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Contribution of intertwined loop to membrane association revealed by Zika virus full-length NS1 structure

Xiaoying Xu, Hao Song, Jianxun Qi, Yuqian Liu, Haiyuan Wang, Chao Su, Yi Shi, George F Gao

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 11 August 2016

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by two referees and their comments are provided below.

As you can see both referees find the structure important. The referees also bring up the Brown et al. NSMB paper that was published very recently. Referee #1 finds that this study compromises the novelty of the present analysis. However, our editorial polices are also such that any related work that is published while a manuscript is under review/revision here is not taken into account when assessing the conceptual advance of the study. As the NSMB study was published after your manuscript was submitted here this paper is not an issue for us. I have also discussed this further with the referee who is in agreement with this point of view.

Referee #1 suggests incorporating mutational analysis to support the NS1 dimer interaction with membranes (point #2). I have looked at this point and while this is a very reasonable point I have also come to the decision that such analysis is not needed for the present study.

The rest of the issues can be resolved with text changes and better explanations.

REFEREE REPORTS

Referee #1:

In this paper Xu et coll. provide the structure of the ZIKV NS1 full-length protein. The structure shows a dimer essentially similar to the ones previously reported by others for DENV and WNV NS1, and the most recent paper on ZIKV NS1, which was published online on July 25th (Brown et al., Nature Structural & Molecular Biology), shows virtually the exact same features as reported

here, in particular the presence of a long intertwined loop in the wing domain that exposes several non conserved aromatic residues to the membrane, thus extending the hydrophobic interfaces between the ZIKV NS1 dimer and cell membranes compared to the DENV and WNV proteins.

Although this paper can therefore no longer apply for publication in The EMBO Journal, perhaps some of the following comments can still be of interest to the authors in their future orientations.

1- The first series of questions relates to the loop described in the wing domain of the ZIKV NS1 dimer. This loop is likely inherently flexible as it appears disordered in the crystals of DENV and WNV NS1. Why does this loop turn out to be less flexible in ZIKV than in DENV and WNV NS1? Can the authors provide structural and/or functional insights into these discrepancies? In particular, is this loop participating in crystal contacts, which could explain why it is ordered in ZIKV and not in DENV and WNV NS1?
What are the b-factors when compared with the rest of the structure? It may be useful to add a panel as supplementary material to show the structure colored by b-factors with an appropriate legend and also to include a panel showing the electron density map in the region around the aromatic residues.

2- It would be important to add some experimental evidence to support the assumption that the aromatic residues contribute to strengthening the interaction of NS1 dimers with membranes, for instance by carrying out flotation experiments with wild-type and mutant proteins and/or deletion mutants of the intertwined loop?

3- The last comment concerns the comparison of the electrostatic surfaces of the ZIKV, DENV and WNV NS1. The authors must explain in detail how they calculate these surfaces, in particular at which pH the values have been obtained. Also, they should show a proper legend to the figure, with a bar indicating the values of the potentials and the values at the limits for the red and blue scales? Are all panels consistent, i.e. can the values in red for WNV be compared with the ones of ZIKV NS1.
The authors should repeat the analysis using the same procedure with APBS at neutral pH, choosing a consistent interval for the three structures.

Referee #2:

Xu et al. report the crystal structure at 2.85 A resolution of Zika virus (ZIKV) NS1. NS1 is arguably the most versatile - and still somewhat enigmatic - protein among the non-structural proteins encoded by the flavivirus genome. For the related dengue virus (DENV), it has been shown that NS1 is the only non-structural protein to be secreted from virus-infected cells. In this form, NS1 exists as a hexameric, lipid-associated species that circulates at remarkably high levels in the blood of severe dengue patients, and there are indications that it is involved in the pathogenesis of the vascular leaks that occur in dengue-associated hemorrhage. Apart from this important role of the secreted form of NS1, the protein is also essential for replication of the viral RNA and has been proposed to help assemble the viral replication complex at the ER membrane. NS1 is located on the lumenal side of the ER membrane, whereas the viral enzymes NS3 (protease-helicase) and NS5 (methyltransferase - RNA-dependent RNA polymerase) are located on the cytoplasmic side. The remaining non-structural proteins NS2A, NS2B, NS4A, and NS4B span the membrane.

