

Three positively charged binding sites on the eastern equine encephalitis virus E2 glycoprotein coordinate heparan sulfate- and protein receptor-dependent infection

Corresponding Author: Dr William Klimstra

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

"Three positively charged binding sites on the eastern equine encephalitis virus E2 glycoprotein coordinate heparan sulfate- and protein receptor-dependent infection"

In this manuscript, Alcorn et al comprehensively investigate the contributions of residues in the EEEV E2 glycoprotein to cell surface interactions between EEEV and heparan sulfate and protein receptors and their impact on infection. Drawing on this group and the collaborator's previous publications which identified E2 K71-77 as critical in EEEV-HS interactions, as well as R84, R119 and K156-157 clusters, this manuscript defines the interactions of R84, R119 and K156, K157 with HS and protein receptors. Using a thorough step by step analysis of mutant viruses, myriad cell types either deficient in binding partners (glycosaminoglycans or heparan sulfate) or over-expression of protein receptors (VLDLR, ApoER2 isoform 1 and 2), the authors identify the conditions in which these residues impact EEEV infection. Key findings include:

- 1) K71-77 and K156,157 exhibit significant impact on HS-dependent infectivity, more so than mutant 84-119
- 2) Disruption to these HS binding site also impacts protein receptor interactions, again most notably with the 71-77 and 156-167 for all protein receptors (VLDLR, ApoER2 isoforms 1 and 2 and LDLR).
- 3) These residue sites are important for the disruption of the interaction between virus and FLDLR LA(1-2)-Fc in neutralization of viral infection, a finding recapitulated in their mouse studies.
- 4) As the disruption of HS binding site impacted protein receptor interactions, the Fc decoy neutralization was also impacted by HS binding.
- 5) Mutants occurring in cell passaging were dependent on the HS abundance vs protein receptor abundance. Mutations from protein abundance impacted HS and protein receptor dependence infection whereas HS abundance impacts HS binding and HS dependent infection but not necessarily protein receptor utilization.
- 6) Finally, the authors found that the impact of K71-77 and K156-157 mutations in vitro correlate with the impact of the mutations on EEEV disease in mice, as well as the dissemination of virus in mosquitoes.

In this reviewer's impression, the findings would be stronger if the following experiments were conducted:

- Tissue / serum titers by plaque assay or qRT-PCR for the mutant viruses after mouse infections and mosquito infections so that more direct comparisons to the quantitative findings in the in vitro data can be made.

Minor comments:

1. The complexity of Figures 2 is impressive. Rewording lines 189 to 202 may increase readers' ability to follow the science. For example, instead of saying "for all cells over-expressing protein receptors," simply state "for cells over expressing VLDLR, LDLR, ApoR2 isoform1 and 2."
2. On line 194, this sentence was hard to follow but with a comma after whereas, and inserting "a" before "smaller decrease in protein-receptor" makes it more readable.
3. On line 196-197, the statement is made that infectivity results largely recapitulated... However, it is not clear which findings were recapitulated.

The data generated in the authors' study is well-done with appropriate replicates, controls and in a variety of cell types, as well as organisms. Statistical analysis was appropriately applied to the experiments. The significance of these findings by

Alcorn et al. is substantial, adding original and innovative studies to a body of literature that aids in our understanding of how EEEV infection of a cell occurs on the residue level, thus allowing us to see how we may one day arrest such infections via antibodies and vaccines targeting precise residues and interactions.

Reviewer #2

(Remarks to the Author)

The authors investigate the role of heparan sulfate (HS) and protein receptors in facilitating Eastern equine encephalitis virus (EEEV) attachment, infection, and pathogenesis. By analyzing specific positively charged residues on the E2 glycoprotein, they demonstrate that these sites are critical for HS- and protein receptor-binding, with mutations at these sites significantly impacting infectivity, neurovirulence, and mosquito transmission. The findings reveal a structural and functional convergence between HS and protein receptor interactions, underscoring their importance in the virus's lifecycle.

