the fusion model presented in previous figure 7. We agree that despite our new data it is still too speculative at this stage.

Referee #1:
This manuscript focuses on the viral membrane fusogen G from related rhabdoviruses VSV and CHAV and reports two new crystal structures of CHAV G ectodomain. By comparing the two new structures to the available structures of the trimeric prefusion form of VSV G (VSV PRE) and trimeric postfusion forms of CHAV G and VSV G (CHAV POST and VSV POST), authors conclude that these represent two distinct intermediates along the fusogenic refolding pathway, namely, an early intermediate and a late intermediate (EI and LI). Previous work from the same lab reported that in-solution refolding of the trimeric VSV PRE to the trimeric VSV POST proceeds through a monomeric intermediate. Here, the authors obtained further evidence in support of this idea by visualizing monomeric G spikes (resembling the LI structure) on the surface of VSV. The crystal structures and the EM data significantly advances our understanding of the fusogenic refolding pathway in G, a representative of the least understood class of viral fusogens, class III, as well as our knowledge of the fusogenic refolding pathway in general.
This part of the manuscript is solid, does not raise any major concerns, and is appropriate for the EMBO Journal. My main criticisms have to do with the remainder of the manuscript, which focuses on the unexpected ability of G to dimerize. Specifically, in the crystal structure, two EI and two LI molecules pack into a flat dimer of dimers, with extensive interfaces. Dimeric G ectodomain was also observed in solution under certain conditions and found to resemble either EI/LI heterodimer or LI homodimer by EM. While G may dimerize in crystals and in solution, whether this happens in the virus is unclear because only monomers but no dimers were observed on the virions under the "intermediate" triggering conditions. Several mutations that affect fusion map to one of the dimeric interfaces, but in the absence of a more rigorous experimental validation, it cannot be concluded that that such dimers form during fusion and that their formation is necessary for fusion. Such conclusion,
stated several times in the manuscript, is premature because it is not sufficiently supported by the available data and detracts from the central message of the manuscript.

We thank the referee for acknowledging that our data significantly advances our understanding of the fusogenic refolding pathway in G.
Concerning a role for non-trimeric oligomers, we have largely toned down our conclusions. However, the new data show that the same compensatory mutation Q112P rescues mutations of residues located on both sides of the EI/LI FD interface. This is strong genetic evidence for a role of this interface. Note that H80, Q112 (E112 in CHAV G), D121 and E123 are clustered only when this interface is formed (Fig. EV2A) but scattered in all previously known oligomeric forms of G (Fig. EV2BC). It is highly plausible that this dimer is transient and unstable (which is consistent with a function at some intermediate stage of the fusion process) explaining why it is so difficult to observe (without mentioning the viral aggregation that it could be responsible for).

Major criticisms:
1. The conclusion that dimers form during fusion and that dimerization drives fusion is not sufficiently supported by the available data and should be toned down. In the abstract and throughout the manuscript, more emphasis should be placed on the novel and very exciting structures of the fusogenic intermediates rather than on the dimer, the nature and the fusogenic role of which are far less certain.

We have both brought new data to support the involvement in fusion of at least the EI/LI dimeric FD interface and have extensively rewritten the manuscript (including the abstract and the discussion) to restrict our conclusions to what is established by these data. Because of this much reduced

emphasis on dimeric oligomers (a possible tetrameric structure for instance is no longer mentioned) the focus is now squarely on the intermediate structures of EI and LI.

2. The model of fusogenic pathway that involves G dimers is ambiguous and has multiple inconsistencies, leaving the reader with a number of questions. At which point would a dimer form on the flat edge of the rhabdoviral virion? By referring to dimers as intermediates, the authors appear to suggest that prefusion trimers dissociate into monomers that then form dimeric intermediates and, finally, postfusion trimers. It would be helpful if authors described the conformational pathway that they envision in more detail. An EI homodimer, which is not observed in the crystals, is depicted in Fig. 7. Yet, mutations that affect fusion map to the EI/LI interface. The reader has to guess that the proposed sequence is as follows: PRE trimer to EI monomer to EI homodimer to EI/LI heterodimer to LI homodimer to POST, but this is not clearly stated anywhere. How do dimers of dimers fit into this? How would LI homodimers transition to POST? Although the authors do not explicitly say this, they seem to propose that at the flat end of the virion, fusion may be driven by the conformational changes within the dimer, namely, conversion of the EI homodimer to the LI homodimer (see Fig. 7). This provocative idea, although not supported experimentally at this time, is interesting but needs to be outlined more clearly.