NS1 plays an important role in the diagnosis of flavivirus infection (in particular, because it shows up early in infection) and as a component of vaccines. This is because it raises antibodies directed at its surface, in particular at the wing domain. Consequently, the surface properties of NS1 are of outmost importance. Xu et al. show here that some portions of the ZIKV NS1 surface differ in a characteristic way from those of DENV2 and West-Nile Virus (WNV). Their structure further reveals the conformation of a loop which is proposed to interact with the ER membrane. In the crystal structures of the corresponding NS1 proteins from DENV2 and WNV, this loop was not defined by electron density. The structure of ZIKV NS1 revealed by the work of the authors is that of the intracellular dimer; however, Xu et al. use this to build a convincing model of the lipid-associated, secreted hexamer of ZIKV NS1.

There is no doubt concerning the importance of the structure presented here by Xu et al.. This

importance is not affected by the publication of a similar structure of ZIKV NS1 by Brown et al. in NSMB a few days ago. So far, almost all structures of ZIKV proteins were published twice (the exception is, so far, the NS2B-NS3 protease), in Nature, Science, Cell, NSMB, Cell Host Microbe, and Protein & Cell - this impressively shows how "hot" the field is. So it is certainly good for the EMBO Journal to also publish a ZIKV-related structural report, not the least, because these papers will be highly quoted.

I have only a few minor concerns regarding the results reported in the manuscript by Xu et al. and their presentation. In addition, although the manuscript is overall written in acceptable English, there is certainly room for improvement, and I am listing some - not all! - of the mistakes below.

Individual concerns:

(1) Title: I suggest to make it clear in the title that the structure reported here is that of full-length NS1, as opposed to the structure of the C-terminal domain that was reported previously by the same group (Song et al., NSMB 2016). Also, I feel the structure has more highlights than just the contribution of the "intertwined loop" to the membrane-association of NS1. So, perhaps the authors should choose a more general title.

(2) Page 4, line 18: A reference is missing here.

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(4) page 6, lines 11-12: This is the only occasion where the reader gets to know something about the glycosylation of NS1. Can the authors be a little more specific? What is the expected carbohydrate structure at N130 and N207? Is there simply no electron density for a carbohydrate at N130 and for more than a single sugar unit at N207, or do the authors claim that the little carbohydrate that they see reflects the real situation? Are the glycosylation sites conserved in other flavivirus NS1 proteins?

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Figures:

(1) There is no figure showing electron density. I suggest that the authors remove one of their less useful images (e.g., Fig. 3A) and replace it by a figure showing electron density. I suggest to show

the single carbohydrate unit attached to N207 or the residues of the "hydrophobic protrusion" (122-124).

(2) Fig. 1A: The glycosylation sites are not connected to the ribbon but seem to be "free-floating". If graphical connection to the ribbon is too difficult, the authors should not attempt to display the atomic structures but use other symbols.

(3) Fig. 1C: Can the authors label the N- and C-termini? Also, some residue labels such as "H163" seem to be "free-floating" in the image; they are not clearly connected to a specific site in the protein chain.

(4) Fig. 3A. This superimposition of NS1 structures is useless. The structures are so similar that most differences will be hidden by the ribbon. Also, the colors chosen by the authors offer very little contrast. the reader will learn very little from a superimposition figure anyway, in particular, because it is not discussed in the text. This figure should be deleted.

(5) The legend mentions only Fig. 3B, but the figure also has images (C) and (D).

(6) Fig. 4A: Where is the "hydrophobic protrusion" (see text, page 10, line 6)? It is a problem that the nomenclature used by the authors for structural features does not seem to be entirely consistent.

(7) The sequence alignment should be moved to Supplementary Materials. Short sequences comprising residues discussed in the main text (e.g., 122-124) may be shown as an alignment with DENV2 NS1 and WNV NS1 as part of the appropriate figures.

Minor problems & language issues:

(1) Page 3, line 3: Replace "structure of a full-length ZIKV" by "structure of the full-length ZIKV".

(2) Page 3, line 8: replace "but the characteristic is hydrophobic" by "but their common characteristic is hydrophobic" (or similar).

(3) Page 4, line 3: Guillain-Barre (with an accent on the e).

(4) Page 4, line 4: considered a mild pathogen (delete "as")

(5) Page 4, line 11: clinically approved

(6) Page 4, line 13: in a decent way ??

