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## Supplemental Information

# TRAF6 Plays a Proviral Role in Tick-Borne Flavivirus Infection through Interaction with the NS3 Protease

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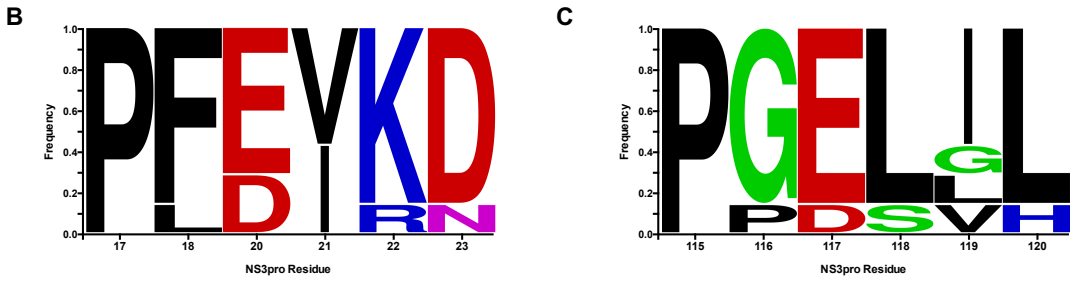
SUPPLEMENTAL FIGURES

A

| | | NS3pro | | TBM1 | | TBM2 | |
|---|---|---|---|---|---|---|---|
| | Accession | % Identity | % Similarity | % Identity | % Similarity | % Identity | % Similarity |
| **TBEV** | AMP18887.1 | 100 | 100 | 100 | 100 | 100 | 100 |
| **LGTV** | NP_620108.1 | 78 | 88 | 50 | 66 | 83 | 99 |
| **OHFV** | NP_878909.1 | 90 | 95 | 83 | 96 | 83 | 99 |
| **LIV** | NP_044677.1 | 92 | 96 | 100 | 100 | 83 | 99 |
| **ALKV** | NC_004355.1 | 79 | 93 | 83 | 96 | 83 | 99 |
| **KFDV** | AFF18436.1 | 79 | 92 | 83 | 96 | 83 | 99 |
| **POWV** | AAL32169.1 | 73 | 86 | 66 | 99 | 16 | 32 |



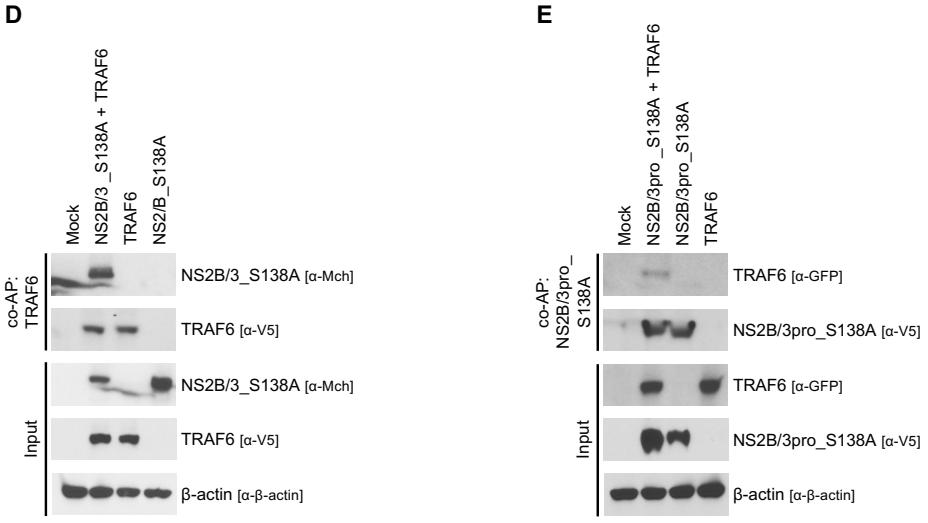




Figure S1. LGTV NS3pro interacts with TRAF6. Related to Figure 1.

(A) Sequence identity and similarity of NS3pro, TBM1, and TBM2 of TBFVs compared to TBEV.

(B) Sequence logo of TBM1.

(C) Sequence logo of TBM2.

(D-E) Co-AP of LGTV NS2B/3_S138A and LGTV NS2B/3pro_S138A constructs with TRAF6. HEK 293 cells were transfected with 2 µg of each indicated plasmid. Cell lysates were pulled down using streptavidin-conjugated beads at 24 hours post-transfection and eluted proteins were analyzed by immunoblot with indicated antibodies. Results representative of three independent experiments.