We agree that the model was ambiguous and have removed it altogether. Indeed we do not know about actual coupled mechanisms of conformational changes, oligomerization and membrane association. We merely point to what is now established by our data, namely that an interface that implies a previously unsuspected self-association of G is involved in fusion.

3. Substitution of several residues at the EI/LI interface affects fusion, but no mechanism is proposed to explain how these substitutions may lead to the observed phenotypes (reduced fusion vs. a change in pH threshold). Further, no explanation is proposed for how suppressor mutations that arose in viruses containing His80 mutations, especially, H80A/Q112P mutant, restore fusogenicity.

What we provide is genetic evidence for an antiparallel interaction between fusion domains. A mechanistic explanation of how the Q112P compensatory mutation may restore fusogenicity is not possible without an explicit model of fusion with the specific role of this interface, something we now refrain from attempting. Once again, we can just state that H80, Q112 (E112 in CHAV G), D121 and E123 are clustered only when the EI-LI interface involving the fusion domains is formed.

4. The available data suggest that several residues found at one of the EI/LI interfaces are somehow important in fusion. Authors conclude that the dimers observed in crystals form during fusion, but the alternative hypothesis that interactions between FD domains observed in crystals may reflect the ability of trimers to interact is not considered.

Indeed, interactions between trimers are necessary in late stages of rhabdovirus fusion (Libersou et al., J. Cell Biol. 2010). We now explicitly point out that H80 is not exposed on the side of G trimers, and thus not available for interaction with D121/E123 of other trimers. Furthermore the anti-parallel orientation of the fusion domains at the EI/LI FD interface is not compatible with the parallel orientation of fusion domains of two neighboring trimers (either PRE or POST). In order to form an antiparallel dimer, the fusion domain has to be parallel to the membrane (which is not the case in both the pre and post-fusion trimeric states). We hope this is explained more clearly in the revised discussion.

5. On multiple occasions, authors speculate that the flat dimer of heterodimers is "ideally suited" to interact with the target membrane. But, fusion loops appear much better exposed in monomers than

in the dimer of heterodimers where only two out of four protomers have exposed loops. If dimers form from monomers, as the authors propose, why would dimers and not monomers be better suited to engage the membrane? In what manner would the dimer of heterodimers be an ideal membrane bridging structure when two of its four protomers (LIs) would have already undergone the fusogenic conformational change? Again, the model needs to be clarified so that the reader does not to have to guess.

We no longer mention the dimer of heterodimers or speculate as to the mechanism behind the involvement of the dimeric interface in fusion.

Minor criticisms:
1. In the abstract, authors state that "no atomic structure of a transitional intermediate is known for any enveloped virus". This is an overstatement. Structures of late intermediates of Dengue E (Klein et al, JVI 2013) and Rift Valley Fever virus Gc (Dessau et al, PNAS 2013) have been reported and should be acknowledged.

This overstatement has been removed. The mentioned articles are now acknowledged in the beginning of the discussion part.

2. Throughout the manuscript, it is not always clear which dimer or dimeric interface the authors are referring to. I suggest that for clarity, they refer to dimers as EI/LI heterodimer, EI homodimer, or LI homodimer and to dimeric interfaces as EI1/LI1, EI1/LI2, etc. Figures showed be labeled accordingly.

We thank the referee for this suggestion which indeed improves the clarity of the text. Figures have been labeled accordingly.

3. Figure EV1. It would be helpful to show VSV G POST structure next to CHAV G POST, to highlight their similarities so as to justify subsequent comparisons of the VSV G PRE with CHAV G POST.

This has been done.