Strength - This study comprehensively analyzes mutations in HS-binding sites (71-77, 84-119, 156-157) using diverse in vitro and in vivo approaches, including infectivity, heparin binding, and receptor binding assays. Distinct mutant phenotypes, such as reduced infectivity and attenuation in vivo, highlight the roles of specific residues in virus-host interactions and virulence. Findings on VLDLR LA(1-2)-Fc decoy receptor neutralization and HS dependence provide insights into therapeutic interventions, while the impact of mutations on mosquito vector infection underscores their relevance in transmission. The attenuated mutants also hold potential for live-attenuated vaccine development, emphasizing the translational significance of the study.

Weakness - The study identifies functional impacts of mutations but lacks clarity on the structural mechanisms linking HS-binding alterations to protein receptor interactions. Reliance on heparin as an HS model and limited exploration of mutant stability, 84-119 mutant phenotypes, and host compensatory mechanisms leave gaps. Inconsistent neutralization results with the VLDLR decoy and overreliance on specific cell lines may reduce relevance to natural infection contexts.

Comments

1. Assess infectivity and dissemination in other mosquito species like *Aedes aegypti* or *Culex* to evaluate whether the attenuation in *Aedes albopictus* generalizes to other vectors.
2. Overreliance on specific cell lines (e.g., CHO-K1, BHK-21) may not fully represent the range of host cell environments, particularly in natural infection contexts. Test the infectivity of WT and mutants in primary human and animal cell types.
3. Use surface plasmon resonance (SPR) to quantify the binding affinities of E2 mutants to HS analogs and VLDLR, providing a detailed kinetic profile.
4. Analyze the immune response in infected animal models by measuring interferon levels to understand how mutations affect immune evasion or activation.
5. Determine if mutants elicit neutralizing antibody responses different from the WT.
6. While the therapeutic potential of receptor decoys is mentioned, please provide more specific insights into how these findings could inform drug or vaccine development.

Reviewer #3

(Remarks to the Author)

In the paper, "Three positively charged binding sites on the eastern equine encephalitis virus E2 glycoprotein coordinate heparan sulfate- and protein receptor-dependent infection" by Alcorn et al. authors investigate the role of a number of previously identified positively charged residues involved in receptor interaction. Mutations are evaluated individually from the three previously identified binding sites (71-77, 84-119 and 156-157) both HS-binding and protein receptor binding is evaluated and a number of different cell types expressing a range of HS or protein receptors are used. Mutant viruses are also passaged in both HS^{high}, protein^{low} and HS^{low}, protein^{high} cells to detect mutations (reversions) in the binding sites, this was followed by sequencing and evaluation with regards to increased preference for HS or protein binding. Finally, the impact of these receptor-interacting motifs is evaluated in the mouse model through IP or IC infection, grading of symptoms and pre-treatment with VLDLR LA(1-2)-Fc as well as in the mosquito host to see increased/decreased dissemination and transmission. Overall conclusions are that there is no clear-cut binding to either HS or protein receptors but rather an overlap which, is rather common when it comes to charge-based interactions. Interestingly as seen in fig 5D, the 156-157 mutant seem to be able to use another "unknown" receptor and by using this receptor the virus is still pathogenic. Characterizing this mutant with regards to cellular interaction would add a lot more novelty to this study.

Concerns

- Novelty of the study could be questioned since authors have already performed similar experiments with the EEEV. There is no clear explanation to why they in this paper use the chimera (SINV/EEEV) to evaluate the mutations since they also have EEEV with the same set of mutations and this virus is also used in the present study.
- The current study is somewhat unfocused with regards to animal and mosquito studies, they are less detailed compared to the cellular work performed in fig 1-3 and it is not clear to the reader what these studies add to the story.

