nature portfolio

Peer Review File

Multi-protomics analysis identified host cellular pathways perturbed by tick-borne encephalitis virus infection

Corresponding Author: Professor Yicheng Zhao

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

Attachments originally included by the reviewers as part of their assessment can be found at the end of this file.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The manuscript addresses TBEV-induced host cell perturbations by a comprehensive approach that includes: (i) characterization of the proteome, phosphoproteome, and acetylproteome of infected cells; (ii) description of a novel pathway where viral NS5 blocks the DNA damage response of the cell (this is the more developed mechanistic part); (iii) description of a novel pathway where viral prM induces authophagy; (iv) characterization of inhibitors targeting kinases, DDR or autophagy. It is a huge amount of work covering various aspects, but some without the necessary depth. For example, role of DNA damage induction and DDR in TBEV pathogenesis like neuroinflammation is not analysed (see more specific comments below). Animal models of TBEV infection are available and would have been important to check for DNA damage and DDR markers in vivo to corroborate in vitro data. In addition to more specific comments below I believe that the paper requires to address the consequences of DNA damage on (neuro)inflammation.

TBEV is an important human pathogen expanding West in Europe.

The omics approach is very comprehensive providing a lot of information that will benefit the scientific community.

The omics is conducted in Vero non-human cells, which is a good cell type for virus replication but is defective of innate immune response thus allowing high levels of virus infection. Some of the innate pathways could be mis-represented in these cells.

The omics part is essentially descriptive and could be reduced or put in supplementary in favor of mechanistic follow up. The sampling at different times post-infection is particularly appreciated because it allows to monitor kinetics of PTMs for example.

Viral protein tagging need more explanation for the postioninig of tags with respect to certain domains such as 2K for example, in same cases it may disrupt function.

Quite surprisingly there is no effect on ER stress/UPR, which have been described for TBEV (Carletti Nat Comm 2019). Contrary to what was shown, data on PKR phosphorylation are showing early upregulation and then downregulation (EIF2AK2) but no data for PERK (EIF2AK3) or EIF-2a/EIF2S1phosphorylation are shown.

The authors then decide to investigate DNA damage and DDR in more detail. They start from omic data also mentioning RRM2 decrease as a potential trigger of DNA damage (page 10). However, this observation is not followed up. For example, it has been shown in SARS-Cov-2 that CHK1 depletion induces RRM2 loss and dNTP shortage with DNA damge induction, DDR, inflammation and senescence both in vitro and in vivo (Gioia Nat Cell Biol 2023). It would be important to show CHK1 and CHK2 levels and phosphorylation status.

More specifically into DDR:
Figure S2A, while DNA-PK and ATM show time dependent increase, phosphorylation of ATR is not so consistent. Better Figure 4a but still ATR poorly activated. I'm also concerned about the statistics of Figure 4b (and other thorughout the

manuscript), significance related to value normalized to 1 seems miscalculated.
It is gH2AX (mis-spelled) and why not induced in HU control? Include CHK1/2 as mentioned above.

Comet assay is an important information, please indicate h.p.i. of the assay.
P21 and MK167 (K167) markers are poorly regulated in the supplementary figure, need IF quantification data. In general lacking IF for markers of DDR. Also NFkB and IL1A need activation data by IF/RT-PCR/WB. A panel of pro-inflammatory cytokines following TBEV infection would be a useful information, as well as induction of senescnece by b-gal staining.

It would be important to exclude apoptosis in the time frame of the DDR assay, please provide such information (i.e. caspase assay).

The increase of G0/1 not very significant by flow cytometry (Figure S15), maybe include bivariate plot showing DNA content (PI) and BrdU incorporation measured by flow cytometry (horseshoe).

P53BP reduction in foci upon infection is would benefit of co-loc with gH2AX and WB of p53BP upon infection.

The background to reach the question is just an hypothesis, in fact the authors then come to the real mechanistic question: we hypothesized that TBEV NS5 might engage with SIRT1 and inhibit SIRT1-mediated deacetylation of Ku70 and KAP1, consequently suppressing the efficiency of NHEJ activity.
Co-IPNS5/SIRT is ok but IF show NS5 in the nucleus. It is known that NS5 from various Flavi has nuclear localization (quite peculiar for a cytoplasmic virus), but for TBEV it is not described to my knowledge, please provide explanation. Also o-loc NS5/SIRt is not very informative being extensive thoughout the cell.

Overexpression of SIRT1 induces deacetylation of Ku70 – evidence? Fig S14J and j-n maybe, not indicated. Data are a bit confused, what is IP:Pan, Pan-acetylation antibody? HA control why showing a band?

Functionally, NS5 reduces NHEJ, ok, and 53BP1foci are reduced, which figure?

The authors use a brain organoid infection model, nice but need introduction.

KD of SIRT no effect but overxpression inhibits TBEV replication. Cathalitically inactive form does not have effect in KD cells, why KD for SIRT background? Wouldn't inhibit also the palsmid?

Conclusion: TBEV NS5 binds to and suppresses the activity of SIRT1, resulting in the elevated acetylation of Ku70 and KAP1, which in turn inhibits DNA damage repairment and promotes virus replication.

Which part of NS5 is required for SIRT1 suppression? Is it nuclear or cyto the site of interaction/inhiibtion? How does it suppress SIRT1 activity? No degrdation is apparent.

Cells used: A549 different from Vero in original proteomic analysis, need comment.
Figure S2b yellow circles are blue circles

Useful to link here the data on the pharmacological modulation of acetylation. However, data don't always reperent text. For example, the HAT inhibitor CPTH2 inhibits TBEV when it is also cytotoxic, need better dose response. Also, HAT inhibitors have pleiotropic effects on transcription for example.
Also for ATM/ATR inhibitors there is a poor dose response. More intriguing reserveratol but where are the data?
Discrepancies between figure 6 and Figure 20s, boh showing Fisetin and Enoxcin but not Resveratol or Selisistat, lots of confusion here in the figure legends.

Then the authors decide to explore also autophagy. Role of autophagy in TBEV infection is tricky because it may have proviral or antiviral roles depending on timing.

In general data support induction of autophagy, although p62 increase is not so evident (Figure S18a,b)
Also acidification is not so evident, lacks in general a sterile induction of autophagy as positive control.

The authors then bring in prM and NS2B3 linked to autophagy regulation.
prM shows more LC3 foci, also quantified in Figure S19, but no quantification for NS3B3. In fact, NS3B3 disappears form the experiments. Is it relevant?

prM induce LC3 puncta and LC3-II/I ratio (still not convinced of statistics) and p62 but not really evident

TBEV infection reduced mTOR phospho and induced early AKT1 phospho and then reduces it – double role of autophagy?

AKT overexpression reduce TBEV replication (include plaque assay?)

LC3-LAMP colocalization in Fig S19d is a bit stretched, same for S19e for VPs11
Better data with coIP with VPS11 and disruption of VPS complex
Targeting autophagy by KD ATG7 reduces viral replciation, but wortmannin autophagy inhibitor induced TBEV replication (hoever data S21/22w don't show dose response and is not the same in the two cell lines). Other autophagy inhibitors inhibit

replication, as did CQ (which could be also affecting entry). Also in this case the inhibitors of autophagy may be introduced in the paragraph related to the mechanism to provide support on the hypothesis rather than in a separate chapter.

The part related to drugs nees refinement. In this context the selective index is important to exclude a general toxic effect. As mentioned above some panels are mislabelled in figures, please check.

Reviewer #2

(Remarks to the Author)
In this manuscript, Sui and colleagues perform a systematic profiling of the global interactome, proteome, phosphoproteome and acetylome of TBEV, to characterize host responses to viral infection in a systematic fashion. To assess the functional relevance of newly or previously reported host targets identified by proteomics, the authors further employ some orthogonal approaches (genetic, imaging or small-molecule inhibitors-based), characterizing selected host interactors bound or modulated at the PTM level in vitro.
Specific examples include:
- a role of NS5 in DNA-damage response via interaction with SIRT1 (read-out co-IP WB, gammaHA2x accumulation in the nuclei and siRNA-meidate KD/OE);
- a role for prM in induction of autophagy (read-out: phosphor-specific WB on AKT and and LC3II/LC3I ratio);
- a TBEV-mediated (mild) dysregulation of cell cycle.

Additionally, the authors tested a subset of host targets from each dataset (27 in total) by pharmacological inhibition, to assess their drug-repurposing antiviral potential. Altogether, these experiments identify 1-3 (depending on the specific cell type and cut-off used) novel inhibitors of TBEV replication in vitro, and confirm the antiviral activity of many other previously reported inhibitors including some associated with anti-IAV, anti-DENV or ZIKV, and anti-SARS-CoV-2 activity or widely described inhibitors with broad pan-viral activity such as Wortmannin or other autophagy-related inhibitors (i.e. spautin, cloroquine, etc.). Among these the most interesting/novel findings are probably those related to SIRT1, for which a few mechanistic data support the mechanism of action of the SIRT1 agonist.
Overall, the study is a muscular tour-de-force both from a technical and a bioinformatics standpoint, and provides a remarkable example of integrative multi-omics. Data are clearly presented and results provided in light of the massive amount of literature existing for different flaviviruses across domains (PPI and Phosphoproteomes above all). Furthermore, the attempt to validate the data via pharmacological inhibition as well as some orthogonal validation by co-IP/WB and immunofluorescence in more relevant model systems (i.e. brain organoids) is notable, and presents some interesting insights. I have only a few comments related to the presentation of some of the statistics and data, and some mechanistic follow-up experiments in vitro (see below).

Major comments

1. In general the study is well-executed and data are well-presented; however all the proteomics dataset and follow-up studies are carried in VeroE6 (African Green Monkey cells) and 293T cells, which both have rather limited physiological relevance for TBEV. While few of the drug-repurposing experiments are also carried in A549 cells or brain organoids, validating some of the key new findings (i.e. SIRT1 over-expression) in more relevant model systems for TBEV (i.e. monocytes, or dermal fibroblasts) will go along the way of increasing their significance in pathogenesis.
2. While this reviewer appreciates the substantial extent of validation already presented in the manuscript, these efforts (both genetic, biochemical or drug-based) almost exclusively focus at corroborating already extensively described pathways or genes previously associated with flavivirus replication (i.e. AKT-mTOR, autophagy flux, Leo1 and PAF1, etc.). In this respect, experimental validation that these pathways or host proteins are also important for TBEV replication is important, but its novelty is limited as no/few new mechanistic insights are presented. Additional experiments addressing the functional relevance of previously unreported genes in TBEV replication (i.e. KD/KO and PFU assay of novel TBEV host factors) or more detailed characterization of the mechanisms underlying previously reported host factors would significantly strengthen the impact and novelty of the study.
3. TBEV PPI Interactome study (Fig.2 and Fig S5): The cloning strategy used for overexpression of epitope-tagged viral protein appears unorthodox, as all proteins are described as cloned with an N-terminal tag (line 197). However, many of the flaviviral ORFs require specific insertion sequence to ensure their correct topology. For instance NS1 requires the E leader sequence to ensure insertion in the ER lumen (so it should be c-terminally tagged); similarly NS4B which has to be expressed alongside with the 2k- signal peptide to ensure correct topology (otherwise the N-terminal tag will be cleaved off mature NS4B, or if 2k not present NS4B will be expressed in the cytoplasm). Similar rationale applies to NS2A, Envelope and prM, each requiring their own leader sequences). Can the authors clarify in more details how exactly these proteins were designed? This aspect is extremely important as it affects correct topology, and ultimately the physiologically-relevant subcellular localization and host interactome of the viral baits.

Minor comments

1. All figures containing ball-networks should be revised to make gene names readable (both supplements and main text; applies to all figures)
2. Figure legends (Fig. 1-3): additional information on the statistical test and cut-off criteria used should be added
3. Pp 5, line 137: enzyme should read “enzymatic”
4. Pp5 line 161: acetylated should read “acetylation”

Comment on statistics

All statistical analyses and gene ontology analysis appear as scientifically sound. Additional information on the cut-off criteria used should be added in figure legends as described above.

Reviewer #3

(Remarks to the Author)
This paper is an extensive body of work that identifies several flavivirus-host interactions using proteomics-based approaches. The authors first identified changes in protein abundance, phosphorylation, and acetylation in response to TBEV infection in Vero cells. Next, all the TBEV proteins were cloned into expression vectors for affinity purification-mass spectrometry (AP-MS) in HEK293T cells, which identified several interaction networks for each of the ten TBEV proteins and NS2B3. The authors then identified two pathways of interest from their considerable amount of data: the DNA damage response and autophagy. The study identified NS5 as essential for preventing DNA damage repair and prM as critical for promoting autophagy. Finally, the authors pharmacologically targeted these pathways to demonstrate their importance in viral replication. Overall, this large body of work is a critical resource to the field as it identifies several key interactions and changes in response to TBEV infection.

While this study has generated large amounts of valuable data on cellular pathways and interactors potentially critical for TBEV infection, the authors mainly focused on the identification of interactions and of changes in protein abundance and post-translational modifications (PTMs). The authors then followed up on the NS5-SIRT1 and prM-AKT1 interactions and their roles in DNA repair and autophagy, which are novel and interesting in the context of TBEV infection. However, interfering with the DNA damage response has been shown for other flaviviruses like ZIKV (Hammack 2019), and promoting autophagy has also been shown for other flaviviruses and RNA viruses (ZIKV and SARS-CoV-2). Elucidating a single mechanism would provide deeper insights into the functional consequences of some of the interactions or PTMs identified during TBEV infection. This could also potentially uncover the distinct and conserved mechanisms among flaviviruses. Taken together, the study provides an excellent resource for the field that will help generate many exciting new hypotheses, but the lack of mechanistic details in the interactions chosen for follow-up along with the lack of rationale for the use of different cell lines and confounding results from inhibitor treatment significantly dampens the impact of the study.

Major Comments:
1. There is little focus regarding the mechanisms and consequences of each interaction or PTM identified. For example, the authors have nicely shown that NS5 and SIRT1 interactions prevent effective DNA damage repair, however it remains unclear how this is beneficial for TBEV replication or production. Moreover, the authors observed most proteins exhibiting hyperacetylation over the course of TBEV infection in contrast to other PTMs such as phosphorylation where both phosphorylated and dephosphorylated proteins were observed. It would be novel and interesting to follow up on the functional consequences of specific hyperacetylation events upon TBEV infection.
2. One major concern is the relevance of cell types used throughout the study. Vero cells are not of human origin, and HEK293T and A549 cells are not physiologically relevant to TBEV infection, thus decreasing the reliability of the findings. Moreover, the authors do not provide rationale for the use and switching among the cell types. For example, the proteomics was done on infected Vero cells in Figure 1, while the AP-MS was done on HEK293T cells expressing individual TBEV proteins in Figure 2. The brain organoid model (Figure 4) is a useful tool for validating the findings, but should be incorporated more cohesively throughout the manuscript. Alternatively, the authors should consider mechanistic studies in neuronal-like cell types such as SNB19 or even primary cells.
3. Pharmacologically targeting the identified pathways is a strong approach to show the importance of these pathways in TBEV replication, however the results raise additional questions. It is important to note that many of the inhibitors used only effectively work in Vero cells, while effectiveness is impaired in A549 cells. Moreover, several inhibitors begin working at concentrations that decrease cell viability, raising concerns regarding the importance of the targeted pathways for TBEV infection. Additionally, two inhibitors that target autophagy, spautin-1 and 3-MA, have opposite effects on TBEV infection, making the role of autophagy unclear. Lastly, it is unclear how the authors rationalized the targeting of the identified pathways. For example, Figure 6e suggests the authors are targeting DNA damage repair, which have larger implications and consequences beyond TBEV infection.
4. There is also a lack of experimental details in the manuscript, specifically regarding the number of independent experiments done for each figure panel, and whether they have done technical or biological replicates. The authors should make this clear in the figure legends and provide rationale for each experiment in the text.
5. The models illustrated in the manuscript are well done, however can be confusing at first. The authors should consider

making a schematic of the cell pathways under normal conditions, followed by a schematic of the pathways when affected by the viral protein. This would make their findings clearer considering the large datasets and many changes that were identified. Additionally, the authors should omit parts of the pathways that are not relevant to the main findings. An example would be to focus on the left portion of the pathway in Figure 4t (should be corrected to Figure 4s).
6. The authors should change the title to reflect the approaches used in the study. “Multi-omics” suggests the use of several -omics approaches including transcriptomics, metabolomics, and proteomics. However, the study only uses different proteomic approaches, and should therefore change the title to “Multi-proteomics”.

Minor comments:
1. Line 126: Change to “conserved regulation of host DNA damage response by flaviviruses”.
2. Lines 236-237: Change “baits” to “preys”.
3. Line 244: Change to “inhibit their recruitment of ISGs”.
4. Line 284: Change to “host innate immune response to TBEV infection”.
5. Line 373: Change to “Specifically”.
6. Line 421: Figure 4t should be changed to Figure 4s.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors addressed all my comments with additional data when necessary. I am happy with the revisions and the amended manuscript that I think is now suitable for publication.

Reviewer #2

(Remarks to the Author)
In the revised version of the manuscript, the authors have addressed one out of my 3 major concerns: the one related to the extent of mechanistic or functional validation of the multi-omic data (Point 1).

In this respect, while I appreciate the additional validation in brain organoids presented in Fig.6 of the rebuttal (substantial and quite laborious, yet elegant system), these experiments still focus entirely on validating extensively published observations (i.e. Autophagy, and DNA damage as judged by the gammaHA2X WB and LC3 puncta/ ratio). The main concern raised in the first revision was indeed that these effects have been previously reported for multiple flaviviruses and in multiple cellular backgrounds. Therefore as such the new experiments provided appear more as an academic exercise rather than an experimental validation on newly discovered pathway, and do not add any knowledge to the existing literature.

Most importantly, this reviewer still strongly feels that the reply to point 3 does not resolve, but rather confirms the issues related to the cloning strategy used to generate the PPI map of TBEV viral proteins-host interactions.
The authors reply that:
“TBEV structural and nonstructural proteins (GenBank Access Number MN615726.1) were cloned into Flag-VR1012, incorporating 2×Strep II affinity and 3×Flag tags at N terminal, according to previous report2) citing the paper by Shah et al. (Shah, P. S. et al. Comparative Flavivirus-Host Protein Interaction Mapping Reveals Mechanisms of Dengue and Zika Virus Pathogenesis. Cell 175, 1931-1945.e1918 (2018).)