(7) Page 4, line 17: of the flavivirus life cycle

(8) Page 5, line 2: providing molecular insight

(9) Page 5, line 5: Replace "structure-known flavivirus NS1 proteins" by "flavivirus proteins with known structure".
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(11) Page 6, line 5: "We got the recombinant NS1 protein successfully" is lab slang and should be expressed in a better way.

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(13) Page 6, line 8: for all amino-acid residues

(14) Page 6, line 10: in either of the WNV or DENV2 structures

(15) Page 7, line 1: The "second wing domain"? There is only one!

(16) Page 7, line 5: amino-acid resides 91 - 130

(17) Page 7, line 8: amino-acid residues 182 - 352

(18) Page 7, line 21: first half of the intertwined loop

(19) Page 8, line 2: The itnertwined loop may contribute

(20) Page 8, line 3: The beta-roll, the connector subdomain, and the second half of the intertwined loop

(21) Page 8, line 5: on one side of the dimer (?)

(22) Page 8, line 8: A reference is missing here. Do the authors quote Youn et al. at this point?

(23) Page 8, lines 13-14: we can clearly see the second half of the intertwined loop

(24) Page 8, line 15: This long loop (containing Y122, F123, V124) forms a hydrophobic protrusion ("spike") that may associate with the membrane.

(25) Page 8, line 16: envelope

(26) Page 8, line 18: we have modeled the ZIKV hexamer structure based on the previously reported...

(27) Page 9, line 1: has a very close distance

(28) Page 9, line 3: the ZIKV NS1 hexamer

(29) Page 9, line 6: can be easily be bound by host factors and reactive antibodies

(30) Page 9, line 7: Comparison with other flavivirus NS1 structures

(31) Page 9, line 11: displays

(32) Page 9, lines 13-14: Why is this a "platform"?

(33) Page 9, line 16: displays a negatively charged

(34) Page 9, line 17: Replace by "DENV2 and WNV NS1 display surfaces of neutral charge"

(35) Page 9, line 19: ZIKV NS1 displays a ...

(36) Page 9, line 20: and the DENV2 NS1 structure contains ...

(37) Modify as follows: In Fig. 4A, the NS1 surface ...

(38) Page 10, line 7: the wing domain is the most variable region

(39) Page 10, line 9: However, irrespective of amino-acid exchanges, ...

(40) Page 10, line 16: NS1-induced

I note that page 11 is empty.

(41) Page 12, line 9: immune-evasive

(42) Page 12, line 18: envelope

(43) Page 12, line 20: In our ZIKV NS1 structure

(44) Page 12, lines 21-22: containing hydrophobic residues Y122, F123, and V124, forms a hydrophobic protrusion

(45) The last sentence on page 12 is redundant and should be deleted.

(46) Page 13, line 2: From the conservation analysis,

(47) Page 13, line 5: ... are most variable, indicating that the latter has divergent characteristics among ...

(48) Page 13, line 9: to ZIKV neutrotropism

(49) Page 13, line 11: flavivirus-infected

(50) Page 13, line 12: ... during early infection has made ...

(51) Page 13, line 20: cross-reactive

(52) Page 13, lines 21-22: could induce a prominent ZIKV-specific ...

(53) Page 14, line 1: ... that the ZIKV NS1 structure ...

(54) Page 15, line 2: Proteins cannot be expressed, only genes can. Change to: "Gene cloning and expression, protein purification". Even the fact that there is a journal called "Protein Expression and Purification" does not make the term "protein expression" correct. It is the responsibility of authors and editors to keep the language of science clear and clean!

(55) Page 15, line 13: Soluble NS1 was recovered

(56) Page 16, lines 2-3: sitting-drop vapor-diffusion method with a Mosquito ...

(57) Page 16, line 6: Tris-HCl

(58) Page 16, line 7: a brief soak

(59) Page 16, line 8: X-ray diffraction data were collected (delete "The")

(60) Page 16, line 14; page 22, lines 13 and 19: The authors should not only quote PDB codes, but also the corresponding publication.

(61) Page 16, line 19: are presented in Table 1.