- In the animal study they use both mutant EEEV and mutant SINV/EEEV viruses with different routes, there is no explanation to why the IC infections are performed with the chimeric virus instead, please motivate and explain this.
- Binding assays: cells are incubated with same number of genomes? Might not correspond to the same number of infectious viral particles?
- The 71-77 and 156-157 mutants show another growth kinetics which might influence the data when authors evaluate "infection" also, in the kinetic experiment they analyse the growth with pfu however, that will skew the data as the viruses infect the BHK cells at different levels, please measure the virus growth with genome equivalent.
- For both mosquito infections and mouse infections it would be very valuable to investigate the adaptation in the viruses with the mutants, are there any mutations acquired in the binding sites and are these similar to what the authors document in cell passaging? Please add sequence data for mutant viruses isolated from tissue.
- For animal studies, please add data regarding viral growth by e.g. qPCR from brain parts and/or peripheral organs to show how these different viruses behave since there is a large variation in viability of the viruses.
- Neutralization assay, please specify how many plaques for each condition were used, since the different viruses infect the cell types with different efficiency.
- Please determine the binding affinities between the different mutant viruses and HS and VLDLR using SPR, BLI or AFM.
- Please state clearly in table 3 what virus background is summarized, if it is EEEV or the chimeric virus. As of now, this table is misleading for the reader as there is a mix of both viral backgrounds.

Minor comments

Line 314: "theover expression cells" should be changed to "the over expression cells"

Line 350: Please rephrase the part of the sentence that "71-77 mutant led to earlier signs of clinical illness" compared to WT, this is not shown in the figure and no statistical analysis has been done.

Inconsistencies: line 894 and 899 please indicate the age and where the mice were purchased now there are inconsistencies in the different spots.

Line 888 and line 720 which body parts of the mosquitos were used in the assay.

For in vitro passaging please specify the number of days for each passage

Please harmonize the figure panels so that the same size fonts are used in all figures throughout, and make sure they are readable.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Alcorn et al have addressed the concerns and the majority of recommendations of this reviewer and others with sound studies and appropriate conclusions. While there were some limitations of availability of facilities, samples and equipment that limited addressing all the recommendations, the underlying concerns were addressed with other experiments where applicable. This reviewer has no further recommendations and recommends acceptance of manuscript.

Reviewer #2

(Remarks to the Author)

The authors have thoroughly addressed reviewer comments by incorporating new experimental data (qRT-PCR, luciferase assays, IVIS imaging, ELISA-based binding assays, interferon response analysis, and mosquito cell infectivity studies). They clearly justified the use of SINV/EEEV chimeras and clarified key methodological details. Limitations related to BSL-3 Select Agent access were acknowledged, particularly for additional mosquito work and virus sequencing, with reasonable alternatives provided. Overall, the revised submission is solid.

Reviewer #3

(Remarks to the Author)

All my concerns have been addressed.

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Response to Reviewers' Comments:

Reviewer 1:

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We appreciate the favorable summary of the paper.

Specific comments:

In this reviewer's impression, the findings would be stronger if the following experiments were conducted:

- Tissue / serum titers by plaque assay or qRT-PCR for the mutant viruses after mouse infections and mosquito infections so that more direct comparisons to the quantitative findings in the in vitro data can be made.

We have added qRT-PCR of mouse serum titers, luciferase assay of early popliteal lymph node (PLN) replication using nanoLuciferase expressing viruses, and IVIS Spectrum CT imaging data for PLN, foot, and head replication following sc. infection of mice (lines 383-425, Figs. 4F-J). Unfortunately, our access to a Select Agent insectary has been interrupted and will not resume until June 2025 at the earliest, and this timing is not guaranteed. Further, based on recommendations from a CDC/DSAT inspection of the University of Texas Medical Branch BSL3/SA laboratories, samples from the previous experiment were destroyed. This type of work can be frequently interrupted,

and inspection recommendations must be followed; we were unaware that this mandate would occur.