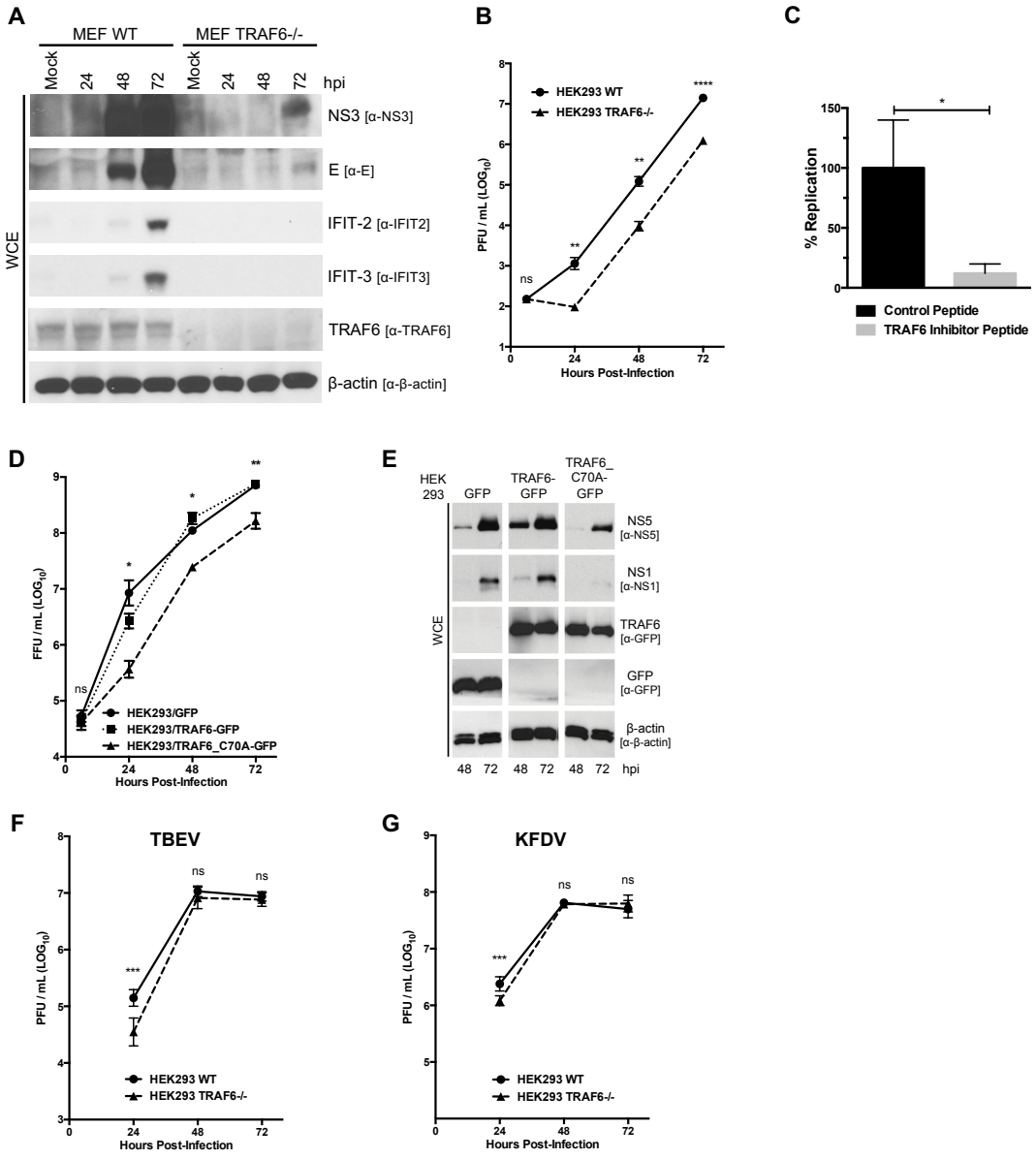



Figure S2. TRAF6 is a proviral factor for TBFVs. Related to Figure 2.

(A) Immunoblot analysis of whole cell extracts from WT or TRAF6$^{-/-}$ MEFs infected with LGTV (MOI 0.1) for indicated times (hr). Blots were probed with antibodies to LGTV NS3, LGTV E, IFIT2, IFIT3, TRAF6, and β-actin. Results representative of three or more independent experiments. Long exposures of LGTV NS3 and LGTV E are included in order to assess viral protein levels during infection of TRAF6$^{-/-}$ MEFs.

**(B)** Titration of infectious particles in the supernatant from WT or TRAF6$^{-/-}$ HEK 293 cells infected with LGTV (MOI of 0.1) for indicated times (hr). Virus titers were determined by plaque assay. Data are representative of three or more independent experiments performed in triplicate and plotted as mean +/- SEM. ns, not significant; **p<0.01; ****p<0.0001.

**(C)** Titration of infectious particles in the supernatant from HEK 293 cells pretreated with 150 μM TRAF6 inhibitor peptide or control peptide for 6 hr prior to infection with LGTV (MOI 1). Supernatants were collected at 48 hpi and titrated by immunofocus assay. Data are presented as percent replication relative to control peptide-treated cells. Data are representative of two independent experiments performed in triplicate and plotted as the mean +/- SEM. *p<0.05.

**(D)** Titration of infectious particles in the supernatant from HEK 293 cells stably expressing GFP, TRAF6-GFP, or TRAF6-C70A-GFP infected with LGTV (MOI of 10) for indicated times (hr). Virus titers were determined by immunofocus assay. Data are representative of three independent experiments performed in triplicate and plotted as mean +/- SEM. ns, not significant; *p<0.05; **p<0.01.