However, in this paper all constructs were cloned with a c-terminal FLAG tag, and not an N-terminal tag:
(excerpt from the paper: “DENV serotype 2 16681, ZIKV French Polynesia 2013 H/FP/2013 (ZIKVfp), and ZIKV Uganda 1947 MR766 (ZIKVug) open reading frames (ORFs) were cloned into pCDNA4_TO with a C-terminal 2xStrep II affinity tag for expression in human cells.”).
Beside this, there is an extensive body of literature (i.e. https://doi.org/10.1038/nrmicro.2017.170, https://doi.org/10.1038/s44298-024-00031-7 , just to cite 2 recent reviews), supporting that only NS2B and NS4A could be also tagged N-terminally without affecting their physiological localisation or topology.
This point is extremely important as erroneous tagging of most of the viral proteins, completely affects their topology (and therefore their interaction with cellular proteins). In fact, the proper insertion in the ER is very specifically mediated by signal sequences located upstream of some of the viral proteins (i.e. Envelope requires the last aa of prM, NS1 requires the last aa of Envelope for proper insertion in the ER; 2k-NS4B will be inserted upside-down in the ER if lacking the 2k peptide; etc..).
See sketch attached for a graphical representation of this issue.

Based on this, all the interaction data related to the remaining 8 viral proteins (Capsid, Envelope, prM, E, NS1, NS2A, NS3, 2k-NS4B and NS5) should be interpreted with extreme caution, and such experiments repeated with appropriately tagged constructs or entirely removed from the manuscript.

Reviewer #3

(Remarks to the Author)
We commend the authors on the large amount of work they put into the revision. We were mostly satisfied with the changes made by the authors, including additional experimentation to show the importance of Ku70 acetylation in the context of TBEV infection, the addition of more relevant neuronal and organoid models that increase the significance of the findings (these results were included in a brand new figure), and modifications made to the schematics and writing.

However, with the additional data provided, we have additional comments that should be addressed/improved:

• A general comment that should be addressed throughout the manuscript is the large number of panels per figure. One example is Figure 6, which contains 23 panels (A through W) and is unnecessarily excessive. This reduces the accessibility of the manuscript to larger audiences, which is critical for the journal. One recommendation for increasing access to this important data is to move redundant or nonessential panels to supplementary section or to other figures. Two examples would be 1) Figure 6J; it is unclear how this is essential to the main message of the figure and can be either removed or put in supplementary, and 2) Figure 6U, V, and W; these would go well in Figure 7 to more cohesively tie in the relevant models to the important findings in Figure 7. These suggestions should be broadly applied to all the figures in this paper.

• Regarding Point 3:
The authors' rationale for why pharmacological targeting of certain pathways is specific to Vero cells but not A549 cells remains unclear. They claim the differences in efficacy may be due to a diminished IFN response but did not directly test this. Moreover, the neuronal cells used in Figure 6U-W presumably have an intact IFN response and yet the inhibitors used show higher efficacy. This again casts doubts on whether the Vero and A549 cells are relevant cell models for testing these inhibitors. Lastly, the inhibitors used on Vero/A549 cells were not used on T98G cells, and the rationale behind this is missing.

The authors briefly mentioned that 3-MA and Spautin-1 have different molecular targets and that may explain why they have opposite effects on TBEV infection, but the authors did not directly address our comment. The role of autophagy remains unclear—why is it that both molecules inhibit autophagy, but 3-MA has less to no effects at higher concentrations (despite no changes in cell viability) while Spautin-1 is antiviral in a dose dependent manner? If there is no scientific explanation for this discrepancy, the authors cannot conclude that inhibitors targeting autophagy exhibit potent antiviral activity.

The authors' response for inhibiting DDR was also confusing. If the virus is inhibiting DDR, what is the rationale behind using DDR inhibitors? Shouldn't DDR be activated to see whether viral infection can be blocked?

• Regarding Point 5:
A suggestion for Figure 5T would be to clarify the effects of the virus on the pathway. One example could be to cross out the inhibitory line from MTOR to the arrow pointing to autophagosome. This would better show that dephosphorylation of MTOR would allow autophagy to proceed.

• Regarding Point 6:
A minor adjustment would be to make the same change from the title into the results section as well (Line 273, Page 9).

Reviewer #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
Major comments:
The authors have placed quite some emphasis on the newly identified and characterized pathways and altogether convincingly show that the revised version of the manuscript is adding to the existing body of evidence (point 1).

Unfortunately, with respect to point 2 (topology of the ectopically expressed viral proteins), this reviewer still strongly feels that incorrect tagging of the majority of the viral proteins invalidates most of the results of their interactome study. Both the IFA data and the Alphafold predictions, added during the revision of the manuscript, do not support correct topology of the viral protein, nor their overall folding, respectively.
As already detailed in the previous revision, erroneous tagging of the viral proteins strongly affects their topology, while not necessarily their localization (i.e. proteins will still be targeted and inserted in the ER membrane, but in the incorrect orientation). Therefore, the additional IF analysis provided does not corroborate correct topology of the tagged proteins, but confirms as expected that they are still localized in the correct compartment. Nonetheless, the consequences for the

interactome remain substantial. Please see the additional drawing attached to clarify again this point, using 2k-NS4B as a test case.
Furthermore, the Alphafold predictions shown as such do not support any conclusions (a) Alphafold performs still poorly on highly transmembrane viral proteins; (b) there is no quantitiative score provided to support similary of folding in the 2 tagging strategy; (c) the structure actually looks very different in many instances.

Minor comments

Methods, Line 892-893: „The employed protein databases encompassed the human proteome (Blast_Chlorocebus_sabaeus_60711_PR_20210928), the mycoplasma proteome (uniprot-reviewed_yes+AND+organism_mycoplasma), and relevant TBEV protein databases“.
Please provide the correct human (293T interactome dataset) and African monkey (VeroE6 dataset) UniProt identifiers used in MaxQuant searches, currently only the African green monkey is provided. Please add also the reference strain for TBEV used in the proteome search.

Dear Editor and Reviewers,

Re: Manuscript NCOMMS-24-14544.R1

On behalf of all co-authors, I would like to thank you and the three **Reviewers** very much for positive and favorable comments, and constructive suggestions on our manuscript (MS) NCOMMS-24-14544. All reviewers liked our MS and considered that our MS is of general and broad interest to the readership of ***Nature Communications***. We are especially grateful to the reviewers for their kindness, generosity and graciousness in providing detailed constructive comments and suggestions on our MS.

We have revised the MS strictly according to the reviewers' comments and suggestions. Two MS files are uploaded: one shows **"tracked changes"** as "**Manuscript with revisions highlighted**", and the other is a "clean file" as "**Manuscript**". In the following, we detail our point-by-point responses to these comments and suggestions.

Responses to the comments and suggestions of Reviewer #1

Point 1: The manuscript addresses TBEV-induced host cell perturbations by a comprehensive approach that includes: (i) characterization of the proteome, phosphoproteome, and acetylproteome of infected cells; (ii) description of a novel pathway where viral NS5 blocks the DNA damage response of the cell (this is the more developed mechanistic part); (iii) description of a novel pathway where viral prM induces autophagy; (iv) characterization of inhibitors targeting kinases, DDR or autophagy. It is a huge amount of work covering various aspects, but some without the necessary depth. For example, role of DNA damage induction and DDR in TBEV pathogenesis like neuroinflammation is not analyzed (see more specific comments below). Animal models of TBEV infection are available and would have been important to check for DNA damage and DDR markers in vivo to corroborate in vitro data. In addition to more specific comments below I believe that the paper requires to address the consequences of DNA damage on (neuro)inflammation.

Response: Thanks very much for the comments. Our analysis of inflammatory factors following TBEV infection revealed an increase in the mRNA expression of inflammatory markers such as *NFKB*, *IL1A*, *IL6*, *IL8*, and *CXCL10* (Figures S15a-b). Additionally, we established a TBEV infection model using BALB/C mice, with the expression of TBEV NS1 confirming successful virus infection (Figures S16d-f). Notably, we observed a significant elevation in the expression level of γH2AX, which also exhibited increased nuclear localization following TBEV infection. Conversely, the nuclear localization of 53BP1 was notably reduced upon TBEV infection. These findings, in conjunction with our in vitro data, provide compelling evidence that TBEV infection modulates the DNA damage response.

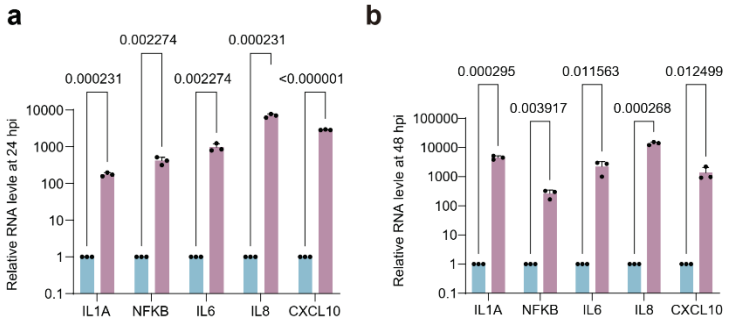


Figure S15 a-b. qPCR assay showing increased levels of genes associated with cellular inflammatory factors (IL1A, NFKB, IL6, IL8, CXCL10) in TBEV-infected cells. A549 cells were infected with TBEV and harvested at 24 hpi (**a**) and 48 hpi (**b**)

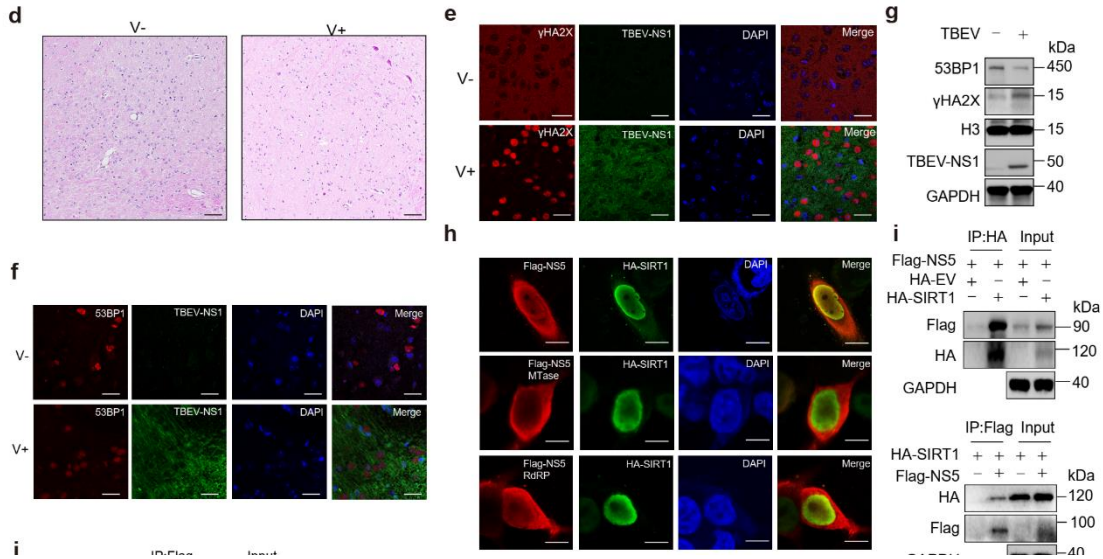


Figure S16.
d. H&E staining of mock and TBEV-infected brain of mice. Scale bar, 50 μm.
e-f. Brain organoids infected with TBEV displayed an increased formation of punctate structures for γH2AX(e) but decreased 53BP1 (f) compared with control brain organoids. Scale bar, 20 μm.
g. Immunoblot assays demonstrated a significant decrease in the levels of 53BP1 but an increased γH2AX protein in brain organoids infected with TBEV compared to control brain organoids.

Point 2: The omics part is essentially descriptive and could be reduced or put in supplementary in favor of mechanistic follow up. The sampling at different times post-infection is particularly appreciated because it allows to monitor kinetics of PTMs for example.

Response: Thanks Reviewer #1 very much for favorable and positive comments on our MS. In response to your suggestions, we have relocated Fig. 1f and 1g to the supplementary section and incorporated the kinase activity into Fig 1f. Additionally, we have conducted a comprehensive screening of significantly altered acetylation sites, identifying 59 sites that demonstrated clear changes at 6, 12, 24, and 48 hours post-infection (hpi), as depicted in Figure S2b. Notably, these sites consistently exhibited elevated levels of acetylation, correlating with the overall increase in acetylation levels following TBEV infection. Among these, the innate immune regulator STAT1 showed a marked increase in acetylation, potentially linked to the activation of innate immunity by TBEV. Furthermore, ER-resident proteins, such as CANX and HSPA5, displayed noticeable acetylation, which possibly associated with TBEV-induced ER stress. Additionally, the receptor of arenaviruses[1], TFRC, demonstrated elevated acetylation at the K399 site, potentially linked to TBEV replication.

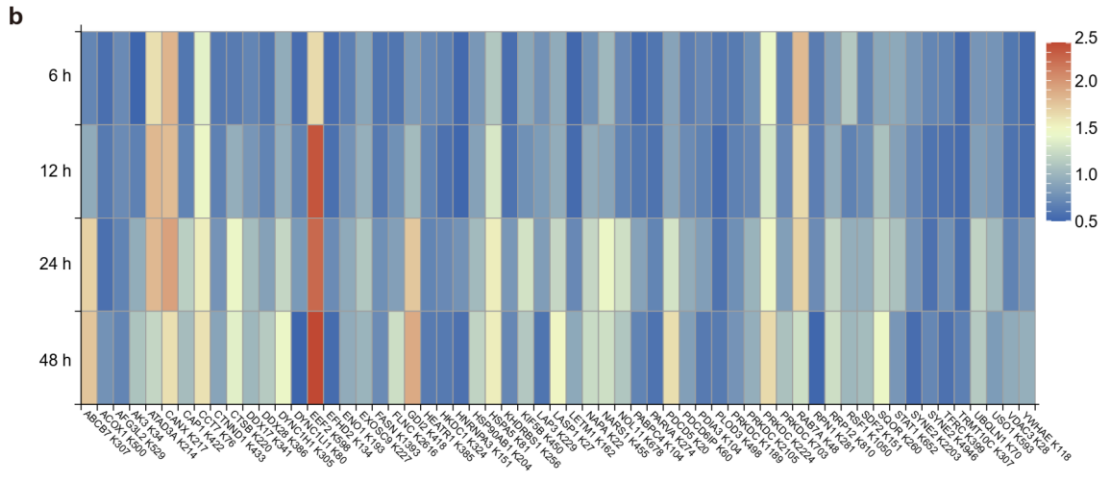


Figure S2. b. Acetylation sites that significantly altered at both 6, 12, 24 and 48 hours upon TBEV infection

Point 3: Viral protein tagging need more explanation for the positioning of tags with respect to certain domains such as 2K for example, in same cases it may disrupt function.

Response: Thanks very much for your comments. The TBEV structural and nonstructural proteins (GenBank Access Number MN615726.1) were cloned into Flag-VR1012, incorporating 2×Strep II affinity and 3×Flag tags at N terminal, according to previous report[2]. You are correct in noting the significance of the 2K protein in the positioning of NS4B. However, in our transcription of NS4B plasmids, we observed the expression of NS4B protein and its proper localization on the ER, which is sufficient for our interaction study.

Point 4: Quite surprisingly there is no effect on ER stress/UPR, which have been described for TBEV (Carletti Nat Comm 2019). Contrary to what was shown, data on PKR phosphorylation are showing early upregulation and then downregulation (EIF2AK2) but no data for PERK (EIF2AK3) or EIF-2a/EIF2S1phosphorylation are shown.

Response: Thanks very much for your comments. The kinase activity analysis mainly focused on identifying significant changes in kinase activity following TBEV infection at multiple time points. Given that the phosphorylation of PERK and EIF-2a only increased during early or late stages of TBEV infection[3], it is possible that their activity changes could be overlooked or dismissed in our analysis. We further investigated the expression of phosphorylated PERK and EIF2a following TBEV infection by immunoblot, revealing upregulation of these phosphorylated proteins, thus demonstrating the impact of TBEV infection on ER stress (Figure S2a). Additionally, our interactome analysis unveiled associations between NS2B, NS2B3, and components involved in ER-associated degradation (ERAD), such as NS2B-SYVN1, NS2B-HERPUD1, and NS2B3-MAN1B1. This interaction may be linked to ER aggregation in cells overexpressing NS2B and NS2B3 (Figure 2b). Moreover, the upregulation of SYVN1 expression suggests a potential disruption of the unfolded protein response (UPR) following TBEV infection (Figure 1c).

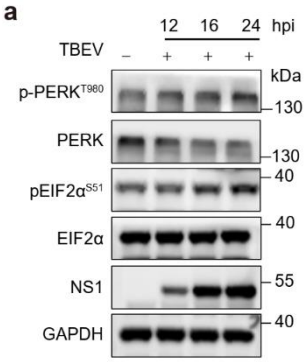


Figure S2. a. Immunoblot assay showed an increase in the phosphorylated forms of PERK (T980) and EIF-2α (S51) in cells infected with TBEV at 12, 16 and 24 hpi.

Point 5: The authors then decide to investigate DNA damage and DDR in more detail. They start from omic data also mentioning RRM2 decrease as a potential trigger of DNA damage (page 10). However, this observation is not followed up. For example, it has been shown in SARS-Cov-2 that CHK1 depletion induces RRM2 loss and dNTP shortage with DNA damage induction, DDR, inflammation and senescence both in vitro and in vivo (Gioia Nat Cell Biol 2023).

Response: Thanks very much for your comments. We also observed a decrease in the expression of RRM2 in the omics data. However, further confirmation through immunoblot assay indicated no significant difference in the expression of RRM2 upon TBEV infection (Figures S17g and h), thereby excluding the activation of DNA damage through the reduction of RRM2. This result has been included in Figure S17 in the revised manuscript.

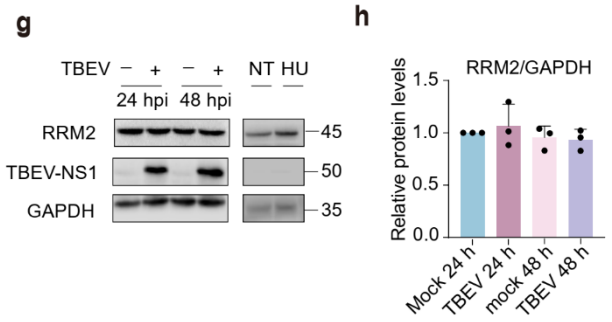


Figure S17 g. Immunoblot assay showed no effect on RRM2 protein levels in TBEV-infected cells. A549 cells were infected with TBEV and harvested at 24 hpi and 48 hpi. Cells not treated (-) or treated with 6 mM HU as positive controls. RRM2 protein levels are normalized by GAPDH and set to 1.00 in control cells.
h. Quantification of RRM2 protein levels shown in (g), values normalized to mock-infected samples.

