HOKKAIDO UNIVERSITY

Title	Dendritic transport of tick-borne flavivirus RNA by neuronal granules affects development of neurological disease
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SI Materials and Methods

Cell culture

Baby hamster kidney-21 (BHK-21) cells were cultured at 37°C in minimum essential medium (MEM) (Thermo Fisher Scientific, Inc., Waltham, MA) supplemented with 8% (v/v) fetal bovine serum (FBS). PC12 cells were purchased from DS Pharma Biomedical Co., Ltd. (Osaka, Japan) and were grown at 37°C in Roswell Park Memorial Institute (RPMI) 1640 medium (Wako Pure Chemical Industries, Ltd., Osaka, Japan) containing 10% (v/v) horse serum, 5% (v/v) FBS, and penicillin/streptomycin. Human embryonic kidney 293T (HEK293T) cells were cultured at 37°C in Dulbecco's Modified Eagle's medium (Thermo Fisher Scientific) supplemented with 10% (v/v) FBS.

Neuronal cultures of the cerebral cortex were prepared from brains of ICR mice embryos (Japan SLC Inc., Shizuoka, Japan) at embryonic day 16−18, as described previously (34). Briefly, the cerebral cortex was dissected from embryonic brains in Hanks' Balanced Salt Solution (HBSS, Thermo Fisher Scientific) supplemented with 10 mM HEPES (Thermo Fisher Scientific) and 1 mM sodium pyruvate (Thermo Fisher Scientific). Tissues were treated with Neural Tissue Dissociation Kit (Miltenyl Biotec K.K., Bergisch Gladbach, Germany), and gently dissociated via trituration in neurobasal medium (Thermo Fisher Scientific) supplemented with 6 mM GlutaMAX (Thermo Fisher Scientific) and 1× B27 supplement (Thermo Fisher Scientific). Dissociated cells were seeded into eight-well glass chamber slides (Matsunami Glass Ind., Osaka, Japan) or 12 well plate (Corning, Inc, Corning ,NY) coated with cell matrix type IC (Nitta Gelatin Inc., Osaka, Japan). The cells were propagated at 37°C and used after 6−7 days of culture.

Viruses

The recombinant viruses of tick-borne encephalitis virus (TBEV) Oshima 5-10 strain (AB062063.2) (35) were recovered from infectious cDNA clones (Oshima-IC) as previously described (36). To construct the TBEV SL-2 loop C-U mutant, a C-to-T mutation was introduced into the Oshima-IC at nt position 120 by PCR and subcloning in a stepwise manner. Working stocks of the viruses were propagated once in BHK-21 cells, and stored at −80 °C. Experiments using live TBEV were conducted in Biosafety Level 3 or Animal

Biosafety Level 3 facilities located at the Graduate School of Veterinary Medicine at Hokkaido University.

Viral titration

Monolayers of BHK-21 cells prepared in multi-well plates were incubated with serial dilutions of viruses for 1 h, and overlaid with MEM containing 2% (v/v) FBS and 1.5% (w/v) carboxymethyl cellulose. Following incubation for 3−5 days, cells were fixed and stained with a solution of 0.25% (w/v) crystal violet in 10% (v/v) buffered formalin. Viral titers were expressed as plaque forming units (PFU)/mL.

Plasmids

The pCMV-TBEV REP plasmid was constructed from the pCMV-TBEV REP-GFP plasmid (kindly provided by Dr. Igarashi, Kyoto University) by deleting the inserted *GFP* gene. Under the control of a cytomegalovirus (CMV) promotor, this plasmid was designed to transcribe a replicon RNA of the TBEV Oshima 5-10 strain in which most of the coding sequences (CDS) for the viral structural proteins (nt 239−2291) had been deleted. The hepatitis delta virus (HDV) ribozyme ribozyme sequence and a poly-A signal were inserted after the replicon sequence. For construction of the other RNA-expressing plasmids, PCR fragments were synthesized by standard fusion-PCR and subcloned into pCMV-TBEV REP.

To construct pCMV-Luc, the *luciferase* gene of pGL4.0 (Promega, Madison, WI) was inserted between the CMV promoter and the HDV ribozyme sequence. Partial CDS of the TBEV Oshima 5-10 strain (nt 132−244; partial CDS for capsid protein, and 2292−4304; from NS1 to NS2A, nt 4206−7007; from NS2B to NS4A or nt 6909−1037; from NS4B to NS5) was inserted into pCMV-Luc to construct plasmids expressing TBEV CDS with *luciferase* sequence. The plasmids expressing flavivirus untranslated regions (UTRs) with the *luciferase* gene were constructed by inserting the 5′ and 3′ UTRs of the TBEV Oshima 5-10 strain or the WNV 6-LP strain (AB185914.2) into the pCMV-Luc plasmid. The sequences of stem-loop (SL) -1 (nt 4−103) and SL−2 (nt 107−128) were deleted from the pCMV-Luc (5′ TBEV/3′ TBEV) plasmid to construct the pCMV-Luc (5′ TBEV dSL-1/3′ TBEV) and (5′ TBEV dSL-2/3′ TBEV) plasmids, respectively. G-to-T mutations at nt positions 114, 115, and 117 were introduced into pCMV-Luc (5′ TBEV/3′ TBEV) to construct

pCMV-Luc (5′ TBEV SL-2 loop G-U/3′ TBEV) plasmid. The pCMV-Luc (5′ TBEV SL-2 loop C-U/3′ TBEV) plasmid was constructed by introducing a C-to-T mutation (120 nt) in pCMV-Luc (5′ TBEV/3′ TBEV). Four mutations (G109U, C110A, G125A, and C126U) were introduced in the pCMV-Luc (5′ TBEV/3′ TBEV) to construct the pCMV-Luc (5′ TBEV SL-2 stem/3′ TBEV) plasmid.

To construct pCXSN-Flag-FMRP, CDS of the *FMR-1* gene from 293T cells was cloned into the pCSXN-Flag plasmid (37). To construct pCSXN-Flag-FMRP (I304N), a T-to-A mutation at nt position 911 was introduced by site-directed mutagenesis.