**(E)** Immunoblot analysis of whole cell extracts from the infected cells in (C) (48 and 72 hpi). Blots were probed with antibodies to LGTV NS5, LGTV NS1, GFP, and β-actin. Results representative of three independent experiments.

**(F-G)** Titration of infectious particles in the supernatant from WT or TRAF6$^{-/-}$ HEK 293 cells infected with TBEV (F) or KFDV (G) (MOI of 0.1) for indicated times (hr). Virus titers were determined by plaque assay. Data are average of three independent experiments performed in triplicate and plotted as mean +/- SEM. ns, not significant; ***p<0.001.

**A**

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     Accession:
LGTV AAF75259  19 DVKNGVYRIYTPGLLWGQRQIGVGYGAKGVLHTMWHVTRGAALLVDGVAVGPYWADVREDVVCYGGAWSLESRWRG-ETVQVHAFPPGRA 107
MVEV CAA27184  71 DTTPGVYRIMARGILG-RYQAGVGVMHEGVFHTLWHTTRGAAIMSGEGRLTPYWGNVKEDRVTYGGPWKLDQKWNGVDDVQMIVVEPGKP 159
                  *   ***** . *.*  . * ***    ** **.** *****..      . ***  *.** *.*** * *. .* * . **.    **.

LGTV AAF75259 108 HETHQCQPGELILENGRKMGAIPIDLAKGTSGSPIMNSQGEVVGLYGNGLKT-NDTYVSSIAQG-EVEKSRPNLPQSVVGTGWTAKGQIT 195
MVEV CAA27184 160 AINVQTKPGIFKTAHG-EIGAVSLDYPIGTSGSPIVNSNGEIIGLYGNGVILGNGAYVSAIVQGERVEEPVP----EAYNPEMLKKRQLT 244
                    . *..**     .*  .**. .*   *******.**.**..******.   * .***.* **  **   *       .     * *.*

LGTV AAF75259 196 VLDMHPGSGKTHRVLPELIRQCVERRLRTLVLAPTRVVLREMERALSGKNVRFHSPAVTEQHANGAIVDVMCHATYVNRRLLPQGRQNWE 285
MVEV CAA27184 245 VLDLHPGAGKTRRILPQIIKDAIQKRLRTAVLAPTRVVAAEMAEALRGLPVRYLTPAVQREHSGNEIVDVMCHATLTHRLMSPLRVPNYN 334
                  ***.***.***.*.**..*. ....**** ********  **  ** *  **. .***  .*... *********  .* . *    *

LGTV AAF75259 286 VAIMDEAHWTDPHSIAARGHLYSLAKENRCAFVLMTATPPGKSEPFPESNGAIASEERQIPDGEWRDGFDWITEYEGRTAWFVPSIARGG 375
MVEV CAA27184 335 LFVMDEAHFTDPASIAARGYIATRVEAGEAAAIFMTATPPGTSDPFPDTNSPVHDVSSEIPDRAWSSGFEWITDYAGKTVWFVASVKMSN 424
                  . .***** *** ****** . .    . .* . ******* *.***..*  .     .***  *  **.***.* *.* *** *.   .

LGTV AAF75259 376 AIARALRQRGKSVICLNSKTFDKEYSRVKDEKPDFVVTTDISEMGANLDVTRVIDGRTNIKP---EEVDGRIELTGTRRVTTASAAQRRG 462
MVEV CAA27184 425 EIAQCLQRAGKRVIQLNRKSYDTEYPKCKNGDWDFVITTDISEMGANFGASRVIDCRKSVKPTILDEGEGRVILSVPSAITSASAAQRRG 514
                   **..*.. ** ** ** *..* ** . *    ***.**********   .**** *  .**   .* .**. *.    .*.********

LGTV AAF75259 463 RVGRQGGRT-DEYIYSGQCDDDDSGLVQWKEAQILLDNITTARGPVATFYGPEQERMTETAGHYRLPEEKRKHFRHLLAQCDFTPWLAWH 550
MVEV CAA27184 515 RVGRNPSQIGDEYHYGGGTSEDDTMLAHWTEAKILLDNIHLPNGLVAQLYGPERDKTYTMDGEYRLRGEERKTFLELIKTADLPVWLAYK 604
                  ****.  .  *** * * . .**. * .* **.******    * **  ****...     * ***  * ** *  *.  .*   *** .

LGTV AAF75259 551 VAANVASVTDRSWTWEGPEENAVDENNGELVTFRSPNGAERTLRPVWRDARMFREGRDIREFVSYASGRR 621
MVEV CAA27184 605 VASNGIQYNDRKWCFDGPRSNIILEDNNE-VEIITRIGERKVLKPRWLDARVYSDHQSLKWFKDFAAGKR 673
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**B**