4. In figure 1, it is difficult to distinguish the four molecules in panels A and B partly because FDs of EI and LI are shown in the same color. The same is true of figures 2 and EV2. Please, change the color scheme and label each molecule, i.e., EI1, EI2, LI1, LI2. Figure EV5 is much clearer in this regard.

We have now used different shades of yellow to distinguish EI1 and LI1 FDs.

5. Authors discuss in detail the interface forming the antiparallel EI/LI heterodimer but not the rest of the interfaces, some of which bury much larger surface areas. Do these other interfaces have any interesting features?

There are several other interesting features. However, those interfaces would be even more difficult to characterize by mutational analysis as most of them are also involved in protomer-protomer interactions in both the pre and post-fusion trimers. Finally, as the three reviewers were concerned by the fact that the tetrameric structure was a crystallization artifact, we do not want to discuss more interfaces that have not been validated.

6. In figure 4, EI, LI, and POST structures should be labeled.

The EM part of figure 4 has now been removed as suggested by referees #2 and #3 who considered that the data were not convincing.

Referee #2:
Gaudin and colleagues present the crystal structures of potential intermediate states of the vesiculovirus CHAV G. The authors describe a new crystal form of CHAV G, which presents new conformations of G different from the prefusion and postfusion. One monomer is similar to the VSV G prefusion protomer with the exception of the position of the R5 region. The second molecule is similar to the CHAV postfusion, but with different orientation of PHD relative to CD. R2 and R3 have the same orientation as in the postfusion conformation, but R1 and R4 are different. The authors claim that they are structural intermediates in the folding pathway from the prefusion to the postfusion conformation. The first conformation, close to the prefusion is being suggested to constitute an early intermediate named E1 and the second one close to the postfusion is a potential late intermediate named L1. Two E1 and two L1 form a tetramer in the asymmetric unit with extensive inter-protomer interactions. The fusion loops of two E1 protomers are exposed at the same side.

Negative staining EM was then used to detect the presence of elongated monomers at pH 6.6, which the authors claim they resemble the LI structure. This may be true, but I think they cannot exclude, at this resolution, that the structure(s) they observe is the prefusion monomer G or even a postfusion monomer with the fusion peptide pointing towards the viral membrane.

The length of elongated monomers (116 +/- 11) completely excludes that they correspond to pre-fusion protomers (7nm height). We agree that at this resolution, it is difficult to exclude that those elongated monomers could correspond to the post-fusion protomer. However, as mentioned in the text (p9), in the averaged subtomogram, the fit of VSV POST protomer resulted in significantly worse statistics than the fit of the CHAV LI structure (see also legend of Fig. 2, Appendix Fig. S6 and Table S4).

They next probed the solution structure at different pH by native mass spectrometry, which revealed the presence of monomers and dimers at pH7.5. At pH 6 only trimers were observed. The effect of the pH between the two experiments requires better explanation. What is the percentage of particles shown in figure 4D and H compared to all imaged particles? Based on the MS data it seems to represent a minor species. Thus dimer and tetramer formation as observed in the crystal must have been favored by the crystallization conditions.

The EM part of figure 4 has now been removed as suggested by both this referee and referee #3 who considered that the data were not convincing.
We agree that the dimer is a minor species. We note that crystals were obtained at pH 7.5 for CHAV-G, i.e conditions were MS detects a mixture of monomers and dimers. The crystallization, which was performed at ~10 fold higher concentration, has most likely shifted the equilibrium toward this form. In this manner we also previously crystallized the VSV G pre-fusion trimer in conditions where it is virtually absent in solution (Roche et al., Science, 2007).
The fact that this dimer is a minor species does not mean that it is irrelevant. Our mutational analysis is consistent with a functional role for a dimer which may be only transiently present at the viral surface.

Mutagenesis of E1-L1 interface residues was shown to abrogate cell-cell fusion and additional mutations in fusion defective VSV G were isolated that compensate for the original mutation and produce infectious particles. In summary, the structures nicely present intermediate conformations that can be attributed to the folding pathway from perfusion to post-fusion. The detection of such intermediates is an important advancement in the field and will be of broad interest.