(62) Page 17, line 8: beamline BL17U

(63) Page 22, line 6: for the NS1 monomer

(65) Page 22, line 8: of the NS1 dimer

(66) Page 22, line 16: dotted lines

(67) Page 23, line 6: colored using the ConSurf server

(68) Page 23, line 11: which consist of

(69) Page 23, line 13: dotted lines

(70) Page 23, line 15: are indicated

Referee #1:

In this paper Xu et coll. provide the structure of the ZIKV NS1 full-length protein. The structure shows a dimer essentially similar to the ones previously reported by others for DENV and WNV NS1, and the most recent paper on ZIKV NS1, which was published online on July 25th (Brown et al., Nature Structural & Molecular Biology), shows virtually the exact same features as reported here, in particular the presence of a long intertwined loop in the wing domain that exposes several non conserved aromatic residues to the membrane, thus extending the hydrophobic interfaces between the ZIKV NS1 dimer and cell membranes compared to the DENV and WNV proteins. Although this paper can therefore no longer apply for publication in The EMBO Journal, perhaps some of the following comments can still be of interest to the authors in their future orientations.

1. *The first series of questions relates to the loop described in the wing domain of the ZIKV NS1 dimer. This loop is likely inherently flexible as it appears disordered in the crystals of DENV and WNV NS1. Why does this loop turn out to be less flexible in ZIKV than in DENV and WNV NS1? Can the authors provide structural and/or functional insights into these discrepancies? In particular, is this loop participating in crystal contacts, which could explain why it is ordered in ZIKV and not in DENV and WNV NS1?*
What are the b-factors when compared with the rest of the structure? It may be useful to add a panel as supplementary material to show the structure colored by b-factors with an appropriate legend and also to include a panel showing the electron density map in the region around the aromatic residues.

Response: Thank you for the insightful suggestion. We have further analyzed the crystal contacts of the intertwined loop in our ZIKV NS1 structure, and indeed we found that the crystal packing helps stabilize the conformation of this intertwined loop. Furthermore, different protein purification methods (the DENV2 and WNV NS1 proteins were purified with detergent) may also affect the conformation of the intertwined loop. We have discussed these possibilities in the Discussion section. We also compared the b-factor of the intertwined loop with the rest of the structure, and the b-factor of this loop is not higher than that of the rest of the structure, indicating that this loop is relatively stable. We have also added a new figure 5 to show the structure colored by b-factors, and included a panel showing the electron density map in the region around the aromatic residues.

2. It would be important to add some experimental evidence to support the assumption that the aromatic residues contribute to strengthening the interaction of NS1 dimers with membranes, for instance by carrying out flotation experiments with wild-type and mutant proteins and/or deletion mutants of the intertwined loop?
Response: We have arranged flotation experiments with the wild-type and mutant proteins. However, it will take time to acquire the data. In order to present the structural data as soon as possible, we suggest combining the flotation data with results from other flavivirus NS1 proteins in a subsequent paper to support the findings from our current manuscript.

3. The last comment concerns the comparison of the electrostatic surfaces of the ZIKV, DENV and WNV NS1. The authors must explain in detail how they calculate these surfaces, in particular at which pH the values have been obtained. Also, they should show a proper legend to the figure, with a bar indicating the values of the potentials and the values at the limits for the red and blue scales? Are all panels consistent, i.e. can the values in red for WNV be compared with the ones of ZIKV NS1.
The authors should repeat the analysis using the same procedure with APBS at neutral pH, choosing a consistent interval for the three structures.
Response: We have added the necessary information for calculating the electrostatic surfaces in the figure legend, including a bar indicating the values of the potentials and the values at the limits for the red and blue scales.

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Response: Thank you for the suggestion. We have now changed the title to the more general "**Zika virus full-length NS1 structure: role of the intertwined loop in its membrane association**".

(2) Page 4, line 18: A reference is missing here.
Response: We apologize for this mistake. We have now added a reference there.

(3) Page 5, line 17: It is conceivable that ZIKV NS1 is involved in the unusual pathogenesis of the virus, as its particular surface properties might lead to interaction with host proteins different from those contacted by DENV or WNV NS1. But the authors should explain this hypothesis by a few more words.
Response: We have now expanded the hypothesis.
The line now reads as follows:
"…Moreover, ZIKV NS1 has unique electrostatic potential maps on both inner and outer faces, which might lead to interaction with host proteins different from those used by DENV and WNV NS1. Our results suggest that ZIKV pathogenesis may be distinct from other flaviviruses and should be considered in the development of specific diagnostic antigens.…"