More specifically into DDR:
Point 6: It would be important to show CHK1 and CHK2 levels and phosphorylation status. Figure S2A, while DNA-PK and ATM show time dependent increase, phosphorylation of ATR is not so consistent. Better Figure 4a but still ATR poorly activated. I' m also concerned about the statistics of Figure 4b (and other throughout the manuscript), significance related to value normalized to 1 seems miscalculated.
It is gH2AX (mis-spelled) and why not induced in HU control? Include CHK1/2 as mentioned above.
Response: Thanks very much for your comments. Our additional findings revealed a significant upregulation in the phosphorylation of CHK1 and CHK2 following TBEV infection, which has been incorporated into Figure 4a.

Regarding the activity of ATR, we observed some inconsistency, as the activity of ATR was not detected at 24 hpi. However, we did note an increase in ATR activity from 12 to 48 hpi, and our immunoblot assay indicated a significant elevation in ATR phosphorylation at 24 and 48 hpi (Figure 4b). We have re-evaluated the protein intensities in Figure 4b, using the 24 h mock as a reference point (set to 1), and the recalculated data has been included in Figure 4b of the revised manuscript.

Furthermore, we conducted a repeat of the HU-treated assay, demonstrating the activation of both rH2AX, CHK1, and CHK2 in response to HU treatment (Figure 4a). We also examined the mis-spelled rH2AX through the MS and revised them.

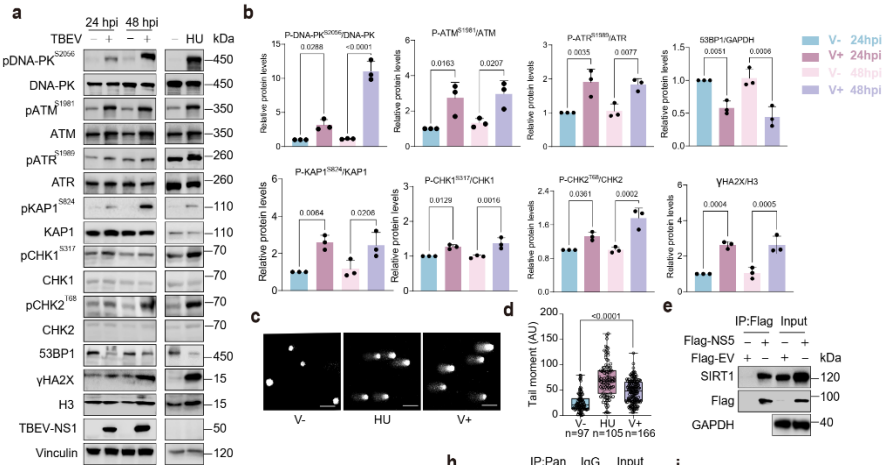


Figure 4.
a. Immunoblot assay showing increased levels of proteins associated with DNA damage and altered DDR

activation in TBEV-infected cells. A549 cells were infected with TBEV and harvested at 24 and 48 hpi (hpi, hours post-inoculation). Lane 2 and lane 4 show cells infected at 24 and 48 hpi at MOI 2.0, respectively. Lane 5 and lane 6 show lysates from A549 cells not treated (-) or treated with 6 mM HU as positive controls. Proteins levels associated with DNA damage and altered DDR activation are normalized by vinculin and set to 1.00 in control cells.
b. Quantification of activated protein levels shown in (**a**), values normalized to mock-infected samples.

Point 6: Comet assay is an important information, please indicate h.p.i. of the assay.
P21 and MK167 (K167) markers are poorly regulated in the supplementary figure, need IF quantification data. In general lacking IF for markers of DDR. Also NFkB and IL1A need activation data by IF/RT-PCR/WB. A panel of pro-inflammatory cytokines following TBEV infection would be a useful information, as well as induction of senescnece by b-gal staining.

It would be important to exclude apoptosis in the time frame of the DDR assay, please provide such information (i.e. caspase assay).
The increase of G0/1 not very significant by flow cytometry (Figure S15), maybe include bivariate plot showing DNA content (PI) and BrdU incorporation measured by flow cytometry (horseshoe).

Response: Thanks very much for your comments. The comet assay was performed at 24 hours post TBEV infection, which was added to the figure legend. We conducted an assessment of pCHK1 for IF staining, revealing an elevated number of pCHK1^{S317} foci per TBEV-infected cell in comparison to uninfected cells (Figures S14e, f), which together with the IF staining for rH2AX and pKAP1, indicated the activated DDR markers upon TBEV infection (Figures S14a-d).

We detected the expression of P21 and KI67 using IFC assay, revealing an increase in P21 levels and a decrease in KI67 levels following TBEV infection (Figures S15d-g). These findings align with our proteomics analysis. Additionally, our investigation indicated that TBEV infection led to the expression of inflammatory factors, including *NFKB*, *IL1A*, *IL6*, *IL8*, and *CXCL10*, as well as an increase in β-gal expression, signifying the induction of inflammation and senescence due to TBEV infection (Figures S15a-c).

We assessed caspase activity at 24 and 48 hpi, concurrently with the detection of DDR markers, and found no significant changes in caspase activity following TBEV infection. This observation excludes apoptosis within the timeframe of the DDR assay (Figure S15l). Furthermore, our analysis of BrdU incorporation indicated G0/G1 cell cycle arrest induced by TBEV infection (Figures S15h, i).

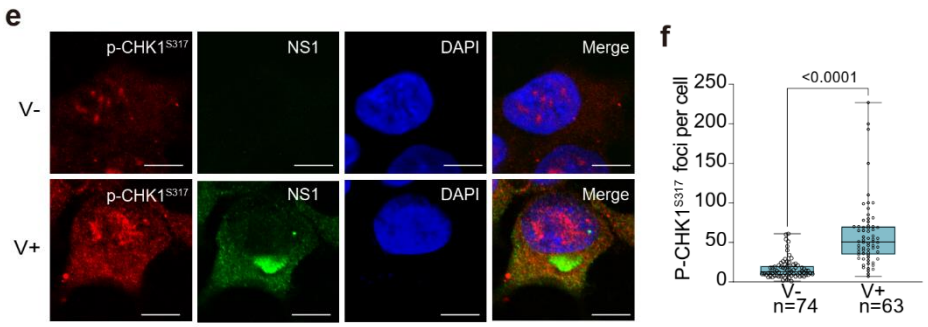


Figure S14 e. Cells infected with TBEV at 24 hpi displayed an increased formation of punctate structures for pCHK1^{S317} compared with control cells. TBEV NS1 protein labelled infected cells. Nuclei were counterstained with DAPI. Scale bar, 10 μm.
f. Quantification of puncta in (**e**) showed a similar trend, with each data point representing a nucleus. Data are shown as mean ± SEM (V-, n=74 cells; V+, n=63 cells).

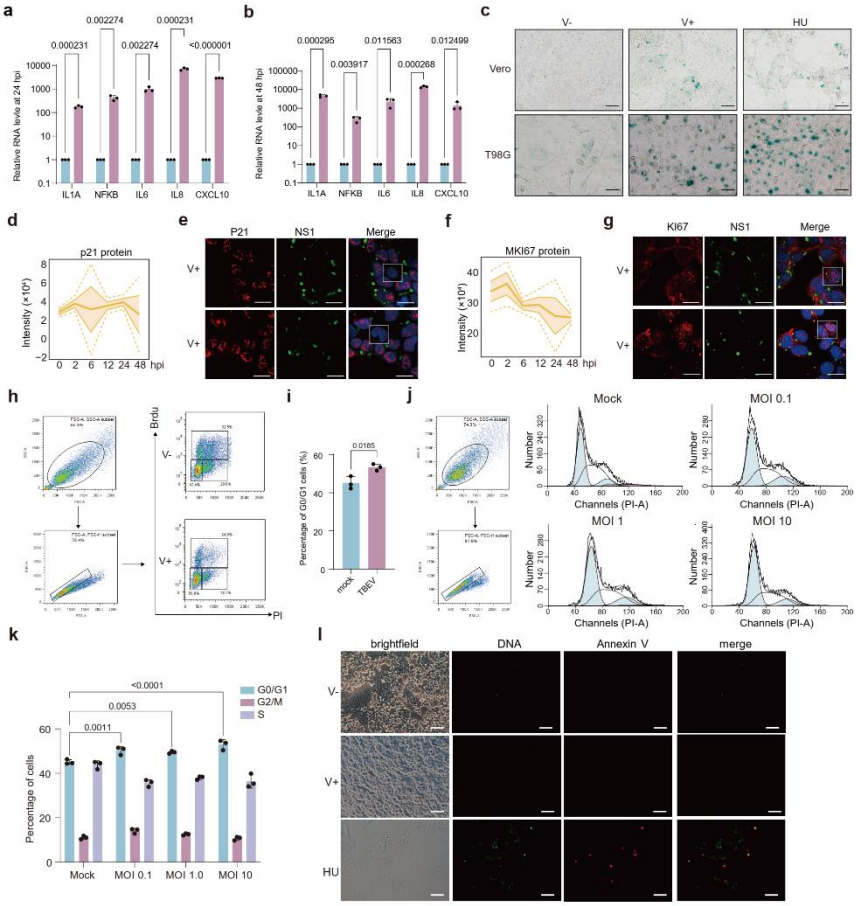


Figure S15. TBEV dysregulates cellular senescence markers and cell cycle.
a-b. qPCR assays showing increased levels of genes associated with cellular inflammatory factors (IL1A, NFKB, IL6, IL8, CXCL10) in TBEV-infected cells at 24 hpi (a) and 48 hpi (b)
c. Cells infected with TBEV 24 hpi showed more senescence-associated β-galactosidase staining compared with control cells. Scale bar, 100 µm.
d-g. Regulation of the protein abundance of P21 and MKI67 evaluated by proteomics (**d, f**) and IFC analysis (**e, g**) in TBEV-infected cells. The graph in **d** and **f** is depicted with the median (line), 50% (shaded region) and 95% (dotted line) confidence intervals, n=3.
h. FACS analysis of TBEV-infected cells by BrdU identify higher percentage of G0/G1 cells upon TBEV infection.
i. Quantification of G0/G1 cells shown in (**h**).
j. FACS analysis of TBEV-infected cells revealed G0/G1 cell cycle arrest.
k. Percentage of cells in G0/G1, S and G2/M was quantified based on the FACS analysis in (**j**).
i. Cells infected with TBEV at 24 hpi showed no effect on Annexin-V/Caspase-3 staining associated with apoptosis compared with control cells. Scale bar, 100 µm.

Point 7: P53BP reduction in foci upon infection is would benefit of co-loc with gH2AX and WB of p53BP upon infection.

Response: We conducted an analysis of the co-localization of 53BP1 and rH2AX and have included the results depicting their co-localization in Figure S16a.

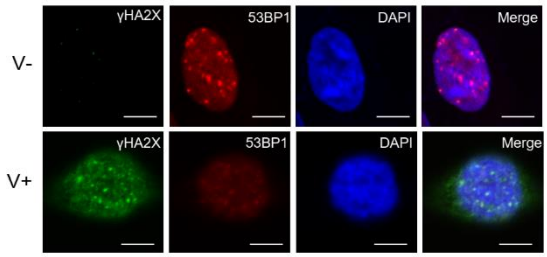


Figure S16a. The colocalization of rH2AX and 53BP1 upon TBEV infection.

Point 8: The background to reach the question is just a hypothesis, in fact the authors then come to the real mechanistic question: we hypothesized that TBEV NS5 might engage with SIRT1 and inhibit SIRT1-mediated deacetylation of Ku70 and KAP1, consequently suppressing the efficiency of NHEJ activity.
Co-IP NS5/SIRT is ok but IF show NS5 in the nucleus. It is known that NS5 from various Flavi has nuclear localization (quite peculiar for a cytoplasmic virus), but for TBEV it is not described to my knowledge, please provide explanation. Also co-loc NS5/SIRt is not very informative being extensive throughout the cell.
Response: Thanks very much for the positive comments and suggestions. We observed that a small amount of TBEV NS5 localized in both the nucleus and cytoplasm. The nuclear localization of TBEV NS5 can be explained in two ways. First, the amino acid similarity of TBEV NS5 with ZIKV and DENV NS5 was 56% and 56.4%, respectively (Table1), which suggests the potential for nuclear localization of TBEV NS5 due to this high similarity. Second, the amino acid sequence $K^{390}R^{391}P^{392}R^{393}$ in ZIKV NS5 is considered crucial for its interaction with importin, leading to nuclear localization. Only when all these amino acids are mutated can the nuclear location of ZIKV NS5 be eliminated [4]. In contrast, TBEV NS5 possesses the sequence $S^{390}R^{391}P^{392}R^{393}$, which supports its nuclear localization. Furthermore, we conducted a repeat of the colocalization analysis of NS5 and SIRT1, demonstrating the colocalization of NS5 with SIRT1 in the nucleus (Figure S16g).

	TBEV	ZIKV	DENV	JEV	WNV	YFV
TBEV	***	56	56.4	56.5	58.8	57.8
ZIKV	65	***	65.9	68.5	69.6	60.7
DENV	64.3	45.2	***	65.1	66.7	59.1
JEV	64.1	40.8	46.8	***	81	59.7
WNV	59.1	39	43.9	22	***	60.4
YFV	61.2	55.2	58.4	57.2	55.7	***

Table 1. Amino Acid Similarity of TBEV NS5 with NS proteins of other Flaviviruses.

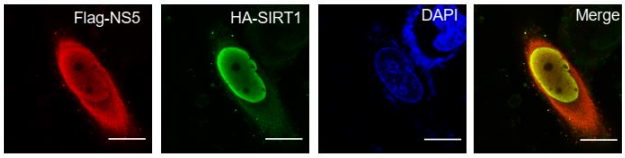


Figure S16. g. Flag-NS5 co-localized with HA-tagged SIRT1, visualized by anti-HA (green) and anti-Flag (red) antibodies. Scale bars, 10 μm.

Point 9: Overexpression of SIRT1 induces deacetylation of Ku70 – evidence? Fig S14J and j-n maybe, not indicated. Data are a bit confused, what is IP:Pan, Pan-acetylation antibody? HA control why showing a band?

Functionally, NS5 reduces NHEJ, ok, and 53BP1foci are reduced, which figure?
The authors use a brain organoid infection model, nice but need introduction.

Response: Thank you very much for your suggestive comments. SIRT1 induced deacetylation of Ku70 was implicated in Fig. S16m in the revised MS, we have indicated this to the manuscript. Additionally, we have updated the figure to specify that "Pan" has been changed to "Pan-Ace" to represent the Pan-acetylation antibody. In relation to Fig. S16m, we observed a band for HA, which might indicate the acetylation of SIRT1. It is worth noting that acetylation of SIRT1 has been demonstrated to regulate its activity and stability [5]. The reduced 53BP1 foci was shown in Figure S16b-c.

To provide a more comprehensive overview of the brain organoids results, we have gathered relevant data and incorporated it into the new Figure 6j-r and S20b-c. The following description has been added to elaborate on these findings.

Brain organoid generated from induced pluripotent stem cells serve as an excellent model for studying neurotropic viruses and the associated neuronal damage. To further elucidate the effects of TBEV on DDR and autophagy, we utilized TBEV-infected brain organoids. Characterization of the brain organoid confirmed the presence of neural progenitor (SOX2), oligodendrocyte (Oligo2), immature neurons (TUJ1), and mature neuronal (NeuN) markers (Figure 6j). A gradual increase in viral titer throughout the infection period confirmed successful infection of TBEV, which primarily localized in mature neuronal cells and astrocytes (Figures 6k and 6l). Concurrently, we observed a significant increase in the intensity of γH2AX in TBEV-infected organoid compared to the mock-infected group, as indicated by both immunoblot and immunofluorescence staining assays. This finding suggests the activation of DNA damage in response to TBEV infection (Figures 6m-p). Conversely, we noted a reduction in the number of 53BP1 foci following TBEV infection, implying a reduction in the repairment of DNA damage (Figures 6q and 6r). Furthermore, our analysis of TBEV-infected brain organoids revealed elevated LC-II/LC3-I ratios and P62 levels, along with an increased abundance of LC3 puncta, supporting the conclusion that TBEV infection induces autophagy (Figures 6s and 6t). Subsequently, we investigated the effects of pharmacological agents targeting DDR and autophagy on TBEV replication. Consistent with results obtained from Vero and A549 cells, treatment with the ATM inhibitor AZD-1390, SIRT1 agonist Fisetin, and autophagic flux inhibitor chloroquine significantly suppressed TBEV transcription (Figure 6u-w). These findings provide further evidence of the regulated DDR and autophagy within an organoid infection model.

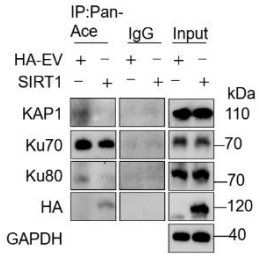


Figure S16. **m.** Co-immunoprecipitation assay demonstrated reduced acetylation of KAP1, Ku70 and Ku80 in SIRT1-overexpressed cells.

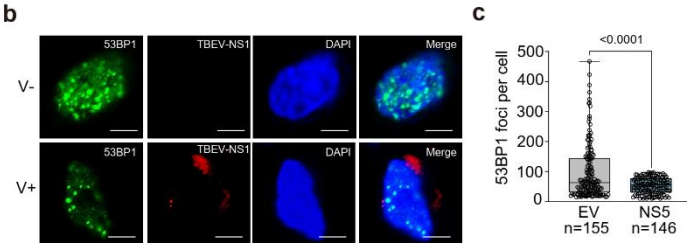


Figure S16. **b.** Cells infected with TBEV at 24 hpi exhibited a decrease formation of punctate structures for 53BP1 compared with control cells. Scale bar, 5 μm. **c.** Quantification of puncta in (b), with each dot representing a nucleus. Data are shown as mean ± SEM (V-, n=155 cells; V+, n=146 cells).