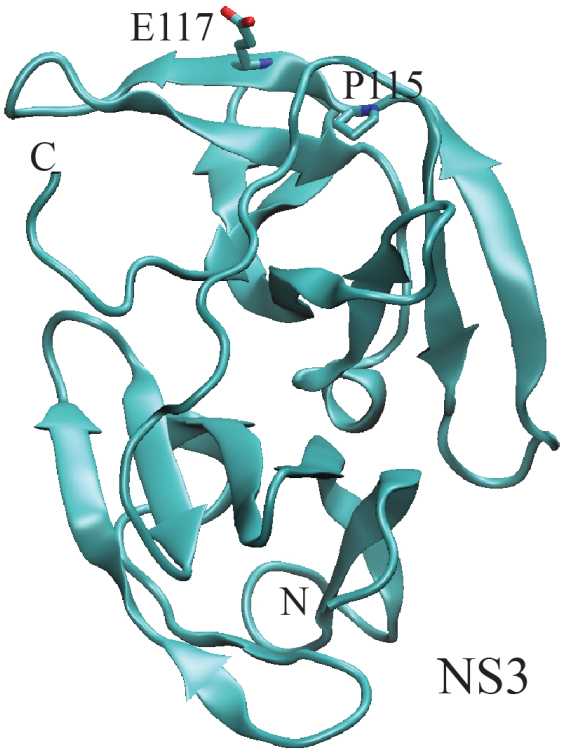


Figure S3. An E117A mutation in LGTV NS3pro disrupts TRAF6 binding and mature protease accumulation. Related to Figure 3.

(A) NS3pro sequence alignment between LGTV and MVEV. Terminal numbers of each sequence refer to residue position within NS3 protein. (*) indicates residues with the same identity; (.) indicates residues with similarity.

(B) The homology model of the LGTV NS3pro based on the 2.75 Å resolution x-ray crystal structure of NS3 from MVEV (PDB: 2WV9). TBM2 of LGTV NS3pro forms a beta strand that situates on the surface of the protein; two key residues P115 and E117 of TBM2 are shown.

## TRANSPARENT METHODS

**Cell culture and reagents.** Human embryonic kidney (HEK) 293 cells, Vero cells, murine embryonic fibroblasts TRAF6$^{+/+}$ (WT MEF), murine embryonic fibroblasts TRAF6$^{-/-}$ (TRAF6$^{-/-}$ MEF) were grown in Dulbecco's modified enrichment medium (DMEM, Thermo Fisher Scientific) containing 10% (vol/vol) fetal bovine serum (FBS, Atlanta Biologicals), 100 units/ml penicillin, and 100 mg/ml streptomycin (Thermo Fisher Scientific) in an atmosphere of 5% $CO_2$ at 37°C. WT and TRAF6$^{-/-}$ MEFs were provided by Dr. S. Best (NIAID, NIH). All cells were counted on a Tali Image Cytometer (Invitrogen).

**Isolation, culture, and immortalization of MEFs.** Fifteen-day old C57BL/6 $^{+/-}$ mouse embryos were collected, washed with PBS, and torsos were removed. Torsos were washed with PBS, minced, placed in 0.05 % trypsin-EDTA (Invitrogen) containing 1 μg/ml DNase I (Ambion) and incubated at 37°C for 15 min. Cells were filtered using a 100 μM nylon strainer, centrifuged (500 x *g*, 5 min), resuspended in complete DMEM, and plated in tissue culture flasks (passage 1). Embryo genotypes were determined by TRAF6-specific PCR and only homozygote TRAF6$^{+/+}$ and TRAF6$^{-/-}$ cells were used in subsequent experiments. Cells were immortalized by knockdown of p53 using commercially-available lentivirus preparations (sc-29436-V, Santa Cruz Biotechnology). All animal experiments were done in compliance with the guidelines of the Animal Care and Use Committee of the NIH.

**Generation of HEK 293 TRAF6 knock-out cells.** WT HEK 293 cells (1 x $10^6$ cells/well of a 6-well plate) were transfected with 2 μg of commercially available TRAF6 CRISPR/Cas9 knock-out plasmids (sc-400117, Santa Cruz Biotechnology) or Control CRISPR/Cas9 plasmid (sc-418922, Santa Cruz Biotechnology) using Lipofectamine 3000 Reagent (100022052, Invitrogen). The TRAF6 CRISPR/Cas9 knock-out plasmids consists of a pool of 3 plasmids, each encoding the Cas9 nuclease, a TRAF6-specific 20 nt guide RNA, and GFP marker. The Control CRISPR/Cas9 plasmid contains a non-targeting 20 nt scramble guide RNA and GFP marker. At 24 hours post-transfection (hpt) GFP was visualized to assess transfection efficiency, which was greater than 95%. Transfected cells were then isolated by serial dilution and plated in a 48-well plate. Individual wells were inspected by microscopy 4 days post-transfection to confirm the presence of a single clone and then allowed to continue to grow until reaching roughly 75% confluency. Cells were then trypsinized, equally divided, and plated in 2 wells of a 6-well plate. When cells reached 75% confluency, whole cell extract of each duplicate well was immunoblotted to confirm the absence of TRAF6 using α-TRAF6 (roughly 10% of clones did not express TRAF6). A single isolate was propagated and used for all subsequent studies. Furthermore, the

isolate was confirmed to have loss GFP expression (because lack of selection) by microscopy and immunoblot.