(4) page 6, lines 11-12: This is the only occasion where the reader gets to know something about the glycosylation of NS1. Can the authors be a little more specific? What is the expected carbohydrate structure at N130 and N207? Is there simply no electron density for a carbohydrate at N130 and for

more than a single sugar unit at N207, or do the authors claim that the little carbohydrate that they see reflects the real situation? Are the glycosylation sites conserved in other flavivirus NS1 proteins?
Response: The N130 and N207 residues are expected to have N-linked glycosylation, and in our ZIKV NS1 structure, only N207 is seen to be glycosylated with one sugar residue in clear electron density. Observation of the little carbohydrate does not reflect the real situation, and our insect-cell-produced NS1 protein have simpler glycosylation patterns than the mammalian-cell-produced NS1 protein. These two glycosylation sites are highly conserved across different flaviviruses.

(5) Page 6, line 14: The r.m.s. deviations should only be specified with two digits; these comparisons do not have the accuracy suggested here.
Response: We have changed the values of r.m.s. deviations to only be specified with two digits.

(6) Page 7, line 11: Figure 1B does not show the dimer!
Response: We have deleted “Figure 1B” in that line to ensure accuracy.

(7) Page 7, line 13: The "spaghetti loop" does have secondary structure (3(10) helices)! What the authors mean is that it lacks repetitive secondary structure (such as alpha-helix or beta-strand).
Response: We have rephrased the description as “it lacks repetitive secondary structures (such as α-helix or β-strand)”.

(8) Page 8, line 19: A reference is missing here!
Response: We have now added a reference to this line.

(9) Page 9, lines 8-9: References are missing here
Response: We have now added a reference to these lines.

(10) Page 9, line 18: How do the authors define "group" here?
Response: We are sorry for the error. We have replaced the term “group-specific” with “different”.

(11) Page 16, line 13: "Bailey, 1994" is an outdated reference for CCP4. Replace by Winn et al., 2011.
Response: We have replaced the citation as requested.

(12) Table 1: The R(merge) for the highest resolution shell (2.95 - 2.85 A) is extremely high. Such high values can be tolerated if the redundancy is high, thereby increasing the precision, as expressed by R(pim). In addition to R(pim), the authors should calculate and report c(1/2). Only if both parameters are in an acceptable range, the resolution can really be claimed to be 2.85 A, otherwise it would probably be lower.
Response: We agree and we thank you for the insightful suggestion. We have now added the values $R_{pim:}$ 0.031(0.269) and $CC_{1/2}$ 0.998(0.819) to Table 1.

Figures:

(1) There is no figure showing electron density. I suggest that the authors remove one of their less useful images (e.g., Fig. 3A) and replace it by a figure showing electron density. I suggest to show the single carbohydrate unit attached to N207 or the residues of the "hydrophobic protrusion" (122-124).
Response: We have now modified the figure as suggested by the reviewer.

(2) Fig. 1A: The glycosylation sites are not connected to the ribbon but seem to be "free-floating". If graphical connection to the ribbon is too difficult, the authors should not attempt to display the atomic structures but use other symbols.
Response: We have now modified the figure as suggested by the reviewer.

(3) Fig. 1C: Can the authors label the N- and C-termini? Also, some residue labels such as "H163" seem to be "free-floating" in the image; they are not clearly connected to a specific site in the protein chain.
Response: We have now modified the figure as suggested by the reviewer.

(4) Fig. 3A. This superimposition of NS1 structures is useless. The structures are so similar that most differences will be hidden by the ribbon. Also, the colors chosen by the authors offer very little contrast. the reader will learn very little from a superimposition figure anyway, in particular, because it is not discussed in the text. This figure should be deleted.
Response: We have deleted the figure as requested by the reviewer.

(5) The legend mentions only Fig. 3B, but the figure also has images (C) and (D).
Response: We have now mentioned these two images in the revised manuscript.

(6) Fig. 4A: Where is the "hydrophobic protrusion" (see text, page 10, line 6)? It is a problem that the nomenclature used by the authors for structural features does not seem to be entirely consistent.
Response: We have now defined the "hydrophobic protrusion. Please see page 8, line 9. In order to maintain consistency in the nomenclature, we have changed the term "hydrophobic protrusion" to "β-roll region". Please see page 10, line 13.