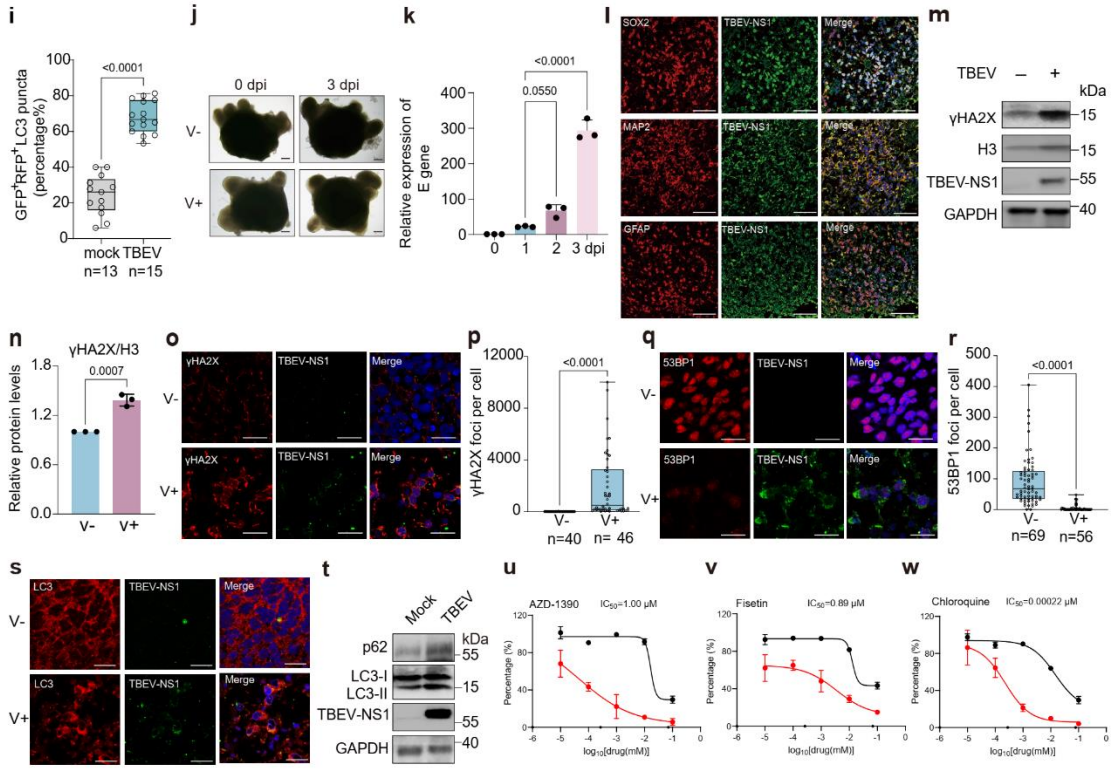


Figure 6.

j. Images of brain organoids, either mock-infected or infected with TBEV at 0- and 3-days post-infection (dpi). Scale bar, 200 μm.
k. Quantification by probe PCR demonstrated a gradual increase in the copies of TBEV E genes with the duration of TBEV infection
l. Immunofluorescence analysis highlighted the colocalization of TBEV NS1 with SOX2 (progenitors), MAP2 (neurons), GFAP (astroglia). Scale bar, 200 μm.
m. Immunoblot assay showing increased expression of γH2AX in TBEV-infected brain organoids.
n. Quantification of increased percentage of γH2AX /H3 in (m).
o. More punctate structures of γH2AX in brain organoids infected with TBEV compared with the control. TBEV NS1 protein used to label infected cells. Nuclei stained with DAPI. Scale bar, 100 μm.
p. Quantification of indicated puncta in (o), each dot is a nucleus.
q. Less punctate structures of 53BP1 in brain organoids infected with TBEV compared with the control. TBEV NS1 protein used to label infected cells. Nuclei were stained with DAPI. Scale bar, 100 μm.
r. Quantification of indicated puncta in (q), each dot is a nucleus.
s. The brain organoid infected with TBEV exhibited an increase number of GFP+LC3 puncta compared to mock-infected organoid. Scale bar, 100 μm.
t. Immunoblot assay of TBEV-infected brain organoids revealed increased expression levels of LC3 and P62.

Point 10: KD of SIRT no effect but overxpression inhibits TBEV replication. Cathalitically inactive form does not have effect in KD cells, why KD for SIRT background? Wouldn’t inhibit also the palsmid?

Response: Thanks very much for the comments. We tested the impact of WT and H363Y SIRT on WT cells, revealing that only the WT, but not the H363Y mutation, exhibited an inhibitory effect on TBEV replication. It is worth noting that the substantial expression of SIRT1 in WT cells may influenced the impact of H363Y. To address this, we further evaluated the effect on SIRT1 knockdown (KD) cells, which demonstrated a more pronounced effect on TBEV replication.

Point 11: Conclusion: TBEV NS5 binds to and suppresses the activity of SIRT1, resulting in the elevated acetylation of Ku70 and KAP1, which in turn inhibits DNA damage repairment and promotes virus replication.

Which part of NS5 is required for SIRT1 suppression? Is it nuclear or cyto the site of interaction/inhiibtion? How does it suppress SIRT1 activity? No degrdation is apparent.

Response: Thanks very much for the comments. TBEV NS5 contains the methyltransferase (MTase) domain on the N terminal and the RNA-dependent RNA polymerase (RdRP) domain on C terminal. We conducted co-immunoprecipitation analysis of NS5 truncated proteins with SIRT1, revealing that the RdRP domain of NS5 could be precipitated with SIRT1 (Figure S16j). Additionally, the colocalization of NS5 truncations and SIRT1 indicated that the MTase domain was primarily located in the cytoplasm, while the C-terminal RdRP domain was found in both the cytoplasmic and nuclear domains, where it colocalized with SIRT1 (Figure S16g).

To gain deeper insights into the mechanism by which NS5 suppresses SIRT1, we performed a protein-protein interaction analysis of SIRT1 and the RdRP domain of TBEV NS5 using HDOCK (Figure S16k). The docking score between SIRT1 and TEBV was -234.47, with eight amino acids of NS5 (ARG^{34}, GLU^{28}, GLU^{24}, GLU^{175}, ARG^{184}, GLU^{17}, ASP^{14}, LEU^{22}) involved in the interaction, suggesting a potential direct interaction between NS5 and SIRT1. This interaction may contribute to the inhibition of SIRT1 activity by NS5.

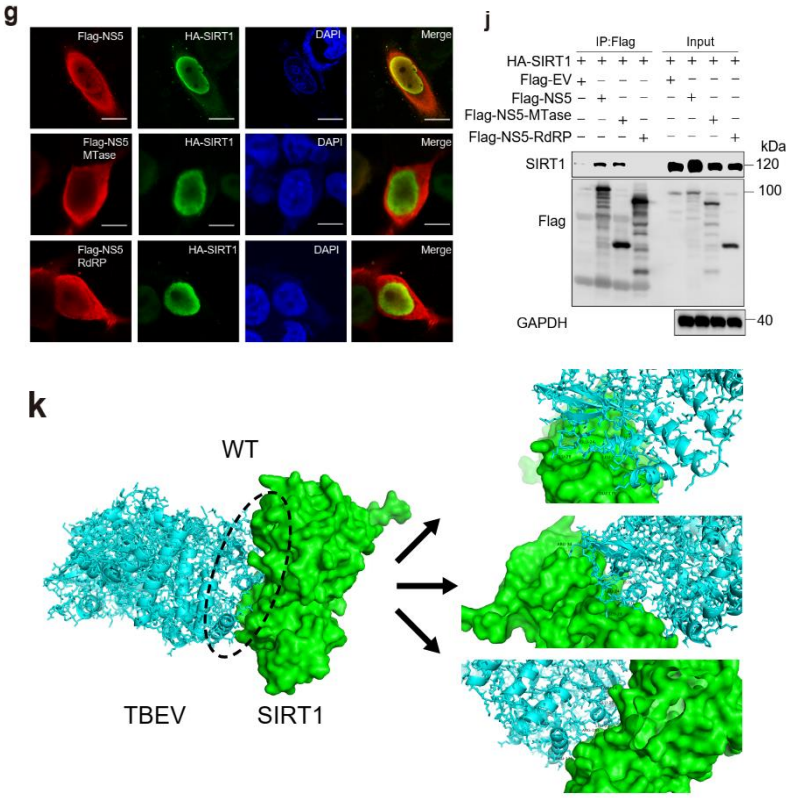


Figure S16. **g.** Flag-NS5 and its truncations co-localized with HA-tagged SIRT1, visualized by anti-HA (green) and anti-Flag (red) antibodies. Scale bars, 10 μm. **j.** Co-immunoprecipitation assay demonstrated that the RdRP domain of NS5 interacted with SIRT1. **k.** Interaction analysis indicated that 8 amino acids on TBEV NS5 (ARG^{34}, GLU^{28}, GLU^{24}, GLU^{175}, ARG^{184}, GLU^{17}, ASP^{14}, LEU^{22}) involved in its interaction with SIRT1.

Point 12: Cells used: A549 different from Vero in original proteomic analysis, need comment. Figure S2b yellow circles are blue circles

Response: We selected Vero cells for the proteomic analysis due to their heightened sensitivity to TBEV replication. However, for the validation analysis, we opted for human-origin A549 cells, which have demonstrated sensitivity to TBEV infection[6]. We have added this comment to “line 370” in the revised MS. We have also changed “yellow” to “blue” in Fig. S2b.

Point 13: Useful to link here the data on the pharmacological modulation of acetylation. However, data don’ t always represent text. For example, the HAT inhibitor CPTH2 inhibits TBEV when it is also cytotoxic, need better dose response. Also, HAT inhibitors have pleiotropic effects on transcription for example.
Also for ATM/ATR inhibitors there is a poor dose response. More intriguing reserveratol but where are the data? Discrepancies between figure 6 and Figure 20s, boh showing Fisetin and Enoxcin but not Resveratol or Selisistat, lots of confusion here in the figure legends.

Response: Thanks very much for your suggestive comments. We incorporated acetylation and DDR inhibitors into Figure 4. Upon re-evaluating the impact of CPTH2 and ATM/ATR inhibitors on TBEV transcription, we observed a dose-dependent inhibition of TBEV replication. Furthermore, Histone acetyltransferase inhibitor II, the other HAT inhibitor, exhibited nearly complete inhibition of TBEV transcription in Vero cells, while showing only slight inhibition in A549 cells. This suggests a potential interferon-mediated inhibition of histone acetyltransferase inhibitor II on TBEV transcription.

We apologize for any confusion regarding the figure placement. The results for resveratol can now be found in Figure 7f and Figure S21f, while those for Selisistat are presented in Figure S22t and S23t. Additionally, we have thoroughly reviewed and verified the figure legends throughout the entire manuscript.

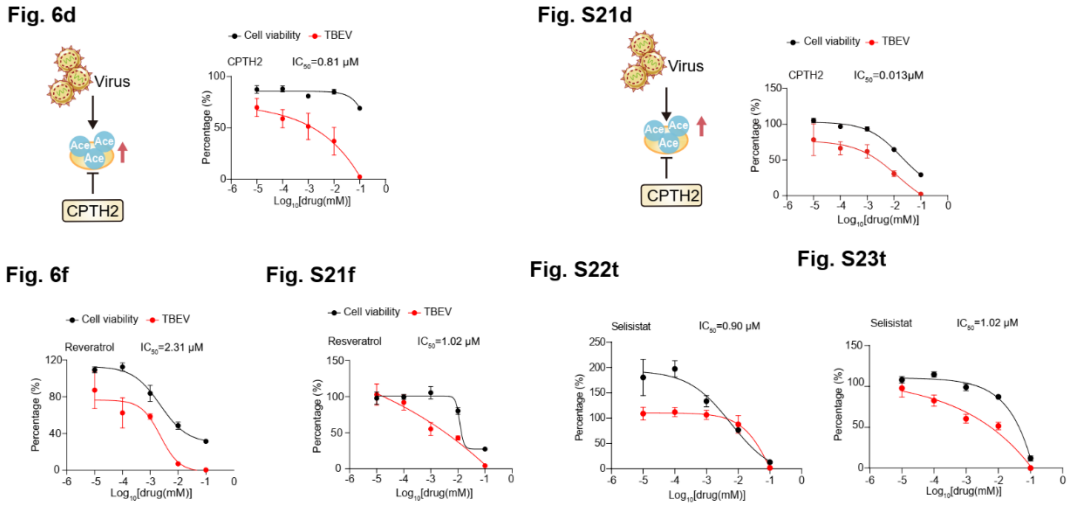


Figure legend. Cells were pretreated with the indicated drugs and then infected with TBEV for 48 h. IC_{50} values were detected by probe PCR. The cell viability was measured by cell counting (CCK-8) assay without viral infection. The red line indicates the effects in infection measured at each drug dose. The black line shows cell viability at each drug dose. Each drug was tested in three independent repeated experiments, and error bars represent the mean ± SEM of three biological replicates.

Point 14: Then the authors decide to explore also autophagy. Role of autophagy in TBEV infection is tricky because it may have proviral or antiviral roles depending on timing.
In general data support induction of autophagy, although p62 increase is not so evident (Figure S18a,b). Also acidification is not so evident, lacks in general a sterile induction of autophagy as positive control.

Response: Thanks very much for the comments. The decrease in P62 levels typically signifies successful autolysosome degradation, while increased or unchanged P62 levels indicate inhibition of autophagic flux. In the case of TBEV infection, we observed only unchanged P62 levels, suggesting inhibition of autophagic flux as a result of the infection. The induction of autophagy by starvation was employed as the positive control and has been include in Figures S18f and S19b in the revised MS.

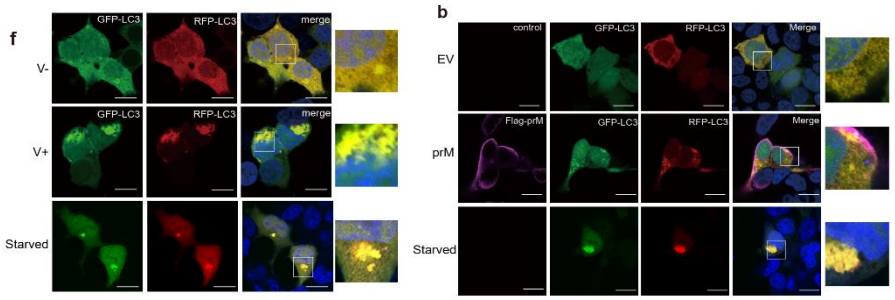


Figure S18. **f**. Cells infected with TBEV displayed more yellow GFP$^+$RFP$^+$LC3 puncta compared to mock-infected cells. Scale bar, 20 μm.

Figure S19. **b**. Cells infected with TBEV displayed more yellow GFP$^+$RFP$^+$LC3 puncta compared to mock-infected cells. Scale bar, 20 μm.

Point 15: The authors then bring in prM and NS2B3 linked to autophagy regulation. prM shows more LC3 foci, also quantified in Figure S19, but no quantification for NS3B3. In fact, NS3B3 disappears form the experiments. Is it relevant?
prM induce LC3 puncta and LC3-II/I ratio (still not convinced of statistics) and p62 but not really evident

Response: Thanks very much for the comments. After observing no significant activation of autophagy by NS2B3 and its interaction with STX17, a key regulator of autophagic flux, we proceeded to assess autophagic flux following NS2B3 overexpression. Our findings revealed that NS2B3 induced a greater amount of GFP$^-$RFP$^+$LC3 compared to the control under starvation conditions (Figure S19f). Co-immunoprecipitation analysis confirmed the interaction between NS2B3 and STX17, with both proteins colocalizing in the cytoplasm (Figures S19g-h). Furthermore, NS2B3 overexpression led to an increase in LC3-II levels and a decrease in P62 expression, indicating that NS2B3 promotes autophagic flux (Figure S19i). These results have been integrated into the revised manuscript.

We re-valuated the expression of LC3 and P62 in prM overexpressed cells, which showed obviously increased LC3-II/I ratio and P62 and punctuated LC3 puncta induced by prM overexpression (Figures 5e-h).

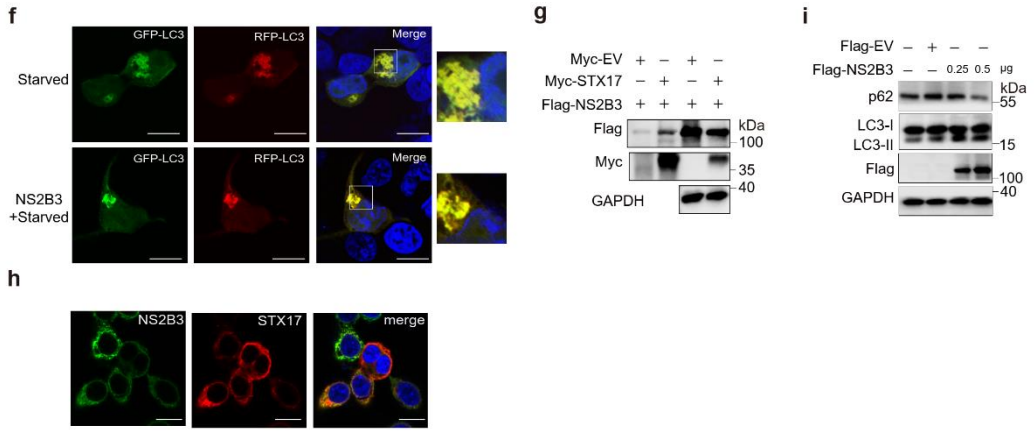


Figure S19. f. Cells expressing NS2B3 exhibited a higher number of GFP$^-$RFP$^+$ LC3 puncta compared to control cells. Scale bar, 10 μm.
g. Co-immunoprecipitation demonstrating the interaction between overexpressed NS2B3 and STX17.
h. Colocalization of TBEV-NS2B3 with STX17 in the cytoplasmic. Scale bar, 10 μm.
i. Immunoblot assay revealed an increase in the expression level of LC3-II while decreased level of P62 in TBEV-

infected cells.