Infection and transfection

For neuronal differentiation, 1.0×10^4 PC12 cells/well were seeded on eight-well glass chamber slides with RPMI 1640 medium containing 0.5% (v/v) horse serum, 0.25% (v/v) FBS and penicillin/streptomycin. Following overnight incubation, the medium was changed with medium supplemented with 50 ng/mL of 2.5s Neuronal growth factor (COSMO BIO co. ltd., Tokyo, Japan) and the cells were incubated for 6−7 days until neurites developed. The differentiated PC12 cells were infected with TBEV at a multiplicity of infection (MOI) of 0.1 or transfected with the plasmids by using X-treameGENE HP Transfection Reagent (Hoffmann-La Roche Ltd., Basel, Switzerland).

Primary neuronal cultures were infected with TBEV at an MOI of 0.1. Following viral adsorption for 1 h, half of the culture medium was replaced. The medium was harvested at 24, 48, and 72 h.p.i. and stored at −80°C.

Antibodies

Polyclonal mouse anti-Langat virus (LGTV) antibodies, which are cross-reactive among the tick-borne flaviviruses, were prepared from ascites of mice repeatedly immunized with the LGTV TP21 strain. Rabbit polyclonal antibodies were prepared by immunization with recombinant TBEV NS3 proteins expressed by *Escherichia coli* as described previously (38). Rabbit anti- RNA granule protein 105 (RNG105) polyclonal antibodies were kindly provided by Dr. Shiina (National Institute for Basic Biology, Okazaki, Japan). Rabbit

anti-Staufen polyclonal antibodies (AB5781), mouse monoclonal anti- fragile X mental retardation protein (FMRP) antibodies (MAB2160), mouse monoclonal ANTI-FLAG M2 antibodies (F3165) and mouse monoclonal anti- Kinesin Heavy chain antibodies (MAB1614) were purchased from Merck KGaA (Darmstadt, Germany). Rabbit polyclonal antibodies against microtubule-associated protein 2 (MAP2; AB32454) were purchased from Abcam (Cambridge, England). Anti-mouse IgG and anti-rabbit IgG antibodies conjugated with AlexaFluor555 were purchased from Thermo Fisher Scientific. Anti-Digoxigenin-Rhodamine, Fab fragments were purchased from Merck. Peroxidase AffiniPure Goat Anti-Mouse IgG (H+L) was purchased from Jackson Immuno Research Laboratories, Inc. (West Grove, PA). Anti-DYKDDDDK tag, Monoclonal Antibodies, Peroxidase Conjugated were purchased from Wako.

Labeled RNA probes

The 3′ UTR of TBEV (nt 10277−11100), partial CDS for NS3 of TBEV (nt 5546−4599), luciferase (nt 31−930), CaMKIIα (nt 483−931), BDNF (nt 2514−2859), and Arc (nt 2968−2354) were cloned into the pGEM-T (Easy) vector (Promega). After the cleavage of the plasmids by restriction enzyme, digoxigenin (DIG-) and fluorescent- labeled RNA were transcribed by using the DIG RNA Labeling Kit (SP6/T7) (Roche) and Fluorescein RNA Labeling Mix (Roche), respectively.

Fluorescent in-situ *hybridization (FISH) and indirect immunofluorescence assay (IFA)*

Cells were fixed in 4% (w/v) paraformaldehyde for 15 min at 37°C and washed with 0.1 M glycine in phosphate-buffered saline (PBS). The cells were permeabilized in 0.5% (v/v) Triton X-100 for 5 min at room temperature and were washed with PBS. The cells were post-fixed with 40% (v/v) formamide in 4 × saline sodium citrate buffer (0.6 M sodium chloride and 0.06 M trisodium citrate dehydrate) for 30 min at room temperature. After washing with PBS, the cells were treated with hybridize buffer (50% (v/v) formamid in 2 × saline sodium phosphate EDTA buffer: 0.36 M sodium chloride, 0.02 M sodium phosphate buffer and 0.002 M EDTA) for 2 h at 45°C. The cells were hybridized overnight at 45°C with hybridization buffer containing 2 μg/mL of anti-sense fluorescein- or DIG- labeled RNA. The hybridized cells were incubated with

hybridization buffer without labeled RNA for 1 h at 45°C and washed.

Following FISH, the cells were washed with PBS and blocked with 2% (w/v) bovine serum albumin. The cells were incubated at room temperature for 2 h with primary antibodies. After extensive washing, the cells were incubated with secondary antibodies conjugated with fluorescent dye and enclosed with a solution of Slowfade Gold antifade reagent with DAPI (Thermo Fisher Scientific). Fluorescent images were taken by using the LSM 700 confocal laser scanning microscopy (Carl Zeiss Microscopy Co., Ltd., Jena, Germany). Images were processed with ZEN 2009 software (Zeiss), IMARIS software (Bitplane AG, Zurich, Switzerland), or Image J (39).

Analysis of fluorescent signal in neurites

To analyze the fluorescent signals in the neurites of PC12 cells, Z-stack images of the cells stained with FISH and IFA were collected with the LSM 700 for 8.8 μm at intervals of 0.88 μm in depth. The three-dimensional images were reconstructed and analyzed with IMARIS software. Briefly, stained regions were assessed as voxels, and overlapping voxels of MAP2 and FISH signals in neurites were defined as the RNA signals in neurites. The measurements were conducted in five microscopic fields that were randomly selected from three biological replicates. Similar analysis was performed to analyze the localization of the RNA-binding proteins (RBPs) in the neurites. Intensity line profiles of the viral proteins and the RBPs were analyzed with ZEN2009 software. To measure the fluorescent signals in the neurites of the primary neurons, neurites positive for the Arc, BDNF or CaMKIIα mRNA were randomly selected in five microscopic fields from three biological replicates, and the fluorescent signals in the area of interest (AOI) were measured with image J. The measurements were conducted in ten independent areas. Similar analysis was performed to measure the co-localized areas of FISH signal and RBPs in the neurites.