**Antibodies.** The following antibodies were used: α-β-actin (AM4302, Ambion); α-GFP (632381, Clontech and ab32146, Abcam); α-DsRed (632496, Clontech); α-V5 (46-0705, Invitrogen); α-HA (16B12, Covance); α-TRAF6 (ab33915, Abcam); α-TBEV (VR79; ATCC); affinity purified chicken α-LGTV NS3 and control IgY antisera (custom prepared by Aves Labs and α-LGTV NS3 (provided by Dr. S. Best, NIAID, NIH, (Taylor et al., 2011)); α-LGTV E and α-LGTV NS1 (provided by Dr. C. Schmaljohn, USAMRIID); α-WNV NS3 (R&D Systems, MAB2907); α-IFIT2 and α-IFIT3 (provided by Dr. S. Chattopadhyay, University of Toledo (Chattopadhyay et al., 2016)).

**Virus infections.** The following viruses were used in this study: Langat virus (LGTV, strain TP21, provided by Dr. A. Pletnev, NIAID, NIH), tick-borne encephalitis virus (TBEV, strain Sofjin, provided by Dr. M. Holbrook, NIAID, NIH), Kyasanur Forest disease virus (KFDV, provided by Dr. S. M. Best, NIAID, NIH), West Nile virus (WNV, strain NY99, provided by Dr. S. M. Best, NIAID, NIH), Kunjin virus (KUNV, provided by Dr. S. M. Best, NIAID, NIH). All virus stocks were propagated on Vero cells. LGTV was titrated by immunofocus assay or plaque assay as indicated and TBEV, KFDV, KUNV, and WNV were titrated by plaque assay. All procedures with WNV were performed under biosafety level-3 (BSL-3) conditions at the Rocky Mountain Laboratories Integrated Research Facility (RML, Hamilton, MT). All procedures with TBEV and KFVD were performed under BSL-4 conditions at RML. Multiplicity of infection (MOI) is represented as focus forming units (FFU) or plaque forming units (PFU) per cell.

**Immunofocus assay.** Cells were infected with LGTV at indicated MOI and viral supernatants collected at various time points post-infection. All samples were stored at -80°C until time of titration. 24 hr prior to set up, 24-well plates were seeded with $2 \times 10^5$ Vero cells per well. 250 µL of 10-fold dilutions of viral supernatants (ranging from $10^0$ to $10^{-8}$, depending on MOI) were added to individual wells. After a 1 hr adsorption period, the inoculum was removed, and the cells were overlaid with DMEM containing 0.8% methylcellulose (w/v) and 2% (vol/vol) FBS. At 5 days post-adsorption, wells were washed twice with DPBS and fixed with 100% methanol for 20 min at room temperature (RT). Plates were washed twice with DPBS and blocked with Opti-MEM (31985-070, Thermo Fisher Scientific) for 30 min at RT. Blocking Opti-MEM was removed and α-LGTV E (1:1000) and α-LGTV NS1 (1:1000) in Opti-MEM added to each well. Plates were incubated for 1 hr at 37°C, washed twice with DPBS, and then incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (1:1000, A28177, Thermo Fisher Scientific) in Opti-MEM for 1 hr at 37˚C. Plates were washed twice with PBS and FFUs

were visualized by adding freshly prepared peroxidase solution containing 0.4 mg/ml 3,3′-diaminobenzidine (D5637, Sigma) and 0.0135% hydrogen peroxide in DPBS.

**Plaque assay.** Cells were infected with KUNV, WNV, KFDV, or TBEV at indicated MOI and viral supernatants collected a various time points post-infection. All samples were stored at -80°C until time of titration. 24 hr prior to set-up, 24-well plates were seeded with 2 x $10^5$ Vero cells per well. 250 µL of 10-fold dilutions of viral supernatants (ranging from $10^0$ to $10^{-10}$, depending on MOI) were added to individual wells. After a 1 hr adsorption period, the inoculum was removed, and the cells were overlaid with Minimum Essential Medium (MEM, 10370-021, Thermo Fisher Scientific) containing 1.5% carboxymethylcellulose (w/v, C4888, Sigma). At 4 days post-adsorption, cells were fixed with 10% formaldehyde for 1 hr at RT and then washed with water to remove all residual overlay media. To visualize plaques, crystal violet solution (1% crystal violet in 25% ethanol) was added to each well and incubated for 15 min at RT and then washed extensively to remove residual crystal violet.

**Immunofluorescence confocal microscopy.** Cells were plated in 8-well Lab-Tek II chamber slides (154534, Thermo Fisher Scientific) and prepared by washing twice with DPBS before fixing with fresh 4% paraformaldehyde (43368, Alfa Aesar) for 20 min at RT. Cells were washed 3 times with DPBS-T (PBS, 0.5% Tween-20) and then incubated with permeabilization buffer (0.1% Triton X-100, 0.1% sodium citrate) for 5 min at RT. Slides were blocked in DPBS, 0.5% BSA, 1% goat serum for 1 hr at RT. Cells were then incubated with primary antibody for 1 hr at RT, washed 3 times with DPBS-T and incubated with appropriate secondary antibodies conjugated to Alexa Fluor 488 or Alexa Fluor 594 (A11029, A11042, Thermo Fisher Scientific) for 1 hr at RT. Slides with GFP were stained with anti-GFP conjugated to Alexa Fluor 488 (A21311, Thermo Fisher Scientific). Slides were washed 3 times with DPBS-T in the dark and overlaid with glass coverslips using Prolong Gold Antifade Reagent with DAPI (P36931, Thermo Fisher Scientific). Stained cells were visualized using an Olympus confocal microscope (Olympus Fluoview FV1000).