We thank the referee for acknowledging that detection of such intermediates is an important advancement in the field and is of broad interest.

The interpretation of the physiological relevance of the observed heterodimers in the crystal, however, needs to be toned down. Although, the strongest evidence that the heterodimer interface is physiologically relevant comes from the mutants that are inactive in fusion. However, because the mutations could affect other steps in the pathway, the authors should consider such possibilities.

The mutational data are now more conclusive. See our previous answers (lines 9-24 and 116-123).

Secondly, the evidence from negative staining that such heterodimers exist in solution is rather poor. Because negative staining also seems to imply that dimers that associate via the fusion peptide exist as well in vitro, the mass spectrometry analysis might have just detected such dimers.

The EM part of figure 4 has been removed as both this referee and referee #3 were not convinced. We cannot absolutely exclude that dimers observed by mass spectrometry interact via their fusion loops. However, the abundance of the dimeric species is dependent on pH for both VSV G and CHAV G (see figure 4, pH 7.5 and 8.8). As there is no protonable residue in the vicinity of the fusion loops, a pH dependent interaction involving the fusion loops seems highly improbable.

Further points that need to be addressed:

The authors use the existence of the large interface between E1-L1 as an argument that such dimers constitute functional intermediates. What is the calculated deltaG of this interaction?

The global DG, as calculated for instance by the PISA program, is highly unreliable in this case. As mentioned in the text (p7, figure EV3), several interfaces involve flexible parts of the molecule (e.g. segments R4 and R5) which means that there is a very strong entropic contribution that is not accounted in DG calculation by those programs.

In principle the EI/LI FD interface, that involves only rigid parts of G, should be more reliably assessed. Still, (i) Differing protonation states are not properly taken into account, a major problem with a pH-dependent association (ii) Actually computing DG for EI1/LI1 and EI2/LI2 FD interfaces yields -6.9 kcal/mol and -4.8 kcal/mol, respectively. This difference is due to small differences in packing leading PISA to count slightly less buried surface area including one less salt bridge and two less H-bonds in the latter version of the interface.

Shouldn't this be a weak interaction that is only formed transiently before refolding completes into the trimeric post-fusion structure? The authors suggest that the heterodimer is positioned parallel between the viral and cellular membrane. How could such a structure proceed to assemble into trimeric post fusion conformations that will bring the membranes into close apposition by positioning the fusion loops and the transmembrane regions at the same end?

The above analysis of EI/LI FD interfaces shows that it is indeed a weak interaction by itself, and one that is highly dependent on precise positioning (and of course protonation states). It is therefore likely transient, which explains much of our results, including our difficulty to observe it by EM at

the viral surface and the native MS data. Of note, the anti-parallel interaction between fusion domains precludes that such a dimer is a direct precursor of the post-fusion trimer. However, our data suggest that this dimer is functional.

Is the hexagonal network observed at pH5.5 related to the dimeric arrangement?

The helical network of the post fusion trimers observed at pH 5.5 is not related to the dimeric arrangement (See also our answer to referee 1 point 4, lines 172-180)

Page 17: . helical network of post fusion trimers such as the one seen in Figure 3F. Figure 3F does not show such a network.

This part of the discussion has been removed (this was a typo and the helical network is in fact seen in Figure 3C).

Figure EV3: The quality of the EM images is quite limited with regard to validate the existence of the heterodimers in solution.

The EM part of figure 4 (and EV3) has now been removed

A figure with the positions of the mutations in the pre-fusion structure and the putative heterodimer structure should be included.

This has been done for both the pre-fusion (Figure EV2B) and the post-fusion states (Figure EV2C).

Mass spectrometry data on the recombinant mutant that prevents cell-cell fusion in vivo could further indirectly validate the existence of the heterodimer in vitro.

We agree with this suggestion. However, for the moment, we do not express recombinant vesiculovirus glycoprotein. We use the virus itself to get a soluble form of G by proteolytic cleavage. Mutant viruses that have lost their fusion properties cannot be grown.