Minor comments:

1. *The complexity of Figures 2 is impressive. Rewording lines 189 to 202 may increase readers' ability to follow the science. For example, instead of saying "for all cells over-expressing protein receptors," simply state "for cells over expressing VLDLR, LDLR, EpoR2 isoform1 and 2."*

We have included these and the following suggestions in editing this section of the text.

2. *On line 194, this sentence was hard to follow but with a comma after whereas, and inserting "a" before "smaller decrease in protein-receptor" makes it more readable.*

3. *On line 196-197, the statement is made that infectivity results largely recapitulated... However, it is not clear which findings were recapitulated.*

The data generated in the authors' study is well-done with appropriate replicates, controls and in a variety of cell types, as well as organisms. Statistical analysis was appropriately applied to the experiments. The significance of these findings by Alcorn et al. is substantial, adding original and innovative studies to a body of literature that aids in our understanding of how EEEV infection of a cell occurs on the residue level, thus allowing us to see how we may one day arrest such infections via antibodies and vaccines targeting precise residues and interactions.

We appreciate the reviewer's comment.

Reviewer 2:

The authors investigate the role of heparan sulfate (HS) and protein receptors in facilitating Eastern equine encephalitis virus (EEEV) attachment, infection, and pathogenesis. By analyzing specific positively charged residues on the E2 glycoprotein, they demonstrate that these sites are critical for HS- and protein receptor-binding, with mutations at these sites significantly impacting infectivity, neurovirulence, and mosquito transmission. The findings reveal a structural and functional convergence between HS and protein receptor interactions, underscoring their importance in the virus's lifecycle.

Strength - This study comprehensively analyzes mutations in HS-binding sites (71-77, 84-119, 156-157) using diverse in vitro and in vivo approaches, including infectivity, heparin binding, and receptor binding assays. Distinct mutant phenotypes, such as reduced infectivity and attenuation in vivo, highlight the roles of specific residues in virus-host interactions and virulence. Findings on VLDLR LA(1-2)-Fc decoy receptor neutralization and HS dependence provide insights into therapeutic interventions, while the impact of mutations on mosquito vector infection underscores their relevance in transmission. The attenuated mutants also hold potential for live-attenuated vaccine development, emphasizing the translational significance of the study.

We appreciate the favorable summary of the strengths of the paper.

Weakness - The study identifies functional impacts of mutations but lacks clarity on the structural mechanisms linking HS-binding alterations to protein receptor interactions. Reliance on heparin as an HS model and limited exploration of mutant stability, 84-119 mutant phenotypes, and host compensatory mechanisms leave gaps. Inconsistent neutralization results with the VLDLR decoy and overreliance on specific cell lines may reduce relevance to natural infection contexts.

Specific Comments:

1. Assess infectivity and dissemination in other mosquito species like Aedes aegypti or Culex to evaluate whether the attenuation in Aedes albopictus generalizes to other vectors.

As stated above, we are unable to perform new mosquito experiments until June 2025 at the earliest. Based on the Reviewer's comments, with which we agree, we have performed *in vitro* infectivity experiments with the commonly used mosquito cell lines, C6/36 and Aag2 (Supp. Fig. 7). C6/36 and Aag2 cells are derived from *Aedes albopictus* and *Ae. aegypti* mosquitoes respectively, representing the species we have used for *in vivo* studies and the species the Reviewers had suggested. These experiments are designed to address the Reviewer's questions regarding the breadth of the receptor utilization deficiency of, in particular, the 156-157 mutant. In the original manuscript, we demonstrated that it is noninfectious for *Ae. albopictus* from a blood meal. Our new data clearly show that this is not the case generally as the 156-157 mutant showed higher infectivity than the wild-type SINV/EEEV for C6/36 cells and similar infectivity for Aag2 cells. We have revised our statements to indicate that the 156A/157A deficiency may be related to gut epithelium infectivity in *Ae. albopictus* or a characteristic of the blood meal infection (lines 463-468).