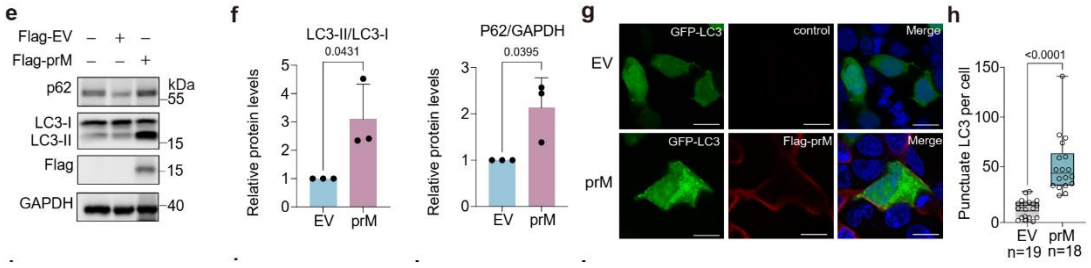


Figure 5. e. Immunoblot assay showing that increased expression of LC3 and P62 in prM overexpressed cells.
f. Quantification of increased percentage of LC3-II/LC3-I and p62/GAPDH in (**e**).
g. Cells expressing prM contained more GFP+LC3 puncta compared with EV transfected cells. Scale bar, 10 μm.
h. Quantification of the number of GFP+ LC3 puncta per cell in (**g**).

Point 16: TBEV infection reduced mTOR phospho and induced early AKT1 phospho and then reduces it – double role of autophagy?
AKT overexpression reduce TBEV replication (include plaque assay?)

Response: Thanks very much for the comments. The activation of autophagy via suppressing AKT1-mTOR is a shared feature with ZIKV and SARS-CoV-2 infections.[7,8] Although TBEV infection initially activates AKT1 at 2 hours, a subsequent reduction is observed at 24 and 48 h after virus infection (Figures S18j and S18k). This phenomenon is akin to observations in HCV-infected cells, where transiently activated AKT1 was suggested to promote HCV entry.[9] Similarly, activated AKT1 has been shown to promote the entry of Ebola virus.[10] It is plausible that TBEV activates AKT1 during the early infection phase to facilitate host cell entry and subsequently suppresses AKT1 to induce autophagy, promoting virus replication during the late infection phase. We have incorporated this in the discussion part.

The overexpression of AKT1 demonstrated a reduction in TBEV replication. This effect was further confirmed using $TCID_{50}$ assay, revealing a decreased TBEV titer following AKT1 overexpression. These findings have been incorporated into Figure 5k in the revised manuscript.

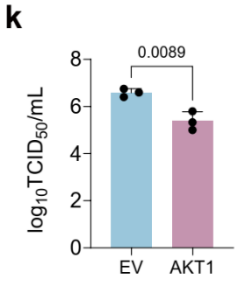


Figure 5k. Titers of virus in supernatants significantly decreased in cells transfected with Myc-AKT1 compared to cells transfected with empty vector.

Point 17: LC3-LAMP colocalization in Fig S19d is a bit stretched, same for S19e for VPs11
Better data with coIP with VPS11 and disruption of VPS complex

Response: Thanks very much for the comments. We have adjusted fig. S19d and S19e to the right scale and replaced co-IP with VPS11 and disruption of VPS complex int the revised MS.

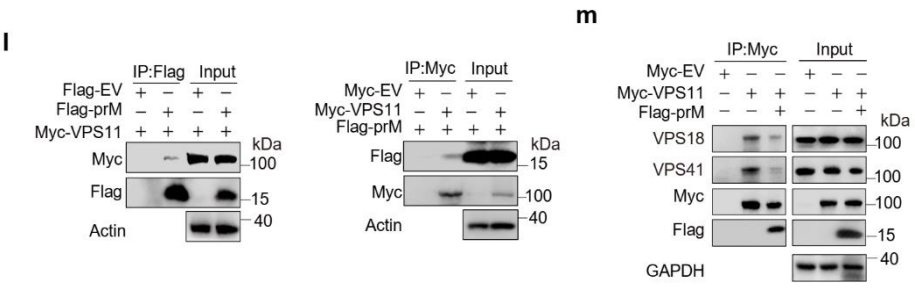
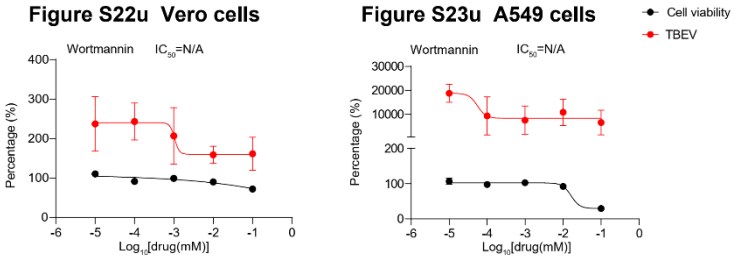


Figure 5. l. Co-immunoprecipitation demonstrating interaction of prM and VPS11 in Flag-prM and Myc-VPS11 overexpressed cells.
m. Co-precipitation showing a reduction in VPS18 and VPS41 co-precipitated by VPS11 in cells overexpressed with prM compared with empty vector transfected cells.

Point 18: Targeting autophagy by KD ATG7 reduces viral replication, but wortmannin autophagy inhibitor induced TBEV replication (however data S21/22w don’ t show dose response and is not the same in the two cell lines). Other autophagy inhibitors inhibit replication, as did CQ (which could be also affecting entry). Also in this case the inhibitors of autophagy may be introduced in the paragraph related to the mechanism to provide support on the hypothesis rather than in a separate chapter.
The part related to drugs needs refinement. In this context the selective index is important to exclude a general toxic effect. As mentioned above some panels are mislabelled in figures, please check.

Response: Thanks very much for your positive suggestions. TBEV infection has been observed to inhibit AKT1 phosphorylation, leading to the promotion of gene transcription. Notably, the overexpression of AKT1 has been shown to inhibit TBEV transcription. While wortmannin has been identified as an inhibitor of AKT1 phosphorylation [11], suggesting its potential to promote TBEV replication. Wortmannin was found to enhance TBEV transcription in both Vero and A549 cells, with lower concentrations showing greater enhancement compared to higher concentrations. We have included the results of CQ in the autophagy mechanism section. Additionally, we have added the selective index of drugs to Table S11 to account for potential general toxic effects. We have thoroughly reviewed the manuscript and corrected any mislabeled figures.



Legend. Effect of Wortmannin on the transcription of TBEV in Vero and A549 cells.

Responses to the comments and suggestions of Reviewer #2

In this manuscript, Sui and colleagues perform a systematic profiling of the global interactome, proteome, phosphoproteome and acetylome of TBEV, to characterize host responses to viral infection in a systematic fashion. To assess the functional relevance of newly or previously reported host targets identified by proteomics, the authors further employ some orthogonal approaches (genetic, imaging or small-molecule inhibitors-based), characterizing selected host interactors bound or modulated at the PTM level in vitro.
Specific examples include:
- a role of NS5 in DNA-damage response via interaction with SIRT1 (read-out co-IP WB, gammaHA2x accumulation in the nuclei and siRNA-meidate KD/OE);
- a role for prM in induction of autophagy (read-out: phosphor-specific WB on AKT and and LC3II/LC3I ratio);
- a TBEV-mediated (mild) dysregulation of cell cycle.

Additionally, the authors tested a subset of host targets from each dataset (27 in total) by pharmacological inhibition, to assess their drug-repurposing antiviral potential. Altogether, these experiments identify 1-3 (depending on the specific cell type and cut-off used) novel inhibitors of TBEV replication in vitro, and confirm the antiviral activity of many other previously reported inhibitors including some associated with anti-IAV, anti-DENV or ZIKV, and anti-SARS-CoV-2 activity or widely described inhibitors with broad pan-viral activity such as Wortmannin or other autophagy-related inhibitors (i.e. spautin, cloroquine, etc.). Among these the most interesting/novel findings are probably those related to SIRT1, for which a few mechanistic data support the mechanism of action of the SIRT1 agonist.
Overall, the study is a muscular tour-de-force both from a technical and a bioinformatics standpoint, and provides a remarkable example of integrative multi-omics. Data are clearly presented and results provided in light of the massive amount of literature existing for different flaviviruses across domains (PPI and Phosphoproteomes above all). Furthermore, the attempt to validate the data via pharmacological inhibition as well as some orthogonal validation by co-IP/WB and immunofluorescence in more relevant model systems (i.e. brain organoids) is notable, and presents some interesting insights. I have only a few comments related to the presentation of some of the statistics and data, and some mechanistic follow-up experiments in vitro (see below).

Point 1: In general the study is well-executed and data are well-presented; however all the proteomics dataset and follow-up studies are carried in VeroE6 (African Green Monkey cells) and 293T cells, which both have rather limited physiological relevance for TBEV. While few of the drug-repurposing experiments are also carried in A549 cells or brain organoids, validating some of the key new findings (i.e. SIRT1 over-expression) in more relevant model systems for TBEV (i.e. monocytes, or dermal fibroblasts) will go along the way of increasing their significance in pathogenesis.

Response: Thank you very much for your positive comments. Given that neuronal cells are particularly sensitive to TBEV infection and are adversely impacted by it, we sought to investigate the effects of TBEV infection on neuronal cells and brain organoids (Figure 6a). T98G, a human brain glioma cell line known for its susceptibility to TBEV infection, was selected for the analysis of DNA damage and autophagy. TBEV infection notably induced the accumulation of γH2A.X (Figures 6b and 6c). Furthermore, the comet assay conducted on TBEV-infected neuronal cells provided additional evidence of DNA fragmentation following TBEV infection (Figures 6d and 6e). Concurrently, our analysis confirmed that TBEV infection led to an increase in the ratio of LC3-II/LC3-I, while P62 levels remained relatively unchanged in TBEV-infected T98G cells (Figures 6f and 6g). Notably, we observed a clear accumulation of GFP+LC3 foci and a higher percentage of $GFP^{+}RFP^{+}$LC3 yellow foci in TBEV-infected T98G cells compared to mock-infected cells (Figures 6h, 6i, S20a and S20b). This observation further supports the notion that TBEV infection induces autophagy while concurrently inhibiting autophagic flux in neuro cells.

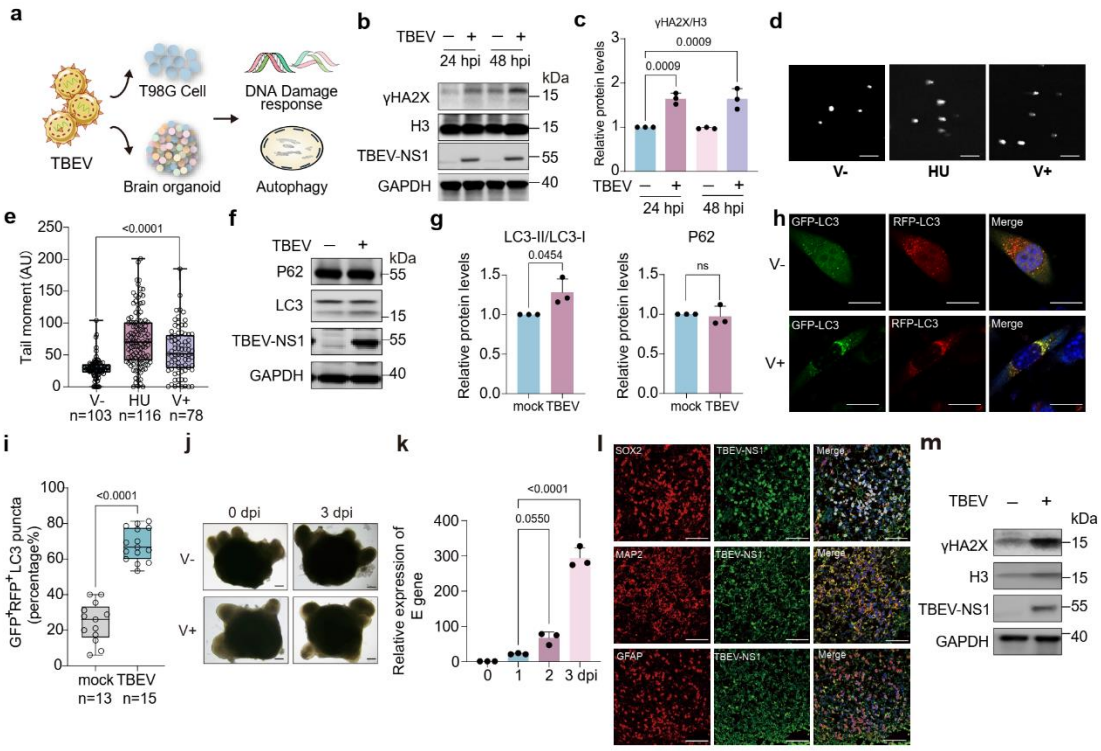


Figure 6.
a. DNA damage response and autophagy were determined in TBEV-infected T98G cells and brain organoids.
b. Immunoblot assay showing increased expression of γH2AX in TBEV-infected T98G cells.
c. Quantification of increased percentage of γH2AX /H3 in (**b**).
d. Images of comet assays from mock or TBEV-infected cells. Scale bar, 20 μm.
e. Quantification of comet tail moment shown in (**d**).
f. Immunoblot assay showing increased expression of LC3-II in TBEV-infected cells.
g. Quantification of increased percentage of LC3-II/LC3-I and unchanged p62/GAPDH in (**f**).
h. TBEV-infected cells exhibited a higher number of GFP+LC3 puncta compared with mock-infected cells. Scale bar, 20 μm.
i. Quantification of the number of GFP+ LC3 puncta per cell confirmed the increase in autophagosomes in TBEV-infected cells.

Point 2: While this reviewer appreciates the substantial extent of validation already presented in the manuscript, these efforts (both genetic, biochemical or drug-based) almost exclusively focus at corroborating already extensively described pathways or genes previously associated with flavivirus replication (i.e. AKT-mTOR, autophagy flux, Leo1 and PAF1, etc.). In this respect, experimental validation that these pathways or host proteins are also important for TBEV replication is important, but its novelty is limited as no/few new mechanistic insights are presented. Additional experiments addressing the functional relevance of previously unreported genes in TBEV replication (i.e. KD/KO and PFU assay of novel TBEV host factors) or more detailed characterization of the mechanisms underlying previously reported host factors would significantly strengthen the impact and novelty of the study.

Response: Thank you very much for your positive comments. Our study project the multi-omics data onto the global cellular interaction network, revealing a substantial impact of TBEV infection on the innate immune response, ribosomal biogenesis, autophagy, DNA damage response (DDR), and cell cycle. Regarding the pathogenic mechanism of TBEV, we discovered that the non-structural protein NS5 of TBEV hinders DNA damage repair by interacting with SIRT1 to suppress the deacetylation of KAP1 and Ku70. We observed that the activation of SIRT1 using overexpressed plasmids or SIRT1 agonist, along with the deacetylated mutation of Ku70 plasmid, both resulted in significant inhibition of TBEV replication. Furthermore, the precursor membrane protein prM induces autophagy by associating with AKT1, while it restricts autolysosome formation through binding to VPS11 and inhibits the formation of the VPS complex. Inhibitors of

autophagy and knockdown of ATG7 both demonstrated suppression of TBEV replication. These findings underscore the important role of DDR and autophagy during TBEV replication, and suggest the significance of SIRT1 and AKT1 as crucial host factors for TBEV.

Point 3: TBEV PPI Interactome study (Fig.2 and Fig S5): The cloning strategy used for overexpression of epitope-tagged viral protein appears unorthodox, as all proteins are described as cloned with an N-terminal tag (line 197). However, many of the flaviviral ORFs require specific insertion sequence to ensure their correct topology. For instance, NS1 requires the E leader sequence to ensure insertion in the ER lumen (so it should be c-terminally tagged); similarly NS4B which has to be expressed alongside with the 2k- signal peptide to ensure correct topology (otherwise the N-terminal tag will be cleaved off mature NS4B, or if 2k not present NS4B will be expressed in the cytoplasm). Similar rationale applies to NS2A, Envelope and prM, each requiring their own leader sequences). Can the authors clarify in more details how exactly these proteins were designed? This aspect is extremely important as it affects correct topology, and ultimately the physiologically-relevant subcellular localization and host interactome of the viral baits.

Response: Thanks very much for your comments. The TBEV structural and nonstructural proteins (GenBank Access Number MN615726.1) were cloned into Flag-VR1012, incorporating 2×Strep II affinity and 3×Flag tags at N terminal, according to previous report[2]. You are right that the 2K protein is very important for the positioning of NS4B, although for the transcription of NS4B plasmids, we detected the expression of NS4B protein and the right location of NS4B on the ER which is enough for the interaction study.

Minor comments

Point 4: All figures containing ball-networks should be revised to make gene names readable (both supplements and main text; applies to all figures)
Response: We have enlarged all the ball-networks contained in the manuscript in the revised MS.

Point 5: Figure legends (Fig. 1-3): additional information on the statistical test and cut-off criteria used should be added
Response: Thanks very much for your comments. We have added the cut-off criteria in the legends of Fig. 1-3.

Point 6: Pp 5, line 137: enzyme should read “enzymatic”
Pp5 line 161: acetylated should read “acetylation”
Response: Thanks very much for your comments. We have carefully checked them and revised according to your suggestions in the revised MS.

Comment on statistics

Point 8: All statistical analyses and gene ontology analysis appear as scientifically sound. Additional information on the cut-off criteria used should be added in figure legends as described above.
Response: Thanks very much for your comments. We have added the cut-off criteria in indicated figure legends.

Responses to the comments and suggestions of Reviewer #3

This paper is an extensive body of work that identifies several flavivirus-host interactions using proteomics-based approaches. The authors first identified changes in protein abundance, phosphorylation, and acetylation in response to TBEV infection in Vero cells. Next, all the TBEV proteins were cloned into expression vectors for affinity purification-mass spectrometry (AP-MS) in HEK293T cells, which identified several interaction networks for each of the ten TBEV proteins and NS2B3. The authors then identified two pathways of interest from their considerable amount of data: the DNA damage response and autophagy. The study identified NS5 as essential for preventing DNA damage repair and prM as critical for promoting autophagy. Finally, the authors pharmacologically targeted these pathways to demonstrate their importance in viral replication. Overall, this large body of work is a critical resource to the field as it identifies several key interactions and changes in response to TBEV infection.

While this study has generated large amounts of valuable data on cellular pathways and interactors potentially critical for TBEV infection, the authors mainly focused on the identification of interactions and of changes in protein abundance and post-translational modifications (PTMs). The authors then followed up on the NS5-SIRT1 and prM-AKT1 interactions and their roles in DNA repair and autophagy, which are novel and interesting in the context of TBEV infection. However, interfering with the DNA damage response has been shown for other flaviviruses like ZIKV (Hammack 2019), and promoting autophagy has also been shown for other flaviviruses and RNA viruses (ZIKV and SARS-CoV-2). Elucidating a single mechanism would provide deeper insights into the functional consequences of some of the interactions or PTMs identified during TBEV infection. This could also potentially uncover the distinct and conserved mechanisms among flaviviruses. Taken together, the study provides an excellent resource for the field that will help generate many exciting new hypotheses, but the lack of mechanistic details in the interactions chosen for follow-up along with the lack of rationale for the use of different cell lines and confounding results from inhibitor treatment significantly dampens the impact of the study.