Analysis of binding of FMRP to TBEV genomic RNA

RNAs of TBEV wild type (wt) or the SL-2 loop C-U mutant were synthesized *in vitro* with MEGAscript SP6 Transcription Kit (Thermo Fisher Scientific). HEK293T cells (4.0×10^5 cells/wel) were

seeded on a six-well plate and transfected with pCXSN-Flag-FMRP or pCXSN-Flag-FMRP (I304N) by using X-treameGENE HP Transfection Reagent. Following a 48 h of incubation, the cells were collected and treated with 250 μL/well of radioimmunoprecipitation assay buffer (50 mM Tris-HCl, 150 mM sodium chloride, 1% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulfate, and 1mM EDTA) containing protease and RNase inhibitor. The synthesized RNAs (1 μg) of the were added to the lysate, and the mixture was pre-cleared with 5 μL of SureBeads Protein G magnetic beads (Bio-Rad Laboratories, Inc., Hercules, CA) for 30 min at 4°C. The lysate was then incubated with 5 μL of magnetic beads with 1 μL of anti-Flag antibody for 1 h at 4°C. Following three washes with PBS, the RNA bound to the beads was extracted from half of the beads with ISOGEN II (Nippon Gene Co., Ltd., Tokyo, Japan). The other half was used to perform Western blotting to detect FMRP with anti-FMRP antibodies, anti-mouse IgG antibodies conjugated with HRP and the Immobilon™ Western Chemiluminescent HRP Substrate (Merck). To detect TBEV RNA in the extracted RNAs, RT-PCR was conducted with SuperScript III (Thermo Fisher Scientific) and Platinum Taq DNA Polymerase (Thermo Fisher Scientific) with TBEV-specific primers. Four biological replicates were performed for each experiment.

Luciferase activity of the construct expressing luciferase mRNA

HEK293T cells were transfected with plasmids expressing luciferase mRNA with or without flavivirus UTRs. After 48 h of incubation, cells were collected and luciferase activity was measured with luciferase assay system (promega). Three biological replicates were performed.

Detection of dendritic mRNAs in primary neuron by RT-PCR

Total RNA in primary neurons seeded on 12 well plate was collected by using ISOGEN II. To detect dendritic mRNAs, Beta-Actin mRNA and TBEV genomic RNA in the extracted RNAs, RT-PCR was conducted with SuperScript III and Platinum Taq DNA Polymerase using specific primers. Three biological replicates were performed for each experiment.

157 *Animal model*

158 Ten five-week-old female C57BL/6 mice (Japan SLC Inc.) were divided randomly into two groups and
159 inoculated with 100 PFUs of virus (TBEV wt or SL-2 loop C-U) intracerebrally. Morbidity was defined as the
160 appearance of 10% weight loss. Surviving mice were monitored for 14 days to obtain survival curves,
161 mortality rates and body weights. The experiment was repeated twice (total $n = 10$). No statistical method was
162 used to estimate the sample size. There were no mice excluded from the analysis. The President of Hokkaido
163 University approved all animal experiments after review by the Animal Care and Use Committee of Hokkaido
164 University (approval no. 13025).

165 Levels of paralysis and neurological symptoms of the infected mice ($n = 5$) were evaluated every day as
166 described previously (40). Briefly, four tests (ledge test, hind limb clasping, gait, and kyphosis) were
167 conducted and scored individually on a scale of 0−3; a score of 0 represented an absence of the relevant
168 phenotype and a score of 3 represented the most severe manifestation. For the ledge test, the mouse was
169 placed on the ledge of cage and the state of movement was observed. To evaluate hind limb clasping, the tail
170 of the mouse was grasped near its base, the mouse was lifted, and the hind limb position was observed. To
171 evaluate the mouse's gate, the mouse was placed on a flat surface and was observed from behind as it walked.
172 The level of kyphosis was observed while the mouse walked after being placed on a flat surface. One mouse
173 infected with TBEV wt and one mouse infected with SL-2 loop C-U were excluded from the analysis at 8 d.p.i.
174 due to death.

175 The brains of the mice were collected at 3 and 6 d.p.i. ($n = 3$) for viral titer analysis and sequencing
176 analysis. They were weighed and homogenized as 10% suspensions (w/v) in PBS with 10% FBS. The
177 suspension was clarified by centrifugation at 12,000 rpm for 5 min at 4 °C and titrated by plaque forming
178 assay in BHK-21 cells.

179 Total RNAs of brains of the dead mice were extracted with ISOGEN II (Nippon Gene). Following
180 amplification by RT-PCR with SuperScript III (Thermo Fisher Scientific) and Platinum Taq DNA Polymerase
181 (Thermo Fisher Scientific) with TBEV-specific primers, SL-2 region was sequenced with 3130 genetic
182 analyzer (Thermo Fisher Scientific).

Prediction of the RNA secondary structure of 5′ UTRs of TBEV

The RNA secondary structures of the 5′ UTRs of TBEV (nt 1−240) were predicted with mfold Web Server provided by University At Albany (41). The RNA secondary structures of the constructs with deletions or mutations in the 5′ UTR were confirmed with mfold prediction.

Statistical analysis

Data are expressed as mean ± the standard errors. Following a one-way analysis of variance test, the Tukey−Kramer test was used to determine the statistical significance of differences in the mean values of the fluorescent signals in neurites (Fig. 1C, 1F, 2C, 2F and 4B), the fluorescent signals in the AOI (Fig. 6B) and the luciferase activity (Fig. S2). Following the F-test to analyze variances, the unpaired two-sided Student's or Welch's *t*-test was used to determine statistical significance between TBEV wt and SL-2 loop C-U mutant at each time point (Fig. 3B, 3D, 3E, S4, S5B and S6B). The Kaplan−Meier survival curves and the log-rank test were used to evaluate the survival of the infected mice (Fig. 3C). Days until onset and average survival time were expressed as 95% confidence intervals. The statistical analysis was performed with SAS University Edition software (SAS Institute Inc., Cary, NC).

Supplemental Figure Legends

Figure S1. Genomic RNA of tick-borne encephalitis virus (TBEV) was transported to the neurites of infected PC12 cells.

(A-C) Differentiated PC12 cells were infected with TBEV, and fixed at 48 or 72 h post-infection. The cells were stained with specific antibodies against a neurite marker (A, green), viral proteins (A and B, magenta), components of neuronal granule (C, magenta), and fluorescent RNA probe against viral genomic RNA (B and C, green). White arrows and arrowheads indicate viral protein and genomic RNA in neurites, respectively.