2. Overreliance on specific cell lines (e.g., CHO-K1, BHK-21) may not fully represent the range of host cell environments, particularly in natural infection contexts. Test the infectivity of WT and mutants in primary human and animal cell types.

We agree that CHO-K1 and BHK-21 cells do not likely represent the ensemble of cells the viruses are likely to encounter *in vivo*. We used them to focus directly on HS utilization, a critical feature of our experiments. These cells are high in HS expression and bind HS binding viruses with high efficiency. Furthermore, we characterized the abundance of the four EEEV protein receptors on these cells, and found all four to be low to negative, such that protein receptor utilization is less of a confounding factor in our experiments. As described in the point above, we have now added two mosquito cell lines.

3. Use surface plasmon resonance (SPR) to quantify the binding affinities of E2 mutants to HS analogs and VLDLR, providing a detailed kinetic profile.

We do not have an SPR instrument at BSL-2 at the University of Pittsburgh. In response to the comment, we have established an HS ELISA and a VLDLR LA(1-2)-Fc ELISA and calculated apparent K_D for each of the four viruses and passaging mutants from the data. These data are now included in Figs. 1E, 2D, 3C, and 3G and the text (lines 162-166, 214-220, 313-316, and 342-347). In general, the ELISA data fit with our conclusions from HS binding to cells and VLDLR LA(1-2)-Fc competition and protection. Notably, our finding that VLDLR LA(1-2)-Fc protected mice from the EEEV 71-77 mutant but it did not inhibit infection *in vitro* was supported by the ELSIA, which showed binding of VLDLR LA(1-2)-Fc to the 71-77 mutant. We have added discussion of this interesting phenomenon to the manuscript (lines 525-529).

4. Analyze the immune response in infected animal models by measuring interferon levels to understand how mutations affect immune evasion or activation.

We have added type I IFN response data to the manuscript (lines 387-394, Fig. 4E)

5. Determine if mutants elicit neutralizing antibody responses different from the WT.

All of the mutants are fatal in the mouse model prior to the time at which significant antibody responses are elicited. We are currently gathering such data using chimeric viruses for a manuscript outlining use of these mutants in vaccine vectors.

6. While the therapeutic potential of receptor decoys is mentioned, please more specific insights into how these findings could inform drug or vaccine development.

Discussion of the receptor decoy utilization is presented in manuscripts from the Diamond group (in collaboration with our laboratory)¹⁻⁴ and the Abraham group^{5,6}. We have added an additional comment in the Discussion addressing the potential for escape from decoy inhibition as in the case of the 156A/157A. However, such mutations may also exert a significant negative impact on virus fitness, which is suggested for 156A/157A in the mosquito infection data.

Reviewer 3:

In the paper, "Three positively charged binding sites on the eastern equine encephalitis virus E2 glycoprotein coordinate heparan sulfate- and protein receptor-dependent infection" by Alcorn et al. authors investigate the role of a number of previously identified positively charged residues involved in receptor interaction. Mutations are evaluated individually from the three previously identified binding sites (71-77, 84-119 and 156-157) both HS-binding and protein receptor binding is evaluated and a number of different cell types expressing a range of HS or protein receptors are used. Mutant viruses are also passaged in both HShigh, proteinlow and HSlow, proteinhigh cells to detect mutations (reversions) in the binding sites, this was followed by sequencing and evaluation with regards to increased preference for HS or protein binding. Finally, the impact of these receptor-interacting motifs is evaluated in the mouse model through IP or IC infection, grading of symptoms and pre-treatment with VLDLR LA(1-2)-Fc as well

as in the mosquito host to see increased/decreased dissemination and transmission. Overall conclusions are that there is no clear-cut binding to either HS or protein receptors but rather an overlap which, is rather common when it comes to charge-based interactions. Interestingly as seen in fig 5D, the 156-157 mutant seem to be able to use another “unknown” receptor and by using this receptor the virus is still pathogenic. Characterizing this mutant with regards to cellular interaction would add a lot more novelty to this study.