Referee #3:

Baquero et al report the crystal structure of the envelope protein G from the vesiculovirus Chandipura virus (CHAV) at pH 7.5. The protein forms a flat tetrameric assembly in the crystal. The CHAV G subunits within the tetramer adopt one of two different conformations, with two subunits is each conformation. Each conformation is significantly different from the pre- and postfusion conformations, which were previously determined, but one conformation is most similar to the prefusion form whereas the other is most similar to the postfusion form. The authors conclude that these conformations respectively correspond to early and late refolding intermediates (EI and LI). The crystallographic data are complemented with a negative-stain EM analysis of recombinant VSV at pH 5.5, 6, 6.6 and 8, including tomographic reconstructions of VSV G spikes at pH 6.6, 4˚C or 37˚C and 35 Å resolution. The reconstructions indicated that VSV G formed postfusion trimers at 37˚C but predominantly monomers in the LI conformation at 4˚C. The CHAV G ectodomain was monomeric at pH > 6.5. At lower pHs, clusters of trimers, and "thin dumbbell-like structures" attributed to tetramers similar to those in the crystallographic asymmetric unit were observed. Mass spec analysis indicated that both VSV G and CHAV G were predominantly monomeric in solution at pH > 6.5, although some dimers and trimers were observed at pH 7.5. Lastly, cells with cell-

surface VSV G with mutations at His80 were found to lack the cell-cell fusion activity seen in wildtype VSV G, and the activity was partially restored by mutations mapping to the EI/LI protein interface within the CHAV G tetramer.

Comparison of the EI and LI conformations of CHAV G in the new crystal form reported here with the pre- and postfusion G structures reveal enough similarities and differences to make the claim credible that the EI and LI conformations represent structures that are sampled during the fusogenic conformational transition. The EM and mass spec data support the concept of monomeric and possibly dimeric fusion intermediates. These data allow the authors to present an attractive and more complete fusion mechanism (Figure 7) that is consistent with previous literature. There are major concerns, however, with the manuscript in its current form. Certain claims are insufficiently supported by data. Specifically, some of the EM and mass spec data are overinterpreted or do not support the authors' conclusions. In particular, the data presented are not sufficiently convincing to claim that the tetramers observed in the crystal form under physiological conditions. The chronology of the appearance of the observed structural intermediates during membrane fusion is also much less clear than the authors claim in the text.

Major concerns
1. CHAV G crystallizes as a tetramer (a dimer of dimers, with G in two different conformations). However, the evidence that these tetramers form under physiological conditions during membrane fusion is unconvincing. The authors point to mass spec and EM data in Figure 4, but the 2-D average shown in panel 4D at pH 6.25 is not at all sufficient to convince this reviewer that CHAV G forms tetramers in solution. Moreover, images collected at pH > 6.5 showed entirely monomeric populations and the tetramer crystals were obtained at pH 7.5.

The EM part of figure 4 (and EV3) has now been removed and is, of course, not discussed anymore in the manuscript.

Regarding the mass spec data, it seems that dimers are indeed present (albeit at low abundance), but not tetramers were observed. Trimers are also clearly visible in the VSV G spectrum at pH 7.5 (Fig. 4B), and upon close examination it appears that CHAV G trimers may also at pH 7.5 (Fig. 4A). The presence of trimers, which is not mentioned in the text at all, suggests that the dimer peak may be a trimer assembly intermediate with G in a conformation closer to the postfusion form. Hence there is no convincing direct evidence in Fig. 4 that the crystallized tetramers form in solution, or indeed that G has any other physiological oligomeric state other than trimer and monomer. Indeed, the EM analysis of virions, in which G is membrane-inserted and at high local concentrations, does not reveal any G dimers or tetramers.

We agree that we have no evidence for tetramers in solution. Therefore, we do not speak of tetramers anymore in the manuscript (except when we describe the crystalline organization).
Concerning the MS data, for VSV G at pH 7.5 there is indeed a detectable subpopulation of trimers along with monomers and dimers. This is explicit in Fig. 3B and is now also mentioned in the text p. 10. For CHAV G however, at pH 7.5 (the pH at which we obtained crystals), only monomers and dimers are detected. We do not conclude from the MS as to the organization of those dimers.