Figure S2. Luciferase activity of the constructs expressing luciferase mRNA with or without flavivirus untranslated regions (UTRs).

Human embryonic kidney 293T cells were transfected with plasmids expressing luciferase mRNA with or without flavivirus UTRs. Following 48 h of incubation, cells were collected and luciferase activity was measured (n = 3). **p < 0.02.

Figure S3. Prediction of the RNA secondary structure of TBEV.

RNA secondary structures of the TBEV Oshima 5-10 strain (nt 1−240) were predicted by mfold. Initiationcodons of the viral coding sequences are underlined and in bold.

Figure S4. Viral growth of TBEV wt and SL-2 loop C-U in primary neuron.

Primary mouse neurons were infected with TBEV wild-type (wt; black squares with continuous lines) or SL-2 loop C-U (white circles with broken lines) at a multiplicity of infection (MOI) of 0.1. The culture supernatant was collected at 24, 48, or 72 h post-infection (h.p.i.) and virus titer was measured with a plaque forming assay. Error bars represent SEM; **p < 0.02 and *p < 0.05.

Figure S5. Detailed Localization of RNA-binding proteins (RBPs) of neuronal granule in dendrites of neurons infected with TBEV.

Primary mouse neurons were infected with TBEV wt or SL-2 loop C-U at a multiplicity of infection of 0.1. and fixed at 48 h post-infection. The cells were stained with antibodies against fragile X mental retardation protein (FMRP), RNA granule protein 105 (RNG105), or Staufen (green), and antibodies against viral proteins (magenta). (A) The differential interference contrast microscope image (DIC, left) and fluorescent image of RNG105 and viral proteins are shown (right). White continuous and broken arrows show the paths of the RNG105-positive and negative neurites, respectively. (B) Antigen accumulations in RNG105 positive or negative neurites were counted in five microscopic fields independently. Statistical differences were assessed with the Student's t-test. Error bars represent SEM; *p < 0.05. (C) Fluorescent Intensity (FI) line profile of the neurites infected with TBEV wt are defined with gray arrows. Scale bars indicate 5 μm in length.

Figure S6. Colocalization of RBPs and viral RNAs in dendrites.

Primary mouse neurons were infected with TBEV wt or SL-2 Loop C-U at a MOI of 0.1. Following fixation at 72 h.p.i., the cells were stained with antibodies against FMRP, RNG105 or Staufen (green) and fluorescent probes against viral RNA (magenta). (A) Fluorescent images of the neurons. Colocalized areas of RBPs and viral genomic RNA are shown in white (right panels). Scale bars indicate 5 μm in length. (B) Colocalized areas of RBPs and viral genomic RNA in dendrites were measured in ten areas of interest (AOI). Error bars represent SEM; **$p < 0.02$ and *$p < 0.05$.

Figure S7. Dendritic mRNAs expression in the primary neuron infected with TBEV.

Primary neurons were infected with TBEV wt or SL-2 Loop C-U. At 72 hours post-infection, total RNAs were extracted. mRNAs for Arc, brain-derived neurotrophic factor (BDNF), and Ca^{2+}/calmodulin-dependent protein kinase II α (CaMKIIα) or beta-Actin, and genomic RNA of TBEV were detected by reverse transcription PCR.

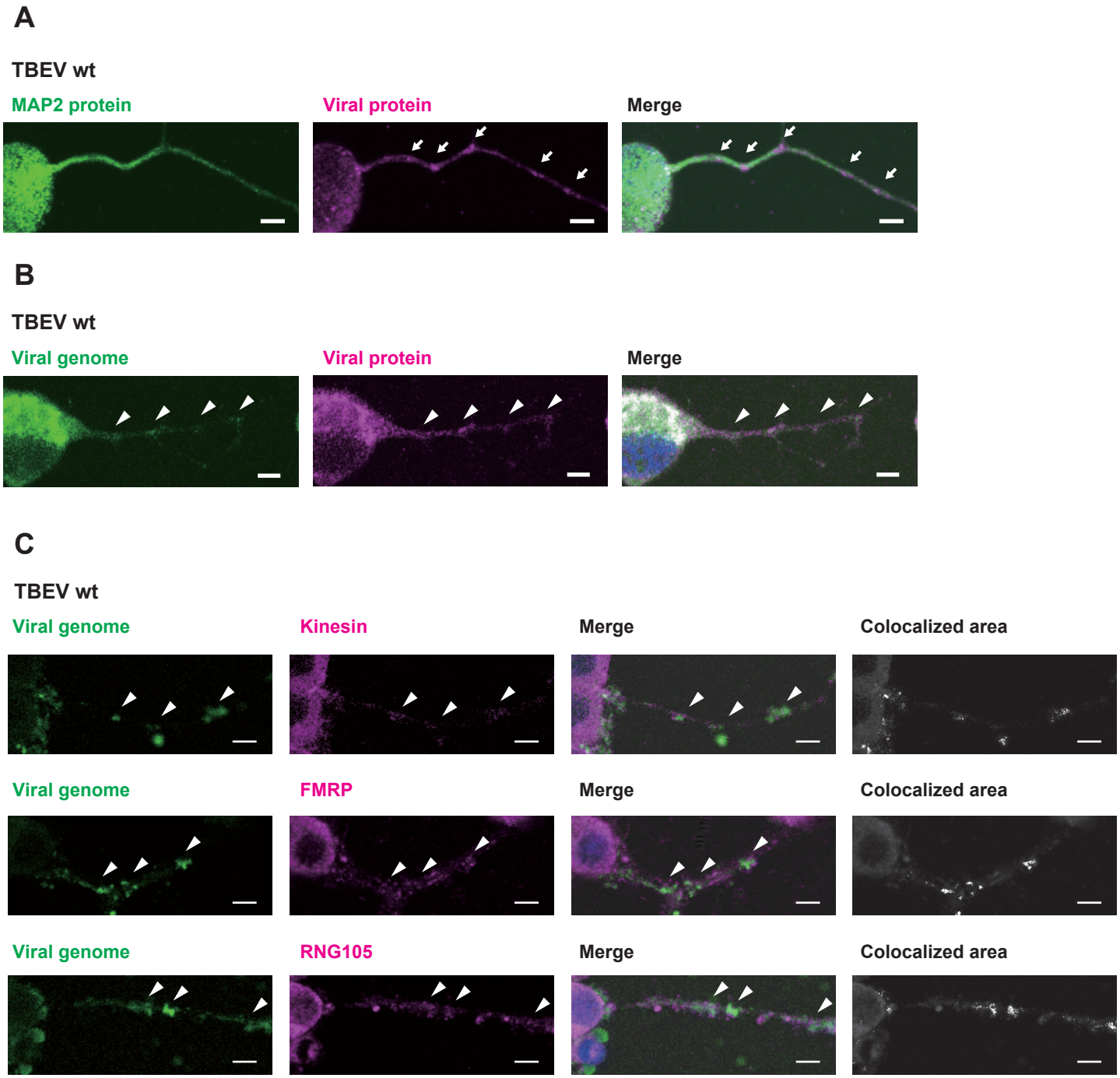
Figure S8. Comparison of the stem loop 2 (SL-2) sequence among flaviviruses.