Specific Comments:

- Novelty of the study could be questioned since authors have already performed similar experiments with the EEEV. There is no clear explanation to why they in this paper use the chimera (SINV/EEEV) to evaluate the mutations since they also have EEEV with the same set of mutations and this virus is also used in the present study.

We feel that the results delineated in the manuscript are novel since, at the time of publication of the original data on the 71-77 mutation, protein receptors for EEEV were unknown. The other two mutants (84A-119A and 156A-157A) have not been characterized, and all three affect protein receptor utilization and HS interactions. This core finding comprises the novelty of the manuscript.

SINV chimeras are well established in the literature as surrogates for parental BSL-3/Select Agent alphaviruses. In our opinion, there has been no data (e.g., cryo-EM reconstructions⁷⁻⁹, neutralization assays^{1,10-13}, infectivity assays (current studies), or binding assays) where they perform differently from the parental viruses, when the structural proteins are being interrogated. They allow us to perform experiments in a more reasonable timeframe at BSL-2, including those that could not be performed practically in the Select Agent BSL-3 laboratory (e.g., due to lack of equipment or possible selection of adaptive viruses).

- The current study is somewhat unfocused with regards to animal and mosquito studies, they are less detailed compared to the cellular work performed in fig 1-3 and it is not clear to the reader what these studies add to the story.

Per other comments above, we have added additional mouse replication and innate immune response data and mosquito cell line assays.

- In the animal study they use both mutant EEEV and mutant SINV/EEEV viruses with different routes, there is no explanation to why the IC infections are performed with the chimeric virus instead, please motivate and explain this.

We have added additional justification for the ic. studies with SINV/EEEV (lines 370-375)

- Binding assays: cells are incubated with a same number of genomes? Might not correspond to the same number of infectious viral particles?

For the binding assays the output is measured in genomes, which we are using as a measure of total virus particles (infectious and non-infectious but all containing genomes). To directly compare binding between the different viruses we need to use equal genomes/particles. This is generally equivalent to a radiolabeled virus particle binding assay where equal CPM of each virus are added to cells¹⁴⁻¹⁷ but genome equivalent measurements may be more relevant to infection characteristics as CPM or virus protein measurements include both genome-containing and non-genome containing virus particles

- The 71-77 and 156-157 mutants show another growth kinetics which might influence the data when authors evaluate "infection" also, in the kinetic experiment they analyse the growth with pfu however, that will skew the data as the viruses infect the BHK cells at different levels, please measure the virus growth with genome equivalent.

We have added IVIS Spectrum CT imaging data for replication in PLNs, foot, and head, as well as qRT-PCR of viremia in mice (lines 387-394 and 405-425, Figs. 4F, and H-J). These data indicate that the mutants are able to infect and replicate efficiently in vivo. BHK-21 cells were infected with genome equivalents; the purpose of doing the replication kinetics was to show that the mutant viruses still replicate and produce infectious viruses, albeit to lower titers on BHK-21 cells. While we can adjust the BHK-21 cell infectivity based on BHK-21 cell GE:PFU as an indicator of replication, this may not be a true representation of the replication kinetics, as the GE:PFU may change during the course of the infection.

- For both mosquito infections and mouse infections it would be very valuable to investigate the adaptation in the viruses with the mutants, are there any mutations acquired in the binding sites and are these similar to what the authors document in cell passaging? Please add sequence data for mutant viruses isolated from tissue.

We have added IVIS Spectrum CT in vivo imaging of nanoLuciferase expressing viruses to the manuscript (lines 405-425, Figs. 4H-J). Sequencing BSL-3, Select Agent alphaviruses is a difficult proposition. The positive-sense genome of the virus is a Select Agent and cannot be removed from Select Agent space and sent to a sequencing facility even if the virus particle is fully inactivated. We have a manuscript in preparation (Terada Y., Gilliland T., Sun C., Sattler R.A., Weaver S.C., and W. B. Klimstra) where we have performed RNAseq for wild-type EEEV in samples from mice, mosquitoes, birds and non-human primates. We have identified multiple mutations, some interestingly conserved between species, and have characterized their effects on HS and VLDLR/ApoER2/2, and LDLR-mediated infectivity. However, this work was done entirely in BSL-3 SA space at UTMB in the National Biocontainment Laboratory. As specified above we will not have access to this space until June at the earliest, and even this timing is not guaranteed.