Point 1: There is little focus regarding the mechanisms and consequences of each interaction or PTM identified. For example, the authors have nicely shown that NS5 and SIRT1 interactions prevent effective DNA damage repair, however it remains unclear how this is beneficial for TBEV replication or production. Moreover, the authors observed most proteins exhibiting hyperacetylation over the course of TBEV infection in contrast to other PTMs such as phosphorylation where both phosphorylated and dephosphorylated proteins were observed. It would be novel and interesting to follow up on the functional consequences of specific hyperacetylation events upon TBEV infection.

Response: We found that NS5 could interact with SIRT1, which inhibited SIRT1 mediated deacetylation of Ku70 and KAP1, resulting the inhibition of DNA damage repair. We further confirmed that NS5 overexpression induced the acetylation of Ku70 and KAP1, to demonstrated the role of Ku70 acetylation in virus replication. We constructed the deacetylation mutation of Ku70 and found that deacetylation of Ku70 obviously inhibited TBEV replication, indicating the proviral role of hyperacetylation events during TBEV replication.

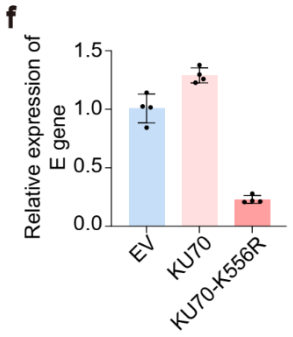


Figure S17f. Probe PCR analysis indicated a reduction in the mRNA level of TBEV E genes in ku70-K556R overexpressed cells compared with empty vector transfected cells.

Point 2: One major concern is the relevance of cell types used throughout the study. Vero cells are not of human origin, and HEK293T and A549 cells are not physiologically relevant to TBEV infection, thus decreasing the reliability of the findings. Moreover, the authors do not provide rationale for the use and switching among the cell types. For example, the proteomics was done on infected Vero cells in Figure 1, while the AP-MS was done on HEK293T cells expressing individual TBEV proteins in Figure 2. The brain organoid model (Figure 4) is a useful tool for validating the findings, but should be incorporated more cohesively throughout the manuscript. Alternatively, the authors should consider mechanistic studies in neuronal-like cell types such as SNB19 or even primary cells.

Response: Thanks very much for your comments. Although Vero cells are not human origin, they are susceptible to virus and present as a good model for multi-omics analysis of virus infection. We chose HEK-293T for AP-MS analysis as it is human origin and was sensitive to transfection of plasmids. We have added the rationale for changing cell lines in the revised MS.

We also tested the DDR and autophagy response in the neuro cells T98G, together with the results of brain organoids were incorporated into the new manuscript as Figure 6.

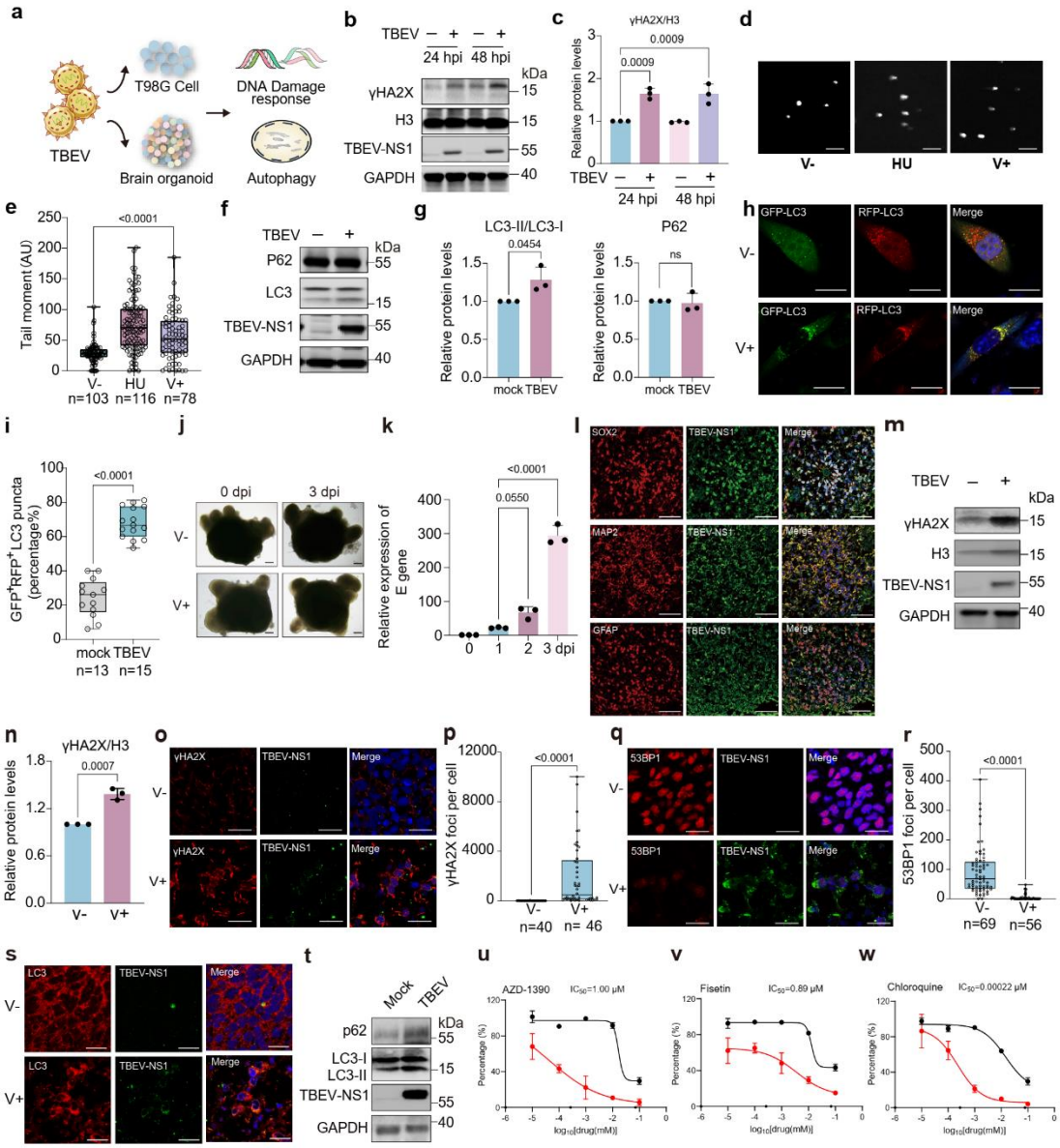


Figure 6. TBEV infection regulates the DNA damage response and autophagy in neuronal cells and brain organoids

a. DNA damage response and autophagy were determined in TBEV-infected T98G cells and brain organoids. **b.** Immunoblot assay showing increased expression of γH2AX in TBEV-infected T98G cells. **c.** Quantification of increased percentage of γH2AX /H3 in (b). **d.** Images of comet assays from mock or TBEV-infected cells. Scale

bar, 20 μm. **e.** Quantification of comet tail moment shown in (d). **f.** Immunoblot assay showing increased expression of LC3-II in TBEV-infected cells. **g.** Quantification of increased percentage of LC3-II/LC3-I and unchanged p62/GAPDH in (f). **h.** TBEV-infected cells exhibited a higher number of GFP+LC3 puncta compared with mock-infected cells. Scale bar, 20 μm. **i.** Quantification of the number of GFP+ LC3 puncta per cell confirmed the increase in autophagosomes in TBEV-infected cells. **j.** Images of brain organoids, either mock-infected or infected with TBEV at 0- and 3-days post-infection (dpi). Scale bar, 200 μm. **k.** Quantification by probe PCR demonstrated a gradual increase in the copies of TBEV E genes with the duration of TBEV infection. **l.** Immunofluorescence analysis highlighted the colocalization of TBEV NS1 with SOX2 (progenitors), MAP2 (neurons), GFAP (astroglia). Scale bar, 200 μm. **m.** Immunoblot assay showing increased expression of γH2AX in TBEV-infected brain organoids. **n.** Quantification of increased percentage of γH2AX /H3 in (m). **o.** More punctate structures of γH2AX in brain organoids infected with TBEV compared with the control. TBEV NS1 protein used to label infected cells. Nuclei stained with DAPI. Scale bar, 100 μm. **p.** Quantification of indicated puncta in (o), each dot is a nucleus. **q.** Less punctate structures of 53BP1 in brain organoids infected with TBEV compared with the control. TBEV NS1 protein used to label infected cells. Nuclei were stained with DAPI. Scale bar, 100 μm. **r.** Quantification of indicated puncta in (q), each dot is a nucleus. **s.** The brain organoid infected with TBEV exhibited an increase number of GFP+LC3 puncta compared to mock-infected organoid. Scale bar, 100 μm. **t.** Immunoblot assay of TBEV-infected brain organoids revealed increased expression levels of LC3 and P62. **u-w.** ATM inhibitor (AZD-1390), SIRT1 agonist (Fisetin), and autophagic flux inhibitor (chloroquine) significantly inhibited the transcription of TBEV E genes in T98G cells. Data are represented as mean ± SEM of 3 independent experiments. Source data are provided as a Source Data file.

Point 3: Pharmacologically targeting the identified pathways is a strong approach to show the importance of these pathways in TBEV replication, however the results raise additional questions. It is important to note that many of the inhibitors used only effectively work in Vero cells, while effectiveness is impaired in A549 cells. Moreover, several inhibitors begin working at concentrations that decrease cell viability, raising concerns regarding the importance of the targeted pathways for TBEV infection. Additionally, two inhibitors that target autophagy, spautin-1 and 3-MA, have opposite effects on TBEV infection, making the role of autophagy unclear. Lastly, it is unclear how the authors rationalized the targeting of the identified pathways. For example, Figure 6e suggests the authors are targeting DNA damage repair, which have larger implications and consequences beyond TBEV infection.

Response: Thanks very much for your suggestive comments. Given that different cell lines may exhibit varied responses to the same drug, we opted to test the efficacy of the inhibition of drugs using both Vero and A549 cell lines. Our results consistently demonstrated the efficacy of the drugs across both cell lines, indicating the reliability of our drug experiments. You are correct in noting that certain drugs showed greater effectiveness in Vero cells, possibly due to the diminished interferon signaling in these cells, which may enhance the drugs' efficacy. Additionally, we have included the selective index of drugs in Table S11 to consider potential general toxic effects.

Spautin-1 primarily inhibits autophagy by targeting USP10 and USP13, while 3-MA acts on VPS34 and PI3Kr to achieve autophagy inhibition. The distinct molecular targets of these drugs may lead to different effects on TBEV infection. While both spautin-1 and chloroquine exhibited significant inhibition of TBEV transcription, the knockdown of ATG7 also notably inhibited TBEV, collectively confirming the crucial role of autophagy during TBEV infection.

In relation to the drugs selected in Figure 6e, the ATM/ATR inhibitors indeed affect DNA damage repair. However, TBEV infection initially induces the activation of ATM, ATR and DNA-PK, subsequently inhibits DNA damage repair. Therefore, the inhibitors of ATM/ATR are supposed to possess the ability to suppress TBEV replication.

Point 4: There is also a lack of experimental details in the manuscript, specifically regarding the number of independent experiments done for each figure panel, and whether they have done technical or biological replicates. The authors should make this clear in the figure legends and provide rationale for each experiment in the text.

Response: We have carefully checked them and revised accordingly.

Point 5: The models illustrated in the manuscript are well done, however can be confusing at first. The authors should consider making a schematic of the cell pathways under normal conditions,

followed by a schematic of the pathways when affected by the viral protein. This would make their findings clearer considering the large datasets and many changes that were identified. Additionally, the authors should omit parts of the pathways that are not relevant to the main findings. An example would be to focus on the left portion of the pathway in Figure 4t (should be corrected to Figure 4s).

Response: Thanks very much for the positive comments and suggestions, which are very valuable for us to improve the quality of the MS. We have changed the model in Fig.4s and 5o into the pattern you suggested in the revised MS.

s

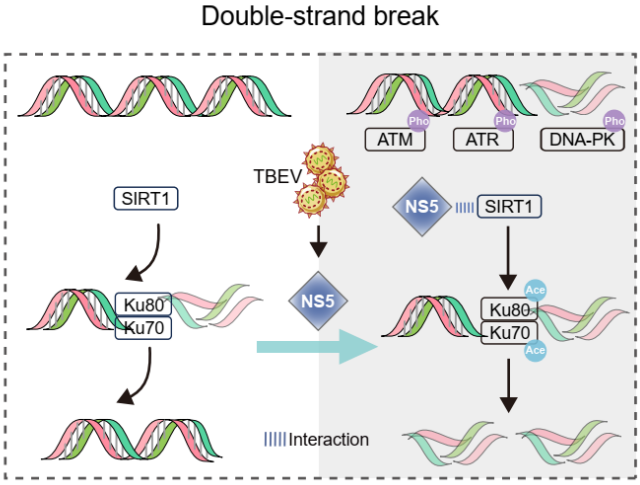


Figure 4s. Subnetworks revealing associations between TBEV and host targets and factors implicated in the modulation of DNA damage.

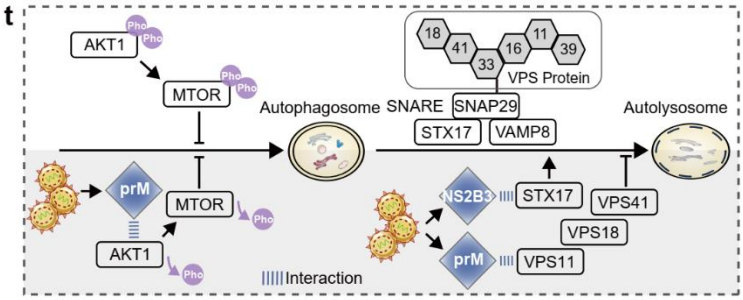


Figure 5t. Outline of host-cell autophagy regulated by TBEV prM and NS2B3.

Point 6: The authors should change the title to reflect the approaches used in the study. "Multi-omics" suggests the use of several -omics approaches including transcriptomics, metabolomics, and proteomics. However, the study only uses different proteomic approaches, and should therefore change the title to "Multi-proteomics".

Response: Thanks very much for your comments. We have changed the title accordingly.

Minor comments:

Point 7: Line 126: Change to "conserved regulation of host DNA damage response by flaviviruses".

Lines 236-237: Change "baits" to "preys".

Line 244: Change to "inhibit their recruitment of ISGs".

Line 284: Change to "host innate immune response to TBEV infection".

Line 373: Change to "Specifically".

Line 421: Figure 4t should be changed to Figure 4s.

Response: Thanks very much for your comments and suggestions, and we have revised the MS accordingly.

References

1 Kerr, S. A. *et al.* Computational and Functional Analysis of the Virus-Receptor Interface Reveals Host Range Trade-Offs in New World Arenaviruses. *J Virol* **89**, 11643-11653 (2015).

2 Shah, P. S. *et al.* Comparative Flavivirus-Host Protein Interaction Mapping Reveals Mechanisms of Dengue and Zika Virus Pathogenesis. *Cell* **175**, 1931-1945.e1918 (2018).

3 Carletti, T. *et al.* Viral priming of cell intrinsic innate antiviral signaling by the unfolded protein response. *Nat Commun* **10**, 3889 (2019).

4 Zhao, Z. *et al.* Nuclear localization of Zika virus NS5 contributes to suppression of type I interferon production and response. *J. Gen. Virol.* **102** (2021).

5 García-Martínez, J. M. *et al.* Vitamin D induces SIRT1 activation through K610 deacetylation in colon cancer. *Elife* **12** (2023).

6 Chmielewska, A. M. *et al.* The Role of IFITM Proteins in Tick-Borne Encephalitis Virus Infection. *J Virol* **96**, e0113021 (2022).

7 Hou, P. *et al.* The ORF7a protein of SARS-CoV-2 initiates autophagy and limits autophagosome-lysosome fusion via degradation of SNAP29 to promote virus replication. *Autophagy* **19**, 551-569 (2023).

8 Liang, Q. *et al.* Zika Virus NS4A and NS4B Proteins Deregulate Akt-mTOR Signaling in Human Fetal Neural Stem Cells to Inhibit Neurogenesis and Induce Autophagy. *Cell Stem Cell* **19**, 663-671 (2016).

9 Liu, Z. *et al.* Transient activation of the PI3K-AKT pathway by hepatitis C virus to enhance viral entry. *J Biol Chem* **287**, 41922-41930 (2012).

10 Kuroda, M., Halfmann, P. & Kawaoka, Y. HER2-mediated enhancement of Ebola virus entry. *PLoS Pathog* **16**, e1008900 (2020).

11 Kim, J. S., Kang, C. G., Kim, S. H. & Lee, E. O. Rhapontigenin suppresses cell migration and invasion by inhibiting the PI3K-dependent Rac1 signaling pathway in MDA-MB-231 human breast cancer cells. *J. Nat. Prod.* **77**, 1135-1139 (2014).

We have carefully examined the MS and corrected other mistakes and typo-errors.

We sincerely hope that the MS has been revised to your satisfaction. We are looking forward to seeing the acceptance of the revised MS for publication in your esteemed journal.

Sincerely yours,

Prof. Quan Liu, PhD, Prof. Yicheng Zhao, PhD
State Key Laboratory for Diagnosis and Treatment of Severe Zoonotic Infectious Diseases
Department of Infectious Diseases and Center of Infectious Diseases and Pathogen Biology
Key Laboratory of Organ Regeneration and Transplantation of the Ministry of Education
The First Hospital of Jilin University, Changchun 130061, China

Dear Editor and Reviewers, Re: Manuscript NCOMMS-24-14544.R1

On behalf of all co-authors, I would like to thank you and the two **Reviewers** very much for positive and favorable comments, and constructive suggestions on our manuscript (MS) NCOMMS-24-14544. All reviewers liked our MS and considered that our MS is of general and broad interest to the readership of ***Nature Communications***. We are especially grateful to the reviewers for their kindness, generosity and graciousness in providing detailed constructive comments and suggestions on our MS.

The data description of this work has now been submitted to Scientific data with the title "Multi-proteomics dataset of TBEV-infected host cells and TBEV-host interactome (**SDATA-24-01381**)" and under the **second revision** now. All co-authors have seen a copy of the manuscript and agree to its submission.