**Immunoblotting.** Cells were washed twice in DPBS and harvested in 1 mL radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1 % SDS, 1% NP-40, 0.5% Na-deoxycholate) with Pierce Protease Inhibitor Tablets, EDTA Free (PI, 88266, Thermo Fisher Scientific). To ensure complete lysis, samples were subjected to three snap freeze/thaw cycles in liquid nitrogen. Samples were treated with 10 units of Turbo DNAse (AM2238, Thermo Fisher Scientific) and insoluble cellular debris was removed by centrifugation (10000 x *g* for 10 min at 4°C). Protein concentration of samples was determined by DC Protein Assay (5000111, Bio-Rad). Equal amounts (10-

30 µg) of protein in sample buffer (2X SB, 62.5 mM TRIS pH 6.8, 10% glycerol, 15 mM EDTA, 4% 2-ME, 2% SDS, and bromophenol blue) were incubated for 10 min at 95°C and loaded on a 10% polyacrylamide gel and resolved by electrophoresis. Protein was transferred onto a nitrocellulose membrane using the iBlot Gel Transfer Device (Thermo Fisher Scientific) and blocked in 5% milk in PBS-T. Membranes were probed with specific primary antibodies overnight at 4˚C followed by a secondary incubation with HRP-conjugated goat anti-mouse antibody (1:3,000, A28177, Thermo Fisher Scientific), anti-rabbit IgG (1:3,000, A27086, Thermo Fisher Scientific), or anti-chicken antibodies (1:3,000, H-1004, Aves Laboratory) for 1 hr at RT. Immunoreactive proteins were detected using Pierce ECL Plus Western Blotting Substrate (32132, Thermo Fisher Scientific) and exposed to film. Quantification of immunoblot bands was performed using ImageJ software (version 1.50i, (Campbell and Pletnev, 2000)).

Co-immunoprecipitation assay (co-IP). HEK 293 cells (1 x1 $0^6$ cells/well of a 6-well plate) were transfected with 2 µg of indicated plasmid with Lipofectamine 3000 reagent (100022052, Invitrogen). At 24 hpt, cells were infected with LGTV or KUNV at a MOI of 10. At 24 hours post-infection (hpi), cells were washed twice with DPBS and harvested in 1 mL immunoprecipitation wash buffer (WB1, 50 mM Tris-HCl pH 7.5, 150mM NaCl, 1% NP-40, and 0.5% Na-deoxycholate, and PI). To ensure complete lysis, samples were subjected to three snap freeze/thaw cycles in liquid nitrogen. Samples were treated with 10 units of Turbo DNAse and insoluble cellular debris was removed by centrifugation (10000 x *g* for 10 min at 4°C). 100 µL of sample was reserved for input analysis. Samples were incubated with Pierce Control Agarose Resin (26150, Thermo Fisher Scientific) for 3 hr at 4°C with rotation as a pre-clear step. After pre-clearing, the samples were incubated with indicated 2 µg of primary antibody for 1 hr at 4°C with rotation then incubated with 50 µL of Protein A-conjugated Agarose Beads (15918-014, Invitrogen) overnight at 4°C with rotation. For co-IP using chicken antibodies, PrecipHen beads (Aves Labs) were used for both pre-clear and precipitation steps. The beads were washed 3 times in RIPA buffer for 15 min at 4°C with rotation followed by elution in 50 µL 2X SB and incubated for 15 min at 95°C. The eluted samples were assayed by immunoblotting as described above.

Co-affinity purification assay (co-AP). As described previously (Taylor et al., 2011), HEK 293 cells (1 x $10^6$ cells/well of a 6-well plate) were transfected with 2 µg of indicated plasmids with Lipofectamine 3000 Reagent (100022052, Invitrogen). Vector DNA was used as a transfection filler where appropriate. At 24 hpt, cells were then washed twice with DPBS and harvested in 1 mL WB1 with PI. To ensure complete lysis, samples were subject to three snap freeze/thaw cycles in liquid nitrogen. Samples were treated with 10 units of Turbo DNAse and insoluble cellular debris was removed by centrifugation

(10000 x *g* for 10 min at 4°C). 100 µL of sample was reserved for input analysis. Samples were incubated with Pierce Control Agarose Resin for 3 hr at 4°C with rotation as a pre-clear step. After pre-clearing, the samples were incubated with streptavidin-conjugated agarose beads (S951, Thermo Fisher Scientific) overnight at 4°C with rotation. The beads were washed 3 times in RIPA buffer for 15 min at 4°C with rotation followed by elution in 50µL 2X SB incubated for 15 min at 95°C. The eluted samples were assayed by immunoblotting as described above.