Alignment of the SL-2 sequences of tick-borne and mosquito-borne flaviviruses. LGTV, Langat virus; LIV, looing ill virus; OHFV, Omsk hemorrhagic fever virus; KFDV, Kyasanur forest disease virus; POWV, Powassan virus; AHFV, Alkhurma hemorrhagic fever virus; DENV2, dengue virus serotype 2; and ZIKV, Zika virus. Squared region indicates conserved sequences.

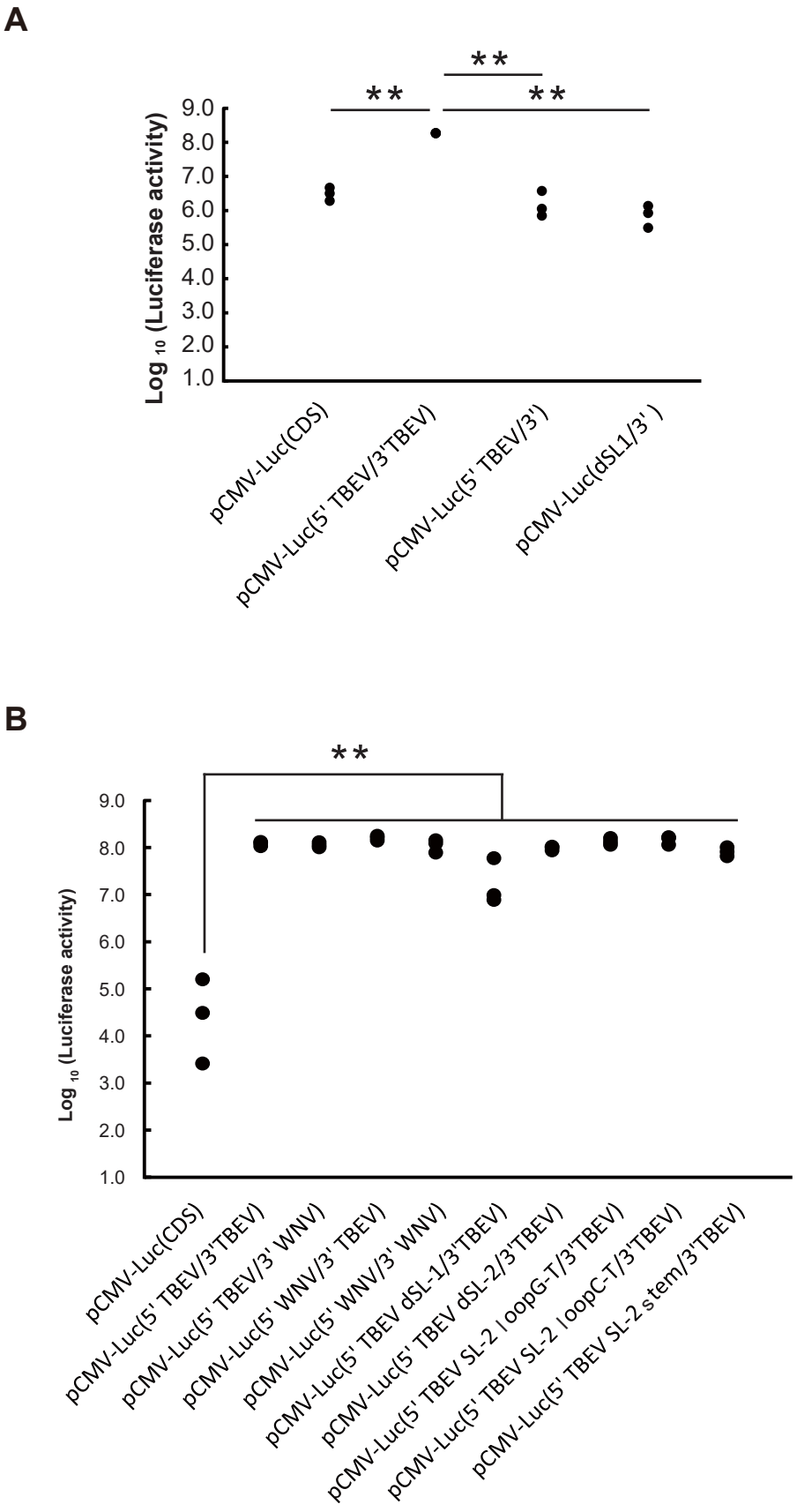
Figure S9. Model of TBEV genomic RNA transport and dendritic dysfunction.

Dendritic mRNAs bound to RBPs of the neuronal granule are transported and locally translated in dendrites for maintenance of synaptic plasticity. In neurons infected with TBEV, viral genomic RNAs bind to RBPs (such as FMRP) via the SL-2 region, and are transported to dendrites. This transport disturbed the transport of dendritic mRNAs. The transported viral genomic RNAs alter RBP distribution, and, the local replication initiates the degeneration of dendrites, resulting in neuronal dysfunction.