We have revised the MS strictly according to the reviewers' comments and suggestions. Two MS files are uploaded: one shows **"tracked changes"** as "**Manuscript with revisions highlighted**", and the other is a "clean file" as "**Manuscript**". In the following, we detail our point-by-point responses to these comments and suggestions.

Responses to the comments and suggestions of Reviewer #2

In the revised version of the manuscript, the authors have addressed one out of my 3 major concerns: the one related to the extent of mechanistic or functional validation of the multi-omic data (Point 1).

Point 1: In this respect, while I appreciate the additional validation in brain organoids presented in Fig.6 of the rebuttal (substantial and quite laborious, yet elegant system), these experiments still focus entirely on validating extensively published observations (i.e. Autophagy, and DNA damage as judged by the gammaHA2X WB and LC3 puncta/ ratio). The main concern raised in the first revision was indeed that these effects have been previously reported for multiple flaviviruses and in multiple cellular backgrounds. Therefore as such the new experiments provided appear more as an academic exercise rather than an experimental validation on newly discovered pathway, and do not add any knowledge to the existing literature.

Response: Thank you very much for your constructive suggestions and comments. As you noted, most viruses can cause autophagy and DNA damage in infected cells; however, the underlying mechanisms often vary among different viruses, which are crucial for understanding viral pathogenesis. In our research, we discovered that TBEV NS5 hinders DNA damage repair by interacting with SIRT1, thereby inhibiting the deacetylation of KAP1 and Ku70. Additionally, the prM protein promotes autophagy through its association with AKT1, but it also restricts autolysosome formation by binding to VPS11. Notably, these findings have not been reported in relation to other flaviviruses.

Furthermore, we have explored the roles of additional factors that may be significant during TBEV infection. For example, UHRF1, an E3 ubiquitin-protein ligase that acts as a key epigenetic regulator in chromatin modification, exhibited a marked reduction following TBEV infection. The overexpression of UHRF1 significantly suppressed TBEV replication (Figures S2c-e). Moreover, SYVN1, a component of the ER-associated degradation, was found to interact with NS2B, and showed a pronounced increase upon TBEV infection (Figures S5e and 1c). Overexpression of

SYVN1 enhanced TBEV replication (Figures S5i-k), while silencing SYVN1 resulted in a substantial decrease in TBEV replication (Figures S5l-n). These findings suggest that various cellular processes and pathways are involved in TBEV infection, highlighting their potential as targets for antiviral intervention.

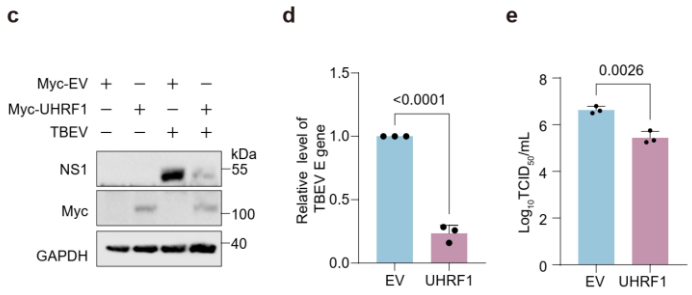


Figure S2. c-e. In cells transfected with Myc-UHRF1, TBEV NS1 protein (**c**), E genes (**d**) and viral titers (**e**) significantly decreased compared to cells transfected with Myc-EV (empty vector).

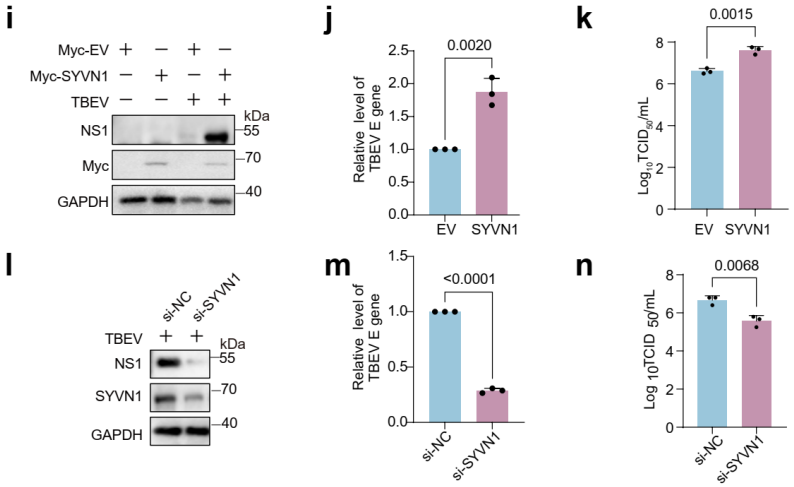


Figure S5. i-k. In cells transfected with Myc-SYVN1, TBEV NS1 protein (**i**), E genes (**j**) and viral titers (k) significantly increased compared to cells transfected with Myc-EV (empty vector).
l-n. In cells transfected with si-SYVN1, TBEV NS1 protein (**l**), E genes (**m**) and viral titers (**n**) significantly decreased compared to cells transfected with si-NC (negative control).

Point 2: Most importantly, this reviewer still strongly feels that the reply to point 3 does not resolve, but rather confirms the issues related to the cloning strategy used to generate the PPI map of TBEV viral proteins-host interactions.
The authors reply that:
“TBEV structural and nonstructural proteins (GenBank Access Number MN615726.1) were cloned into Flag-VR1012, incorporating 2×Strep II affinity and 3×Flag tags at N terminal, according to previous report2) citing the paper by Shah et al. (Shah, P. S. et al. Comparative Flavivirus-Host Protein Interaction Mapping Reveals Mechanisms of Dengue and Zika Virus Pathogenesis. Cell 175, 1931-1945.e1918 (2018).)

However, in this paper all constructs were cloned with a c-terminal FLAG tag, and not an N-terminal tag:
(excerpt from the paper: “DENV serotype 2 16681, ZIKV French Polynesia 2013 H/FP/2013

(ZIKVfp), and ZIKV Uganda 1947 MR766 (ZIKVug) open reading frames (ORFs) were cloned into pCDNA4_TO with a C-terminal 2xStrep II affinity tag for expression in human cells.”).
Beside this, there is an extensive body of literature
(i.e. https://doi.org/10.1038/nrmicro.2017.170, https://doi.org/10.1038/s44298-024-00031-7 , just to cite 2 recent reviews), supporting that only NS2B and NS4A could be also tagged N-terminally without affecting their physiological localisation or topology.
This point is extremely important as erroneous tagging of most of the viral proteins, completely affects their topology (and therefore their interaction with cellular proteins). In fact, the proper insertion in the ER is very specifically mediated by signal sequences located upstream of some of the viral proteins (i.e. Envelope requires the last aa of prM, NS1 requires the last aa of Envelope for proper insertion in the ER; 2k-NS4B will be inserted upside-down in the ER if lacking the 2k peptide; etc..). See sketch attached for a graphical representation of this issue.
Based on this, all the interaction data related to the remaining 8 viral proteins (Capsid, Envelope, prM, E, NS1, NS2A, NS3, 2k-NS4B and NS5) should be interpreted with extreme caution, and such experiments repeated with appropriately tagged constructs or entirely removed from the manuscript.

Response: Thank you for your constructive suggestions and comments, and we acknowledge that tagging viral proteins for some viruses may affect their topology and cellular localization. However, for Zika virus, no significant difference in endoplasmic reticulum (ER) localization has been observed between N-terminal and C-terminal tags, suggesting that the tagging moieties do not substantially influence their localization within the ER. (please see the reference: PMCID PMC6210227, Page 2246).

TBEV is genetically related to Zika virus, both belonging to the *Flaviviridae* family. Therefore, we think that tagging viral proteins of TBEV may not significantly affect their topology and cellular localization, consistent with Zika virus.

We also examined the N-terminal tagged viral proteins for TBEV, with all TBEV viral proteins except NS5, were colocalized with the ER (Figure S6). These results are similar to those of other flaviviruses (please see the reference: PMCID PMC6474419).

We also utilized AlphaFold to analyze the topological structures of N- and C-terminally tagged viral proteins, as well as those without tags, and found no significant impact of tagging on the proteins' structural integrity (Figures S25, S26).

We acknowledge the potential for unforeseen effects resulting from N-terminal tagging of viral proteins, and we have emphasized application of PPI results with caution in the discussion section. Moreover, we will evaluate the potential effects of N-terminal and C-terminal tagging viral proteins on cellular localization, topology and functions for TBEV in future investigations.

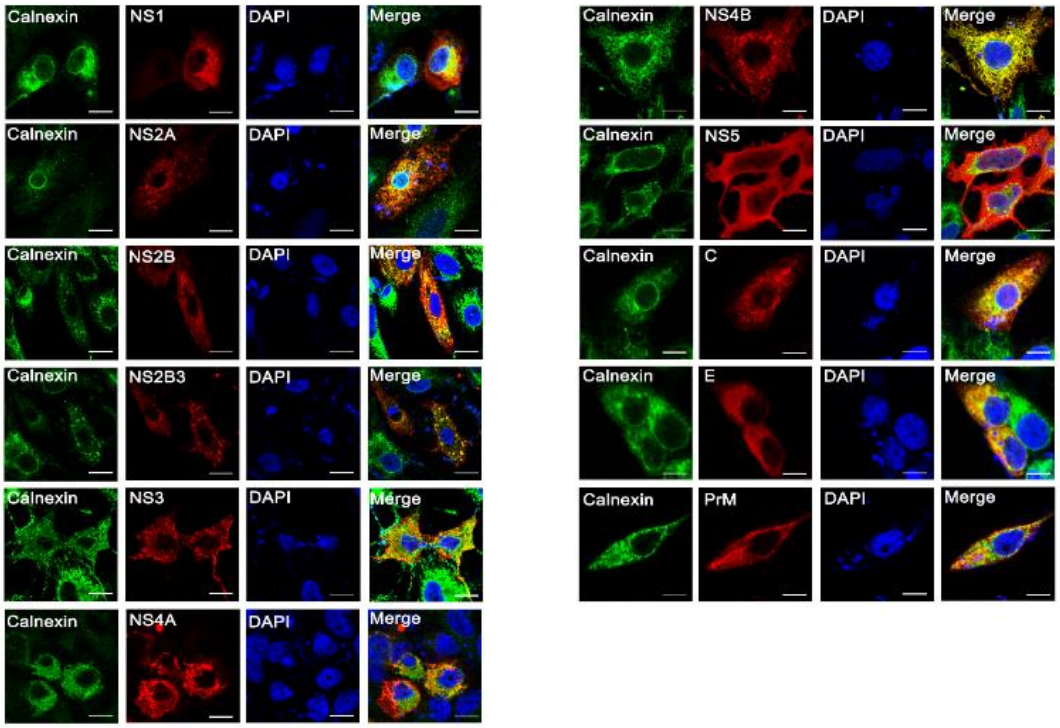


Figure S6. Localization analysis of TBEV proteins on endoplasmic reticulum

The Flag-tagged TBEV viral proteins were overexpressed in Vero cells for 24 h. The endoplasmic reticulum was labeled with anti-Calnexin antibody (red), viral proteins were labeled using an anti-Flag antibody (green) and nuclear was labeled with DAPI (blue). Scale bar, 20 μm.

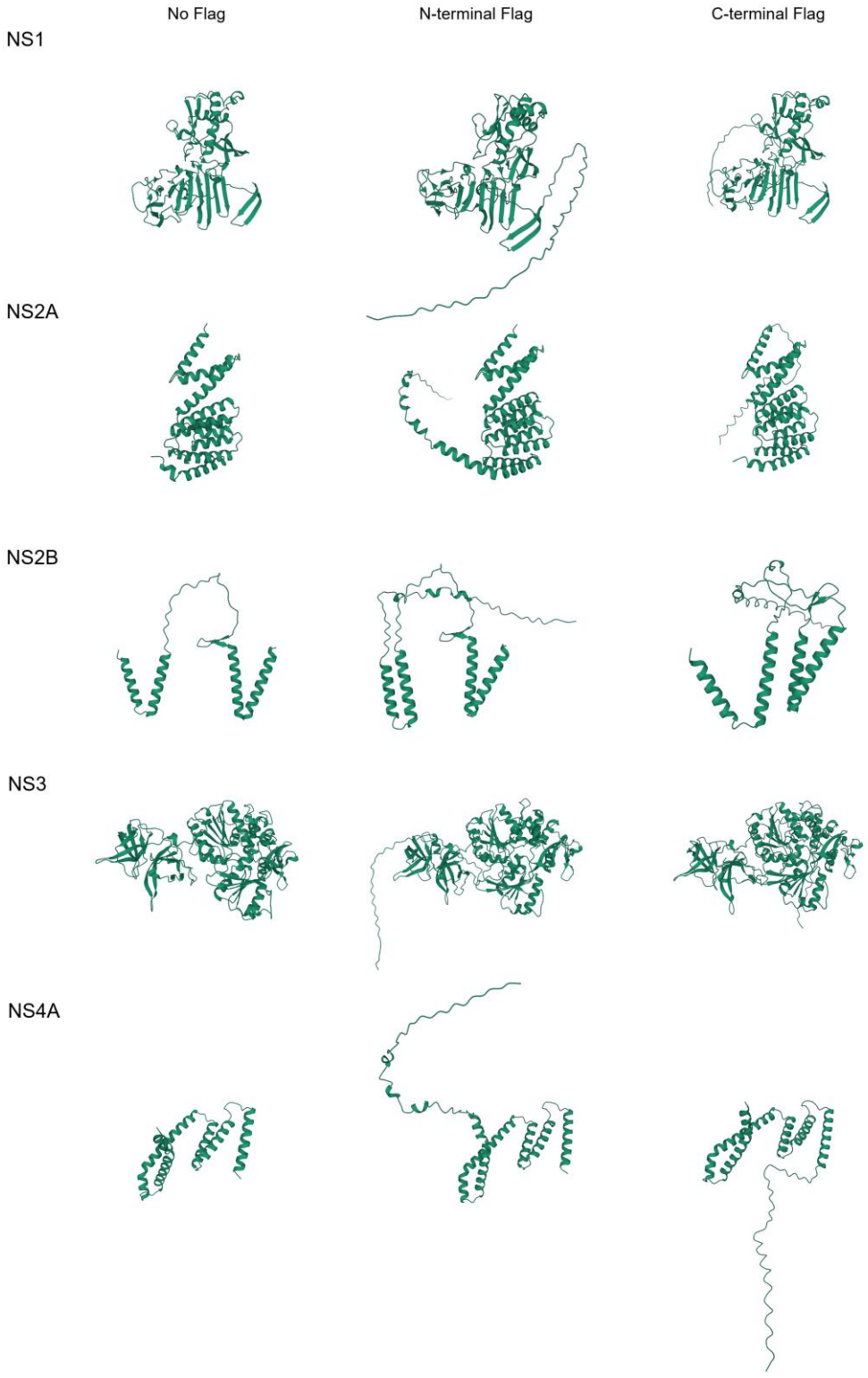


Figure S25. Predicted structures of TBEV proteins with N-terminal and C-terminal 3x Flag-2x strep-linker-tag. The structural models were generated using AlphaFold3 and visualized with Protein Viewer.

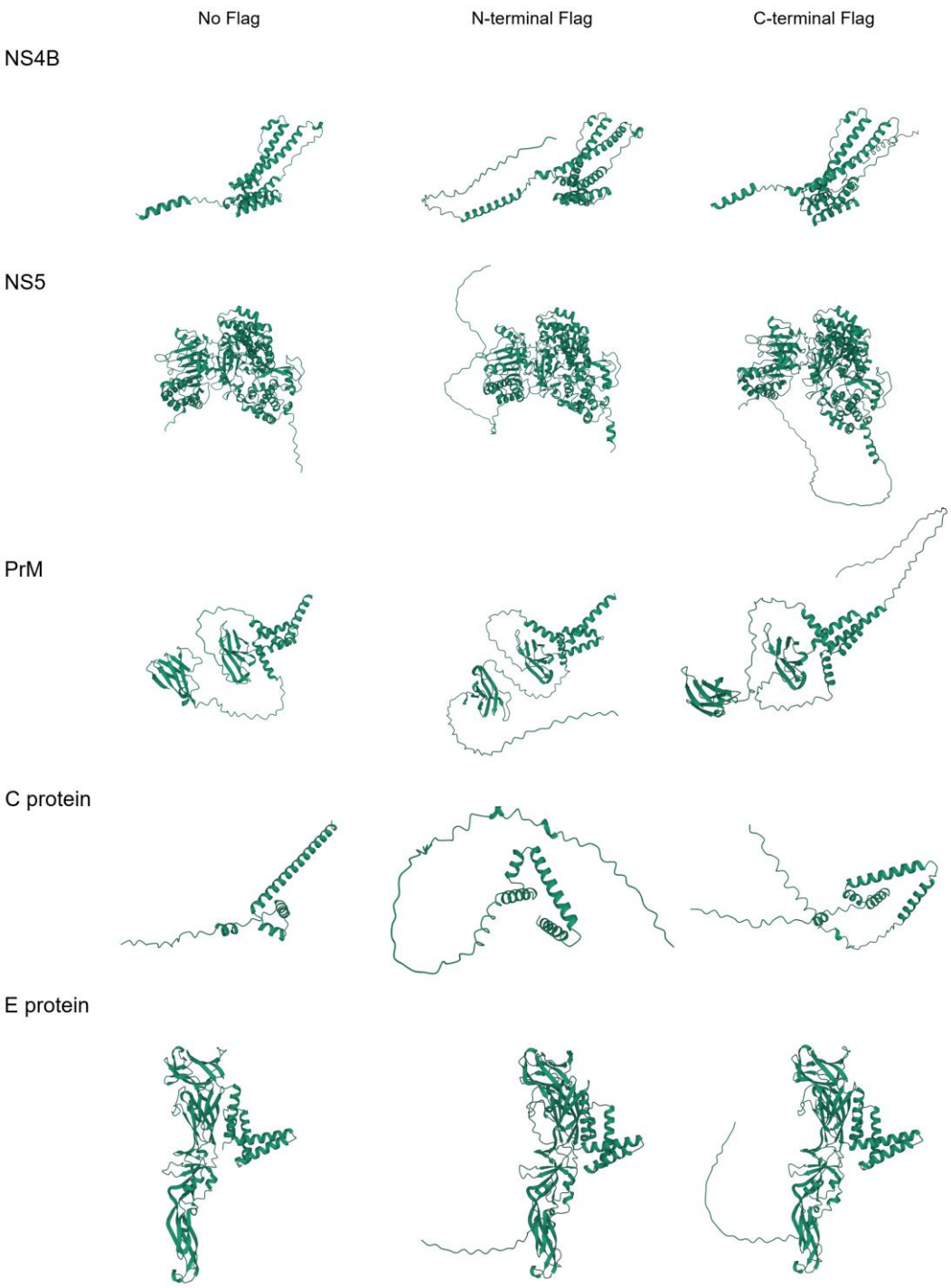


Figure S26. Predicted structures of TBEV proteins with N-terminal and C-terminal 3x Flag-2x strep-linker-tag. The structural models were generated using AlphaFold3 and visualized with Protein Viewer.