Enzyme linked immunosorbant assay (ELISA). Interferon-β (IFN-β) in culture supernatants of LGTV-infected cells was quantified by VeriKine Mouse IFN-β ELISA Kit (42400-1, PBL Assay Science) according to manufacturer's protocol. ELISA plates were read on a CLARIOstar plate reader (BMG Labtech).

Infectious clone technology. A sub-cloning vector was constructed by PCR amplification of the region between the endogenous restriction sites, NsiI and SpeI found in the full-length plasmid DNA p61-E5 corresponding to LGTV strain E5 (provided by Dr. A. Pletnev, NIAID, NIH, (Campbell and Pletnev, 2000; Pletnev, 2001; Rumyantsev et al., 2006)) followed by ligation into the Gateway entry vector, pENTR-SD-D-TOPO (46-0823,Thermo Fisher Scientific) according to manufacturer's protocol. Site-directed mutagenesis of sub-cloning vector was performed with QuikChange II Site-Directed Mutagenesis Kit (200523-5, Agilent Technologies). Desired mutations were confirmed by sequencing followed by cloning of mutation into the LGTV infectious clone. Mutated full-length infectious clone was linearized by EcoRV digestion for 4 hr at 37 ˚C and cleaned up by ethanol precipitation to remove contaminants. Linearized DNA was used as a template for *in vitro* transcription using the MEGAscript SP6 polymerase kit (AM1330, Ambion) with 15 mM $m^7G$ cap analog (AM8048, Ambion) according to the manufacturer's protocol. The resultant RNA was treated with DNAse to remove DNA contamination. HEK 293 cells were transfected with viral RNA using Lipofectamine 3000. At 6 days post-transfection cell culture supernatant was collected, filtered, and concentrated by ultracentrifugation to produce mutant virus stocks. Each mutant viral stock was titrated by immunfocus assay on Vero cells. Additionally, viral RNA was isolated from each stock using QIAamp Viral RNA Mini Kit (52906, Qiagen), reverse transcribed using Superscript Reverse Transcriptase, and sequenced to confirm presence of mutation.

Bioinformatics. Viral sequences were obtained from the National Center for Biotechnology Information databases (https://www.ncbi.nlm.nih.gov). Accession numbers for each sequence are provided in corresponding figure or table. Eukaryotic Linear Motif (ELM) analysis of the TBEV polyprotein was performed with the ELM

Resource for Functional Sites in Proteins (http://elm.eu.org, (Dinkel et al., 2013)). Sequence alignments and calculations of sequence percent identity/similarity were made using MacVector version 12.7.0. Sequence logos of TBMs were generated with WebLogo version 2.8.2 (http://weblogo.berkeley.edu/logo.cgi, (Crooks et al., 2004)).

**Molecular modeling of the TRAF6-NS3pro binding.** A 3D structural model for the TRAF6-NS3pro complex was generated using a two-stage protocol. For the first stage, the three-dimensional structure for NS3pro from LGTV was predicted by means of homology modeling using the SWISS-MODEL Workplace (Arnold et al., 2006) because no crystal structure exists for any TBFV NS3pro. Murray Valley encephalitis virus (MVEV) NS3pro was used as a template for modeling because it was found to have the highest sequence identity (46%) among solved MBFV NS3pro crystal structures (Figure S3A). This high sequence homology lays the foundation for the generation of a reliable homology model for LGTV NS3pro based on the 2.75 Å resolution x-ray crystal structure of NS3pro from MVEV (PDB: 2WV9, (Assenberg et al., 2009)). Figure S3B displays the modeled structure of the LGTV NS3pro.

During the second stage, TBM2 of the model LGTV NS3pro structure was docked with the C-terminal MATH domain of TRAF6 from *Homo sapiens* by means of quantum mechanics/molecular mechanics (QM/MM) optimization to form the TRAF6-LGTV NS3pro complex. Initially, TBM2 of the model LGTV NS3pro structure was placed into the binding site of TRAF6 utilizing the crystal structure of a previously reported TRAF6-MAVS complex (PDB: 4Z8M, (Shi et al., 2015)) as a template. The QM layer contained TBM2 and its directly interacting protein residues of TRAF6 and was treated at the PM3 level. The MM layer included the rest of the TRAF6 protein and was described by the AMBER force field. The QM/MM optimization was performed using the Gaussian 09 package (Frisch et al., 2009). The QM/MM two-layer partitioning was carried out as previously described (Liu and Hu, 2006). The modeled structure of the complex of the TBM2 motif of LGTV NS3pro with the C-terminal MATH domain of TRAF6 is shown in Figure 3E.

The intermolecular interaction energies between E117 of LGTV NS3pro and its interacting protein partners in TRAF6 were calculated at the MP2/6-311++G** level using the supermolecular approach as previously described (Mao et al., 2003). In the supermolecular approach, the intermolecular interaction energy between the two monomeric molecules (e.g. A and B) is computed as the difference between the energy of the interacting dimer $E_{AB}$ and the energies of the monomers $E_A$ and $E_B$: $\Delta E_{int} = E_{AB} - E_A - E_B$. The basis set superposition error (BSSE) was corrected by the Boys and Bernardi Counter Poise Method (Boys and Bernardi, 1970). All calculations were carried out using the Gaussian09 software (Frisch et al., 2009). For the gas phase interaction energy $\Delta E^{gas}$, the electronic energies for all molecular species ($E_i$, i=AB, A, B) are calculated directly. For

the aqueous phase interaction energy $\Delta E^{solution}$, the Polarizable Continuum (PCM) model was adopted. In a similar manner, the intermolecular interaction energies between the mutated A117 of NS3 and its interacting protein partners were calculated.