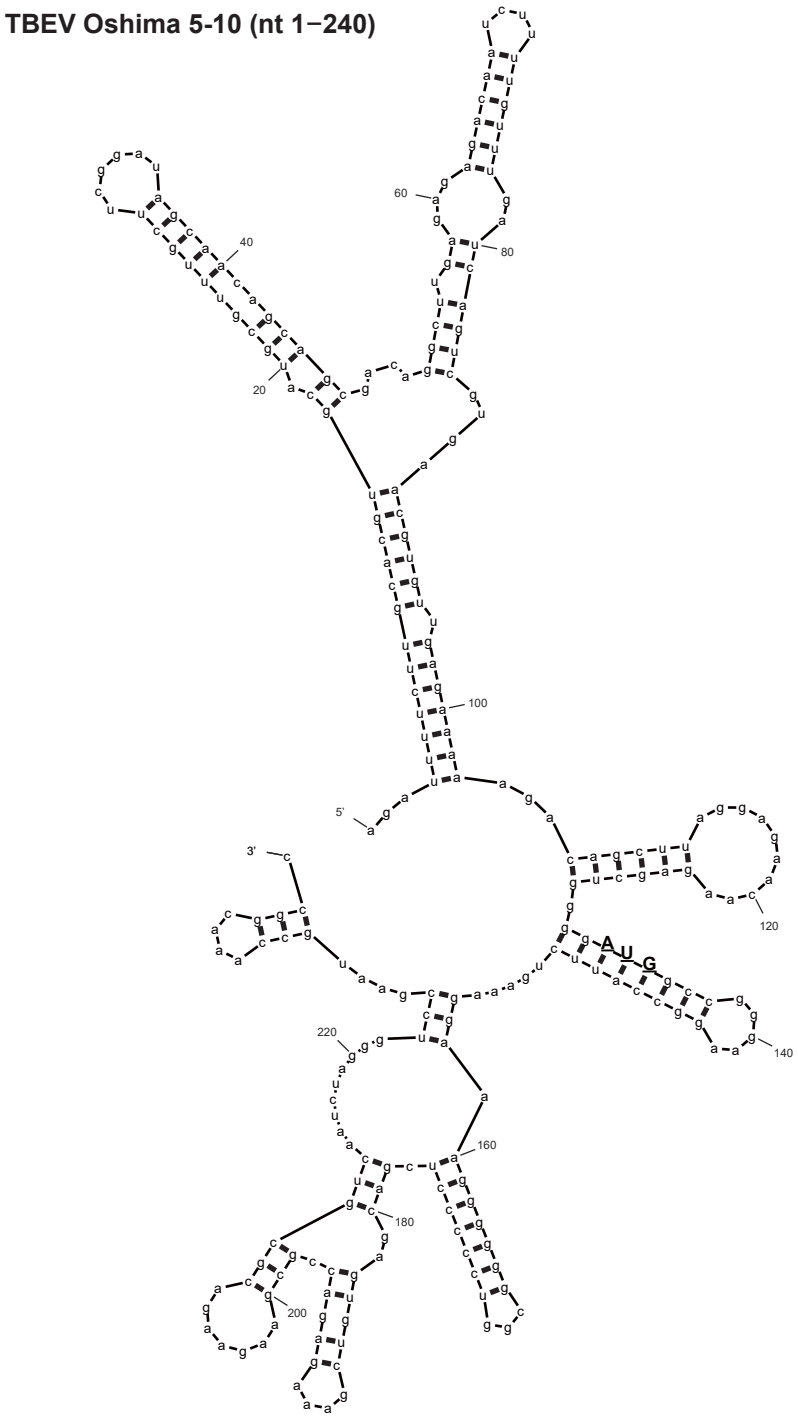
Supplemental Figure 1



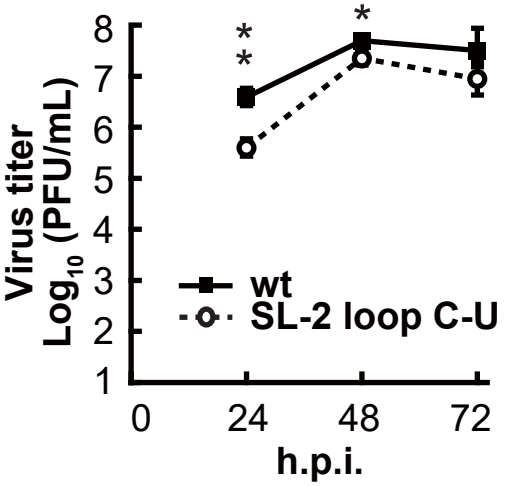
Supplemental Figure 2