- For animal studies, please add data regarding viral growth by e.g. qPCR from brain parts and/or peripheral organs to show how these different viruses behave since there is a large variation in viability of the viruses.

As mentioned above, we have added titration and IVIS data to the manuscript. We feel that the ic. infections with the SINV/EEEV chimera are indicative of the potential for brain replication without the confounding effects of replication and spread after a sc. infection. We have added several comments and additional figure panels related to pathogenesis of the WT and mutants in mice.

- Neutralization assay, please specify how many plaques for each condition were used, since the different viruses infect the cell types with different efficiency.

This information has been added (line 981).

- Please determine the binding affinities between the different mutant viruses and HS and VLDLR using SPR, BLI or AFM.

As mentioned above, we have developed an HS and VLDLR LA(1-2)-Fc ELISA and generated apparent affinity binding data.

- Please state clearly in table 3 what virus background is summarized, if it is EEEV or the chimeric virus. As of now, this table is misleading for the reader as there is a mix of both viral backgrounds.

We have addressed this comment by indicating whether SINV/EEEVs or EEEVs were used in the assay.

Minor comments

Line 314: “theover expression cells” should be changed to “the over expression cells”

This has been corrected.

Line 350: Please rephrase the part of the sentence that “71-77 mutant led to earlier signs of clinical illness” compared to WT, this is not shown in the figure and no statistical analysis has been done.

Clinical signs caused by the 71-77 mutant include data in this manuscript and a previous publication from our laboratory¹⁸. We have now cited the publication.

Inconsistencies: line 894 and 899 please indicate the age and where the mice were purchased now there are inconsistencies in the different spots.

We have added the timing for inoculation of mice (4-6 weeks of age). With BSL-3 SA viruses, the mice are purchased at 4 weeks of age, and they must then be transferred from standard housing to the containment laboratories which may cause a week's delay in experiment initiation. However, infections with all four viruses are done at the same time, so the mouse ages are normalized between viruses. Importantly, we do not see significant differences in EEEV mortality between 4 and 6 weeks of age.

Line 888 and line 720 which body parts of the mosquitos were used in the assay.

We have clarified this point in both sections of the text (lines 803-804 and 1036-1040).

For in vitro passaging please specify the number of days for each passage

We have added this information (lines 1017-1018 and 1019).

Please harmonize the figure panels so that the same size fonts are used in all figures throughout, and make sure they are readable.

We have confirmed that the figures are harmonized and use the same font size in all the figures and panels. However, there does appear to be an occasional issue when copying the figures into the Word document that makes it appear like font sizes are different or blurred. We will work with the editor to ensure that the final product in the manuscript is clear and up to the resolution standards of the journal.