The authors also point to the lack of cell-cell fusion activity of VSV G with a mutation within the tetramer interface (H80A) and to "complementing" mutations that recover this activity. However, the mutations that restore partial cell-cell fusion activity are actually either pseudo-revertants (H80K) or compensatory (Q112P) rather than truly complementing. The mutational data is hence also insufficient to support the claim that the crystallized tetramer is physiologically relevant.

We agree that our previous mutational data were not sufficient. Our now improved mutational analysis is consistent with the conclusion that the EI/LI FD interface of the crystal is physiologically relevant, implying an anti-parallel interaction between fusion domains (see our answers above, lines 9-24 and 116-123). We no longer speculate about the nature of the oligomer involved and do not discuss a role for a putative tetramer anymore.

Due to the concerns listed above, it is necessary for the authors to remove the claim that the tetramers represent a physiological fusion intermediate (while maintaining the claim that the EI and LI conformations are intermediates), or provide additional data to back it up. Removing the claim would mean moving Fig. 2 to an appendix, removing Fig. 4D and 4H, and modifying the abstract, Results and Discussion throughout to remove claims that the tetramer and its internal protein-protein interfaces are physiologically relevant fusion intermediates. Alternatively, the authors should provide convincing evidence that dimers or tetramers form in solution or in virions (other than by fusion loop clustering, which is a detergent artifact), and/or identify a truly complementing mutation pair (eg. a salt bridge charge reversal) that maintains cell-cell fusion activity.

We have removed the claim that the tetramer represents a physiological fusion intermediate. We have provided new mutational data strongly supporting the conclusion that an anti-parallel interaction between fusion domains is required for fusion.
We have moved previous figure 2 into figure S3 of the appendix and removed the EM of figure 4.
The text has been largely modified accordingly.

2. The authors make specific claims about the CHAV G structural intermediates being "early" or "late" intermediates, but the chronology of the appearance of these structural intermediates during membrane fusion is unclear. In the absence of kinetic studies of membrane fusion, and with experiments performed at 4˚C and with purified, proteolytically cleaved ectodomains, it is not possible to claim with any certainty that structural intermediate occur early or late in the fusion transition. The text should be modified throughout to reflect this, including on p. 5 and p. 7.

Structurally, EI is very similar to the pre-fusion protomer with a single change at the level of the R5 segment. LI has R5 in the same orientation as EI and, furthermore, R2 and R3 already in the same conformation as the one found in the post-fusion protomer.
At the viral surface, the pre-fusion trimer is only detected at high pH in equilibrium with EI-like conformation whereas LI is detected at lower pH (6.6).
By far, the simplest interpretation is that LI is much further along the transition temporally, as it is structurally. We say nothing more in the discussion (p.14, "it is reasonable to infer...").

3. More emphasis could have been placed on how the EI and LI conformations contribute to our understanding of how the structural fusogenic transition proceeds from (prefusion to LI to EI to post-fusion).

We already described in detail the transition (pre-fusion to EI, to LI to post-fusion) in the discussion and in figure 6. The removal of the discussion on the tetramer naturally places the emphasis on the transition and on the interaction between fusion domains.

4. Crystallographic data collection, refinement and validation statistics should be provided in the main paper or as an extended view item.

This has been done. Crystallographic data are now presented in table 2.

Minor points
1. The last paragraph on p. 4 and the paragraph after that should be deleted or shortened significantly.

We have shortened those two paragraphs.

2. p. 5 Change "molecular basis of a model" to "molecular basis of a more detailed model"

This has been done.

3. p. 6, 1st paragraph. Specify the pH at which the structure was determined here.

This has been done.

4. p. 16. Points (i) and (ii) seem redundant.

This was in a part of the manuscript which is now removed.

5. p. 16. Delete "(Figure 7)" from the subheading.

This part of the discussion and figure 7 have been removed.

6. p. 17. The proposal that some fusogenic conformational transitions go through flat dimers/oligomers and others don't is not supported by data (see also above).