Responses to the comments and suggestions of Reviewer #3

We commend the authors on the large amount of work they put into the revision. We were mostly satisfied with the changes made by the authors, including additional experimentation to show the importance of Ku70 acetylation in the context of TBEV infection, the addition of more relevant neuronal and organoid models that increase the significance of the findings (these results were included in a brand new figure), and modifications made to the schematics and writing.

However, with the additional data provided, we have additional comments that should be addressed/improved:

Point 1: A general comment that should be addressed throughout the manuscript is the large number of panels per figure. One example is Figure 6, which contains 23 panels (A through W) and is unnecessarily excessive. This reduces the accessibility of the manuscript to larger audiences, which is critical for the journal. One recommendation for increasing access to this important data is to move redundant or nonessential panels to supplementary section or to other figures. Two examples would be 1) Figure 6J; it is unclear how this is essential to the main message of the figure and can be either removed or put in supplementary, and 2) Figure 6U, V, and W; these would go well in Figure 7 to more cohesively tie in the relevant models to the important findings in Figure 7. These suggestions should be broadly applied to all the figures in this paper.

Response: Thank you for your constructive feedback. We have implemented the following revisions:

Figure 6j has been removed.

Figures 6k and 6m have been relocated to the supplementary materials.

Figures 6U-W have been placed after Figure 7 to the supplementary materials.

We have conducted a thorough reviewed all other figures. Due to the limited number of panels in Figures 1-3, we have consolidated the panels in Figures 4 and 5. The drug-related results have been integrated and transferred to Figure 7 or the corresponding supplementary figures.

We appreciate your guidance and have made these changes to enhance the clarity and coherence of our presentation.

Point 2: Regarding Point 3:
The authors' rationale for why pharmacological targeting of certain pathways is specific to Vero cells but not A549 cells remains unclear. They claim the differences in efficacy may be due to a diminished IFN response but did not directly test this. Moreover, the neuronal cells used in Figure 6U-W presumably have an intact IFN response and yet the inhibitors used show higher efficacy. This again casts doubts on whether the Vero and A549 cells are relevant cell models for testing these inhibitors. Lastly, the inhibitors used on Vero/A549 cells were not used on T98G cells, and the rationale behind this is missing.

Response: Thank you very much for your positive feedback. In our study, we have chosen various inhibitors that target the same pathway but act on different host factors. For example, among the autophagy inhibitors, Spautin-1 specifically targets USP10 and USP13, whereas 3-MA exerts its effect on VPS34 and PI3Kr to inhibit autophagy. Wortmannin primarily targets the PIK3 family, while chloroquine not only inhibits autophagy and lysosome function but may also suppress the expression of Toll-like receptor-9 (TLR9). The unique molecular targets of these inhibitors could result in distinct impacts on TBEV infection. At the same time, the diverse origins of cell lines are associated with variations in cellular signaling, metabolistic status, and interferon responses,

which contribute to differences in sensitivities to viral infections and drug treatments. This diversity may account for the observed differences in efficacy among different cells when exposed to the same drugs or inhibitors targeting the same pathway. Based on this, we opted to test the antiviral efficacy of drugs using both Vero, A549 and T98G cell lines, which could provide more comprehensive data, facilitating a thorough analysis that is crucial for our ongoing research.

You are correct in noting that certain drugs showed greater effectiveness in Vero cells. To delve into this, we selected chloroquine (CQ), which demonstrated superior antiviral efficacy in Vero cells when compared to A549 cells. We then assessed the impact of CQ on interferon signaling in response to TBEV infection. Notably, the expression levels of interferon-stimulated genes (ISGs), including IFIT2 and IFIT3, were considerably lower in Vero cells compared to those in A549 cells upon TBEV infection, aligning with the reduced IFN response observed in Vero cells. However, following the addition of CQ, the expression of ISGs in Vero cells increased to 3-4 times the original level, while in A549 cells, it only increased 1.2-1.5 times comparted to the original level (Figures S24d, S24e). Based on the established direct antiviral activity of IFIT2 and IFIT3 on flaviviruses[1,2], these activated ISGs might contribute to the greater antiviral efficacy of CQ exhibited in Vero cells.

In addition to interferons, the direct antiviral effects of drugs, such as regulating virus release, synthesis and assembly, and even targeting directly on viral proteins or RNA, as well as the effects of drugs on host cells, including different cellular signaling pathways, can all lead to differences in antiviral efficiency among various cell types[3,4]. We did not observe a differential induction of ISGs between A549 and T98G cells in response to CQ (Figures S24e, S24f), suggesting that CQ may achieve higher antiviral efficiency in T98G cells through other pathways. Although we have tested 37 drugs in our study, the range of pathways they target is even broader, which is why we intend to investigate the mechanisms behind the varying efficacies of these drugs in our upcoming research.

Vero and A549 cells were usually chosen for the evaluation of antiviral efficacy of drugs for flaviviruses [5,6]. In our study, we chose Vero cells for antiviral examination due to they are routinely used for TBEV isolation, A549 cells were chosen because their susceptibility for TBEV infection[1,7]. Additionally, neuroblastoma cells T98G were included in the selection because TBEV tends to target the central nervous system. Our results consistently demonstrated the efficacy of the drugs across both cell lines, indicating the reliability of our drug experiments. While for some of the inhibitors tested on Vero and A549 cells that were not applied to T98G cells, we plan to evaluate these in our upcoming studies.

Additionally, the points raised have been incorporated into the discussion section of the revised manuscript.

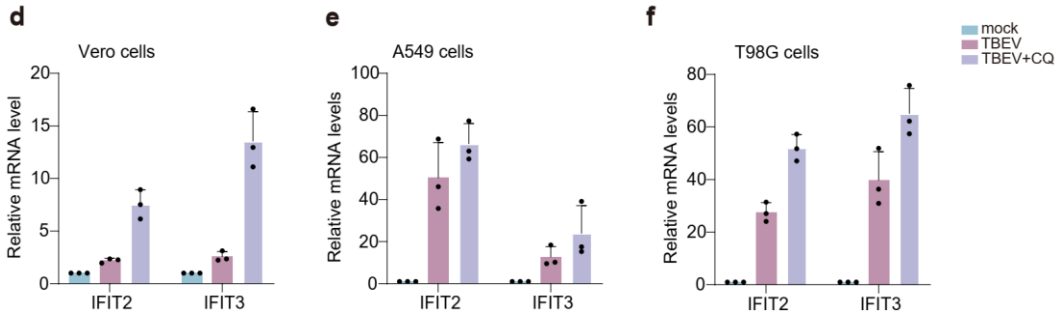


Figure S24 d-e. Vero (d), A549 (e) and T98G cells (f) were either mock-infected or infected with TBEV, along with some cells were pre-treated with 1 μM chloroquine (CQ) prior to TBEV infection. Following these treatments, the cells were harvested, and the mRNA expression levels of IFIT2 and IFIT3 were assessed.

References

1 Jiang, D. et al. Identification of Five Interferon-Induced Cellular Proteins That Inhibit West Nile Virus and Dengue Virus Infections. J. Virol. 84, 8332-8341 (2010).
2 Cho, H., Shrestha, B., Sen, G. C. & Diamond, M. S. A role for Ifit2 in restricting West Nile virus infection in the brain. J Virol 87, 8363-8371 (2013).
3 Wei, S. Y. et al. Identification of KU-55933 as an anti-atherosclerosis compound by using a hemodynamic-based high-throughput drug screening platform. Proc Natl Acad Sci U S A 121, e2318718121 (2024).
4 Loaiza-Cano, V., Monsalve-Escudero, L. M., Filho, C. d. S. M. B., Martinez-Gutierrez, M. & Sousa, D. P. d. Antiviral Role of Phenolic Compounds against Dengue Virus: A Review. Biomolecules 11 (2020).
5 Li, Z. et al. Antiviral effects of simeprevir on multiple viruses. Antiviral Res. 172 (2019).
6 Mumtaz, N. et al. Cell-line dependent antiviral activity of sofosbuvir against Zika virus. Antiviral Res 146, 161-163 (2017).
7 Overby, A. K., Popov, V. L., Niedrig, M. & Weber, F. Tick-borne encephalitis virus delays interferon induction and hides its double-stranded RNA in intracellular membrane vesicles. J Virol 84, 8470-8483 (2010).

Point 3: The authors briefly mentioned that 3-MA and Spautin-1 have different molecular targets and that may explain why they have opposite effects on TBEV infection, but the authors did not directly address our comment. The role of autophagy remains unclear—why is it that both molecules inhibit autophagy, but 3-MA has less to no effects at higher concentrations (despite no changes in cell viability) while Spautin-1 is antiviral in a dose dependent manner? If there is no scientific explanation for this discrepancy, the authors cannot conclude that inhibitors targeting autophagy exhibit potent antiviral activity.

Response: Thanks very much for your insightful comments. Although 3-MA and spautin-1 exhibited distinct responses during TBEV infection, the dose-dependent inhibition observed with spautin-1 suggests a potential role for autophagy in the viral infection process. However, as you rightly pointed out, we cannot definitively conclude that autophagy-targeting inhibitors exhibit significant antiviral activity based solely on these findings. In response to your feedback, we have removed this statement in the abstract. Additionally, we reviewed the manuscript and eliminated any statements that may have overstated the antiviral potential of these inhibitors.

Point 4: The authors' response for inhibiting DDR was also confusing. If the virus is inhibiting DDR, what is the rationale behind using DDR inhibitors? Shouldn't DDR be activated to see whether viral infection can be blocked?

Response: Thanks very much for your suggestive comments. TBEV infection elicits distinct regulatory effects across various phases of DNA damage response. Upon TBEV infection, we observed an initial activation of this response, characterized by a significant increase in the phosphorylation of key proteins, such as ATM, ATR, and DNA-PK. Based on these findings, we selected specific inhibitors targeting these proteins for further investigation.

Subsequent to the induction of DNA damage, our research demonstrated that TBEV infection hinders the repair of DNA damage, as evidenced by reduced nuclear localization of 53BP1 and diminished efficiency of the NHEJ. In this context, we discovered that Enoxacin, an activator of DNA damage repair, significantly inhibited TBEV replication. These findings underscore the potential of modulating the DNA damage response as a strategy to counteract TBEV infection.

Point 5: Regarding Point 5:
A suggestion for Figure 5T would be to clarify the effects of the virus on the pathway. One example could be to cross out the inhibitory line from MTOR to the arrow pointing to autophagosome. This would better show that dephosphorylation of MTOR would allow autophagy to proceed.

Response: We have done the deletion accordingly.

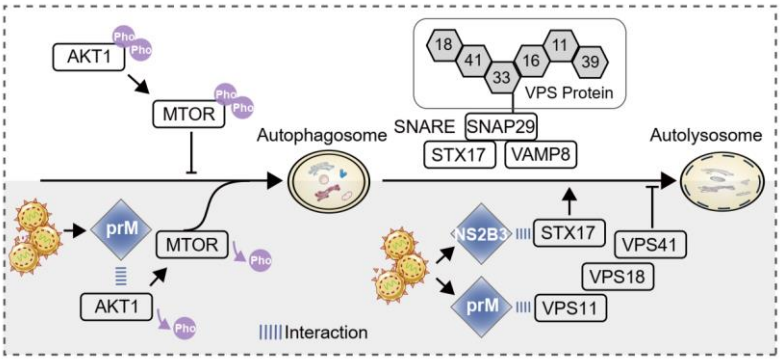


Figure 5r. Outline of host-cell autophagy regulated by TBEV prM and NS2B3.

Point 6: Regarding Point 6:
A minor adjustment would be to make the same change from the title into the results section as well (Line 273, Page 9).

Response: Thanks very much for the positive comments and suggestions, we have changed the title accordingly.

We have carefully examined the MS and corrected other mistakes and typo-errors.

We sincerely hope that the MS has been revised to your satisfaction. We are looking forward to seeing the acceptance of the revised MS for publication in your esteemed journal.

Sincerely yours,
Prof. Quan Liu, PhD
State Key Laboratory for Diagnosis and Treatment of Severe Zoonotic Infectious Diseases Department of Infectious Diseases and Center of Infectious Diseases and Pathogen Biology Key Laboratory of Organ Regeneration and Transplantation of the Ministry of Education The First Hospital of Jilin University, Changchun 130061, China https://orcid.org/0000-0003-3411-3196

Responses to the comments and suggestions of Reviewer #2

The authors have placed quite some emphasis on the newly identified and characterized pathways and altogether convincingly show that the revised version of the manuscript is adding to the existing body of evidence (point 1).

Unfortunately, with respect to point 2 (topology of the ectopically expressed viral proteins), this reviewer still strongly feels that incorrect tagging of the majority of the viral proteins invalidates most of the results of their interactome study. Both the IFA data and the Alphafold predictions, added during the revision of the manuscript, do not support correct topology of the viral protein, nor their overall folding, respectively.
As already detailed in the previous revision, erroneous tagging of the viral proteins strongly affects their topology, while not necessarily their localization (i.e. proteins will still be targeted and inserted in the ER membrane, but in the incorrect orientation). Therefore, the additional IF analysis provided does not corroborate correct topology of the tagged proteins, but confirms as expected that they are still localized in the correct compartment. Nonetheless, the consequences for the interactome remain substantial. Please see the additional drawing attached to clarify again this point, using 2k-NS4B as a test case.
Furthermore, the Alphafold predictions shown as such do not support any conclusions (a) Alphafold performs still poorly on highly transmembrane viral proteins; (b) there is no quantitiative score provided to support similary of folding in the 2 tagging strategy; (c) the structure actually looks very different in many instances.
Response: Thank you very much for your constructive suggestions and comments. We have deleted the interactome results in the revised MS accordingly.

Point 2: The employed protein databases encompassed the human proteome (Blast_Chlorocebus_sabaeus_60711_PR_20210928), the mycoplasma proteome (uniprot-reviewed_yes+AND+organism_mycoplasma), and relevant TBEV protein databases".
Please provide the correct human (293T interactome dataset) and African monkey (VeroE6 dataset) UniProt identifiers used in MaxQuant searches, currently only the African green monkey is provided.
Please add also the reference strain for TBEV used in the proteome search.
Response: Thank you for your comments, we have added the reference strain for TBEV used in the proteome search.

We have carefully examined the MS and corrected other mistakes and typo-errors.

We sincerely hope that the MS has been revised to your satisfaction. We are looking forward to seeing the acceptance of the revised MS for publication in your esteemed journal.

Sincerely yours,

Prof. Yicheng Zhao, PhD
China-Japan Union Hospital of Jilin University, Changchun, 130031, China
School of Pharmaceutical Sciences, Jilin University, Changchun 130061, China

Editorial Note: Overleaf is appended the Reviewer #2 Version 1 Review Attachment (credit SwissBioPics under a Creative Commons Attribution 4.0 International (CC BY 4.0) License), and the Reviewer #2 Version 2 Review Attachment

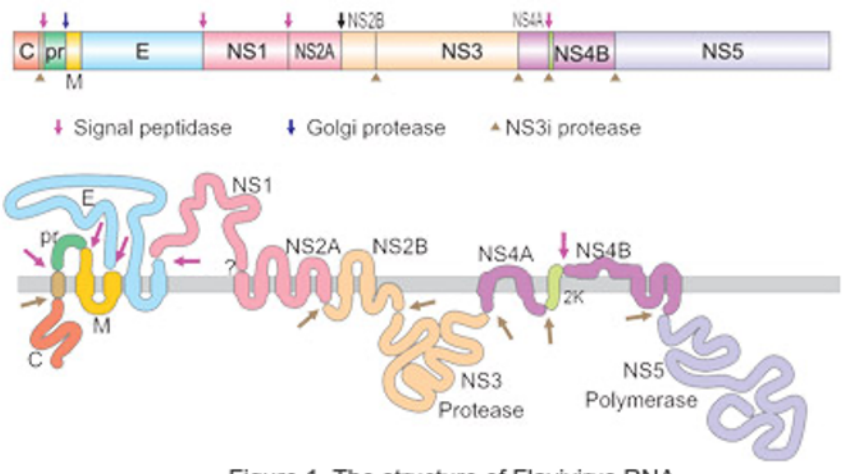
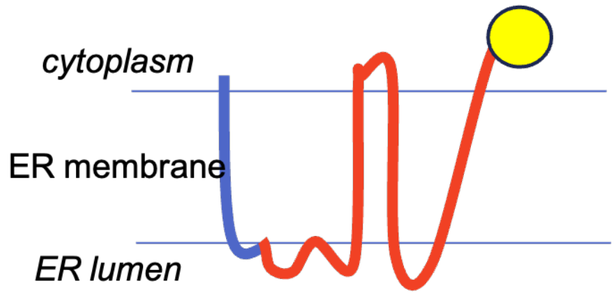
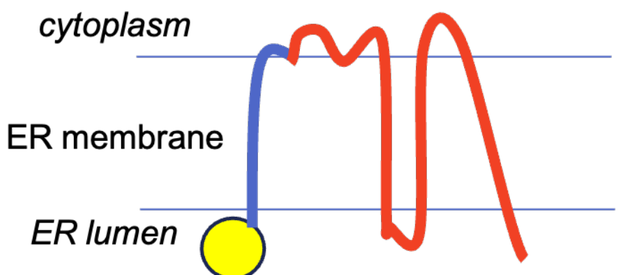


Figure 1. The structure of Flavivirus RNA

*This diagram is derived from viralzone.expasy.org

Tagging strategy *(Example of 2k-NS4B)*	Topology	Localization	Interactors
Correct tagging  *Tag at C-term = correct insertion in the ER lumen*		At the ER membrane	Cytosolic side of ER
Incorrect tagging  *Tag at N-term = incorrect insertion in the ER lumen*		Still at the ER membrane	Luminal side of ER? Transmembrane orientation not physiological!