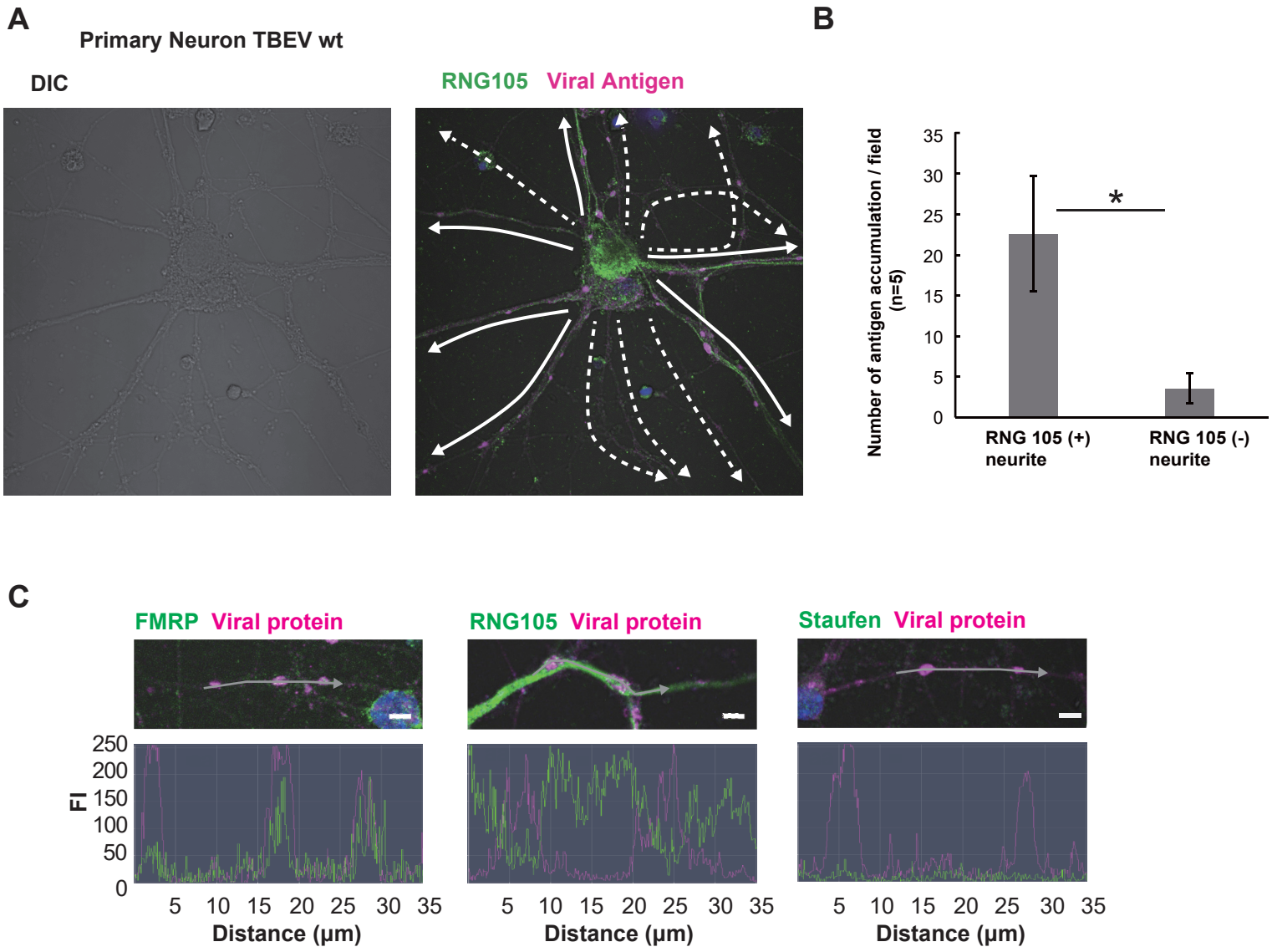
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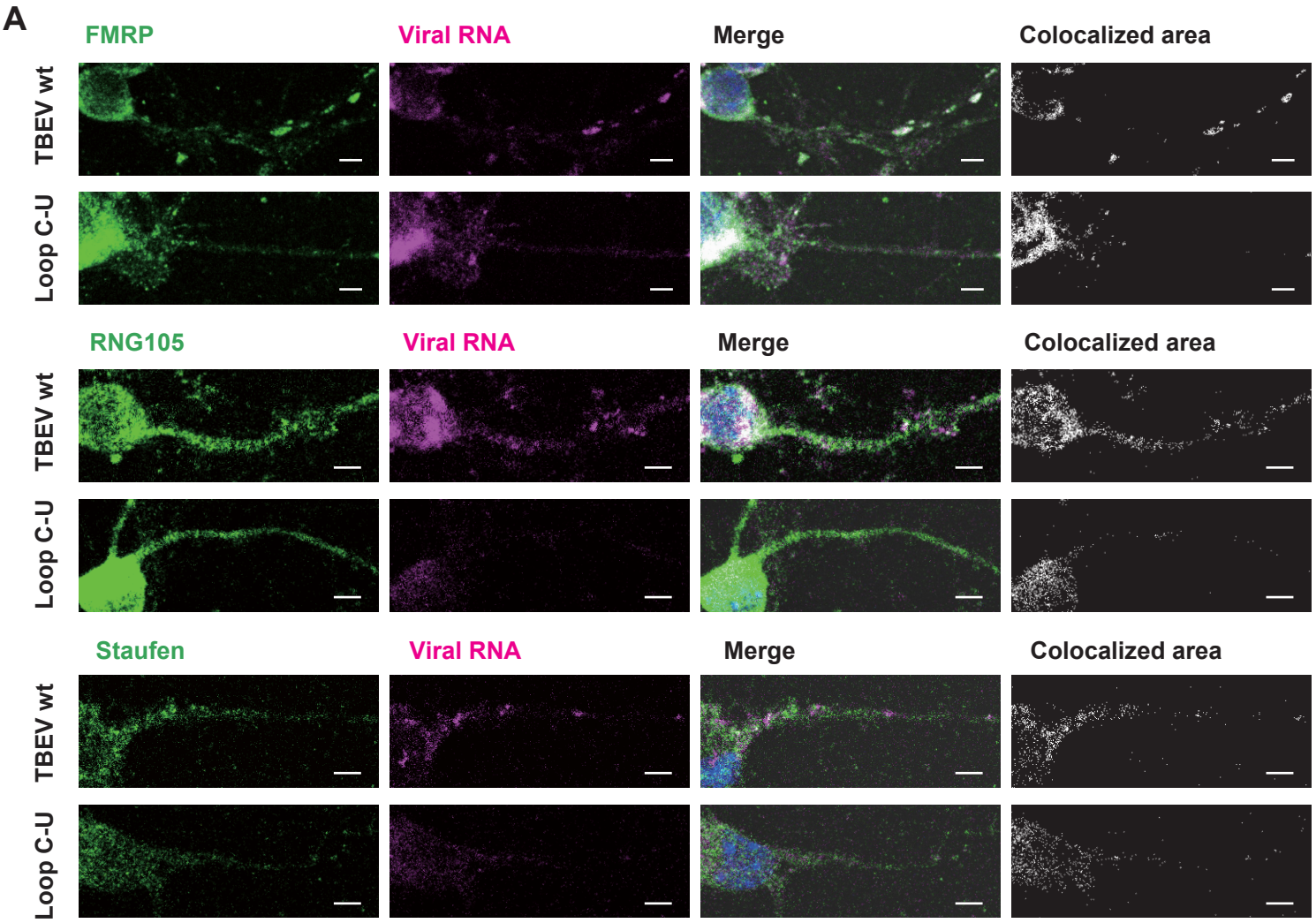
Supplemental Figure 4