**Statistical analysis.** Data were analyzed by unpaired *t* tests using GraphPad Prism 6 software.

## SUPPLEMENTAL REFERENCES


Arnold, K., Bordoli, L., Kopp, J., Schwede, T., 2006. The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. Bioinformatics 22, 195–201. doi:10.1093/bioinformatics/bti770

Assenberg, R., Mastrangelo, E., Walter, T.S., Verma, A., Milani, M., Owens, R.J., Stuart, D.I., Grimes, J.M., Mancini, E.J., 2009. Crystal Structure of a Novel Conformational State of the Flavivirus NS3 Protein: Implications for Polyprotein Processing and Viral Replication. Journal of Virology 83, 12895–12906. doi:10.1128/jvi.00942-09

Boys, S.F., Bernardi, F., 1970. The calculation of small molecular interactions by the differences of separate total energies. Some procedures with reduced errors. Molecular Physics 19, 553–566. doi:10.1080/00268977000101561

Campbell, M.S., Pletnev, A.G., 2000. Infectious cDNA Clones of Langat Tick-Borne Flavivirus That Differ from Their Parent in Peripheral Neurovirulence. Virology 269, 225–237. doi:10.1006/viro.2000.0220

Chattopadhyay, S., Kuzmanovic, T., Zhang, Y., Wetzel, J.L., Sen, G.C., 2016. Ubiquitination of the Transcription Factor IRF-3 Activates RIPA, the Apoptotic Pathway that Protects Mice from Viral Pathogenesis. Immunity 1–12. doi:10.1016/j.immuni.2016.04.009

Crooks, G.E., Hon, G., Chandonia, J.-M., Brenner, S.E., 2004. WebLogo: a sequence logo generator. Genome Research 14, 1188–1190. doi:10.1101/gr.849004

Dinkel, H., Van Roey, K., Michael, S., Davey, N.E., Weatheritt, R.J., Born, D., Speck, T., Kruger, D., Grebnev, G., Kuban, M., Strumillo, M., Uyar, B., Budd, A., Altenberg, B., Seiler, M., Chemes, L.B., Glavina, J., Sanchez, I.E., Diella, F., Gibson, T.J., 2013. The eukaryotic linear motif resource ELM: 10 years and counting. Nucleic Acids Research 42, D259–D266. doi:10.1093/nar/gkt1047

Frisch, M.J., Trucks, G., Schlegel, H., Scuseria, G.E., Robb, M.A., Cheeseman, J.R., Scalmani, G., Barone, G.A., Petersson, G.A., Nakatsuji, H., Caricato, M., Li, M., 2009. Gaussian 09.

Liu, Y.M., Hu, X.H., 2006. Molecular determinants for binding of ammonium ion in the ammonia transporter AmtB - A quantum chemical analysis 110, 1375–1381. doi:10.1021/jp054261c

Mao, L.S., Wang, Y.L., Liu, Y.M., Hu, X.C., 2003. Multiple intermolecular interaction modes of positively charged residues with adenine in ATP-binding proteins. J. Am. Chem. Soc. 125, 14216–14217. doi:10.1021/ja036096p

Pletnev, A.G., 2001. Infectious cDNA Clone of Attenuated Langat Tick-Borne Flavivirus (Strain E5) and a 3′ Deletion Mutant Constructed from It Exhibit Decreased Neuroinvasiveness in Immunodeficient Mice. Virology 282, 288–300. doi:10.1006/viro.2001.0846

Rumyantsev, A.A., Murphy, B.R., Pletnev, A.G., 2006. A Tick-Borne Langat Virus Mutant That Is Temperature Sensitive and Host Range Restricted in Neuroblastoma Cells and Lacks Neuroinvasiveness for Immunodeficient Mice. Journal of Virology 80, 1427–1439. doi:10.1128/JVI.80.3.1427-1439.2006

Shi, Z., Zhang, Z., Zhang, Z., Wang, Y., Li, C., Wang, X., He, F., Sun, L., Jiao, S., Shi, W., Zhou, Z., 2015. Structural Insights into mitochondrial antiviral signaling protein (MAVS)-tumor necrosis factor receptor-associated factor 6 (TRAF6) signaling. Journal of Biological Chemistry 290, 26811–26820. doi:10.1074/jbc.M115.666578

Taylor, R.T., Lubick, K.J., Robertson, S.J., Broughton, J.P., Bloom, M.E., Bresnahan, W.A., Best, S.M., 2011. TRIM79**a**, an Interferon-Stimulated Gene Product, Restricts Tick-Borne Encephalitis Virus Replication by Degrading the Viral RNA Polymerase. Cell Host Microbe 10, 185–196. doi:10.1016/j.chom.2011.08.004