(7) The sequence alignment should be moved to Supplementary Materials. Short sequences comprising residues discussed in the main text (e.g., 122-124) may be shown as an alignment with DENV2 NS1 and WNV NS1 as part of the appropriate figures.
Response: We have created a new figure 5 as the reviewer suggested, and moved the original figure 5 of sequence alignment to Expanded View Figures as Figure EV1.

Minor problems & language issues:

(1) Page 3, line 3: Replace "structure of a full-length ZIKV" by "structure of the full-length ZIKV".
Response: Done**.**

(2) Page 3, line 8: replace "but the characteristic is hydrophobic" by "but their common characteristic is hydrophobic" (or similar).
Response: Done**.**

(3) Page 4, line 3: Guillain-Barre (with an accent on the e).
Response: Done.
(4) Page 4, line 4: considered a mild pathogen (delete "as")
Response: Done.

(5) Page 4, line 11: clinically approved
Response: This phrase has now been corrected.

(6) Page 4, line 13: in a decent way ??
Response: We have changed the line as follows: "… to understand the pathogenesis of ZIKV".

(7) Page 4, line 17: of the flavivirus life cycle
Response: This phrase has now been corrected.

(8) Page 5, line 2: providing molecular insight
Response: This phrase has now been corrected.

(9) Page 5, line 5: Replace "structure-known flavivirus NS1 proteins" by "flavivirus proteins with known structure".
Response: Done.

(10) Page 6, line 4: full-length
Response: This phrase has now been corrected.

(11) Page 6, line 5: "We got the recombinant NS1 protein successfully" is lab slang and should be expressed in a better way.
Response: Done.

(12) Page 6, line 8: Throughout the manuscript, in particular in the Materials & Methods section, the authors should not only quote PDB codes, but also the corresponding publications.
Response: We have added the references. Please see page 6, line 8, page 16, line 14, and page 23, line 4.

(13) Page 6, line 8: for all amino-acid residues
Response: This phrase has now been corrected.

(14) Page 6, line 10: in either of the WNV or DENV2 structures
Response: This phrase has now been corrected.

(15) Page 7, line 1: The "second wing domain"? There is only one!
Response: This phrase has now been corrected.

(16) Page 7, line 5: amino-acid resides 91 - 130
Response: This phrase has now been corrected.

(17) Page 7, line 8: amino-acid residues 182 - 352
Response: This phrase has now been corrected.

(18) Page 7, line 21: first half of the intertwined loop
Response: We have corrected the phrase as “first half part of the intertwined loop”.

(19) Page 8, line 2: The itnertwined loop may contribute
Response: This phrase has now been corrected..

(20) Page 8, line 3: The beta-roll, the connector subdomain, and the second half of the intertwined loop
Response: This phrase has now been corrected.

(21) Page 8, line 5: on one side of the dimer (?)
Response: We have corrected the phrase as “on the inner side of the dimer”.

(22) Page 8, line 8: A reference is missing here. Do the authors quote Youn et al. at this point?
Response: Here we just wanted to describe the facts, and have now rephrased this sentence to avoid misunderstanding.

(23) Page 8, lines 13-14: we can clearly see the second half of the intertwined loop
Response: This phrase has now been corrected.

(24) Page 8, line 15: This long loop (containing Y122, F123, V124) forms a hydrophobic protrusion ("spike") that may associate with the membrane.
Response: This phrase has now been corrected.

(25) Page 8, line 16: envelope
Response: This phrase has now been corrected.

(26) Page 8, line 18: we have modeled the ZIKV hexamer structure based on the previously reported...
Response: This phrase has now been corrected.

(27) Page 9, line 1: has a very close distance
Response: This phrase has now been corrected.

(28) Page 9, line 3: the ZIKV NS1 hexamer
Response: This phrase has now been corrected.

(29) Page 9, line 6: can be easily be bound by host factors and reactive antibodies
Response: This phrase has now been corrected.

(30) Page 9, line 7: Comparison with other flavivirus NS1 structures
Response: This phrase has now been corrected.

(31) Page 9, line 11: displays
Response: This phrase has now been corrected.

(32) Page 9, lines 13-14: Why is this a "platform"?
Response: We have now changed “platform” to “surface”.

(33) Page 9, line 16: displays a negatively charged
Response: This phrase has now been corrected.

(34) Page 9, line 17: Replace by "DENV2 and WNV NS1 display surfaces of neutral charge"
Response: Done.

(35) Page 9, line 19: ZIKV NS1 displays a ...
Response: This phrase has now been corrected.