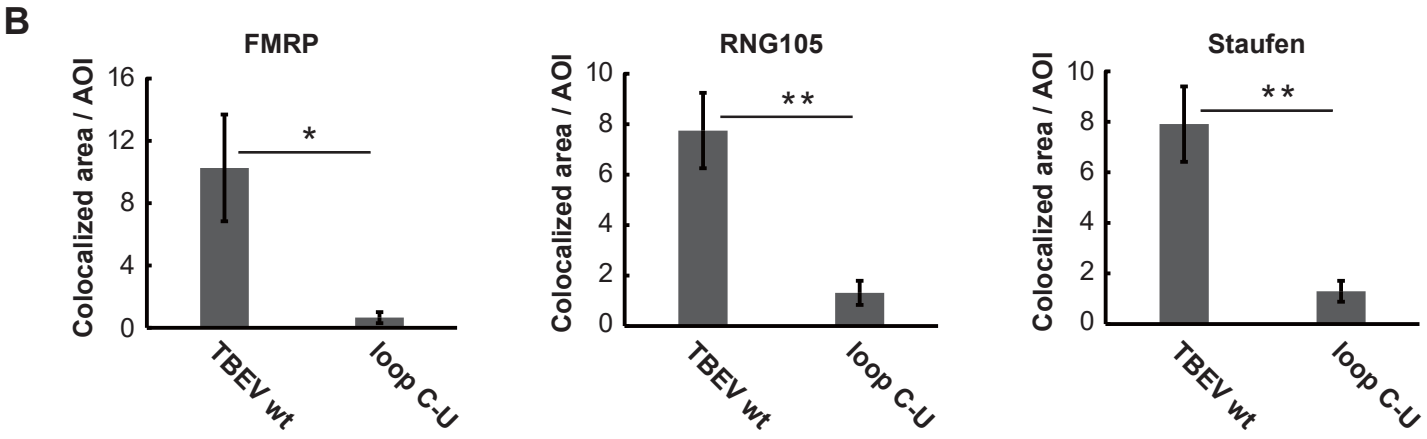


Supplemental Figure 5

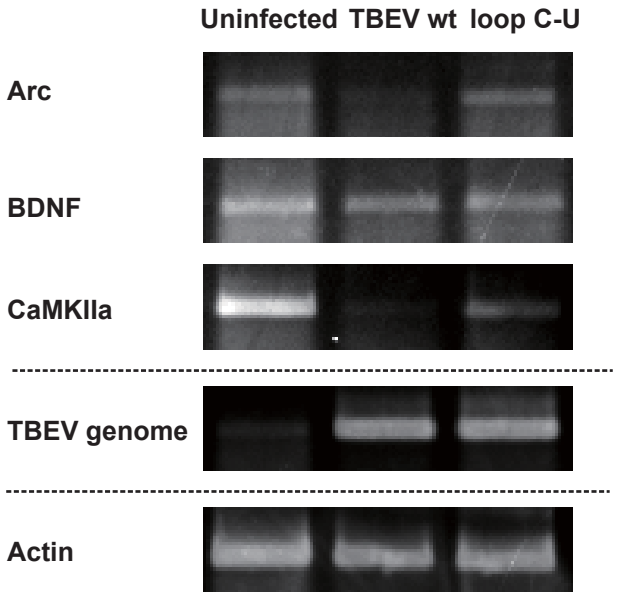


Supplemental Figure 6

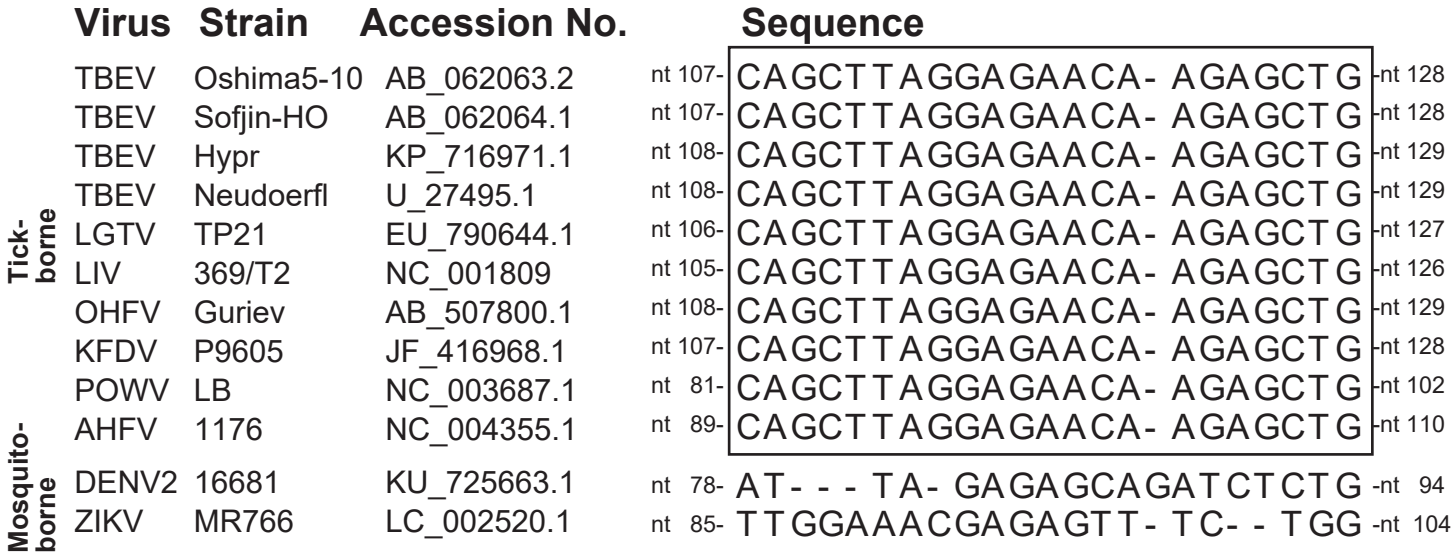




Supplemental Figure 7



Supplemental Figure 8



Supplemental Figure 9