References

- 1 Ma, H. *et al.* LDLRAD3 is a receptor for Venezuelan equine encephalitis virus. *Nature* **588**, 308-314, doi:10.1038/s41586-020-2915-3 (2020).
- 2 Raju, S. *et al.* Structural basis for plasticity in receptor engagement by an encephalitic alphavirus. *Cell*, doi:10.1016/j.cell.2025.02.036 (2025).
- 3 Palakurty, S. *et al.* The VLDLR entry receptor is required for the pathogenesis of multiple encephalitic alphaviruses. *Cell Rep* **43**, 114809, doi:10.1016/j.celrep.2024.114809 (2024).
- 4 Adams, L. J. *et al.* Structural and functional basis of VLDLR receptor usage by Eastern equine encephalitis virus. *bioRxiv*, 2023.2011.2015.567188, doi:10.1101/2023.11.15.567188 (2023).
- 5 Fan, X. *et al.* Molecular basis for shifted receptor recognition by an encephalitic arbovirus. *Cell*, doi:10.1016/j.cell.2025.03.029 (2025).
- 6 Li, W. *et al.* Shifts in receptors during submergence of an encephalitic arbovirus. *Nature* **632**, 614-621, doi:10.1038/s41586-024-07740-2 (2024).
- 7 Hasan, S. S. *et al.* Cryo-EM Structures of Eastern Equine Encephalitis Virus Reveal Mechanisms of Virus Disassembly and Antibody Neutralization. *Cell Rep* **25**, 3136-3147.e3135, doi:10.1016/j.celrep.2018.11.067 (2018).
- 8 Chen, C. L. *et al.* Cryo-EM structure of eastern equine encephalitis virus in complex with heparan sulfate analogues. *Proc Natl Acad Sci U S A* **117**, 8890-8899, doi:10.1073/pnas.1910670117 (2020).
- 9 Chen, C. L. *et al.* Cryo-EM structures of alphavirus conformational intermediates in low pH-triggered prefusion states. *Proc Natl Acad Sci U S A* **119**, e2114119119, doi:10.1073/pnas.2114119119 (2022).
- 10 Trobaugh, D. W., Sun, C., Dunn, M. D., Reed, D. S. & Klimstra, W. B. Rational design of a live-attenuated eastern equine encephalitis virus vaccine through informed mutation of virulence determinants. *PLoS Pathog* **15**, e1007584, doi:10.1371/journal.ppat.1007584 (2019).

- 11 Adams, L. J. *et al.* Structural and functional basis of VLDLR usage by Eastern equine encephalitis virus. *Cell*, doi:10.1016/j.cell.2023.11.031 (2024).
- 12 Williamson, L. E. *et al.* Therapeutic alphavirus cross-reactive E1 human antibodies inhibit viral egress. *Cell* **184**, 4430-4446.e4422, doi:10.1016/j.cell.2021.07.033 (2021).
- 13 Zimmerman, O. *et al.* Vertebrate-class-specific binding modes of the alphavirus receptor MXRA8. *Cell*, doi:10.1016/j.cell.2023.09.007 (2023).
- 14 Klimstra, W. B., Ryman, K. D. & Johnston, R. E. Adaptation of Sindbis virus to BHK cells selects for use of heparan sulfate as an attachment receptor. *J Virol* **72**, 7357-7366 (1998).
- 15 Klimstra, W. B., Nangle, E. M., Smith, M. S., Yurochko, A. D. & Ryman, K. D. DC-SIGN and L-SIGN can act as attachment receptors for alphaviruses and distinguish between mosquito cell- and mammalian cell-derived viruses. *J Virol* **77**, 12022-12032, doi:10.1128/jvi.77.22.12022-12032.2003 (2003).
- 16 Smit, J. M. *et al.* Adaptation of alphaviruses to heparan sulfate: interaction of Sindbis and Semliki forest viruses with liposomes containing lipid-conjugated heparin. *J Virol* **76**, 10128-10137, doi:10.1128/jvi.76.20.10128-10137.2002 (2002).
- 17 Bernard, K. A., Klimstra, W. B. & Johnston, R. E. Mutations in the E2 glycoprotein of Venezuelan equine encephalitis virus confer heparan sulfate interaction, low morbidity, and rapid clearance from blood of mice. *Virology* **276**, 93-103, doi:10.1006/viro.2000.0546 (2000).
- 18 Gardner, C. L., Ebel, G. D., Ryman, K. D. & Klimstra, W. B. Heparan sulfate binding by natural eastern equine encephalitis viruses promotes neurovirulence. *Proc Natl Acad Sci U S A* **108**, 16026-16031, doi:10.1073/pnas.1110617108 (2011).