(36) Page 9, line 20: and the DENV2 NS1 structure contains ...
Response: This phrase has now been corrected.

(37) Modify as follows: In Fig. 4A, the NS1 surface ...
Response: This phrase has now been corrected.

(38) Page 10, line 7: the wing domain is the most variable region
Response: This phrase has now been corrected.

(39) Page 10, line 9: However, irrespective of amino-acid exchanges, ...
Response: This phrase has now been corrected.

(40) Page 10, line 16: NS1-induced
I note that page 11 is empty.
Response: This phrase has now been corrected.

(41) Page 12, line 9: immune-evasive
Response: This phrase has now been corrected.

(42) Page 12, line 18: envelope
Response: This phrase has now been corrected.

(43) Page 12, line 20: In our ZIKV NS1 structure
Response: This phrase has now been corrected.

(44) Page 12, lines 21-22: containing hydrophobic residues Y122, F123, and V124, forms a hydrophobic protrusion
Response: This phrase has now been corrected.

(45) The last sentence on page 12 is redundant and should be deleted.
Response: We have now deleted the sentence.

(46) Page 13, line 2: From the conservation analysis,
Response: This phrase has now been corrected.

(47) Page 13, line 5: ... are most variable, indicating that the latter has divergent characteristics among ...
Response: This phrase has now been corrected.

(48) Page 13, line 9: to ZIKV neutrotropism
Response: This phrase has now been corrected.

(49) Page 13, line 11: flavivirus-infected
Response: This phrase has now been corrected.

(50) Page 13, line 12: ... during early infection has made ...
Response: This phrase has now been corrected.

(51) Page 13, line 20: cross-reactive
Response: This phrase has now been corrected.

(52) Page 13, lines 21-22: could induce a prominent ZIKV-specific ...
Response: This phrase has now been corrected.

(53) Page 14, line 1: ... that the ZIKV NS1 structure ...
Response: This phrase has now been corrected.

(54) Page 15, line 2: Proteins cannot be expressed, only genes can. Change to: "Gene cloning and expression, protein purification". Even the fact that there is a journal called "Protein Expression and Purification" does not make the term "protein expression" correct. It is the responsibility of authors and editors to keep the language of science clear and clean!
Response: We apologize for the error and have now made the appropriate changes.

(55) Page 15, line 13: Soluble NS1 was recovered
Response: This phrase has now been corrected.

(56) Page 16, lines 2-3: sitting-drop vapor-diffusion method with a Mosquito ...
Response: This phrase has now been corrected.

(57) Page 16, line 6: Tris-HCl
Response: This phrase has now been corrected.

(58) Page 16, line 7: a brief soak
Response: This phrase has now been corrected.

(59) Page 16, line 8: X-ray diffraction data were collected (delete "The")
Response: Done.

(60) Page 16, line 14; page 22, lines 13 and 19: The authors should not only quote PDB codes, but also the corresponding publication.
Response: We have now added the relevant publications to the manuscript.

(61) Page 16, line 19: are presented in Table 1.
Response: This phrase has now been corrected.

(62) Page 17, line 8: beamline BL17U
Response: This phrase has now been corrected.

(63) Page 22, line 6: for the NS1 monomer
Response: This phrase has now been corrected.

(65) Page 22, line 8: of the NS1 dimer
Response: This phrase has now been corrected.

(66) Page 22, line 16: dotted lines
Response: This phrase has now been corrected.

(67) Page 23, line 6: colored using the ConSurf server
Response: This phrase has now been corrected.

(68) Page 23, line 11: which consist of

Response: This phrase has now been corrected.

(69) Page 23, line 13: dotted lines
Response: This phrase has now been corrected.

(70) Page 23, line 15: are indicated
Response: This phrase has now been corrected.

EMBO PRESS

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: George F. Gao, Yi Shi
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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?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - 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).

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	NA
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
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For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
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5. For every figure, are statistical tests justified as appropriate?	NA
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA
Is there an estimate of variation within each group of data?	NA
Is the variance similar between the groups that are being statistically compared?	NA

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
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http://ClinicalTrials.gov
http://www.consort-statement.org
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http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
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http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
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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).	NA
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* 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
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
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17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

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	Coordinates and structrue factors have been deposited in the Protein Data Bank under accession code PDB 5GS6.
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	NA
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

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