The previous model on figure 7 has been removed. This idea only remains as a possibility in the last paragraph of the discussion.

7. p. 19. Remove "carefully".

This is now removed.

8. Remove Fig. 4D/H and associated discussion (see above).

This has been removed.

9. Some of the extended view figures are unnecessary or not obviously related to main figures, including (EV2, EV3 and EV5).

Figures EV3 and EV 5 have been removed. Figure EV2 is now in the appendix.

2nd Editorial Decision 28 November 2016

Thank you for submitting a revised version of your manuscript. It has now been seen by the three original referees and their comments are shown below.

As you will see the referees generally find that all major criticisms have been sufficiently addressed

and they recommend the manuscript for publication, pending clarification and rephrasing of a few minor points. In particular, you will see that referee #3 still finds that more clarification and explanation is required regarding the occurrence and possible functional role for the dimeric form. This referee also asks if you could speculate on a possible basis for the compensatory effect of the Q112P.

Based on the referee comments I would like to invite you to submit a final revised version of the manuscript in which you address the remaining, minor points via text revision. In addition, there are a few editorial issues with text and figures that I would also ask you to address:

-> Please add a scale bar in the microscopy images in figs 4 and EV3 and indicate the size of the scale bar in the figure legends.

-> Please include the pdb accession number for the presented structures.

-> I have made a few minor suggestions and edits to the abstract (included below), would you agree to this version?

'Vesiculoviruses enter cells by membrane fusion, driven by a large, low-pH-induced, conformational change in the fusion glycoprotein G that involves transition from a trimeric pre-fusion toward a trimeric post-fusion state via monomeric intermediates. Here, we present the structure of the G fusion protein at intermediate pH for two vesiculoviruses, vesicular stomatitis virus (VSV) and Chandipura Virus (CHAV), which is responsible for deadly encephalopathies. First, a CHAV G crystal structure shows two intermediate conformations forming a flat dimer of heterodimers. On virions, electron microscopy (EM) and tomography reveal monomeric spikes similar to one of the crystal conformations. In solution, mass spectrometry shows dimers of G. Finally, mutations at a dimer interface, involving fusion domains associated in an antiparallel manner to form an intermolecular β-sheet, affect G fusion properties. The location of the compensatory mutations restoring fusion activity strongly suggests that this interface is functionally relevant. This work reveals the range of G structural changes and suggests that G monomers can re-associate, through antiparallel interactions between fusion domains, into dimers that play a role at some early stage of the fusion process.'

REFEREE REPORTS

Referee #1:

The authors have adequately addressed my earlier concerns.

Referee #2:

The authors have responded to all of my previous concerns. They have included more mutagenesis data to validate the physiological role of the dimer interface observed in the crystal structure and they toned down and removed some of the previous claims. Overall, the work is complete and highlights important fusion intermediate conformations. The only concern I still have is on the role of the dimer interface. I agree with the authors that their data shows that the interface is physiologically relevant. The mutagenesis data and the Mass spec data, however, do not prove that it is the dimer observed in the crystal that is important. While the dimer observed in the crystal could well be the dimer formed in vitro and relevant in vivo, the possibility that the interface is involved in other interactions cannot be completely excluded. This possibility should be mentioned in the abstract and the discussion.

Referee #3:

Baquero et al report the crystal structures of the envelope protein G from Chandipura virus (CHAV) in a dimeric configuration with each protomer in a different and novel conformation proposed to

correspond to early and late fusion intermediates, respectively. The authors have revised their manuscript to remove speculation that inter-dimer contacts observed in the crystals but not in solution were physiologically relevant. They have also generated an additional fusion-defective VSV strain, which was found to acquire the same compensatory mutation as the previously describe fusion-defective strain. The defect causing mutations and compensatory mutation all map to the interface between the two protomers within the G dimer. The now expanded set of mutations cluster in close proximity of each other in the structure reported here, but not in previously reported structures of vesiculovirus G proteins. Moreover, each individual mutation inhibits membrane fusion but does not prevent protein folding. With this slightly expanded genetic analysis dataset the authors provide some additional support for their proposal that dimers containing subunits in the early and late intermediate conformations form at an early stage during the fusion process and may be important in fusion catalysis.

Overall the revisions and additional mutational data have address most of my concerns. The following concerns still remain:

1-The only evidence for the formation of G dimers in solution is from the mass spectrometry analysis. However the relative abundance of dimers is low, and VSV G trimers are also present in the case of VSV G, suggesting that the dimers in the mass spectrum could potentially represent a minor artifactual species (eg. from fusion loop-fusion loops interactions or partial posfusion trimer formation) rather than representing an important fusion intermediate as the authors argue. This concern is compounded by the observation of posftusion hexamers at pH6 for VSV G "corresponding to postfusion trimers associated through their fusion loops", and by the absence of visible dimers in any of the electron micrographs.

2-Although the fit for late intermediate into the EM density is better than for postfusion G, the difference is not fully convincing at this resolution.

3-It is unclear from structure why the Q112P mutation is compensatory. From the position of residue 112, it appears that mutation to proline would remove atoms in the wt residues (Gln/Glu) that are capable of forming intermolecular hydrogen bonding across the dimer interface. Why would this restore fusion activity in the fusion-deficient mutants? Perhaps the authors could speculate on this.

Minor points
1-In the abstract, it should be mentioned that monomers and trimers are seen by mass spec in addition to dimers as the current statement could be misread as implying dimers are the dominant species. Also, "This work reveals the chronological order of G structural changes..." should be changed to "This work reveals novel structural intermediates of G..." or something similar to reflect the fact that the exact chronology remains unclear (eg. it is not clear that fusion always proceeds through a dimeric intermediate).

2-The last paragraph of the introduction is largely redundant with the abstract. And could be shortened. Also, the authors should state change the text to "...two distinct conformations apparently corresponding to early and late refolding state..."

3-In the last sentence of the Results, the text should be changed to "...beta-sheet may be functional relevant to fusion" as there is no direct evidence that the beta sheet is disrupted in the fusion-defective mutants.

4-In Fig. EV2A in the inset panel the view should be zoomed in further and the main chain atoms of residues participating in the shown beta-sheet hydrogen bonds should be shown.

2nd Revision - authors' response 02 December 2016

We would like to thank you for your positive decision. Here are our specific answers to the minor points that were raised:

Your major comment was "You will see that referee #3 still finds that more clarification and explanation is required regarding the occurrence and possible functional role for the dimeric form. This referee also asks if you could speculate on a possible basis for the compensatory effect of the Q112P."

We have added a short paragraph that addresses these two connected points in the discussion (End of the paragraph on p16). We point out that any explanation of compensatory mutation Q112P is speculative but provide three plausible hypotheses for the effect of the mutation on dimer function.

Concerning the other remarks of referees #2 and #3, we feel that they were already sufficiently addressed in the previous revision. However, we have further attenuated some sentences according to the requirement of the referees.

Referee #3 also asked for some modifications in figure EV2 stating that "In Fig. EV2A in the inset panel the view should be zoomed in further and the main chain atoms of residues participating in the shown beta-sheet hydrogen bonds should be shown."

We have produced alternate versions of this panel following his suggestion. However, we are not convinced that they improve the clarity of the panel. We keep the previous version in our resubmission but also provide the best alternate version for your appraisal.

We have also corrected all editorial issues you raised and approved your changes to the abstract.

3rd Editorial Decision 06 December 2016

Thank you for submitting your final revised manuscript for our consideration. I am writing to inform you that we have now accepted it for publication in The EMBO Journal.

EMBO PRESS

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

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Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
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  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

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1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
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6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Anti-G ectodomain antibody (KeraFAST, 8G5F11). Goat anti-mouse Alexa Fluor 488 (Invitrogen)
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	BSR cells (clone of BHK21 cells), HEK-293T (Human Embryonic Kidney expressing SV40T-Antigen, ATCC CRL-3216) cells.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
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16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
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F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	The crystal structure will be deposited next week.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	This will be done as soon as we will have the PDB number.
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA