

Development of an attenuation mechanism targeting the structure-function of the West Nile virus
envelope protein

by

Bailey Elizabeth Maloney

B.S., University of Denver, 2019

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine/Pathobiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2023

Abstract

Flaviviruses cause human diseases. Live-attenuated vaccines (LAVs) that elicit long-lasting immunity with one immunization are only available for yellow fever virus (YFV) and Japanese encephalitis virus. Developing candidate LAVs for flaviviruses is a public health priority, and the rational design of candidate LAVs can ensure safety. This dissertation examined the flavivirus conserved structure-function of the envelope (E) protein. We used West Nile virus (WNV) and YFV as models to generate the knowledge of how the E protein drives membrane fusion. The conformational change of the E protein from dimer to trimer induces the membrane fusion process. Formation of the E protein trimer requires rearranging three domains (EDI, EDII, and EDIII). Dissociation of the E protein dimer is due to the movement of EDII against EDI, forming an intermediate structure that is stabilized by the movement of EDIII against EDI. Therefore, the dimer-to-trimer transition involves two interdomain movements called the EDI-EDII hinge region and EDI-EDIII linker.

We hypothesize that mutations of the flavivirus conserved amino acids in the EDI-EDII hinge region or EDI-EDIII linker can attenuate the virulence phenotype of multiple flaviviruses in mice. Mutations of flavivirus-conserved residues in each interdomain region were characterized using the infectious clones of the WNV NY99 strain and YFV Asibi strain. The E-A54I and E-Y201P mutations in the EDI-EDII hinge region and the E-L295S mutation in the EDI-EDIII linker can fully attenuate the mouse neuroinvasive phenotype of WNV NY99. The E-V188T mutation in the EDI-EDII hinge region can fully attenuate the virulence phenotype of YFV Asibi. Our findings demonstrated that the EDI-EDII hinge region and the EDI-EDIII linker are crucial for the structure-function of the E protein, and identified residues are worthwhile investigating for a flavivirus-common attenuation strategy.

Development of an attenuation mechanism targeting the structure-function of the West Nile virus envelope

by

Bailey Elizabeth Maloney

B.S., University of Denver, 2019

A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine/Pathobiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2023

Approved by:

Major Professor
Dr. Dana Vanlandingham

Copyright

© Bailey Maloney 2023.

Abstract

Flaviviruses cause human diseases. Live-attenuated vaccines (LAVS) that elicit long-lasting immunity with one immunization are only available for yellow fever virus (YFV) and Japanese encephalitis virus. Developing candidate LAVs for flaviviruses is a public health priority, and the rational design of candidate LAVs can ensure safety. This dissertation examined the flavivirus conserved structure-function of the envelope (E) protein. We used West Nile virus (WNV) and YFV as models to generate the knowledge of how the E protein drives membrane fusion. The conformational change of the E protein from dimer to trimer induces the membrane fusion process. Formation of the E protein trimer requires rearranging three domains (EDI, EDII, and EDIII). Dissociation of the E protein dimer is due to the movement of EDII against EDI, forming an intermediate structure that is stabilized by the movement of EDIII against EDI. Therefore, the dimer-to-trimer transition involves two interdomain movements called the EDI-EDII hinge region and EDI-EDIII linker.

We hypothesize that mutations of the flavivirus conserved amino acids in the EDI-EDII hinge region or EDI-EDIII linker can attenuate the virulence phenotype of multiple flaviviruses in mice. Mutations of flavivirus-conserved residues in each interdomain region were characterized using the infectious clones of the WNV NY99 strain and YFV Asibi strain. The E-A54I and E-Y201P mutations in the EDI-EDII hinge region and the E-L295S mutation in the EDI-EDIII linker can fully attenuate the mouse neuroinvasive phenotype of WNV NY99. The E-V188T mutation in the EDI-EDII hinge region can fully attenuate the virulence phenotype of YFV Asibi. Our findings demonstrated that the EDI-EDII hinge region and the EDI-EDIII linker are crucial for the structure-function of the E protein, and identified residues are worthwhile investigating for a flavivirus-common attenuation strategy.

Table of Contents

Table of Contents	vi
List of Figures	x
List of Tables	xv
Acknowledgments	xviii
Chapter 1 - An Introduction to Flaviviruses and Their Vaccine Development	1
1.1 Arboviruses	1
A. Flaviviruses	3
B. Alphaviruses	8
C. Bunyaviruses	11
1.2 Medically important flaviviruses	14
A. Epidemiology and ecology	15
YFV serocomplex	16
DENV serocomplex	28
JEV serocomplex	30
TBEV serocomplex	42
B. Flavivirus structure-function	45
C. Flavivirus intracellular lifecycle	57
1.3 Flavivirus vaccines	60
A. Empirically derived live attenuated vaccines	63
B. Chimeric vaccines	72
C. Inactivated vaccines	77
D. Rational design of live attenuated vaccines	82
1.4 Structure-function of the E protein and the rational design of candidate live-attenuated vaccines	87
A. Structure-function of the E protein involved in the flavivirus-host cell membrane fusion process	87
B. Flavivirus conserved E protein residues and antibody-mediated neutralization	96
1.5 Central hypothesis, model systems, and rationale of this project	99
A. Central hypothesis	99

B. Model system #1: West Nile virus	100
C. Model system #2: yellow fever virus	101
1.6 Specific aims of this project.....	102
Chapter 2 - Materials and Methods.....	106
2.1 Cell lines and competent cells	107
A. Bacterial competent cells	107
B. Mammalian cells	107
C. Mosquito cells	108
2.2. Reverse genetics systems and molecular cloning procedures	108
A. West Nile virus cDNA infectious clone and PCR-site-directed mutagenesis.....	108
B. Yellow fever virus cDNA infectious clone and PCR site-directed mutants	126
C. Procedures for the recovery of recombinant WNV and YFV mutants	131
D. Purification of plasmids	133
E. Ligation and linearization of WNV two-plasmid reverse genetics system	135
F. Linearization of YFV infectious clone	137
G. <i>In vitro</i> transcription.....	138
H. Rescue of recombinant WNV and YFV.....	139
2.3 Characterization of WNV and YFV mutants in cell culture.....	141
A. Nucleotide sequencing analysis	141
B. Determination of infectivity and multiplication kinetics	144
C. Genome-PFU ratio analysis	146
D. Serial passaging of WNV mutants	147
E. Hemagglutination activity of WNV mutants.....	147
2.4 Virulence phenotype of WNV and YFV mutants in mice.....	149
A. WNV neuroinvasive disease mouse model.....	149
B. Wild-type YFV viscerotropic disease mouse model.....	150
C. Analysis of mouse tissues	151
2.5 Statistical analysis.....	152
2.6 Generation of 3D protein models with PyMOL	153
Chapter 3 - Engineering of Attenuating Mutations in the WNV EDI-EDII Hinge Region.....	154
3.1 Introduction.....	156

A. Configuration of the EDI-EDII hinge region	156
B. Attenuation of the flavivirus EDI-EDII hinge region	161
C. Amino acid sequence homology of four motifs that constitute the EDI-EDII hinge region	163
3.2 Results.....	172
A. Recovery and infectivity of WNV EDI-EDII mutants.....	172
B. Reduction in multiplication kinetics of WNV E-A54 and E-Y201 mutants in Vero and BHK-21 cells	175
C. Single E-A54S and E-Y201P mutations impair WNV infection in cells.....	177
D. Serial passage of E-A54S and E-Y201P mutants in Vero cells	180
E. Next-generation sequencing of WNV mutants.....	181
F. Attenuation of the mouse neuroinvasive phenotype of WNV by E-A54S and E-Y201P mutants	194
G. Recovery and infectivity of WNV E-A54 and E-Y201 optimized mutants	203
H. Engineering of the E-Y201P mutation based on the codon bias of WNV.....	217
I. Attenuation of the mouse neuroinvasive phenotype of optimized WNV E-A54 and E-Y201 mutants	218
3.3 Discussion	233
Chapter 4 - Engineering of Attenuating Mutations in the WNV EDI-EDIII Linker.....	239
4.1 Introduction.....	240
A. Location of the EDI-EDIII linker in the E protein.....	241
B. The EDI-EDIII linker is crucial for the infectivity of multiple flaviviruses	246
C. Site-directed mutagenesis strategy of flavivirus conserved residues in the EDI-EDIII linker	247
4.2 Results.....	250
A. Recovery and infectivity of WNV EDI-EDIII mutants	250
B. Optimization of the infectivity of the WNV E-L295 and E-V304 mutants	252
C. Attenuation of the mouse neuroinvasive phenotype of WNV by E-L295 and E-V304 mutant	258
4.3 Discussion.....	267
A. Attenuation of the WNV mouse neuroinvasive phenotype by the E-L295 mutants.....	268

B. Attenuation of the WNV mouse neuroinvasive phenotype by the E-V304 mutants	270
Chapter 5 - Demonstration of Attenuating Effects by YFV Mutants in the EDI-EDII Hinge Region.....	272
5.1 Introduction.....	273
A. Yellow fever virus represents the ancestral mosquito-borne flavivirus.....	273
B. EDI-EDII hinge region is among the conserved motifs within the E protein	274
5.2 Results.....	280
A. Recovery and infectivity of YFV mutants	280
B. Reduction in multiplication kinetics of YFV mutants in Vero and BHK-21 cells.....	283
C. Mutations of the flavivirus conserved hydrophobic residues in the EDI-EDII hinge region increase the genome-PFU ratio of YFV Asibi (ic) in Vero cell.....	285
D. Attenuation of the mouse viscerotropic phenotype of YFV E-V188T mutant	287
5.3 Discussion.....	292
Chapter 6 - Identification of Putative Mechanisms of Attenuation in the E Protein Interdomain Regions	297
A. Structural-functional importance of the flavivirus EDI-EDII hinge region conserved residue and putative mechanism of attenuation	299
B. Putative mechanism of attenuation by mutations in the EDI-EDIII linker	314
Chapter 7 - Discussion and Future Directions	321
A. Structural variations of the EDI-EDII hinge region between different flaviviruses need to be considered for the rational design of candidate live-attenuated vaccines	321
B. Attenuating mutations in the EDI-EDIII linker of WNV and formation of the E protein trimer.....	325
C. Selection of attenuating mutations in the E protein interdomain regions with utility for the rational design of candidate LAVs	326
D. Translation of the attenuated phenotype from mosquito-borne flaviviruses to tick-borne flaviviruses.....	328
E. Future directions for the development of a broadly effective attenuating strategy based on the common structure-function of class II fusion proteins	330
References.....	345

List of Figures

Figure 1-1: <i>Aedes aegypti</i> mosquito	16
Figure 1-2: Yellow fever virus phylogenetic tree	20
Figure 1-3: Transmission cycle of YFV in Africa and South America	23
Figure 1-4: Annual number of YF cases before and after the introduction of the 17D vaccine...	26
Figure 1-5: Worldwide distribution of <i>Aedes</i> species mosquitoes.....	29
Figure 1-6: Transmission cycle of flaviviruses in the JEV-serocomplex	32
Figure 1-7: West Nile virus phylogenetic tree.....	35
Figure 1-8: Transmission cycle of WNV	37
Figure 1-9: Genome organization and proteolytic processing of the flavivirus polyprotein.....	46
Figure 1-10: Cross-section model of the structure of the flavivirus virion.....	47
Figure 1-11: Crystal structure of DENV-2 capsid homodimer (PDB ID: 6VSO).....	48
Figure 1-12: Crystal structure of the DENV-2 E protein monomer (PDB ID: 1OAN).....	49
Figure 1-13: Crystal structure of the DENV-2 pr-E protein heterodimer (PDB ID: 3C5X)	50
Figure 1-14: Crystal structure of DENV-1 NS1 homodimer (PDB ID: 4OIG).....	52
Figure 1-15: Crystal structure of DENV-4 NS3 protein (PDB ID: 2VBC) and DENV-2 NS2B/NS3 protease (PDB ID: 2FOM).....	54
Figure 1-16: Crystal structure of DENV-2 NS5 (PDB ID: 5ZQK)	56
Figure 1-17: Lifecycle of flaviviruses.....	58
Figure 1-18: Fusion of the viral membrane with the cellular membrane by the flavivirus class II fusion protein	90
Figure 1-19: The crystal structure of the flavivirus E protein dimer	91
Figure 1-20: Crystal structure of the conformational change of the DENV-2 E protein monomer	94
Figure 1-21: Herringbone pattern of JEV E protein dimers on the virion surface (PDB ID: 3J27)	97
Figure 2-1: Plasmid maps of pWN-AB and pWN-CG	109
Figure 2-2: In-fusion cloning reaction to insert single amino acid mutation.....	124
Figure 2-3: Molecular cloning strategy for combining WNV infectious clone plasmids pWN-AB and pWN-CG.	125

Figure 2-4: Plasmid maps of the YFV subcloned plasmid and the YFV infectious clone	127
Figure 2-5: Molecular cloning strategy for combining inserting the mutated YFV fragment from pGEM plasmids into the YFV infectious clone	130
Figure 2-6: Sanger sequencing cDNA fragments for verification of WNV mutations	142
Figure 2-7: Sanger sequencing cDNA fragments for verification of YFV mutations	143
Figure 2-8: Mouse study design for outbred Swiss Webster mice intraperitoneally injected with WNV	150
Figure 2-9: Mouse study design for immunocompromised AG129 mice intraperitoneally injected with YFV	151
Figure 3-1: The crystal structure of the flavivirus E protein dimer of DENV-2 with EDI-EDII hinge region (PDB ID: 1OAN)	156
Figure 3-2: The crystal structure of a flavivirus E protein trimer of TBEV in the post-fusion conformation after transition from a dimer to a trimer (PDB ID: 1URZ)	157
Figure 3-3: Configuration of the EDI β -sheets in DENV- 2 E protein (PDB ID: 1OAN)	158
Figure 3-4: Configuration of the EDII β -sheets and α -helices in DENV-2 E protein (PDB ID: 1OAN).....	159
Figure 3-5: The relative location of the kID ₀ and the gfeah β -sheets in the crystal structure of the WNV E protein monomer in the pre-fusion structural conformation (PBD ID: 2HG0)	160
Figure 3-6: Conformational change of the kID ₀ and the gfeah β -sheets in the post-fusion crystal structure of the EDI-EDII hinge region in the DENV-2 E protein (PBD ID: 1OK8).....	161
Figure 3-7: Locations of the eight flavivirus conserved hydrophobic residues in the EDI-EDII hinge region of the WNV E protein monomer.....	171
Figure 3-8: Infectivity of WNV mutants containing single mutations in the EDI-EDII hinge region	174
Figure 3-9: Growth curves of WNV mutants over 7 days in Vero and BHK-21 cells	176
Figure 3-10: PFU-genome ratio of WNV EDI-EDII hinge region mutants	178
Figure 3-11: Serially passaged WNV EDI-EDII hinge region mutants over 5 passages	180
Figure 3-12: Single nucleotide variants of recovered P0 and P1 WNV mutants.....	183
Figure 3-13: Attenuation of the neuroinvasive phenotype of WNV in four-week-old mice.....	195
Figure 3-14: Viral RNA loads in mice infected with WNV EDI-EDII hinge region mutants ...	201

Figure 3-15: Serum-neutralizing antibodies in mice receiving the IP challenge with the E-A54S or E-Y201P mutant.	202
Figure 3-16: Infectivity of WNV E-A54 mutants in Vero cells	208
Figure 3-17: Multiplication kinetics of the E-A54 mutants in Vero cells between 0 and 3 dpi .	209
Figure 3-18: PFU-genome ratio of WNV E-A54 mutants.....	211
Figure 3-19: Infectivity of WNV E-Y201 mutants in Vero cells	213
Figure 3-20: Reduced multiplication kinetics of the E-Y201C and E-Y201D mutants in Vero cells between 0 and 3 dpi.	215
Figure 3-21: PFU-genome ratio of WNV E-Y201 mutants.....	216
Figure 3-22: Infectivity comparison of WNV E-Y201P mutants.....	218
Figure 3-23: Attenuation of the neuroinvasive phenotype of WNV E-A54 mutants in four-week-old mice.....	219
Figure 3-24: Comparison of brain viral loads in groups of mice infected with WNV NY99ic, the E-A54E mutant, and the E-A54I mutant.....	222
Figure 3-25: Comparison of serum neutralizing titers in mice that survived the IP challenge with the E-A54E and E-A54I mutants.	224
Figure 3-26: Survival curve of four-week-old outbred Swiss Webster mice receiving IP challenge with WNV NY99ic, the E-Y201C mutant, and the E-Y201D mutant.....	225
Figure 3-27: Comparison of brain viral load in mice infected with WNV NY99ic, the E-Y201C mutant, and the E-Y201 mutant.	229
Figure 3-28: Survival curves of four-week-old outbred Swiss mice challenged with WNV NY99ic and the codon-optimized E-Y201P mutant	230
Figure 4-1: The crystal structure of the flavivirus E protein dimer of DENV-2 with EDI-EDIII linker before membrane fusion (PDB ID: 1OAN).....	240
Figure 4-2: The crystal structure of a flavivirus E protein trimer of TBEV with EDI-EDIII linker after membrane fusion (PDB ID: 1URZ).....	241
Figure 4-3: Relative location of the pre-fusion DENV-2 EDI-EDIII linker between the A ₀ C ₀ D ₀ E ₀ F ₀ and B ₀ I ₀ G ₀ H ₀ β-sheets of EDI and ABDE and CFG β-sheets of EDIII (PDB ID: 1OAN).....	242

Figure 4-4: Relative location of the post-fusion DENV-2 EDI-EDIII linker between the A ₀ C ₀ D ₀ E ₀ F ₀ and B ₀ I ₀ G ₀ H ₀ β-sheets of EDI and ABDE and CFG β-sheets of EDIII (PDB ID: 1OK8)	243
Figure 4-5: Stabilization movement of the EDI-EDIII linker during the formation of the E protein trimeric structure.	245
Figure 4-6: Locations of the eight flavivirus conserved hydrophobic residues in the EDI-EDIII linker of the WNV E protein monomer	250
Figure 4-7: Infectivity of WNV E-L295 mutants	256
Figure 4-8: Infectivity of WNV E-V304 mutants	258
Figure 4-9: Attenuation of the neuroinvasive phenotype of WNV E-L295A, E-L295M, E-L295S, E-L295T, and E-L295V mutants in four-week-old mice.....	259
Figure 4-10: Attenuation of the neuroinvasive phenotype of WNV E-L295F and E-V304N mutants in four-week-old mice	260
Figure 5-1: Comparison between WNV and YFV E protein monomer	275
Figure 5-2: Locations of the flavivirus conserved hydrophobic residues in the EDI-EDII hinge region of the YFV E protein monomer	279
Figure 5-3: Infectivity of YFV EDI-EDII hinge region mutants	282
Figure 5-4: Multiplication kinetics of YFV Asibi (ic) and mutants containing single amino acid substitutions of flavivirus conserved hydrophobic residues in the EDI-EDII hinge region in Vero cells	283
Figure 5-5: Growth curves of YFV mutants over 5 days in BHK-21 cells	284
Figure 5-6: PFU-genome ratio of YFV EDI-EDII hinge region mutants	286
Figure 5-7 Attenuation of the viscerotropic phenotype of YFV in six-week-old mice	288
Figure 6-1: Seven flavivirus conserved hydrophobic residues crucial for the structure-function of the E protein	298
Figure 6-2: Location of the E-A54 and E-Y201 residues in the EDI-EDII hinge region (PDB ID: 2HG0)	300
Figure 6-3: Location of WNV E-A54 residue on the periphery of the E protein dimer, modeled using JEV E protein (PDB ID: 3J27).	301
Figure 6-4: Mutations of the WNV E-A54 residue in the peripheral region of the “gfeah” β-sheet of the E protein (PDB ID: 2HG0)	303

Figure 6-5: Hydrophobic triad of the fg hairpin of the E protein (PDB ID: 2HG0).....	306
Figure 6-6: Mutation of the WNV E-Y201 residue at the edge of the fg hairpin of the E protein (PDB ID: 2HG0)	308
Figure 6-7: Mutation of the YFV and WNV residues located within the 2 nd and 3 rd motifs that are important to the gfeah rotation around the fg hairpin of the E protein	310
Figure 6-8: Mutations in the kl hairpin of WNV E protein	312
Figure 6-9: WNV E-L281 and YFV E-L276 residues kl hairpin of the E protein	314
Figure 6-10: Location of WNV E-L295 and E-V304 residues in the EDI-EDIII linker before and after membrane fusion	315
Figure 6-11: Mutation with large hydrophobic amino acids of the WNV E-L295 residue at the N- terminus of the EDI-EDIII linker (PDB ID: 2HG0)	317
Figure 6-12: Mutations of the WNV E-L295 residue at the N-terminus of the EDI-EDIII linker that produced an attenuated neuroinvasive phenotype (PDB ID: 2HG0).....	318
Figure 6-13: Mutations of the WNV E-V304 residue of the EDI-EDIII linker (PDB ID: 2HG0)	320
Figure 7-1: Comparison of different flavivirus E protein crystal structures.....	323
Figure 7-2: Locations of the E-L107F, E-E138K, and E-Q264H virulence determinants in the JEV SA14-14-2 E protein (PDB ID: 3P54)	324
Figure 7-3: Comparison of the E protein crystal structure of mosquito-borne flaviviruses and tick-borne flaviviruses	329
Figure 7-4: Cartoon depiction of the class II fusion protein in the pre-fusion conformation.....	332
Figure 7-5: Comparison of the flavivirus E protein, alphavirus E1 protein, and bunyavirus Gc protein in its pre-fusion conformation	333
Figure 7-6: Comparison of the flavivirus E protein, alphavirus E1 protein, and bunyavirus Gc protein in its post-fusion conformation.....	334
Figure 7-7: Structural homology between the glycoproteins of different arboviruses	336
Figure 7-8: Proposed model for the hinge effect to control the EDI-EDII interdomain movement	343

List of Tables

Table 1-1: Current status of flavivirus vaccines	61
Table 1-2: Comparison of the complete genome sequences of JEV SA14 and SA14-14-2.....	69
Table 1-3: Fab HM14c10 epitope on DENV-1 E protein.....	98
Table 2-1: Amino acid sequences of the flavivirus EDI-EDII hinge regions and EDI-EDIII linker among different flaviviruses	110
Table 2-2: Hydrophobicity of the amino acids	112
Table 2-3: Mutations of the eight conserved hydrophobic residues in the WNV EDI-EDII hinge region.	114
Table 2-4: Mutations of the E-A54 residues in the WNV EDI-EDII hinge region for optimization of attenuation.	115
Table 2-5: Mutations of the E-Y201 residues in the WNV EDI-EDII hinge region for optimization of attenuation.	116
Table 2-6: Mutations of the eight conserved hydrophobic residues in the WNV EDI-EDIII linker.	117
Table 2-7: Mutations of the E-L295 residues in the WNV EDI-EDIII linker for optimization of attenuation.	118
Table 2-8: Mutations of the E-V304 residues in the WNV EDI-EDIII linker for optimization of attenuation.	119
Table 2-9: Overlapping primer sets for site-directed mutagenesis of WNV mutants.....	121
Table 2-10: Mutations of the eight conserved hydrophobic residues in the YFV EDI-EDII hinge region.	128
Table 2-11: Overlapping primer sets for site-directed mutagenesis of YFV mutants	129
Table 2-12: Buffer quantities for simulated hemagglutination in various pH environments	148
Table 3-1: Acknowledgements for collaborative projects in Chapter 3	155
Table 3-2: Sequence of the 1 st motif of the EDI-EDII hinge region between flaviviruses.....	164
Table 3-3: Sequence of the 2 nd motif of the EDI-EDII hinge region between flaviviruses.....	166
Table 3-4: Sequence of the 3 rd motif of the EDI-EDII hinge region between flaviviruses	168
Table 3-5: Sequence of the 4 th motif of the EDI-EDII hinge region between flaviviruses	170
Table 3-6: Viability and stability of WNV EDI-EDII mutants.....	173

Table 3-7: Optimal pH for the HA activity of WNV NY99ic and each single mutant	179
Table 3-8: Summary of SNVs >1% identified in the P0 stocks of WNV NY99ic and the respective mutants.....	184
Table 3-9: Summary of SNVs >1% identified in the P1 stocks of WNV NY99ic and the respective mutants.....	189
Table 3-10: Average survival times of mice infected with WNV EDI-EDII mutants.....	196
Table 3-11: Individual weights for mice infected with WNV EDI-EDII mutants	198
Table 3-12: Viability and stability of WNV E-A54 mutants.....	204
Table 3-13: Viability and stability of WNV E-Y201 mutants.....	205
Table 3-14: Comparison of average survival times between mice challenged with WNV NY99ic, the E-A54E mutant, and the E-A54I mutant.....	220
Table 3-15: Comparison of weights for mice infected with WNV NY99ic, the E-A54E mutant, and the E-A54I mutant.....	221
Table 3-16: Comparison with average survival times of four-week-old outbred Swiss mice challenged with WNV NY99ic, the E-Y201C mutant, or the E-Y201D mutant.....	226
Table 3-17: Weight change of mice infected with WNV NY99ic, the E-Y201C mutant, and the E-Y201D mutants	227
Table 3-18: Comparison of average survival time between mice challenged with WNV NY99ic and the codon-optimized WNV E-Y201P mutant at 500 and 3,000 PFU	231
Table 3-19: Individual weights for mice infected with WNV NY99ic or the codon-optimized WNV E-Y201P mutants	232
Table 4-1: Acknowledgements for collaborative projects in Chapter 4	239
Table 4-2: Amino acid sequence alignment of the EDI-EDIII linker between flaviviruses.....	248
Table 4-3: Phenotype of WNV mutants encoding single alanine substitutions in the EDI-EDIII linker	251
Table 4-4: Viability and stability of WNV E-L295 mutants	253
Table 4-5: Viability and stability of WNV E-V304 mutants.....	254
Table 4-6: Average survival times of mice infected with WNV E-L295A, E-L295M, E-L295S, E-L295T, and E-L295V mutants	262
Table 4-7: Average survival times of mice infected with WNV E-L295F and E-V304N mutants	263

Table 4-8: Individual weights for mice infected with WNV E-L295A, E-L295M, E-L295S, E-L295T, and E-L295V mutants	264
Table 4-9: Individual weights for mice infected with E-L295F and E-V304N mutants	266
Table 5-1: Acknowledgements for collaborative projects in Chapter 5	272
Table 5-2: Comparison of the sequence of the 1 st through 3 rd motifs of the EDI-EDII hinge region between WNV and YFV	277
Table 5-3: Comparison of the sequence of the 4 th motifs of the EDI-EDII hinge region between WNV and YFV	278
Table 5-4: Viability and stability of YFV EDI-EDII mutants	281
Table 5-5: Summary of average survival times of AG129 mice challenged with YFV Asibi (ic) and single-site mutants in the flavivirus conserved residues in the EDI-EDII hinge region	289
Table 5-6: Summary of weight challenge in groups of four male and four female mice receiving the IP challenge of YFV Asibi (ic) or single mutants containing amino acid substitutions of flavivirus conserved hydrophobic residues in the EDI-EDII hinge region.....	290
Table 6-1: Amino acid sequence alignment of the hydrophobic triad in EDII.....	305
Table 7-1: Conserved sequences of the EDI-EDII hinge region and EDI-EDIII linker across the different serocomplexes of flaviviruses	331
Table 7-2: Sequences of the DI-DIII linker in the alphavirus E1 proteins	338
Table 7-3: Amino acid sequences alignment of the DI-DIII linker of hantaviruses.....	339
Table 7-4: Amino acid sequences alignment of the DI-DIII linker of phleboviruses	340
Table 7-5: Amino acid sequences alignment of the DI-DIII linker of orthobunyaviruses	341

Acknowledgments

I want to extend my deepest gratitude to the many people that helped me through these last few years. First, I would like to thank my major professor, Dr. Dana Vanlandingham, as well as my committee members, Dr. Stephen Higgs, Dr. Yan-Jang Scott Huang, Dr. Alan Barrett, and Dr. Phil Hardwidge, for providing their wisdom, support, guidance, and friendship throughout my entire degree. I also want to thank Susan Hettenbach for her help and support while conducting this research and editing this dissertation.

I want to acknowledge the support I received in completing this dissertation work. I want to thank the past and present lab members of the Vanlandingham lab, especially Ashley Bilyeu, Dr. Danielle Saunders, Dr. Natalia Costa Ball, and Adrienne Pohl, for their help throughout my time in the lab, helping me with the large number of WNV and YFV mutants that needed construction and analysis. For their help with the completion of the WNV mouse studies, I would like to thank Ashley Bilyeu, Dr. Danielle Saunders, Dr. Natalia Ball, Adrienne Pohl, Dr. So Lee Park, Dr. Emily Mantlo, and Alexis Schneider for giving me an extra pair of hands to keep the studies running smoothly. I want to thank CEZID and Dr. Jürgen Richt of the Department of Diagnostic Medicine/Pathobiology at Kansas State University for providing their lab space for our use during our analyses. I would also like to thank the Biosecurity Research Institute staff and the Comparative Medicine Group staff at Kansas State University College of Veterinary Medicine for their assistance in providing a place to conduct my work and working with our group as partners to ensure procedures are done accurately and safely.

I am also sincerely grateful to our outside collaborators who helped with this research. I want to thank Dr. Claire Huang at the Division of Vector-Borne Infectious Diseases at the Centers for Disease Control and Prevention, who provided the WNV cDNA infectious clone and processed

mouse tissue samples for our analysis. To our collaborators at the University of Texas Medical Branch, I would like to thank the lab of Dr. Alan Barrett and Kassandra Carpio for completing next-generation sequencing for our WNV analysis. I am also extremely grateful to our collaborators at Bioqual, Dr. Swagata Kar, Dr. Brian Backstedt, and Dr. Hanne Anderson, for running the YFV mouse study for us.

The research reported in this dissertation was supported by the National Institute of General Medical Sciences (NIGMS) of the National Institutes of Health under award number P20GM130448 and the State of Kansas National Bio and Agro-Defense Facility Transition Fund.

Chapter 1 - An Introduction to Flaviviruses and Their Vaccine

Development

1.1 Arboviruses

In 1942, a new group of viruses was defined as “arthropod-borne viruses” to describe a group of human and animal viruses that replicated within a hematophagous arthropod and then were transmitted to a new vertebrate host (Hammon 1947, WHO 1985). Characteristics of “arthropod-borne viruses” include replicating and producing viremia within a vertebrate host, multiplying within the tissues of arthropods, and transmitting the virus to a new host through arthropod bites. After their discovery, the term “arthropod-borne viruses” was shortened to “arboviruses” and was officially utilized by the International Subcommittee on Viral Nomenclature in 1963 (WHO 1967, WHO 1985). Over 800 arboviruses have been recognized by the Centers for Disease Control and Prevention (CDC), with over 130 viruses capable of causing human disease (CDC 2017, CDC 2021a). The majority of arboviruses that are important to human and veterinary health belong to one of five different virus families: *Flaviviridae*, *Bunyaviridae*, *Togaviridae*, *Reoviridae*, and *Rhabdoviridae*.

Since their official description in 1942, arboviruses that are important to public health are transmitted via biological transmission, which is when the causative agent of disease transmission plays an essential role in the life cycle of the pathogen (Marquardt 2005). Biological transmission by arthropods was demonstrated in 1876 by Sir Patrick Manson when he demonstrated that mosquitoes could transmit filarial nematodes (Manson-Bahr and Alcock 1927). Studies since identified several groups of arthropods that are competent for the transmission of arboviruses to vertebrate hosts, such as ticks, mosquitoes, fleas, biting midges, black flies, sand flies, and tabanid flies (Marquardt 2005).

Horizontal transmission between arthropods and vertebrate hosts most commonly occurs via saliva transfer during blood feeding, but in some cases, vertical transmission can occur (Eldridge and Edman 2003, Marquardt 2005). Vertical transmission (or transovarial transmission) involves the passage of the virus from one infected female vector to both the male and female offspring due to the infection of the ovaries after systemic infection by horizontal transmission (Lequime and Lambrechts 2014). Although vertical transmission is not known to cause human or animal disease, the lack of viremic hosts sustains viral maintenance in adverse ecological conditions such as drought or winter months, where biological transmission cycles cannot be maintained. This transmission has been proven essential for the biology of several bunyaviruses, including Rift Valley Fever virus (RVFV) and La Crosse virus (LACV) (Borucki et al. 2002, Lumley et al. 2017). Maintenance of RVFV and LACV can occur through mosquitos ovipositing infected eggs that can withstand desiccation for months to years until the ecological conditions are optimal again, leading to long periods between epidemics. Once the eggs hatch and release the infected progeny, the new mosquitoes can continue the cycle of horizontal transmission (Borucki et al. 2002, Lumley et al. 2017).

Zoonotic arboviruses are maintained between viremic animal reservoirs and competent arthropod vectors. Arboviruses have evolved with humans for centuries, resulting in medically important viruses known for causing large epidemics (Powers et al. 2001, Holmes and Twiddy 2003, Beck et al. 2013). Some arboviruses that cause hemorrhagic fever or arthritis in humans often emerged from sylvatic cycles involving non-human primates (Valentine et al. 2019). Humans began to enter the forest areas as agricultural activity developed and proliferated during the rise of civilization, providing the opportunity for the emergence of arboviruses from the sylvatic cycle, as seen with yellow fever virus (YFV) and chikungunya virus (CHIKV) (Barrett and Higgs 2007,

Valentine et al. 2019). Arboviruses circulate among wild animals, domesticated animals, and humans as replicating hosts that maintain the virus. However, some arboviruses also cause spillover transmission into incidental, dead-end hosts, which play little or no role in transmission even though they can succumb to disease. This is seen in viruses that cause encephalitis, such as Japanese encephalitis virus (JEV), West Nile virus (WNV), and equine encephalitis virus (Ciota and Kramer 2013, Mackenzie et al. 2022, Luethy 2023). Urban transmission of these viruses can cause thousands of human cases in population centers throughout the tropical or subtropical regions. Most infections only lead to subclinical or mild diseases, creating large numbers of viremic hosts to sustain silent transmission. Therefore, many of these viruses can cause large-scale outbreaks without epizootic spillover (Weaver and Reisen 2010).

A. Flaviviruses

The genus *Flavivirus* belongs to the *Flaviviridae* family, although it is also known as the genus *Orthoflavivirus* by the International Committee on Taxonomy of Viruses (ICTV) as of 2022 (ICTV 2022). They can be distinguished from members of the genera *Pestivirus*, *Hepacivirus*, and *Pegivirus* within the *Flaviviridae* family based on the transmission cycle and genome structure, particularly the number of envelope glycoproteins in the genome. Pestiviruses are RNA viruses that encode three surface glycoproteins: Erns, E1, and E2. These viruses infect mammals, including cattle, sheep, goats, and swine, and can be transmitted via contact with feces or bodily fluids like saliva and nasal secretions. The most notable virus of this family is classical swine fever virus (Schulz et al. 2017, Riedel et al. 2021). Hepaciviruses are also RNA viruses, but their genome encodes for only two surface glycoproteins: E1 and E2. The viruses infect various mammals, including horses, rodents, bats, cows, and humans. Transmission often occurs through contact with blood through injection, blood transfusion, and parenteral exposure. In nature, transmission can

occur through contaminated food, fomites, or aerosols. One of the most notable viruses of this genus and the only one to cause infection in humans is hepatitis C (Pybus and Thézé 2016, Hartlage et al. 2017, Li et al. 2021). Pegiviruses are similarly structured to hepaciviruses with an RNA genome that encodes for two glycoproteins: E1 and E2. Pegiviruses include blood-borne or sexually transmitted infections that can affect mammals such as porcine, equine, bats, rodents, and humans. Two pegiviruses known for causing human diseases include hepatitis G virus and human pegivirus (Stapleton et al. 2011, Yu et al. 2022).

Flaviviruses are the largest group of viruses within the *Flaviviridae* family and can be distinguished into three groups based on their transmission. The first are vertebrate-specific flaviviruses, such as Modoc virus and Rio Bravo virus, which are exclusively isolated from rodents and bats. There is no known vector for these flaviviruses. The next group is the insect-specific flaviviruses maintained with the vector population, such as sandflies, midges, and mosquitoes. The final and most extensive group of flaviviruses are the vector/vertebrate flaviviruses that can transmit from ticks or mosquitoes to various vertebrate hosts (Blitvich and Firth 2015). Many of these vector/vertebrate flaviviruses have evolved to infect humans as amplifying or dead-end hosts.

Flavivirus is derived from the Latin word *flavus*, meaning yellow, which refers to YFV, the prototype virus (Fenner et al. 1974, Mukhopadhyay et al. 2005). Flaviviruses have become major public health concerns over the last several decades. Over half of the world's population is at risk for the infection of at least one flavivirus (Gubler 2002, Guarner and Hale 2019). The flavivirus genus can be split into two groups based on tissue tropisms and clinical manifestations: encephalitis or hemorrhagic fever.

Flaviviruses that belong to the JEV and tick-borne encephalitis virus (TBEV) serocomplexes cause encephalitis and other neurological diseases. Members of the JEV serocomplex are primarily vectored by *Culex* species mosquitoes that exhibit ornithophilic feeding behavior. Consequently, enzootic transmission of JEV, WNV, and St. Louis encephalitis virus (SLEV) primarily involves avian species. The epizootic spillover of the respective encephalitic flaviviruses is often due to the spread of viruses from viremic avian species to humans or equids (Buescher et al. 1959, Scherer and Buescher 1959, Wimberly et al. 2014, Kleinschmidt-DeMasters and Beckham 2015, Ortiz-Martínez et al. 2017, Haddow et al. 2020). The *Ixodes* species ticks are vectors for multiple neurotropic flaviviruses that belong to the TBEV serocomplex, including TBEV and Powassan virus (POWV), which are maintained through enzootic transmission in rodent species (Mansfield et al. 2009, Kemenesi and Bányai 2019). It is important to note that maintenance of tick-borne flaviviruses does not always require viremic rodents. As engorgement of blood meals by ticks often lasts for multiple days and saliva of infected ticks can deliver large quantities of infectious viruses in the feeding site, co-feeding of infected and susceptible ticks may be sufficient to result in non-viremic or direct transmission of tick-borne flaviviruses (Marquardt 2005).

Except for Zika virus (ZIKV), which can cause neonatal microcephaly, flaviviruses transmitted by the *Aedes* species mosquitoes cause hemorrhagic fever in humans, including dengue virus (DENV) and YFV. Compared to encephalitic flaviviruses, DENV and YFV serocomplexes members have a narrower host range, maintaining transmission between only vector species and susceptible primates, including humans (Guarner and Hale 2019). Dengue virus is one of the biggest global health concerns, with an estimated 390 million infections yearly (WHO 2022a). Dengue virus also has four serotypes (DENV-1, -2, -3, -4) known for causing various

clinical diseases, from febrile illness to severe dengue (WHO 2022a). Yellow fever virus is a significant public health concern in South America and Africa, with an estimated 200,000 cases of the disease yearly. It was named after one of its characteristic symptoms, jaundice, which causes the skin to turn yellow (CDC 2018).

At least three tick-borne flaviviruses are known to cause hemorrhagic fever, including Kyasanur Forest disease virus (KFDV), Alkhurma virus, and Omsk hemorrhagic fever virus (OHFV). Kyasanur Forest disease virus is a viral hemorrhagic fever unique to the Western Ghats of India for the last 60 years, known for transmitting from monkeys to humans through a tick vector (Růžek et al. 2010). Alkhurma virus was discovered in 1994 in Saudi Arabia and is commonly associated with feeding and slaughtering animals, food processing, and tick transmission. Alkhurma virus is a concern locally due to high levels of hospitalization, and it is known for causing fatality rates of up to 25% (Alzahrani et al. 2010). Omsk hemorrhagic fever virus is an acute hemorrhagic disease in some regions of Western Siberia in Russia. Unlike other hemorrhagic fevers, OHFV is known to have a low mortality rate of 0.4-2.5% (Munivenkatappa et al. 2018). A list of some of the significant flaviviruses with human public health importance is summarized in section 1.2.A.

All flaviviruses are evolutionally related and share similar genome organization, polyprotein processing, and virion structure (Araujo et al. 2020). Flaviviruses are enveloped positive-sense single-stranded RNA viruses with an 11-Kilobase viral genome that encodes for ten proteins. These genes are divided into three structural genes, capsid (C), pre-membrane (prM), and envelope (E), and seven non-structural (NS) genes, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5. The 5' and 3' untranslated regions (UTRs) are at the ends of the viral genome (Lindenbach et al. 2007). The flavivirus virion is approximately 50 nm in diameter and comprises 180 copies

of the E protein monomer, forming an icosahedral lattice with T=3 symmetry (Zhang et al. 2004). The E proteins are assembled in the mature virion into a homodimer formation parallel to the virion surface. The E protein homodimer is crucial in binding to the host-cell receptor and initiating the membrane fusion process. Once membrane fusion is initiated, the E protein undergoes a conformational change from a dimer to a trimer. It fuses the cell and viral membranes to allow the viral RNA to enter the cell. Once the RNA is in the cell cytoplasm, the RNA is translated and transcribed in replication complexes within the endoplasmic reticulum (ER). Once fully assembled in the ER, the immature virion moves through the ER and the Golgi apparatus, where the acidic pH of the Golgi initiates the final conformational change of the flavivirus virion to create a mature virion. The virion is then released from the cell to infect neighboring cells (Pierson and Diamond 2020). Further details of the flavivirus structure, replication, and assembly will be discussed in section 1.2.

The flavivirus E protein is a major structural protein in the virion and plays a significant role in various stages of the viral life cycle. The protein has been well characterized among many flaviviruses and exhibits up to 40% amino acid sequence homology between them. The E protein monomer consists of three distinct domains that are encoded in discontinuous sequences: domain I (EDI), domain II (EDII), and domain III (EDIII), based on their location and function. Many of the crystal structures for the E protein have been determined, including for WNV, YFV, DENV, and TBEV (Rey et al. 1995, Kanai et al. 2006, Klein et al. 2012, Lu et al. 2019). The fusion properties of the flavivirus E protein structurally resemble other class II fusion proteins (Harrison 2015). The overall structures of flavivirus E proteins resemble one another, with only minor variations in the structures existing. Functional characterization of the different domains of flavivirus E proteins provides information critical to understanding the biology of the viruses as

well as their application to develop preventive and therapeutic measures for the diseases. Conformational changes in the E protein occur in a pH-dependent manner. The changes are essential for viral entry and the secretion of viral RNA into the cell cytoplasm (Modis et al 2014).

A mature flavivirus virion comprises 180 copies of the E protein monomer arranged in a homodimer formation parallel to the virion surface. In addition to an antiparallel dimeric arrangement of the E proteins, the surface particles also contain 3-fold and 5-fold symmetry throughout the virion surface (Kuhn et al. 2002). Each of the three domains of the E protein plays a vital structural and functional role in the E protein. Flavivirus structure and function will be discussed more in section 1.4.

B. Alphaviruses

Alphaviruses are mosquito-borne viruses that constitute one of the two genera in the *Togaviridae* family. Serological assays demonstrated at least seven antigenic complexes within the *Alphavirus* genus, and the classification is consistent with the evolutionary relationship established using contemporary molecular virology techniques (Porterfield 1975, Powers et al. 2001). Despite the differences between the seven complexes, analysis of the nucleotide and amino acids sequences and the antigenic properties suggests significant homology within the genomes of the different alphaviruses (Powers et al. 2001, Knipe and Howley 2013). The public health significance of alphaviruses has grown significantly in the 21st century, as observed with the emergence of the CHIKV in the Indian Ocean Archipelago between 2006 and 2007 and in Latin America in 2014 and 2015. Other alphaviruses have also been designated with high biodefense research priority, such as Venezuelan equine encephalitis virus (VEEV), Eastern equine encephalitis virus (EEEV), and Western equine encephalitis virus (WEEV) (NIAID 2018). Those

alphaviruses that concern human and veterinary public health often include emerging and zoonotic pathogens and are identified based on their specific characteristics.

Pathogenic alphaviruses can be classified based on tissue tropism and clinical manifestations. Arthritogenic alphaviruses are primarily circulating in the Old World, with the most widely distributed being the prototypic alphavirus, Sindbis virus (SINV), which was isolated in 1952 (Taylor et al. 1954). Exceptions to this pattern include chikungunya virus, found in the New and Old World, and Mayaro virus (MAYV), which can only be found in the Americas and the Caribbean (Go et al. 2014). Encephalitic alphaviruses primarily circulate in the Americas, hence New-World alphaviruses (Skidmore and Bradfute 2023). The most notable examples are EEEV, VEEV, and WEEV, which are all most commonly isolated from encephalitic horses but can cause severe central nervous system infections in humans. All three viruses are known for causing high mortality rates in horses, but only EEEV is known for causing high cases of human mortality at 50-75% (Zacks and Paessler 2010, Stromberg et al. 2020). The divide between these two groups of alphaviruses is becoming less valuable as there are now several New-World viruses that do not cause any disease, and the discovery of MAYV in the New-World with a disease phenotype that is similar to an Old-World virus (Go et al. 2014, Acosta-Ampudia et al. 2018).

All alphaviruses are evolutionarily related and share the same coding strategy in the single-stranded positive-sense RNA that is approximately 12 Kilobases in length (WHO 1985). The genome codes for four nonstructural proteins (nsP1, nsP2, nsP3, and nsP4) and four structural proteins (capsid, E1, E2, and 6k/TF) (Jose et al. 2009, Kim and Diamond 2022). The 5' and 3' UTRs are at the ends of the viral genome. The 5' UTR encodes for a type 0 cap (N7mGppp), while the 3' UTR encodes for the poly(A) tail. Both the 5' UTR and its complement in the 3' UTR of the negative strand RNA contain parts of the promoter elements known as conserved sequence

elements, which are recognized by the alphavirus replication complex (Hyde et al. 2015). Additionally, between the nonstructural and structural proteins lies a subgenomic promoter. This promoter produces 26S RNAs translated to express the structural proteins capsid, E2, and E1 glycoproteins that form the virus particles (Steel et al. 2013).

The alphavirus virion is approximately 70 nm in diameter. The virion surface is composed of a host lipid bilayer decorated with 240 copies of E1 and E2 protein, which form an icosahedral lattice with T=4 symmetry. Every three neighboring copies of the E1-E2 heterodimer are assembled into 80 spike trimers (Hasan et al. 2021). The E1-E2 heterodimer plays a key role in the binding and membrane fusion with the host cell. Specifically, the E2 protein contains a receptor-binding domain that shares amino acid sequence homology with the domain III of the flavivirus E protein (Myles et al. 2003). The E1 protein consists of three domains (DI, DII, and DIII), following the overall molecular architecture of class II fusion proteins. The class II fold is characteristically comprised of a discontinuous central sandwich of two antiparallel β -sheets (DI): the “outer” $A_0C_0D_0E_0F_0$ β -sheet and the “inner” $B_0I_0G_0H_0$. The segments connecting β -strands D_0 to E_0 in the outer sheet and H_0 to I_0 in the inner sheet are very long and elaborate segments that comprise the portions of DII. These two strands are comprised of 12 β -strands and two short α -helices, creating an elongated shape with two subdomains. The first subdomain is a central open β -barrel near DI made of β -strands $klaefgj$, and the second subdomain is the “distal tip.” The distal tip, comprised of a β -sandwich between the bcd β -sheet and the ij β -hairpin, is a standard feature of arbovirus class II proteins known as the fusion loop (Gibbons et al. 2004, Guardado-Calvo et al. 2016, Serris et al. 2020). Finally, after the I_0 β -strand, DI connects via a linker to DIII. Due to the function of the DI-DIII linker as a stabilizer for the conformational change, the DI-DIII linker is always an intrinsically disordered region of the glycoprotein.

Free energy provided by the protonation of endosomes leads to the conformational change of the E1 protein. The E1 trimer inserts the alphavirus-conserved fusion loop at the tip of DII. It triggers the virus-cell membrane fusion process and instigates the release of RNA into the cell cytoplasm (Roussel et al. 2006). The nonstructural (P1234) and structural polyproteins are generated upon entry into the cell. Initial cleavage of nsP4 from the polyprotein causes the formation of an unstable replication complex that triggers the synthesis of negative-strand RNA for genomic and subgenomic synthesis. Cleavage of nsP1 creates a more stable replication complex within cytopathic vacuoles and initiates the generation of genomic RNA. After full cleavage of nsP1, nsP2, nsP3, and nsP4, subgenomic RNA is generated. Concurrently as the nonstructural polyprotein is cleaved, the structural polyprotein is cleaved into its proteins with the E1 and E2 heterodimers using the Golgi apparatus to congregate in the membrane at the cell surface. Interactions between C and E2 proteins drive the budding process, with E1 and E2 forming the envelope around the nucleocapsid-like particles before releasing them into the extracellular space (Leung et al. 2011, Kim and Diamond 2022). Although alphaviruses are classified under *Togaviridae*, alphaviruses and flaviviruses share similar receptor binding and membrane fusion mechanisms, showing a conserved mechanism for a wide variety of arboviruses (Harrison 2015).

C. Bunyaviruses

Another major category of arboviruses are the bunyaviruses. These viruses are part of the order *Bunyavirales* and were formerly classified under the family *Bunyaviridae*, which consists of the families *Nairoviridae*, *Hantaviridae*, and *Tospoviridae* and the genera *orthobunyavirus* and *Phlebovirus* (CDC 2021b, ICTV 2022). *Orthobunyaviruses*, *phleboviruses*, and *nairoviruses* are considered arthropod-borne viruses, whereas *hantaviruses* only infect vertebrate hosts, and

tospoviruses are plant pathogens. The most recent ICTV release designates the genus orthobunyavirus in the family Peribunyaviridae as well as the genus phlebovirus in the family Phenuiviridae (ICTV 2022). Most bunyaviruses are transmitted by an arthropod vector, except for hantaviruses, which are primarily transmitted by rodents (Knipe and Howley 2013).

Orthobunyaviruses are a large number of arboviruses that use different arthropod vectors. For example, bunyaviruses under the California encephalitis serogroup cause pediatric encephalitis in North America and are mainly vectored by *Ochlerotatus triseriatus* mosquitoes. Members of the Simbu serogroup utilize either mosquitoes or biting midges as vectors, including Cache Valley virus transmitted by *Anopheles quadrimaculatus*, Oropouche virus transmitted by *Culicoides paraensis*, and Schmallerberg virus transmitted by *Cs. Obsoletus* (Blackmore et al. 1998, Mignotte et al. 2021, Files et al. 2022). Many orthobunyaviruses are known for causing diseases in humans and livestock, such as Batai virus (BATV), which is an emerging arbovirus initially detected in Malaysia. However, it has now spread to three continents (Asia, Europe, and Africa) due to transmission through a wide range of mosquito, mammalian and avian species. Although it is often associated with Crimean-Congo hemorrhagic fever, BATV is known for causing high fever and hemorrhages in the skin (Mansfield et al. 2022).

Members of the *Phlebovirus* genus are found worldwide except in Australia and are known for being transmitted by mosquitoes, ticks, and sandflies. Well-known phleboviruses include RVFV and Toscana virus (TOSV). Rift Valley fever virus is known to infect both humans and livestock through mosquito transmission. Large epizootic epidemics of RVFV have caused severe economic loss due to the high abortion rates amongst infected ruminants in Africa and some Asian and European countries. In humans, RVFV can cause miscarriages and other severe diseases, including encephalitis, ocular disease, and hemorrhagic disease (Wright et al. 2019). Sandflies

transmit TOSV and are commonly found in Mediterranean Europe and Africa. Toscana virus is the only sandfly-borne phlebovirus frequently associated with aseptic meningitis (Lambert and Hughes 2021). Nairoviruses are a group of tick-borne viruses, with its most essential viruses included in the Crimean-Congo hemorrhagic fever (CCHF) serocomplex. Crimean-Congo hemorrhagic fever virus is the most important virus in this group from a human perspective. Spreading across sub-Saharan Africa, Europe, and Asia, CCHFV is known for causing hemorrhagic fever in humans, which results in up to 30% mortality. After DENV, CCHFV is the most widespread arbovirus pathogenic to humans (Lasecka and Baron 2014).

The bunyavirus genome consists of three single-stranded negative-sense RNA segments. The single open reading frame in the large (L) segment encodes the RNA-dependent RNA polymerase. The medium (M) segment encodes the glycoproteins Gc and Gn. The small (S) segment encodes the nucleocapsid protein and the virulence factor NSs protein. It is important to note that orthobunyaviruses, phleboviruses, and nairoviruses vary in the coding strategies in the M and S segments. As a precursor to the structural glycoproteins, orthobunyaviruses, phleboviruses, and nairoviruses sometimes encode a non-structural protein of unknown function termed NSm, which is probably co-translationally processed. Most bunyaviruses have negative sense RNA genomes, but some genera, like *Phlebovirus*, have some ambisense RNA fragments. The bunyavirus genome encodes for non-structural proteins, including an RNA-dependent RNA polymerase, and several structural proteins, including nucleocapsid and glycoproteins Gn and Gc (Walter and Barr 2011, Hulswit et al. 2021). The bunyavirus virions are all approximately 80-120 nm in diameter. The surface of the virion is composed of a lipid bilayer decorated with Gn and Gc glycoproteins assembled in spikes to form an icosahedral structure possessing T=12 symmetry (Guo et al. 2011).

Cell entry of bunyaviruses requires the Gc glycoprotein to act as a class II fusion protein, resembling the membrane fusion process triggered by the flavivirus E protein and alphavirus E1 protein. The Gc protein is a class II fusion protein that undergoes a conformational change after exposure to the low pH environment of the early endosome. The low pH environment initiates viral membrane fusion with the cellular membrane in the late endosome, releasing the genomic material into the cell cytoplasm (Guardado-Calvo et al. 2016, Hulswit et al. 2021). The three segments of genomic RNA are transcribed after cap-snatching when a host mRNA cleaves through the endonuclease activity of the L protein to obtain 10-18 nucleotide-long primers for transcription. The regular transcription of negative-sense RNA leads to proteins for replication of the genome and the construction of new virions. Transcription of the ambisense RNA leads to inhibition of the host immune responses (Bouloy and Weber 2010, Barker et al. 2023). The envelope glycoprotein Gn-Gc is translated into the endoplasmic reticulum and attached to the membrane. In some bunyaviruses, Gn traffics to the Golgi apparatus on its own. At the same time, Gc is retained in the endoplasmic reticulum, but further progression through the Golgi apparatus requires both Gn and Gc proteins. After a congregation of enough Gn-Gc heterodimer, the genomic RNA is packaged in the virion. However, little is known about how this occurs and is transported in vesicles through the Golgi apparatus, where the virion changes from an immature state to a mature virion. Unlike most negative sense RNA viruses, bunyaviruses do not typically have a matrix protein, so it is proposed that the Gn-Gc heterodimer acts as a matrix surrogate. Once matured, the virion is exocytosed to the extracellular space (Bouloy and Weber 2010, Barker et al. 2023).

1.2 Medically important flaviviruses

The virus taxonomy released by ICTV in 2022 listed 53 species under the flavivirus genus (ICTV 2022). It is believed that flaviviruses evolved from precursor RNA virus(es) with no known

vectors, followed by the emergence of flaviviruses that exclusively infect bats and rodents, tick-borne flaviviruses, and mosquito-borne flaviviruses (Kuno et al. 1998). Two major clades of tick-borne flaviviruses exist, including the Tyulenyi virus serocomplex and TBEV serocomplex. Mosquito-borne flaviviruses include YFV, the prototype flavivirus, the DENV serocomplex, the JEV serocomplex, and insect-specific flaviviruses. Members of the TBEV serocomplex circulate between seabirds and the *Ixodes* species ticks and insect-specific flaviviruses are not known to cause disease in vertebrate hosts (Gaunt et al. 2001, Lindquist and Vapalahti 2008). In addition to YFV, the prototype flavivirus, members DENV serocomplex and the JEV serocomplex stand out due to their public health importance.

A. Epidemiology and ecology

Over 50% of flaviviruses identified to date are pathogenic in humans and/or animals, causing tens of thousands of deaths annually worldwide. Mosquito-borne flaviviruses account for most human infections by arboviruses, as observed by their presence in all six significant continents. Several tick-borne flaviviruses are considered emerging pathogens, including POWV in the United States (U.S.) (Gould and Solomon 2008, Vogels et al. 2023). Epidemiology and human disease caused by the respective flaviviruses are discussed as follows.

Flaviviruses transmitted by *Aedes* species mosquitoes emerged in evolution prior to the emergence of those transmitted by *Culex* species mosquitoes. Specifically, *Aedes* (*Ae.*) *aegypti* is highly important for transmitting flaviviruses, and is the principal vector for urban transmission of YFV, the prototype flavivirus (Figure 1-1) (Gaunt et al. 2001, Mukhopadhyay et al. 2005). The modern population of *Ae. aegypti* can trace back to the ancestral *Ae. formosus* in Africa. *Aedes formosus* feeds on non-human primates in the jungle, making it one of the species involved in the sylvatic transmission of YFV and other flaviviruses such as DENV and ZIKV. It is believed

that *Ae. aegypti* co-evolved with human civilization in Africa, leading to the anthropophilic feeding preference and making it one of the most competent vectors for the transmission of arboviruses between humans (Mattingly 1957). To date, three flaviviruses transmitted by mosquitoes have been known to cause large outbreaks in urban areas, including YFV, DENV, and ZIKV.



Figure 1-1: *Aedes aegypti* mosquito

Image used with permission from Dr. Stephen Higgs, Ph.D., F.R.E.S., F.A.S.T.M.H.

YFV serocomplex

Yellow fever virus is the first arbovirus discovered to be transmitted by mosquitoes, and it is considered a significant public health issue. In addition to viral hemorrhagic disease, the virus is known for causing jaundice in patients. It is estimated that there are 200,000 cases of the disease and 30,000 deaths yearly (CDC 2018, Gaythorpe et al. 2021). Although it is now regarded as a rare and exotic disease for the U.S., historically, YFV caused epidemics throughout the U.S., with its first appearance in Spanish Florida in 1649-1650. Geographically, YF disease was isolated to the southern U.S. and major port cities. The first significant outbreak of YFV occurred in Philadelphia in 1793, killing 9% of the city's population. However, the largest YFV outbreak

occurred from 1874 to 1878, resulting in 16,000 to 20,000 deaths spreading from Memphis, Tennessee, to cities along the Mississippi River. The last time a YFV epidemic was reported in the U.S. was during the 1905 epidemic in New Orleans, where 437 people died (Patterson 1992).

During the last 1500 years, circulating strains of YF arose in Africa, with the first epidemics witnessed in the 17th century (Tomori 2004). During the 17th century, YFV was introduced to the Americas, likely through the slave trade, and spreading rapidly due to the sub-tropical climate and the wide variety of susceptible mosquitoes in Central America. During the 19th and 20th centuries, international maritime trade commercial ships played a role in the spread of YF. It became widely feared when ships would arrive at port flying a yellow flag to announce the ship was under quarantine, giving YFV the name “yellow jack” (Douam and Ploss 2018, Tuells et al. 2022). During this time, YFV also caused epidemics in some European countries, including Italy, France, and Spain, with a Spanish YF epidemic in 1800 killing 60,000 people (Tomori 2004).

The first idea that mosquitoes could serve as agents for the transmission of YF was presented by Josiah Nott in 1848 (Tomori 2004). In 1900, army physician Walter Reed was chosen to head a team to research mosquito transmission in Havana, Cuba. By 1901 Reed had discovered that a filterable agent caused YFV and that *Ae. aegypti* mosquitoes could transmit the disease (Tuells et al. 2022). Although the mode of transmission was identified, the cause of YF was not identified until 1927 when members of the Rockefeller Foundation Yellow Fever Commission isolated the etiological agent of yellow fever by serial passaging in rhesus macaques the serum from the blood of a Ghanaian man named Asibi who had developed a mild case (Stokes et al. 2001). Two essential strains for vaccine production, the Asibi strain, and the French viscerotropic strain, were isolated in the 1920s. The Asibi strain later became the starting material in the serial passage experiments by Max Theiler to produce the attenuated 17D strain (Theiler and Smith

1937b). The French viscerotropic virus (FVV) became the parental strain for the empirically derived French neurotropic vaccine (FNV) (Peltier 1947).

During the 1940s and 1950s, YFV epidemics were reduced significantly due to the protection provided by the French West and Equatorial Africa vaccine campaigns with the FNV and YFV 17D vaccines (Peltier 1947). However, vaccine coverage was not universal and led to some dramatic outbreaks in the area, including Nigeria in 1950 and 1952, which caused 12,000 cases and 500-600 deaths. Other outbreaks were more substantial. For example, the Ethiopia outbreak in 1961 caused more than 100,000 cases and 30,000 deaths, with an estimated infection rate of 10% of the total population (Douam and Ploss 2018).

South America experienced recurrent epidemics with high case fatality rates of 40-60%. Due to the high death toll and abundant competent mosquitoes and non-human primate reservoirs, Brazil continued to make the YFV vaccine part of their standard vaccination procedure even when cases dropped significantly after the 1960s. However, the re-emergence of the virus began in the last two decades. In 2008-2009, outbreaks occurred in non-endemic regions with a 39-43% case fatality rate. Despite no major outbreaks from 2010-2015, the most significant epidemic of sylvatic YFV occurred between 2017 and 2018, resulting in 805 cases in 2017 and 1307 cases in 2018, leading to over 700 deaths in total (Douam and Ploss 2018, de Oliveira Figueiredo et al. 2020, WHO 2022c).

Overall in the 21st century, the geographic distribution of YF in the Americas and Africa has shown that between 2005 and 2022, areas at risk of YFV transmission in Africa have decreased while regions in South America have significantly expanded. Re-emergence of YFV in South America led to epidemics in Brazil, Paraguay, Argentina, and Venezuela, resulting in the resurgence of mass emergency vaccination campaigns. This led to a severe reduction in the YFV

vaccine stockpile typically used for travelers (Roukens and Visser 2008, Tuells et al. 2022). Although most of the increase in disease is located in South America, epidemics are still emerging in Central Africa with Angola, Nigeria, and the Democratic Republic of Congo. The Democratic Republic of Congo had the largest African outbreak in 2020, with 529 cases (Mokaya et al. 2021, WHO 2022c).

There are seven different genotypes of YFV, five in Africa and two in Latin America (Figure 1-2). They are defined by their pairwise nucleotide comparisons of greater than 9% for the prM and E protein gene sequence (Ellis and Barrett 2008). The most recent common ancestor of the YFV dates back to the year 1007 CE before eventually diverging in the 1400s into the first genotype we are familiar with today: the Angola genotype (Beck et al. 2013). As the first genotype to branch out from the most recent common ancestor, Angola genotype YFV is highly divergent, with 17-23% nucleotide variation from the other African genotypes (Mutebi and Barrett 2002). As the name suggests, the Angola genotype is primarily found in Angola and consists of the single strain Angola71 (Mutebi et al. 2001).

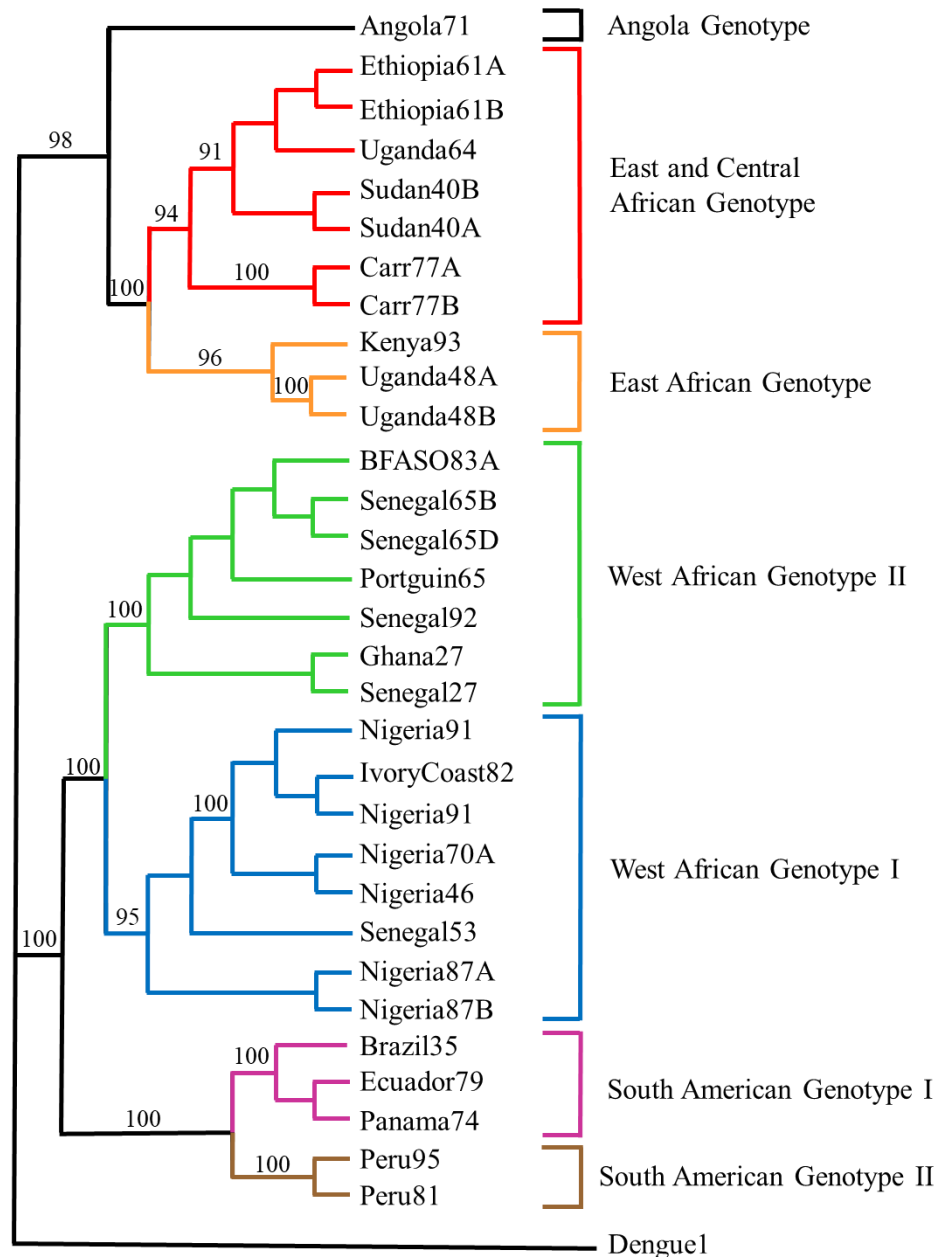


Figure 1-2: Yellow fever virus phylogenic tree

The image was modified in PowerPoint from an image published in *Microbes and Infection*, Volume 4, JP Mutebi and ADT Barrett, "The epidemiology of yellow fever in Africa." 1459-1468. (2002); with permission from Elsevier.

The West African strains diverged from the East African ones about three centuries ago, but the complete divergence between the West African genotypes 1 and 2 did not occur until the

mid-1800s (Mutebi et al. 2001, Beck et al. 2013, Chippaux and Chippaux 2018). West African genotypes 1 and 2 are commonly found in Nigeria and Ivory Coast, while genotype 2 is also in Senegal. Most of the YFV activity observed in the last 15 years resulted from the western lineages, resulting in 1315 cases and 297 deaths. Although the West African strains are known to cause many cases of YFV, they are also the source of the Asibi and FVV strains, which became the parent strains of two live-attenuated vaccines (LAVs) in the 1930s (Mutebi and Barrett 2002, von Lindern et al. 2006).

The Eastern genotypes diverged in the mid-1800s (Beck et al. 2013). There is an overlapping geographical distribution between the East African and the East/Central African genotypes, with East African strains being found in Kenya and Uganda and the East and Central African strains being found in the Democratic Republic of the Congo, Ethiopia, Central African Republic, and Uganda (von Lindern et al. 2006, Ellis and Barrett 2008). Due to their geography, the two genotypes are more closely related genetically than the West African ones (Mutebi et al. 2001).

The two Latin American genotypes are South American genotypes 1 and 2. South American genotype 1 is typically found in Brazil, Columbia, Panama, and Trinidad, while genotype 2 is predominantly in Peru (von Lindern et al. 2006). The American strains are phylogenetically closer to the West African strains than the East African strains, suggesting that the South American strains evolved from West Africa. However, the genome's 3' non-coding region indicates the virus may have originated in the jungle areas of East/Central Africa. The East/Central Africa genotype has only two repeat sequences (RYFs) in its 3' non-coding region (RYF1 and RYF3), with no evidence that RYF2 existed within the genotype. West African genotypes have all three RYFs, but the South American genotypes only have one: RYF3. This

genetic differentiation, in addition to phylogenetic analysis, suggests that the South American genotypes may have originated in East/Central Africa, extended their range into West Africa, and then were transported from West Africa to South America (Mutebi and Barrett 2002, Chippaux and Chippaux 2018).

Since its discovery in *Aedes* species mosquitoes, YFV has been discovered to be transmitted between non-human primates and humans through three transmission cycles: the sylvatic or jungle cycle, the savannah or rural cycle, and the urban cycle (Figure 1-3). The sylvatic cycle involves the transmission of YFV between non-human primates and wild-infected mosquitoes. In Africa, transmission is primarily through *Ae. africanus* mosquitoes and Old-World primates that typically show no sign of infection. In South America, transmission between primates occurs primarily between three species of mosquitoes: *Haemagogus leucocelaenus*, *H. janthinomys*, and *Sabethes chloropterus*. New World primates, such as howler monkeys, infected by these mosquitoes often succumb to infection and suffer from fatal diseases (Barrett and Higgs 2007, Gianhecchi et al. 2022). The sylvatic transmission cycle for Africa and South America always occurs in homogeneous rainforest environments (Douam and Ploss 2018).

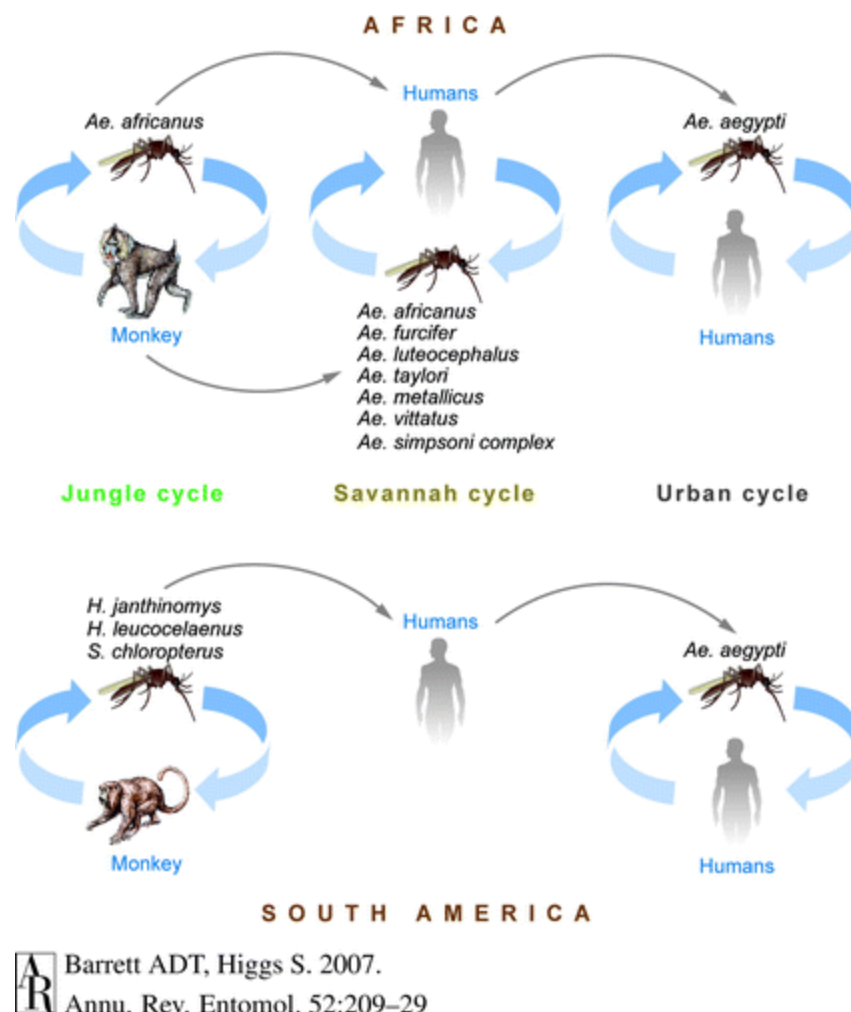


Figure 1-3: Transmission cycle of YFV in Africa and South America

Image taken from *Annual Reviews, Inc.*, from “Yellow fever: A disease that has yet to be conquered,” ADT Barrett and S Higgs, Volume 52 (2007); permission conveyed through Copyright Clearance Center, Inc.

YFV passes from non-human primates to humans through zoophilic mosquitoes as bridge vectors in the savannah or rural transmission cycle. This transmission type usually occurs in a region known as the zone of emergence on the fringe of the forest where the sylvatic transmission cycle occurs, and human dwellings leads to spillover events (Gabiane et al. 2022). In this cycle, many vectors are responsible for the transmission from non-human primates through opportunistic

encounters with humans: *Ae. luteocephalus*, *Ae. furcifer*, *Ae. metallicus*, *Ae. opok*, *Ae. taylori*, *Ae. vittatus*, and members of *Ae. simpsoni* (Barrett and Higgs 2007). As the name suggests, the savannah cycle occurs in the forest-savannah ecotone or moist savannah of Africa, where humans and *Aedes* mosquitoes co-exist at a higher rate (Douam and Ploss 2018). The savannah transmission cycle only occurs in Africa, not South America, so epidemics in Africa are usually of higher intensity than in South America. Therefore, the sylvatic transmission cycle accounts for most cases in South America (Hamlet et al. 2021).

The urban or domestic cycle involves the transmission of YFV between humans and *Ae. aegypti* mosquitoes. This form of transmission relies on relatively high-titer viremia being present and the ability of the virus to infect and disseminate in *Ae. aegypti*, so it can be transmitted through saliva (Barrett and Higgs 2007). Transmission occurs in Africa in the dry savannah regions and urban areas in South America (Douam and Ploss 2018).

Yellow fever is the prototypic virus for viral hemorrhagic fever, which is known for causing up to 20% to 50% lethality if the patients are not adequately treated. The YF disease expresses itself in three stages: infection, remission, and intoxication. Severe disease is primarily documented as lesions in the liver and other viscerotropic symptoms, but most notably jaundice (Monath 2008, WHO 2022c). Yellow fever virus is introduced subcutaneously into the primate host by injecting saliva from an infected mosquito. During symptomatic infection of wild-type YFV strains, the infection incubation involves the development of viremia 3 to 6 days after exposure, followed by an abrupt onset of symptoms at the peak of viremia levels (Waggoner et al. 2018). In mild cases, signs of infection are typically nonspecific, manifesting as fever and headache. Patients usually recover in a few days with no lasting symptoms. In more severe cases, the symptoms resemble other viral hemorrhagic fevers and viral diseases, including fever of 39°C,

chills, nausea, vomiting, myalgia, and malaise, often manifesting in Faget's sign (increasing temperature with decreasing pulse rate) (Gardner and Ryman 2010). The infection stage is usually followed by a period of remission where symptoms abate for 2 to 24 hours. During this period virus is cleared from the blood, and IgM antibodies appear in the serum. At this point, many YF infections will resolve without other symptoms, leading to the patient's recovery (Monath 2001, Waggoner et al. 2018).

In approximately 20% of symptomatic infections, the illness will reappear after remission and enter the period of intoxication. For 3 to 8 days, a more severe form of infection presents symptoms, including high fever, vomiting, epigastric pain, prostration, and dehydration. Some of the most severe disease manifestations include severe hemorrhagic manifestations such as jaundice, major bleeding, hematemesis or "black vomit," melaena, or metrorrhagia. Damage is also frequently noted within the kidneys, leading to acute renal failure (Gardner and Ryman 2010). After 3 to 8 days, the patient can begin to recover and enter convalescence for 2 to 4 weeks, characterized by prolonged weakness and fatigue (Monath 2001).

However, late central nervous system (CNS) manifestations occur, such as confusion, seizure, and coma. The patient's death likely occurs within 7 to 10 days of onset (Gardner and Ryman 2010). The mortality of patients with hepatorenal diseases can range from 20% to 50%, and deaths in the recovery phase have also been described in the past (Lefevre et al. 2004). During intoxication, the cell-mediated immune response kicks in, starting with the seroconversion of IgG-neutralizing antibodies and triggering various cytokine responses. The presence of elevated levels of serum alanine transaminase (ALT) and aspartate transaminase (AST) are the most common characteristics of liver damage and apoptosis caused by YF (Monath 2001, Waggoner et al. 2018). Although the hematological characteristics vary among patients, thrombocytopenia, prolonged

clotting times, and platelet dysfunction have also been reported. In addition, neutropenia and renal damage may lead to superimposed bacterial infections and pneumonia or require dialysis due to the necrosis in the renal tissues (Monath 2001, Lefevre et al. 2004).

Controlling the spread of YFV has been most successful by using two live attenuated vaccines (LAVs) introduced in the 1930s: the YFV 17D vaccine and FNV. Of the two vaccines, the most successful was the 17D vaccine, which was found to reduce the virulent phenotype of the wild-type YFV. The YFV 17D strain was derived from the YFV Asibi strain in 1937 by passaging the virulent virus in mouse brain and chicken tissues (Theiler and Smith 1937a). The 17D vaccine significantly decreased the disease burden and is still used today (Figure 1-4). The 17D vaccine will be discussed more in-depth in section 1.3.A.

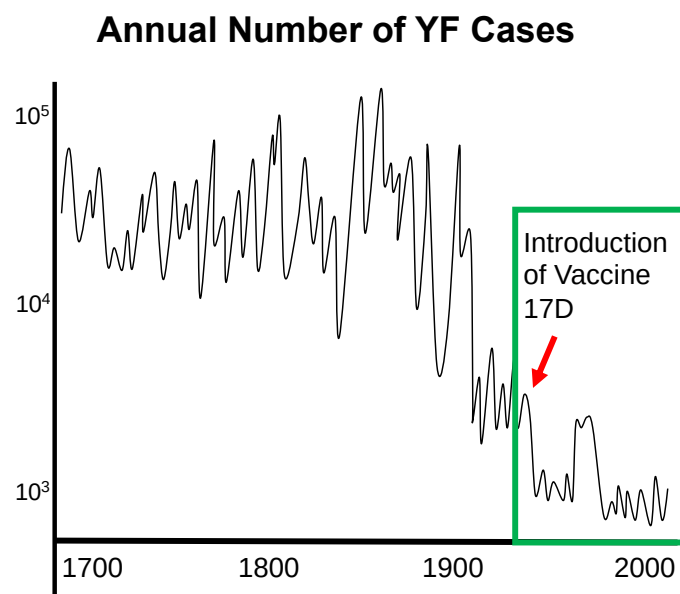


Figure 1-4: Annual number of YF cases before and after the introduction of the 17D vaccine

Image used with permission from Dr. Thomas Monath. The image is from his 2008 speech for the American Committee for Arthropod-Borne Viruses at the American Society of Tropical Medicine and Hygiene Annual Meeting.

The development of FNV took place concurrently with the YFV 17D LAV. The parental wild-type FVV was isolated from the same outbreak with the prototype Asibi strain in 1927 and was serially passaged 128 times in mouse brains. Like the 17D strain, FNV does not exhibit viscerotropism in humans or nonhuman primates and has lost the ability to be transmitted by mosquitoes (Beck and Barrett 2015). Despite the nearly identical genomes of the Asibi strain and FVV, FNV has a very different set of attenuating mutations than the 17D vaccine (Beck and Barrett 2015). The FNV strains from various collections differ from FVV with a total of 60-122 nucleotide changes, 19 of which resulted in common amino acid substitutions. Two consensus mutations are shared between the FNV and 17D vaccines, including the M-L36F and NS4B-I94M mutations (Wang et al. 1995, Beck et al. 2014). Interestingly, both the M-L36F and NS4B-I94M mutations contributed to the loss of the 17D strain to be transmitted via mosquitoes (McElroy et al. 2006b). It is likely that the same mechanism also contributes to the attenuated phenotype of FNV.

The FNV was delivered via scarification, allowing large immunization campaigns to be administered with the smallpox vaccine (Barrett 1997, Frierson 2010). While the FNV vaccine is highly immunogenic, the serial passage in mouse brains led to the neuroinvasive property of FNV. More than 80 million doses of FNV were administered to residents in French-speaking African countries. The incidence of FNV-associated encephalitis was estimated to be 3-4 per 1,000 vaccines (MacNamara 1953). The mortality rate in children under 14 could be up to 40% (Fox 1956, Barrett 1997, Frierson 2010). Such a risk was deemed unacceptable and led to the discontinuation of FNV in 1982.

Although the introduction of YFV LAVs has reduced the spread of the virus after the 1930s, a reemergence of the YFV began in the last two decades and has become a public health problem. In 2008-2009, outbreaks occurred in non-endemic regions with a 39-43% case fatality

rate. Despite no major outbreaks from 2010-2015, the most significant epidemic of sylvatic YFV occurred between 2017 to 2018, resulting in 805 cases in 2017 and 1307 cases in 2018, leading to over 700 deaths in total (Douam and Ploss 2018, de Oliveira Figueiredo et al. 2020, WHO 2022c). To combat the reemergence, the WHO implemented the Eliminate Yellow Fever Epidemics Strategy starting in 2017. The program has three main plans to reduce outbreaks. The first stage to contain the spread of the virus and protect the population was completed in 2020 through the initiation of mass vaccination and implementation of outbreak emergency plans. The second phase is ongoing until 2024, which is to build resistance and readiness through ongoing routine vaccination and increasing diagnostic and surveillance capabilities. The final and longest stage started in 2018 and is planned to be completed in 2026. This step eliminates the risk of new YFV epidemics by building up the health systems capacity, urban resilience, ongoing surveillance, and improved disease control practices (WHO 2022b).

DENV serocomplex

Another major flavivirus that is a public health concern is DENV, of which there are four serotypes (DENV-1, DENV-2, DENV-3, and DENV-4). About half of the world's population is at risk of DENV infection. It is estimated that 100-400 million people are at risk of dengue infection yearly in over 100 countries across Africa, the Americas, the Eastern Mediterranean, South and East Asia, and the Western Pacific (WHO 2022a). Isolation of sylvatic DENV strains from *Aedes* mosquitoes and nonhuman primates suggests that DENV emerged from the jungle population. Since the end of World War II, the incidence of DENV infection has continued to increase, and viremic humans are now the host species responsible for the widespread transmission of DENV by *Ae. aegypti* and *Ae. albopictus* (Figure 1-5) (Holmes and Twiddy 2003, Guzman et al. 2015).

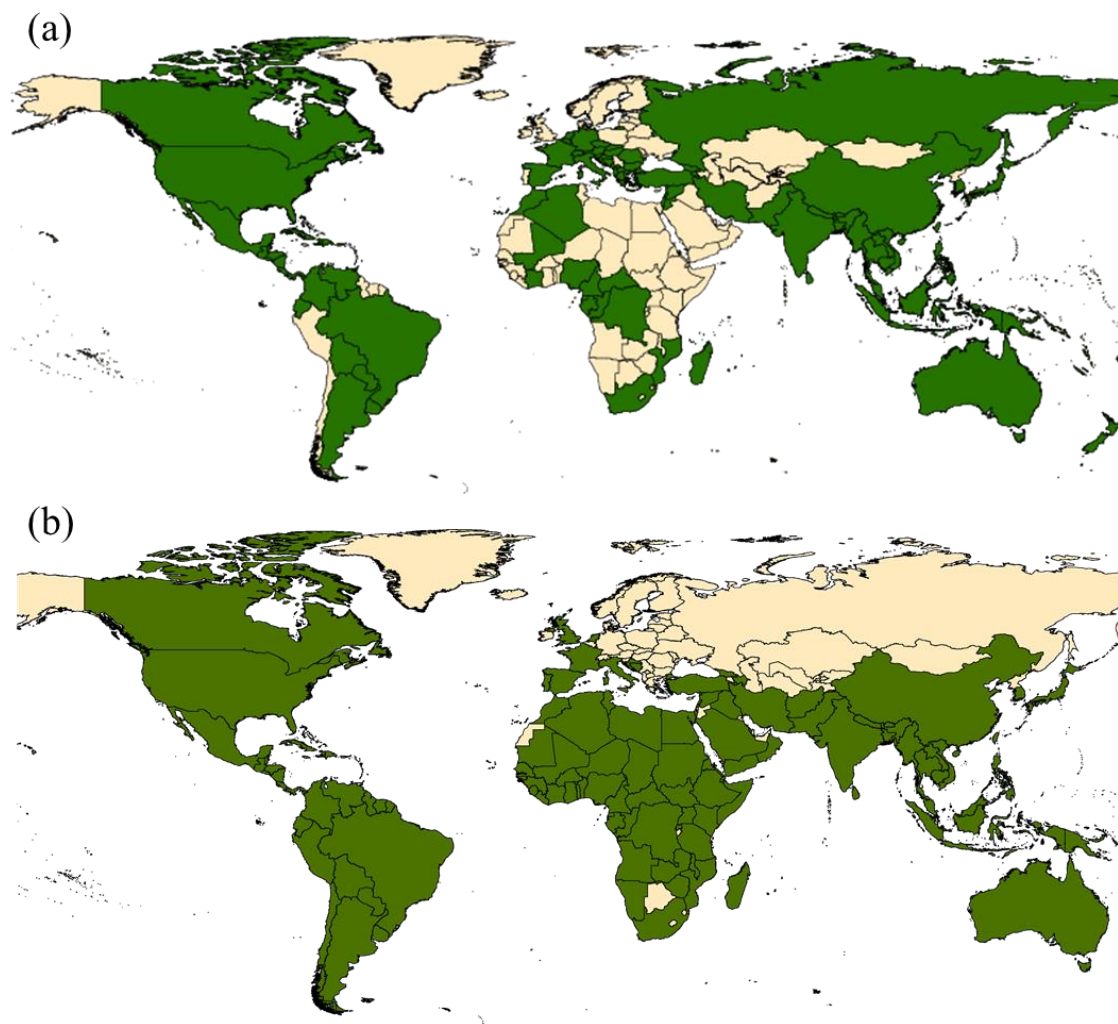


Figure 1-5: Worldwide distribution of *Aedes* species mosquitoes

(a) The geographical distribution of *Ae. albopictus*. (b) The geographical distribution of *Ae. aegypti*. Green represents the regions where the *Aedes* mosquitoes are found. Image taken from the Walter Reed Biosystematics Unit (WRBU 2021a, WRBU 2021b).

Human DENV infection can lead to dengue fever and severe dengue, formerly known as dengue hemorrhagic fever and dengue shock syndrome. The mechanism leading to severe dengue remains poorly understood. Neither the antibody-dependent enhancement nor the virulence hypothesis can fully explain the pathogenic mechanism of severe dengue. Antibody-dependent enhancement hypothesis suggests that the antibodies produced during an initial DENV infection

are cross-reactive and bind to another DENV serotype from a secondary infection. However, they lack neutralizing activity and are unable to prevent infection. Instead, these antibody-virus complexes are internalized into the cells and elicit infection in more target cells, potentially leading to higher viral production and more severe disease (Guzman and Vazquez 2010). Alternatively, the virulence hypothesis suggests that severe dengue may result from a secondary DENV infection caused by a more virulent strain or serotype than the original DENV infection (Prommalikit and Thisyakorn 2015). To control the spread of DENV, developing dengue vaccines has been a global health priority, especially ones targeting multiple DENV serotypes. Two licensed DENV vaccines, including DENGvAXIA and QDenga, will be discussed more in-depth in section 1.3.

JEV serocomplex

The epidemiology of flaviviruses transmitted by *Culex* species mosquitoes differs significantly from those transmitted by *Aedes* mosquitoes. Humans do not develop high levels of viremia or support transmission and are among the incidental hosts that can succumb to encephalitis or neurological disease (Chen and Wilson 2005, Higgs et al. 2005). Flaviviruses transmitted by *Culex* species mosquitoes belong to the JEV serocomplex and represent a group of important zoonotic pathogens, including JEV, WNV, and SLEV. All three encephalitic flaviviruses use avian species as amplifying hosts and can cause equine encephalitis, making them important veterinary pathogens.

Japanese encephalitis virus is the prototype member of the JEV serocomplex and is the leading cause of pediatric encephalitis in the Asian Pacific region. Although there are multiple licensed JE vaccines, e.g., SA14-14-2 LAV, IMOJEV chimeric LAV, and formalin-inactivated vaccines, it is estimated that there are 68,000 clinical cases of human JE and 13,600-20,400 death every year (WHO 2019). Most JEV isolates have been derived from mosquitoes, avian species,

domestic pigs, and humans, reflecting that JEV is maintained in the enzootic egret/heron-*Culex* (*Cx.*) *tritaeniorhynchus* mosquito cycle. There is a high degree of overlap in the locations of JEV isolates from domestic pigs and humans, suggesting that domestic pigs are sources of viruses for the epizootic spillover of JEV to humans. Phylogenetic analyses showed that JEV potentially emerged from the jungle of Southeast Asia and has evolved into five different genotypes (GI, GII, GIII, GIV, and GV). The first JEV isolate was the Nakayama strain derived from a pediatric case during the 1935 outbreak of summer encephalitis in Japan (Solomon et al. 2003). Most JEV strains isolated before the 1970s belong to GIII, followed by the switch of the predominantly circulating genotype to the clade b of GI since the 1980s.

The emergence of GIV and GV has recently been reported in Australia and Northeast Asia. The outbreak in Australia in 2022 was the first time that GIV JEV strains were detected outside Southeast Asia and caused human disease (van den Hurk et al. 2022). The GV JEV has begun to emerge in South Korea since it was first isolated from human patients in 2015 (Woo et al. 2020). JEV remains an important zoonotic flavivirus as genotypes emerge in new regions where past JEV immunity may no longer be protective. Although prominently in Eastern and Southern Asia, JEV spreads throughout Indonesia and into Australia. *Culex tritaeniorhynchus* is the primary vector for transmission, although other *Culex* species mosquitoes can also transmit the virus. JEV can be maintained through avian species such as the Ardeid water birds like the black-crowned night heron (*Nycticorax nycticorax*). However, JEV has a unique ecology and requires swine species as an amplifying host. Humans and horses are considered incidental or dead-end hosts (Figure 1-6) (Mackenzie et al. 2022). There are three available vaccines: the live-attenuated 14-14-2 vaccine, the formalin-inactivated vaccine IC51 and the ChimeriVax-JE vaccine (van den Hurk et al. 2009, Satchidanandam 2020).

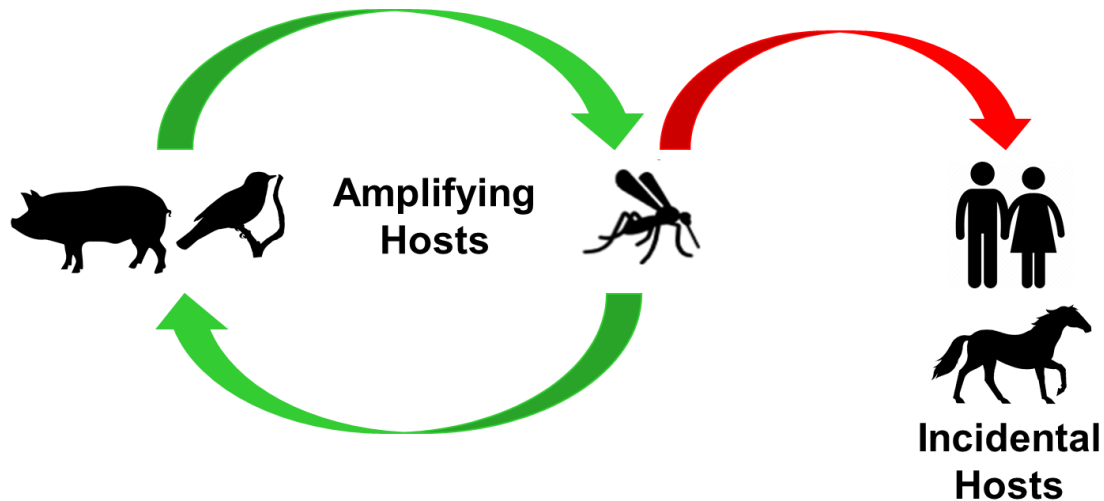


Figure 1-6: Transmission cycle of flaviviruses in the JEV-serocomplex

Image was generated in PowerPoint.

West Nile virus has existed in nature for less than 2000 years and originated from African lineages. However, it was not isolated until 1937 from a native woman who lived in the West Nile district of Uganda suffering from a febrile illness (Smithburn et al. 1940, Zanotto et al. 1996). Although the virus was isolated in 1937, significant clinical disease was recognized in the 1950s when epidemics began in Israel from 1951 to 1952 and Egypt from 1951 to 1954. The start of sporadic outbreaks of febrile illness began to increase from the 1950s to the 1980s. The virus quickly spread across the continents into Africa, Australia, Europe, and the Middle East (Sejvar 2003). Israel had more outbreaks in 1957 and 1962, but the 1957 outbreak was unique. The outbreak occurred among a group of elderly residents in a nursing home. They developed severe neurological disease, which became the first report of such clinical manifestations (Hayes and Monath 1989). Before this outbreak, very few cases of neurological illness were ever reported, and only among patients undergoing experimental infections. Additional outbreaks occurred in France in 1962-1965 and South Africa in 1974 and 1983-1984. Patients in both countries during the initial

outbreaks developed meningitis or encephalitis, although the majority of cases were mild, self-limited febrile diseases (Sejvar 2003).

Severe incidents of WNV began to develop in the 1990s, when cases of human disease gained frequency, with significant outbreaks across Africa, Europe, and the Middle East. One of the most notable outbreaks of this period occurred in Bucharest, Romania. The Romanian outbreak was the first WNV outbreak in a predominantly urban area, and it was the first outbreak where many cases involved CNS infection. Studies showed that the city was highly susceptible to WNV infection due to the population being serologically naïve to WNV and the abundance of domestic fowl close to the population (Campbell et al. 2001). Following the outbreak in Romania, several epidemics showed relatively high rates of CNS infection, including Morocco in 1996, Tunisia in 1997, and Italy and Israel in 1998. The Tunisia outbreak had 173 patients hospitalized with meningitis or meningoencephalitis, with 8 of those cases resulting in death. Other significant outbreaks during this period included Algeria in 1994 and 1997, Russia in 1999, and France in 2000 (Sejvar 2003, Mann et al. 2013, Kleinschmidt-DeMasters and Beckham 2015).

In 1999, WNV emerged in New York and quickly became one of North America's most significant arboviruses for human and veterinary public health. A cluster of severe encephalitis cases was noted in August 1999 in the Queens area of New York City. Initially, health officials could only tell that it was an encephalitic disease accompanying muscle weakness and axonal neuropathy. It was initially believed to be SLEV as WNV had not yet been seen in North America (Jordan et al. 2009). Before and during the investigation into the human cases, a high rate of bird fatalities was noted in and around New York City. Four weeks after the first human case was reported, genomic sequencing of specimens from New York City birds, infected mosquitoes

collected in Connecticut, and human brain tissue from a fatal case of encephalitis led to the identification of WNV as the etiologic agent of this outbreak (Sejvar 2003, Roehrig 2013).

By the end of the summer of 1999, there were 62 confirmed cases of acute WNV infection, with 59 of those cases resulting in hospitalization. Similar to other outbreaks in the 1990s, the New York City outbreak was associated with high levels of neurological disease (Sejvar 2003). Although the mechanism of how WNV was introduced into New York City is unknown, the source of the virus originated from the Middle East. There was a simultaneous outbreak of WNV during this time in Israel, and genomic sequencing of the two viruses resulted in a very high amount of genomic homology (Giladi et al. 2001). Introduction into the U.S. led to the rapid spread through the country. By the following summer in 2000, 21 cases of WNV were identified in 10 counties in the northeastern states. By the summer of 2001, 66 cases were identified in 38 counties in 10 states.

In 2002, the number of cases of WNV in North America became unprecedented in the largest West Nile meningoencephalitis outbreak ever recorded and the largest outbreak of arboviral meningoencephalitis ever in the western hemisphere. The outbreak spread from the Mississippi River to the Pacific Coast in one year. As of January 2003, there had been 4156 cases of WNV, with 2354 cases of meningoencephalitis and 284 deaths (CDC 2002). The number of WNV cases peaked in the U.S. in 2003 with 9862 total cases and 264 deaths. Since then, there have been several hundred to a few thousand cases of WNV every year, with most of the cases now residing in the Midwest and along the Rocky Mountains (CDC 2023c). Over the last quarter of a century, WNV has caused over 51,000 human clinical cases and over 2,300 deaths since its introduction, displaying this pathogen's immense public health importance (Ronca et al. 2021).

There are five different lineages of WNV, although lineage 1 and lineage 2 encompass the majority of the WNV strains (Figure 1-7). Each of the five lineages can have as much as 20-25%

genomic variation between them, often associated with their geographical point of isolation (Bialosuknia et al. 2022). Lineages 1, 2, and 5 are the most important public health concerns because they have been associated with outbreaks in humans (Chancey et al. 2015).

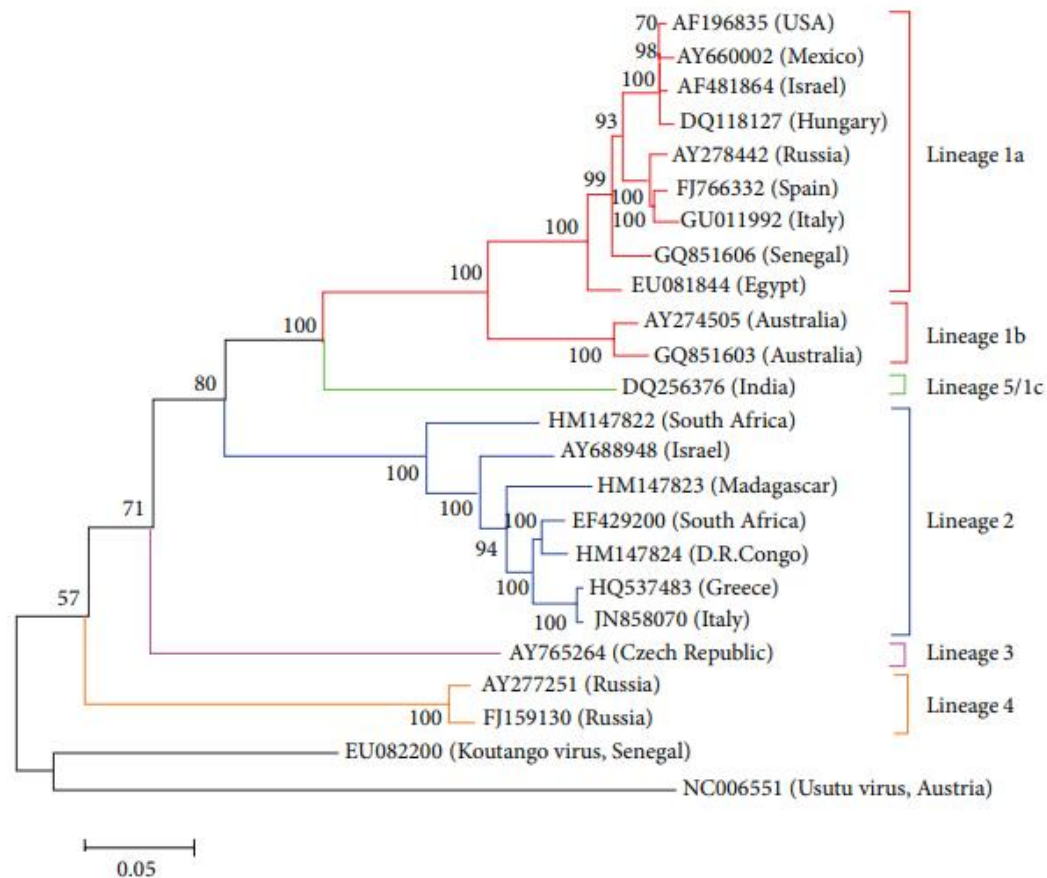


Figure 1-7: West Nile virus phylogenetic tree

Image taken from Chancey et al., 2015; published under the Creative Commons Attribution License.

Lineage 1 is the WNV lineage that is most widely distributed worldwide, and it can be further categorized into two clades. Clade 1a encompasses isolates from Africa, Europe, the Middle East, Asia, and the Americas. Phylogenetic analysis indicates that this clade likely originated from sub-Saharan or Northern Africa. Clade 1a spread northwards in the 1970s and 1980s using the eastern bird migration route that connects Northern Africa to countries in the Middle East and

Central Europe, then further into Western Europe and other African countries like Morocco in the 1990s. Eventually, in 1999, the WNV lineage 1a was transported, most likely from the Middle East to the Americas, where it quickly spread across North and South America (Zehender et al. 2011, Chancey et al. 2015). Clade 1b includes the Australian Kunjin virus (KNV), which was isolated from a local aboriginal tribe of Kowanyama in North Queensland, Australia. Despite KNV being known since the 1960s, it was only classified as a WNV strain in the early 2000s (Hall et al. 2006, Chancey et al. 2015). There was previously lineage 1c, but this has been reidentified as Lineage 5 and consists primarily of isolates from India, dating from 1955 to the present. Of the 13 Indian isolates, only two do not belong to lineage 5 (Bondre et al. 2007, Chowdhury et al. 2014).

Lineage 2 viruses are historically endemic to sub-Saharan Africa and Madagascar. However, it is associated with the more recent avian and human outbreaks in southern and eastern Europe on at least two separate occasions (Burt et al. 2002, Chancey et al. 2015). Lineage 3 includes only two isolates from mosquitoes on the Czech Republic border near Rabensburg, Austria, in 1997 and 1999. This strain of WNV is known as Rabensburg virus. The isolates share 89-90% nucleotide homology with members of the WNV lineages 1 and 2 but are most similar antigenically to the Egyptian Eg101 strain from lineage 1. There is no direct link between RABV and human infections (Aliota et al. 2012). Lineage 4 contains viruses circulating Russia since 1988; these include a tick isolate from the southwest Caucasus and isolates collected from mosquitoes and reptiles near the Volga River. This lineage is another lineage that has never been associated with human disease (Chancey et al. 2015).

West Nile virus has caused the most widespread arthropod-borne viral disease in the world as well as the largest outbreaks of neuroinvasive disease ever seen in the U.S. The overall transmission is partially caused by its transmission cycle, although this occurs most frequently in

warmer months from July to October (Soverow et al. 2009). West Nile virus replicates within an enzootic transmission cycle that is primarily maintained between *Culex* species mosquitoes and various avian species which are its reservoir host (Figure 1-8). Almost 300 avian species are susceptible to WNV. However, in November 1997, a highly virulent strain of WNV became prevalent in Israel, resulting in a significant number of bird deaths from encephalitis and paralysis, linking WNV to avian mortality and morbidity (Banet-Noach et al. 2003, Sejvar 2003). In some avian species, WNV can cause neurological disease, particularly in crows, ravens, and magpies, while other avian species develop various levels of morbidity and mortality. The high mortality observed in wild birds in the U.S. is unique to North America. However, the mortality of birds was noted in both the Israeli and Egyptian outbreaks (Ciota and Kramer 2013). Overall, it is thought that migratory birds are the principal agents for spreading WNV across North America and the rest of the globe. During the largest outbreak in the U.S. in 2002-2003, WNV was detected in 19 species of migrant birds (Dusek et al. 2009).

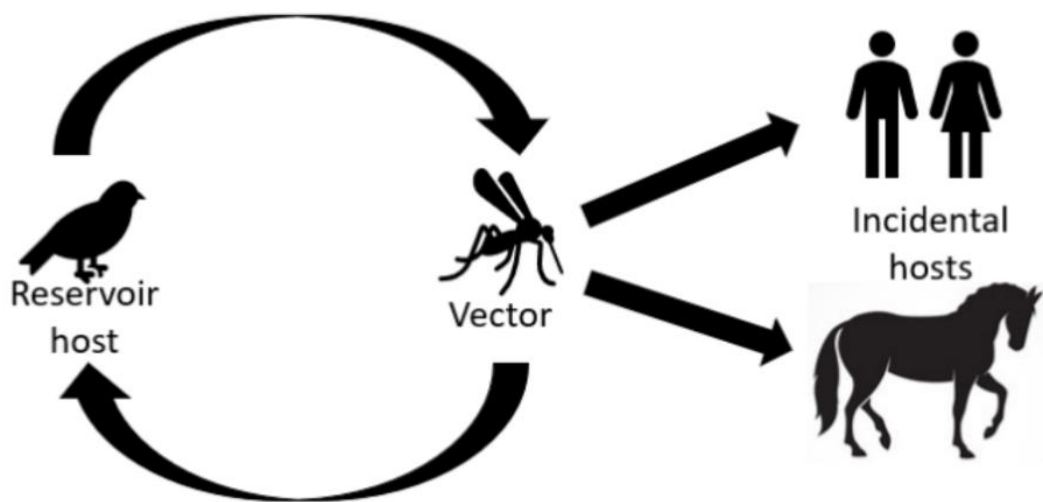


Figure 1-8: Transmission cycle of WNV

Image used with permission from Ashley Bilyeu from her 2023 Master's thesis (Bilyeu 2023).

Transmission between avian species occurs due to mosquitoes and is another significant cause of the spread of WNV. The spread of WNV by competent mosquitoes begins when a mosquito feeds on an infected bird or another competent host. The virus will then disseminate within the mosquito for several days from the midgut to the mosquito's salivary glands. Transmission of WNV occurs when a mosquito with a disseminated virus bites a human or other susceptible vertebrate host (Hayes et al. 2005). A total of almost 60 species of mosquitoes in North America alone can transmit WNV. It is suspected that *Cx. pipiens* species like *Cx. tarsalis* and *Cx. restuans* may be responsible for up to 80% of WNV infections in the U.S. Still, worldwide the spread of WNV is primarily *Cx. pipiens*, *Cx. quinquefasciatus*, *Cx. australicus*, and *Cx. globocoxitus* (Ciota and Kramer 2013).

Although the primary transmission cycle is between mosquitoes and birds, mosquitoes also transmit WNV to other mammals. This transmission often happens with horses and humans, but neither develop high enough viremia to allow transmission, so they are dead-end hosts due to the epizootic spillover. A few mammals not seen in the primary transmission cycle have been shown to have viremia levels adequate for infecting mosquitoes in laboratory settings, namely small mammals such as tree squirrels, eastern gray squirrels, eastern chipmunks, and Eastern cottontail rabbits. It remains to be seen if these are genuinely natural, competent hosts (Ciota and Kramer 2013). This transmission cycle is widely accepted, but it is important to note that a tiny portion of human infections have occurred through other means of transmission. Some transmission has occurred through organ transplants, blood transfusions, breast milk, and even one reported case of transplacental transmission (Sejvar 2003). Due to the possibility of alternative transmission, WNV and ZIKV are the only flaviviruses tested for during blood transfusions in the U.S. (Araujo et al. 2020). There has been no evidence of human-to-human transmission (WHO 2017).

With WNV being an emerging flavivirus, there are increased clinical cases. A significant increase in WNV cases in European countries occurred in 2018, with over 1,500 human cases compared to the previous year, which saw over 200 human cases (European Centre for Disease Prevention and Control 2017, Rizzo et al. 2020). One of the most critical virulence factors is the continuous evolution of WNV, which leads to the emergence of new genetic lineages that are transmitted more efficiently in nature. The emergence of new viral strains is due to the function of the RNA-dependent RNA polymerases, which are prone to error. A single amino acid substitution can increase virulence in specific hosts. For example, the amino acid substitution of T249P in the NS3 helicase increased virulence in the American crow. It replaced the V159A in the E protein in the NA/WN02 strain, shortened extrinsic incubation in *Culex* mosquitoes, and led to a higher prevalence of the virus among mosquitoes (Yu and Cheng 2022). Another strain, NY10, which originated in the U.S. in 2010, saw amino acid substitutions of R1331K and I2513M, which led to increased cases of human infection and a higher prevalence of the virus seen in mosquitoes (Bialosuknia et al. 2022).

It is thought that although WNV can cause serious illness, around 80% of infections end up being asymptomatic, with 20% resulting in West Nile fever or severe West Nile disease and 5% of those clinical cases resulting in death (WHO 2017, CDC 2023a). West Nile virus can cause significant human disease and considerable economic loss through epizootic outbreaks. Before the severe outbreaks in the 1990s, West Nile fever was often called the “summer flu” and was considered primarily a febrile illness (Bai et al. 2019). When WNV does lead to symptomatic West Nile fever, the symptoms can include fever, fatigue, head and body aches, nausea and vomiting, swollen lymph nodes, and occasionally rash that will develop 3 to 14 days after exposure. A rash is observed in about 50% of infected individuals but occurs most often in children (Gould and

Solomon 2008, Guarner and Hale 2019). The development of viremia after infection can first be detected very early, 1 to 2 days after exposure, but the viremia rarely lasts for more than two weeks (Busch et al. 2008, Zhang et al. 2009). By the time the onset of the symptomatic disease occurs, WNV is rarely detected due to the low levels of viremia or absence due to symptomatic infections often occurring after the resolution of viremia (Lustig et al. 2016).

Roughly 1 in every 150 cases will further develop neuroinvasive disease; however, since the outbreaks in 2002 in the U.S., the occurrences of neuroinvasive disease have risen to almost 50% of all reported cases. Although neuroinvasive disease can occur in anyone, young children and elderly patients over 50 are more at risk for developing severe disease and mortality. Additionally, people who are insulin-dependent diabetics, have hypertension, or are immunocompromised have a higher chance of developing the severe disease (Guarner and Hale 2019). Severe disease symptoms can be divided into major and sometimes overlapping categories. The first is meningitis which usually is accompanied by headaches and neck stiffness. The second is encephalitis which includes disorientation, stupor, coma, and convulsions. The final and most severe group is acute flaccid paralysis, including poliomyelitis with frequent tremors, myoclonus, parkinsonism, ataxia and limb atrophy, or Guillain-Barre-like syndrome (Guarner and Hale 2019, Rizzo et al. 2020). Patients experiencing neurological symptoms have a fatality rate of 5-10%. Pathology observed in fatal cases of WNV infections is primarily localized within the central nervous system (Gould and Solomon 2008, Bai et al. 2019).

Severe WNV infection of the CNS can cause severe damage that can lead to dysfunction of the neural circuits. When the dysfunction continues past the resolution of the acute phase of infection, the neurological deficiencies are termed “sequelae,” and they can have long-term effects on the patient’s health. Generally, long-term sequelae are present in 30-60% of survivors. It

includes deficiencies such as paralysis, memory loss, depression, fatigue, dementia, cognitive and motor impairment, and epilepsy that can last for months or the rest of a patient's life (Patel et al. 2015, Fulton et al. 2020).

The ability of WNV to infect a broad range of avian and mosquito species contributes to its rapid dispersal. For example, common house mosquitoes, i.e., *Cx. pipiens* and *Cx. pipiens quinquefasciatus* are competent vectors for WNV in both the Old and New Worlds. Additionally, *Cx. tarsalis* transmitting zoonotic arboviruses in the Western U.S. can also transmit WNV (Philip and Smadel 1943, Hurlbut et al. 1956). Similarly, WNV has been detected in more than 326 species of birds (Ciota and Kramer 2013, Fiacre et al. 2020). It is anticipated that WNV will continue to cause human and equine encephalitis worldwide.

St. Louis encephalitis virus was the most important mosquito-borne flavivirus in Canada and the U.S. before the emergence of WNV in New York in 1999. The largest epidemic occurred during its original discovery in 1933 in St. Louis, Missouri, where 1,095 cases were reported, with an almost 20% mortality rate. Since then, outbreaks have occurred across the U.S., but none have been as significant as the 1933 outbreak. In the last 20 years, only sporadic cases have been reported, with the largest being 49 cases in 2003 (CDC 2023b). SLEV is maintained in the bird populations in urban areas, such as the house sparrow, pigeon, blue jay, and robin, but transmission to humans occurs through mosquito vectors. *Culex tarsalis* is believed to be the primary vector for SLEV in the western U.S., *Cx. pipiens* and *Cx. quinquefasciatus* in the eastern U.S., and *Cx. nigripalpus* in Florida. Humans do not produce viremia titers high enough for mosquito transmission, but there have been rare cases of the infection being transmitted through blood transfusion (Diaz et al. 2018, CDC 2021c).

After the introduction of WNV to the U.S., there has been debate over the continued transmission of SLEV, given the serologic cross-reactivity of the two viruses in avian hosts, including the house finch. Studies have demonstrated that SLEV neutralizing immunity in finches prevented SLEV reinfection but only reduced WNV viremia titers. Conversely, WNV prevented reinfection of both WNV and SLEV, which suggests that WNV could competitively exclude SLEV from amplification in shared avian species, resulting in reduced transmission of SLEV. Considerable host overlap between SLEV and WNV would be required for competitive exclusion, but it may result in fewer cases in select regions (Fang and Reisen 2006, Diaz et al. 2018).

TBEV serocomplex

Flaviviruses within the TBEV complex are vectored by ixodid (hard) ticks through two modes of transmission, i.e., transstadial and nonviremic (direct) transmission. Unlike mosquitoes that acquire blood meals to complete a gonotrophic cycle at the adult stage, blood feeding occurs at every stage of the tick lifecycle. Unless the feeding is interrupted, a tick normally engorges once from a single host at each stage. An engorged tick must digest the blood meal to complete the molting process and develop into the next life stage, allowing for simultaneous virus transmission to the next vertebrate host. Therefore, tick-borne flaviviruses can be maintained by transstadial transmission. Alternatively, tick-borne flaviviruses can be transmitted by nonviremic or direct transmission due to the prolonged feeding period of ticks. An infected tick can inject many infectious viruses at the feeding site during the multi-day engorgement process. Susceptible ticks can become infected while co-feeding with an infected tick, followed by transmission of tick-borne flaviviruses to the next susceptible vertebrate host (Randolph et al. 1996, Randolph 2011). Tick-borne flaviviruses can infect many tissues, including ovaries, so viruses can be passed on to the eggs of an infected tick, leading to the emergence of infected larvae. Infection can then persist in

a tick for up to two years due to the lifespan of the ticks, making control of the tick populations important (Lindquist and Vapalahti 2008).

Tick-borne encephalitis virus, the prototype member of TBEV serocomplex, is endemic to Europe and the Far East. It is estimated that 10,000-12,000 cases of human TBE are reported annually across almost 40 countries in Europe and Asia. However, many human TBEV infections are likely subclinical or mild, so many cases are undetected. Mild human TBE infection consists of febrile symptoms, myalgia, general malaise, and thrombocytopenia. Nevertheless, severe cases develop neurological disease in up to 15% of patients, including encephalitis and meningitis (CDC 2022b, WHO 2023b). It is assumed that hard ticks of the family *Ixodidae* supports the enzootic transmission of TBEV primarily in small rodents and occasionally large mammals like deer, goats, sheep, and cows. Consumption of raw dairy products is one of the risk factors for acquiring TBEV infection (van den Brom et al. 2020). Similar to other encephalitic flaviviruses, humans are incidental hosts for TBEV (Brackney and Armstrong 2016).

There are three well-established subtypes of TBEV based on their phylogenic and geographical relationships: European, Siberian, and Far-Eastern subtypes (Smura et al. 2021). Control of the different subtypes has been successful using inactivated vaccines such as FSME-Immun®, Encepur®, and EnceVir®. These vaccines will be discussed in more detail in section 1.3.C. The most successful use of vaccination against TBEV occurred in Austria in the 1970s. Before vaccination, Austria recorded the highest morbidity of TBE in Europe, and TBEV caused 50% of all viral meningoencephalitis cases. In 1971, the country prompted the use of the FSME-Immun® inactivated vaccine derived from the Neudörfl strain. After three vaccinations, seroconversion rates were recorded at >99%, prompting a mass vaccination campaign in 1981, resulting in over 90% vaccination coverage in some high-risk areas. Since the vaccine campaign,

the disease burden has been significantly reduced, with only one observed case of TBEV in the age group over 20 (Kunz 2003, EVIP 2013). However, inactivated vaccines are less immunogenic and require multiple doses for adequate protection, resulting in a costly control program. Significant resources have been dedicated to developing a candidate LAV for TBEV. For example, one candidate LAV currently undergoing preclinical evaluation is the T/1674-mirV2 vaccine, a chimeric virus containing the prM and E genes of TBEV combined with a genetic backbone of an antigenically closely related but less pathogenic member of the TBEV complex: Langat virus. The T/1674-mirV2 vaccine has been shown to protect mice against a challenge with wild-type TBEV (Tsetsarkin et al. 2019).

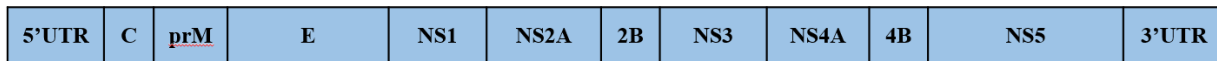
Powassan virus is endemic to Canada and parts of North America. The emergence of POWV has received much attention from the federal government and research community, although there have been less than 300 documented cases in the U.S. since 2004 (CDC 2022a). Phylogenetic analysis showed that available POWV isolates are divided into lineage I and II. Lineage I includes POWV, and lineage II includes deer-tick virus. POWV is commonly found in the northeastern regions of the U.S. and parts of Canada, whereas deer-tick virus is mainly found in the upper midwestern U.S. (Ebel et al. 1999, Robich et al. 2019). POWV is known for causing acute neuroinvasive disease, with 10-15% of neuroinvasive cases resulting in death. For patients that survive the acute infection, 10-30% suffer from long-term or permanent neuropsychological sequelae, including depression, memory loss, hearing impairment, lack of concentration, and partial paralysis (CDC 2022a, Telford and Piantadosi 2023). Transmission occurs through six species of tick from the *Ixodes* and *Deracentor* genera. However, the deer tick (*Ixodes scapularis*) is one of the most important vectors for POWV. Small to medium mammals such as woodchucks, squirrels, and white-footed mice are some of the main amplifying hosts for POWV, whereas

humans do not produce high enough titers for transmission and are an incidental dead-end host (Kemenesi and Bányai 2019).

B. Flavivirus structure-function

All flaviviruses are evolutionally related and have a common genome structure, post-translational processing strategy, and virion structure (Araujo et al. 2020). The positive-sense single-stranded RNA genome is approximately 11 Kilobase long, encapsidated by the C protein and packaged into the lipid bilayer envelope derived from the previously infected host. A single reading frame encodes three structural genes, C, prM, and E on the 5' end and seven non-structural (NS) genes, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 on the 3' end and is flanked by an untranslated region (UTR) on both ends (Figure 1-9). The 5' end of the genome is modified with a cap structure that increases the stability of the viral genome against degradation by host nuclease digestion and allows recognition by the host translation machinery (Daffis et al. 2010). The 3' UTR lacks a poly-A-tail usually found among eukaryotic mRNA (Lindenbach et al. 2007).

(a)



(b)

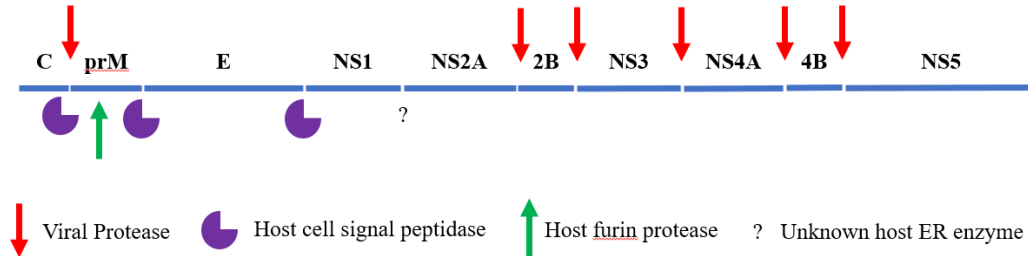


Figure 1-9: Genome organization and proteolytic processing of the flavivirus polyprotein

(a) Depiction of the flavivirus RNA genome. (b) Depiction of the flavivirus polyprotein and the host and viral proteins responsible for processing the individual proteins. Image was generated in PowerPoint.

The positive-sense genome can act as a template for host translation to produce viral proteins following cell entry. Nonstructural proteins constitute the replication complex in the rough endoplasmic reticulum, where newly synthesized genomes from negative-sense replication templates are encapsidated and assembled into progeny virions. A flavivirus virion is approximately 50 nm in diameter and contains three structural proteins. The virion surface is covered by 180 copies of E protein monomers, assembled into homodimers, and exhibiting a herringbone pattern. Each E monomer is anchored to the lipid bilayer envelope via a transmembrane domain and is complexed with the M protein, which acts as a molecular chaperon during the virion maturation process. Inside the sphere of the lipid bilayer envelope is the icosahedral nucleocapsid formed by the C protein and viral RNA (Figure 1-10) (Kuhn et al. 2002, Mukhopadhyay et al. 2003, Kanai et al. 2006, Araujo et al. 2020).

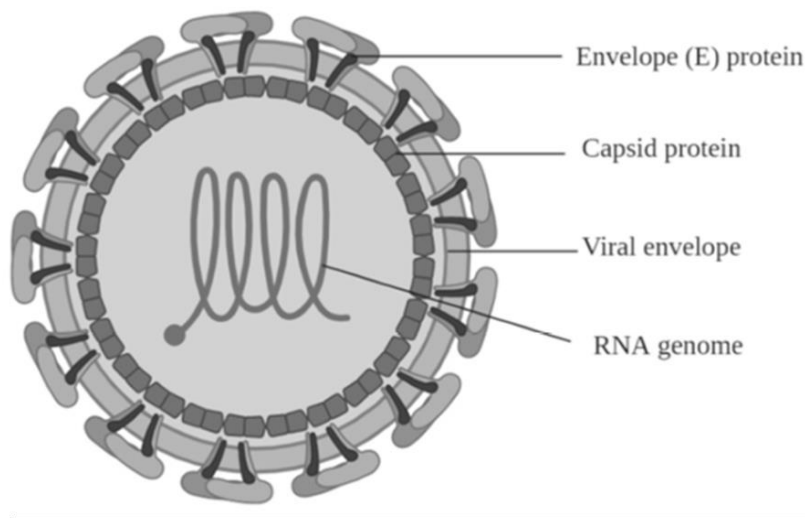


Figure 1-10: Cross-section model of the structure of the flavivirus virion

Image taken from Mandary et al. 2019; published under the Creative Commons Attribution License.

The C protein is 13 Kilodaltons (kDa) and comprises approximately 100 amino acids. Its principal function is to encapsidate the viral genome. Roughly 180 capsid proteins form the electron-dense icosahedral core surrounding one copy of the RNA genome (Figure 1-11) (Zhang et al. 2021). In protecting the viral RNA genome, the C protein exhibits the protease function for polyprotein processing. It involves the reorganization of membrane organelles plus virion assembly and maturation. The C protein acts as a protease and exhibits the autocleavage function involved in the posttranslational processing of the polyprotein. Autocleavage by the C protein is required for the downstream viral and cellular protease cleavage, which ensures the proper folding of each viral protein (Urbanowski and Hobman 2013).

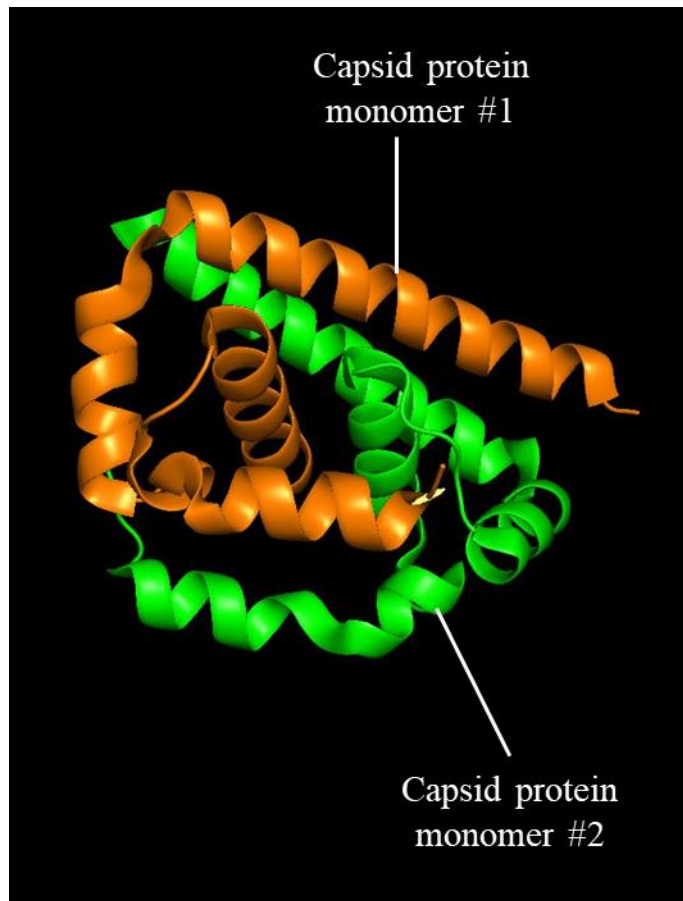


Figure 1-11: Crystal structure of DENV-2 capsid homodimer (PDB ID: 6VSO)

The green and orange colors represent the two separate capsid proteins that form the dimer. The image was generated in PyMOL.

The flavivirus E protein is a class II fusion protein vital for viral membrane fusion and assembly (Harrison 2015). Each of the three domains of the E protein has a functionally important role in the conformational change of the E protein from a dimer to a trimer that instigates membrane fusion activity. Conformational changes in the E protein are pH-dependent and essential for viral entry and the secretion of viral RNA into the cell cytoplasm. The E protein monomer consists of three distinct domains that are encoded in discontinuous sequences: EDI, EDII, and EDIII, based on their location and function (Figure 1-12) (Rey et al. 1995, Kanai et al. 2006, Klein

et al. 2012, Lu et al. 2019). A more detailed discussion of the E protein as a class II fusion protein will be discussed in section 1.4.

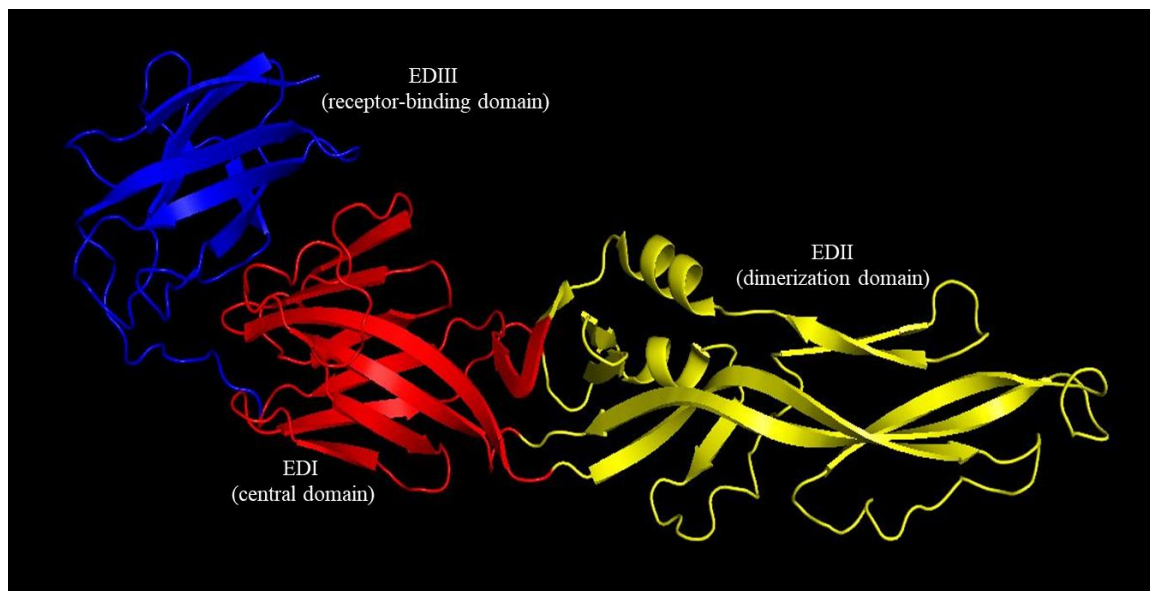


Figure 1-12: Crystal structure of the DENV-2 E protein monomer (PDB ID: 1OAN)

The E protein domains are color-coded with EDI in red, EDII in yellow, and EDIII in blue. The image was generated in PyMOL.

The prM protein is approximately 19-21 kDa. The name prM is due to the pr peptide, which protects the fusion loop structure at the E protein's distal end of the EDII domain and prevents premature membrane fusion in the *Golgi* network during virion maturation (Figure 1-13). The pr peptide is removed by the furin protease prior to the release of virion from the infected cell. The remaining M protein is approximately 10 kDa and contains two C-terminal transmembrane helices that anchor the protein to the lipid bilayer envelope (Yu et al. 2009, Roby et al. 2015). The molecular chaperon function of the prM protein ensures the maturity of progeny virions. The rough surface of immature virions assembled in the ER is due to the protruding E protein nearly perpendicular to the viral envelope. Progeny virions migrate through the acidic *Golgi* network and

exhibit a smooth surface as the prM protein guides the repositioning of the E protein parallel to the virion surface (Lorenz et al. 2002).

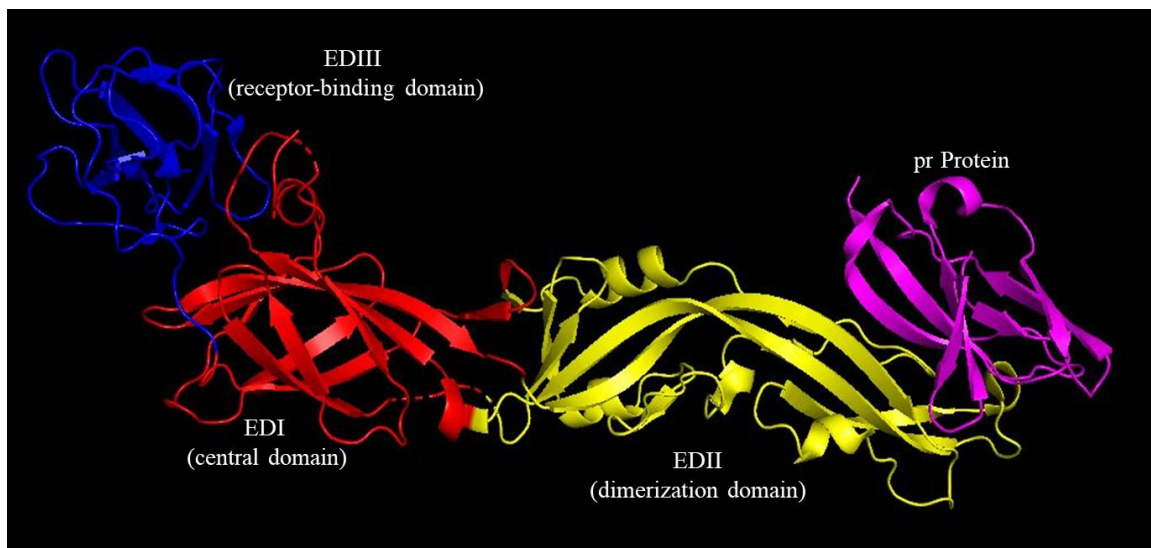


Figure 1-13: Crystal structure of the DENV-2 pr-E protein heterodimer (PDB ID: 3C5X)

The E protein domains are color-coded with EDI in red, EDII in yellow, and EDIII in blue. Magenta represents the pr protein as it sits at the distal end of the EDII domain of the E protein. The image was generated in PyMOL.

The NS proteins function as enzymatic machinery for the propagation of the progeny viruses and antagonists for antiviral immunity. The translation of the genomic RNA leads to the production of a polyprotein, which is subjected to the cleavage of viral and host proteases (Chambers et al. 1990). The NS1 protein is a highly conserved protein with an approximate molecular weight of 40-50 kDa, depending on its glycosylation status (Figure 1-14). It is a multifunctional protein as there are two forms. The cell-associated form takes part in viral replication by causing invaginations in the endoplasmic reticulum membrane to form the vesicles supporting the replication complex (Carpio and Barrett 2021). The second form of NS1 is unusual for a nonstructural protein because it is secreted from the cell. These secreted NS1 proteins modulate the host's innate immune response by binding to factor H, C1s, and C4 to promote the

degradation of C4 to C4b, thereby reducing complement activation (Avirutnan et al. 2010). Recent cell culture and animal studies suggest that NS1 may be a determinant for the tissue tropism of flaviviruses (Puerta-Guardo et al. 2019). This mechanism is studied in detail in the context of DENV infection. The addition of recombinant DENV-2 NS1 protein damaged endothelial HUVEC cell culture. Treatment of *Ifnar*^{-/-} C57BL/6 mice with recombinant DENV-2 NS1 protein increased the permeability of blood vessels, consistent with DENV pathogenesis (Beatty et al. 2015). It has been shown that DENV-2 NS1 protein binds to the negatively charged glycosaminoglycans, e.g., heparan sulfate, and is internalized by endothelial cells. The internalization leads to the activation of cathepsin L in endosomes of the endothelial cells, contributing to the degradation of the extracellular matrix and NS1-induced endothelial hyperpermeability. Alternatively, cathepsin L may be activated via a sequence of molecular signals following DENV NS1 binding to the endothelial cell surface, leading to endothelial barrier dysfunction (Puerta-Guardo et al. 2016, Wang et al. 2019).

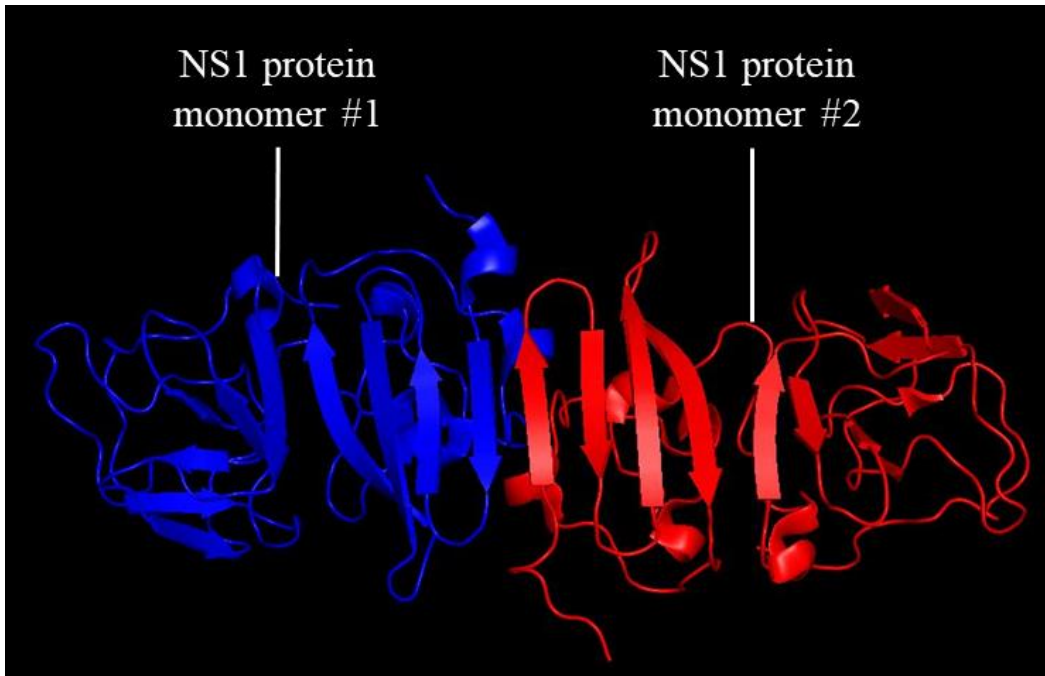


Figure 1-14: Crystal structure of DENV-1 NS1 homodimer (PDB ID: 4OIG)

The red and blue colors represent the two separate NS1 proteins that form the dimer. The image was generated in PyMOL.

The NS2A protein is a small hydrophobic, membrane-associated protein involved in RNA replication. The nonstructural protein, consisting of 231 amino acids, binds highly specifically to the 3' UTR and other components within the replication complex. It is involved in viral RNA replication, the host-antiviral interferon response modulation, and part of the virus particle assembly and secretion process (Leung et al. 2008). The NS2B protein is a small integral membrane protein with a molecular weight of 14 kDa. The NS2B protein consists of three hydrophobic domains and a central hydrophilic domain. Notably, the hydrophilic domain is required to activate the NS3 serine protease domain, making NS2B a cofactor for the protease activity (Wahaab et al. 2022).

The NS3 protein is a nonstructural protein with a molecular weight of approximately 69 kDa (Figure 1-15). It is an essential protein in both polyprotein processing and viral replication,

and it has two key domains: the N-terminal serine protease domain and the C-terminal helicase domain. The protease domain is instrumental in polyprotein processing by cleaving the polyprotein after the C, NS2A, NS2B, NS3, NS4A, and NS4B proteins. The NS3 protein accomplishes this task in the presence of an NS2B cofactor, which sits in the active catalytic site of NS3. Both the region of the NS3 active catalytic site and the NS2B cofactor are highly conserved sequences across the different flaviviruses (Brecher et al. 2013). The helicase domain unwinds the RNA secondary structure of the replication template during viral replication. In addition to the protease and RNA helicase functions, NS3 has one subdomain that exhibits nucleoside 5'-triphosphatase activity. This is required to synthesize the 5'-cap structure on the terminus of viral mRNA that mimics the host cap to facilitate the translation of viral proteins (van den Elsen et al. 2023).

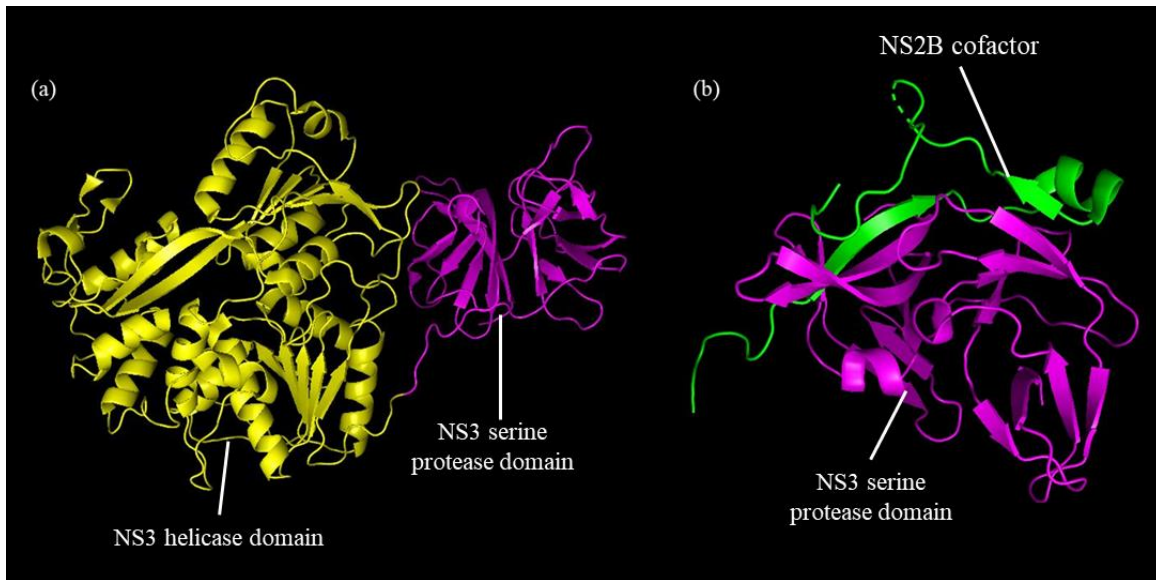


Figure 1-15: Crystal structure of DENV-4 NS3 protein (PDB ID: 2VBC) and DENV-2 NS2B/NS3 protease (PDB ID: 2FOM)

Two different DENV proteins were used to generate the image since the DENV-2 NS3 crystal structure is not available. (a) DENV-4 NS3 protein. The different colors represent the two domains of NS3, with the yellow domain representing the helicase domain and the magenta domain representing the serine protease domain. (b) DENV-2 NS2B/NS3 protease. The two colors represent the protein and substrate. Magenta represents the NS3 serine protease, and green represents the NS2B cofactor. The image was generated in PyMOL.

The NS4A protein is a small transmembrane protein with a molecular weight of approximately 16 kDa. Although the functions of NS4A are poorly understood, the NS4A protein participates in the formation of the replication complex by interacting with host factors and other flavivirus nonstructural proteins, inducing autophagy to prevent cell death, counteracting the cellular interferon responses and hijacking various cellular processes. Cellular interferon responses are counteracted when NS4A manipulates RIG-like-receptor signaling, DDX42 helicase, JAK/STAT signaling, and the unfolded protein response to counteract the IFN- α/β response. The

NS4A protein also hijacks the signaling pathways Akt-mor, ANKLE2/VRK1, JAK/STAT, and Notch signaling, which leads to developmental defects such as microcephaly (Klaitong and Smith 2021). Additionally, NS4A is thought to function as a cofactor that binds to the NS3 helicase domain to optimize the protein's function. NS4A allows NS3hel to sustain the unwinding rate of the viral RNA when presented with conditions of ATP deficiency in the host cell (Shiryaev et al. 2009).

The NS4B protein is another transmembrane protein with a molecular weight of approximately 27 kDa. The NS4B protein acts similarly to NS4A by interacting with the NS3 helicase domain, but NS4B functions by dissociating NS3 from single-stranded RNA after the synthesis of new RNA has occurred (Wang et al. 2022). The NS4B protein also has immunomodulatory functions through a motif near the N-terminal of NS4B that resembles an immunomodulatory tyrosine inhibitory motif found in various components of mammalian cell-signaling cascades. This motif contributes to antagonistic activities against type I interferon and inflammatory cytokine signaling (Xie et al. 2016, Li et al. 2019).

The amino acid sequence of the NS5 protein exhibits the highest level of homology between different flaviviruses due to the two conserved enzymatic domains. The NS5 protein encoded in the viral genome is the most significant NS protein, and the two enzymatic domains are essential to viral genome replication. The N-terminal S-adenosylmethionine-dependent methyltransferase domain drives the 5' cap structure modification (Figure 1-16). During replication, NS5 is directly involved in the capping and replication of the viral genome as the N-terminal methyltransferase domain adds the cap structure in the 5' end of the viral genome. The methyltransferase catalyzes both guanine N-7 and ribose 2-OH methylations during the formation of the viral cap m7GpppA-RNA. The two distinct reactions utilize different side chains in the

active site (K-D-K-E motif) (Egloff et al. 2002, Zhou et al. 2007). The C-terminal RNA-dependent RNA polymerase domain synthesizes both the positive-sense viral genome and the negative-sense RNA template. It is somewhat anticipated that the NS5 protein colocalizes with the NS3 protein because of the involvement of both NS proteins in viral genome replication. Because there is no transmembrane domain in the NS5 protein, the interaction with the NS3 protein also tethers the NS5 protein to the replication complex (Duan et al. 2019, Caldwell et al. 2022).

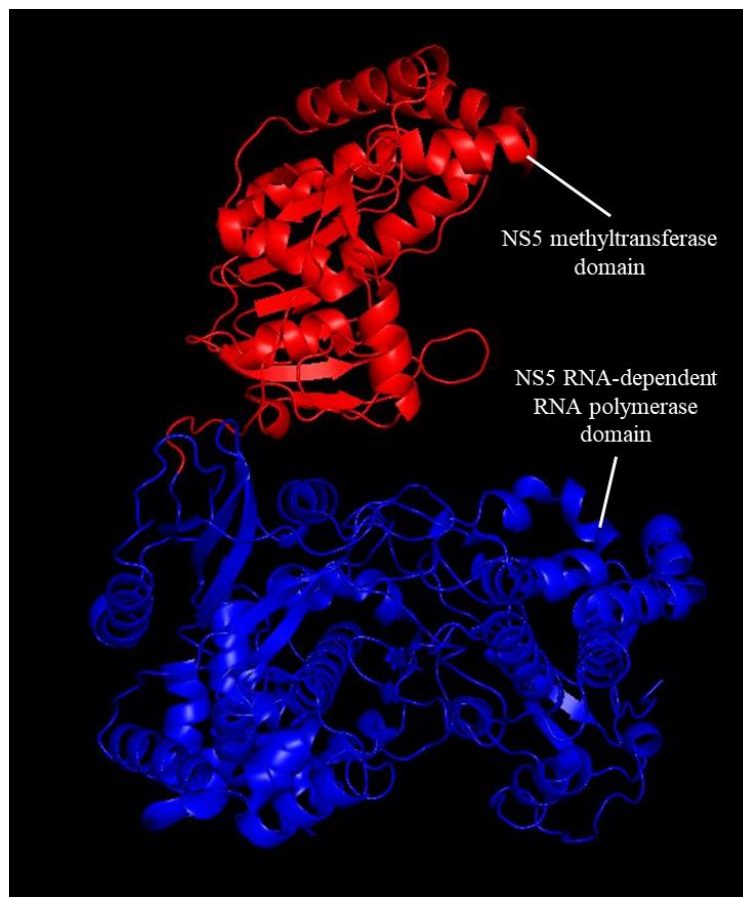


Figure 1-16: Crystal structure of DENV-2 NS5 (PDB ID: 5ZQK)

The red and blue colors represent the two domains of NS5, with the red domain representing the methyltransferase domain and the blue domain representing the RNA-dependent RNA polymerase domain. The image was generated in PyMOL.

C. Flavivirus intracellular lifecycle

Successful establishment of infection of a cell by a flavivirus is initiated by the viral E protein binding to cellular receptors on the surface of the cell (Figure 1-17). Many putative receptors have been identified for flaviviruses. However, the glycosaminoglycans, such as heparan sulfate, and lectins, such as dendritic-cell-specific intercellular adhesion molecule 3-grabbing non-integrin and mannose receptors, are considered to be essential cellular factors for the attachment of flaviviruses. While those are the two main types of cellular receptors for the attachment of flaviviruses to the cell, specific receptors vary considerably between different flaviviruses. Other categories of receptors include heat shock proteins, immunoregulatory proteins, and phosphatidylserine receptors (Chen et al. 1997, Tassaneetrithep et al. 2003, Meertens et al. 2012, Anwar et al. 2022).

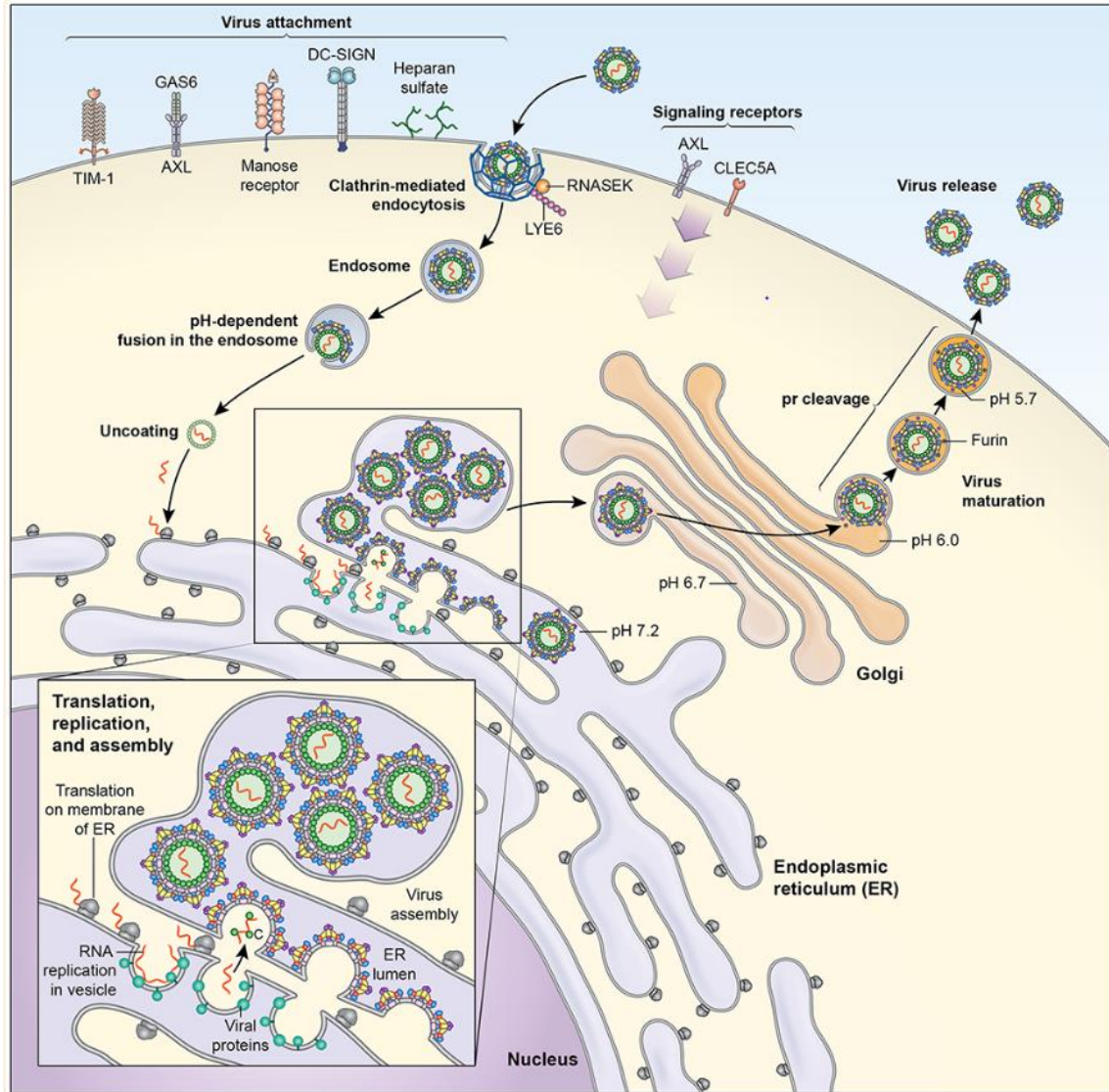


Figure 1-17: Lifecycle of flaviviruses

Image taken from Nature Microbiology, Volume 5, TC Pierson and MS Diamond, "The continued threat of emerging flaviviruses." 796-812. (2020); with permission from Springer Nature.

Receptor binding triggers the clathrin-mediated endocytosis of virions. RNASEK initiates the internalization process by stimulating the tubularization of lymphocyte antigen locus 6. The microtubules formed on the endosome's surface then facilitate the flavivirus virion's internalization. Once in the cell cytoplasm, protonation of the endosomes provides free energy to trigger the conformational change of the E protein from dimer to trimer and leads to the exposure

of the fusion loop. Subsequently, the fusion loop is inserted into the endosomal membrane, establishing the physical interaction required for the virus-cell membrane fusion (Hackett and Cherry 2018). The membrane fusion creates the pore structure that releases the nucleocapsid into the cytoplasm. Degradation of the nucleocapsid exposes the viral genome as the template for translating a single viral polyprotein. Viral and host proteases cleave the polyprotein containing all structural and nonstructural proteins into individual proteins (Pierson and Diamond 2020). Properly folded nonstructural proteins enable the formation of a replication complex, which appears as spherical compartments within the ER in the electron micrograph. Formation of the vesicle packets protects the viral replication process from the detection of cytoplasmic innate immune sensors, e.g., pattern recognition molecules for double-stranded RNA intermediates (Aktepe and Mackenzie 2018).

The replication complexes transcribe the viral RNA into a negative-strand RNA intermediate, yielding genomic positive-strand RNA. This new genomic RNA is packaged within new C, prM, and E proteins, creating an immature virion. The immature virions are secreted in vesicles from the ER to the Golgi apparatus, progressing through decreasing pH (van den Elsen et al. 2021). Each prM-E heterodimer lies perpendicular to the immature virion surface. Three neighboring copies of the prM-E heterodimer are arranged in a trimeric spike. The pr peptide of the prM protein covers the fusion loop of the E protein from premature membrane fusion. Maturation of the flavivirus virion takes place through the *trans*-Golgi network. Decreasing the pH gradient from pH 7.2 to pH 5.7 triggers the reorganization of the E protein to lie flat against the virion in a lattice of head-to-tail E protein dimers, using the prM protein as a molecular chaperone. The rearrangement exposes the furin-cleavage site on the prM protein prior to the secretion of the virion. Upon secretion of the virion through exocytosis and encountering neutral

pH, pr dissociates from E for the last step of maturation, resulting in a fully infectious virus particle (Yu et al. 2008, Pierson and Diamond 2020, Stiasny et al. 2022).

1.3 Flavivirus vaccines

At this time, there are no licensed antiviral therapies for flaviviruses. Like other types of RNA viruses, developing antivirals has proven difficult for flaviviruses, and few antiviral compounds have progressed to clinical trials (Low et al. 2017). The candidate therapeutic monoclonal antibody for WNV MGAWN1 was evaluated in clinical trials but was later terminated due to the difficulty of enrolling subjects (NIH 2022). Vaccination and vector control methods are the most effective measures to control flaviviruses. Although controlling vector populations have a long history of success, the strategies have proven costly, time-consuming, and unsustainable (Reiner Jr. et al. 2016). A significant challenge for vector control is the development of insecticide resistance and the re-infestation after the program's termination. Therefore, vaccination is the best method for reducing the disease burden for flaviviruses (Table 1-1). Human vaccines for five flaviviruses currently exist, including DENV, JEV, KFDV, TBEV, and YFV. Inactivated vaccines are available for JEV, KFDV, and TBEV but require multiple doses due to low immunogenicity. Live-attenuated vaccines can elicit protective immunity with one single immunization. However, only the empirically derived YF 17D and JE SA14-14-2 LAVs are available and implemented for disease control. Two LAVs for DENV Dengvaxia and QDENVGA have been licensed in several countries.

Table 1-1: Current status of flavivirus vaccines

Virus	Type of vaccine	Vaccine	Availability	Reference
YFV	Live-attenuated	17D vaccine	Available worldwide for human use	(Smith and Theiler 1937, Theiler and Smith 1937a)
JEV	Live-attenuated	SA14-14-2	Available in Asia for human use	(Eckels et al. 1988, Liu et al. 1997)
		ChimeriVax-JE (IMOJEV)	Available in Australia and Asia for human use	(Appaiahgari and Vрати 2010, Chokephaibulkit et al. 2015)
	Inactivated	JEBIKV, ENCEVAC, and TC-JEV	Available in Japan and South Korea for human use	(Okada et al. 2012, Yun et al. 2015, Hegde and Gore 2017)
		IC51 (Ixiaro)	Available in the U.S., Australia, and Europe for human use	(Amicizia et al. 2019, Kling et al. 2020)
DENV	Live-attenuated	ChimeriVax-DEN (Dengvaxia)	Available in the U.S. for human use	(Guirakhoo et al. 2002, Guy et al. 2010b)
		DENVax (QDENGGA)	Available in Europe for human use	(Osorio et al. 2011, Takeda 2022)
		DENV-4-3'-Δ-30	Phase III clinical trials in progress	(Durbin et al. 2011, Kallas et al. 2020)
	Subunit	DEN1-80E	Phase I clinical trials in progress	(Torresi et al. 2017)
	Nucleic acid	D1ME100	Phase I clinical trials complete	(Torresi et al. 2017)
		Vaxfectin	Phase I clinical trials complete	(Danko et al. 2018)
	Inactivated	PIV DENV	Phase I clinical trials complete	(Martinez et al. 2015)

Virus	Type of vaccine	Vaccine	Availability	Reference
WNV	Live-attenuated	ChimeriVax-WN02	Phase II clinical trials complete	(Arroyo et al. 2004, NIH 2016)
		WN/DENV-4-3'Δ30	Phase I clinical trials complete	(Durbin et al. 2013, Pierce et al. 2017)
		Recombitek	Available in the U.S. for equine use	(Grosenbaugh et al. 2004, Garch et al. 2008)
	Inactivated	WN Innovator, Vetera, and Prestige	Available in the U.S. for equine use	(Tesh et al. 2002, Chaintoutis et al. 2015)
		HydroVax-001	Phase I clinical trials complete	(NIH 2019, Gould et al. 2023)
	Subunit	WN-80E	Phase I clinical trials complete	(Lieberman et al. 2007)
TBEV	Inactivated	Nucleic Acid VRC-WNVDNA020-00-VP	Phase I clinical trials complete	(Martin et al. 2009)
		FSME-Immun and TicoVac	Available in Russia, Europe, and the U.S. for human use	(Ehrlich et al. 2003, Pfizer 2023)
		Encepur, Moscow vaccine, Tick-E-Vac, and EnceVir	Available in Russia and Europe for human use	(Barrett et al. 2003, Demicheli et al. 2009, Amicizia et al. 2013)
KFDV	Inactivated	Chick embryo fibroblast vaccine	Available in India for human use	(Dandawate et al. 1994, Kasabi et al. 2013)

A. Empirically derived live attenuated vaccines

The first successful development of a flavivirus LAV was spearheaded by Max Theiler, who undertook the empirical passage of the wild-type Asibi strain to obtain the attenuated 17D vaccine strain. The empirical derivation included 176 passages in mouse brains and chicken embryos (Smith and Theiler 1937). The resulting 17D strain was subcutaneously injected into macaques and exhibited the loss of viscerotropism. While a low level of viremia was detected, immunized primates can develop serum-neutralizing antibody responses, which later were proven to correlate with protection (Mason et al. 1973). Approximately 30 individuals were involved in the initial evaluation of the safety and immunogenicity of the 17D vaccine. Nearly all subjects developed neutralizing antibody titers within two weeks after vaccination without signs of viscerotropic disease (Theiler and Smith 1937b, Theiler and Smith 1937a). In addition to being safe and immunogenic in humans, the 17D vaccine has repeatedly been shown to lose the ability to replicate in *Ae. aegypti*. The attenuated phenotype in mosquitoes ensures the safety of the deployment of the 17D vaccine in endemic areas because *Ae. aegypti* is unable to transmit the 17D vaccine strain and cause outbreaks even if vaccines can develop approximately 2 logs genome equivalent of viremia (Whitman 1939). This was the first vaccine to prove a loss of viscerotropism in primates and further found non-transmissibility in mosquitoes (McElroy et al. 2005).

The attenuated phenotype of the 17D strain is due to the genetic differences compared to the parental wild-type Asibi strain. Sanger sequencing identified 67 nucleic acid changes, leading to 31 amino acid substitutions between the Asibi strain and the 17D-204 strain (Hahn et al. 1987). Further, the 17D vaccine strain consists of a stable homogenous RNA population compared to the diverse quasispecies of the Asibi strain and other wild-type YFV strains (Beck et al. 2014, Beck et al. 2018). The original materials that gave rise to the 17D strain are no longer available, making

investigating attenuation mechanism(s) difficult. Although the sequence differences have been mapped out, molecular determinants for virulence attenuation are still poorly understood. It is assumed that multigenic mutations contribute to the attenuated phenotype of the 17D strain. A comparison of the Asibi and 17D genomes revealed that the E glycoprotein is the most heavily mutated among the ten viral proteins, harboring 12 amino acid changes. The remaining proteins had various amino acid changes, with six amino acid changes maximum. Therefore, based on known rates of mutation and known function of the proteins, published studies have primarily focused on the examination of putative attenuating mutations in the E, NS1, NS4B, and NS5 proteins (Hahn et al. 1987, McElroy et al. 2006a, Fernandez-Garcia et al. 2016).

Studies using mutants derived from the 17D vaccine strain suggested that multiple mutations in the E protein can act as the determinants for the mouse neurovirulence phenotype, such as the E-G52R and E-V173T attenuating mutations in the EDI domain. The E-G52R mutation can alter the pH conformational change, and the E-V173T mutation can alter the conformational change at the MAb 864-defined epitope, resulting in attenuation of the mouse neurovirulence phenotype (Schlesinger et al. 1996, Ryman et al. 1997, Ryman et al. 1998). Another line of research focused on examining the putative attenuating mutations in the receptor binding domain, EDIII. One such mutation is the E-T380R mutation in the EDIII of the 17D strain. The E-T380R mutation increases the positive charge of EDIII and binding affinity with negatively charged glycosaminoglycans on the cell surface. While the E-R380T reversion engineered in the 17D strain was shown to restore the virulence phenotype of YFV in immunocompromised mice partially, the E-T380R mutation has no known attenuating effect in *Ae. aegypti* (Nickells and Chambers 2003, Lee and Lobigs 2008, Huang et al. 2014).

Deployment of the 17D vaccine in Sub-Saharan Africa and tropical South America began after the end of World War II. It is shown that over 90% of patients can seroconvert within two weeks after immunization, and neutralizing antibody responses can last for several decades (Gotuzzo et al. 2013). The success of the 17D vaccine led to a significant decline in human YF cases in the 20th century. It changed from a significant health concern to a neglected tropical disease (Frierson 2010). The vaccine elicited an innate and adaptive response by activating Toll-like receptor (TLR) signaling, followed by proinflammatory cytokines. Long-term persistent immunity was also observed over a decade after the initial vaccination, shown in the CD8(+) T-cell memory responses by the T-cell epitopes on the NS proteins (Querec et al. 2006, Akondy et al. 2009). The 17D vaccine later elicited a phenomenal long-term immune response, with neutralizing antibodies in the blood for 30-35 years after vaccination (Poland et al. 1981).

Two substrains are used for manufacturing the YFV 17D vaccine: 17D-204 and 17DD. The 17D-204 substrain was obtained based on the 204th passage, and the 17DD substrain was further passaged in eggs in South America from the 195th passage (Barrett 1997). Both substrains are immunogenic, but their nucleotide composition is 99% identical resulting in slight variations in immunogenicity and reactogenicity (Santos et al. 1994). For example, 17D vaccines activate various TLRs. The 17DD vaccine activates TLRs 3 and 9, but 17D-204 activates TLRs 3 and 9, in addition to TLRs 2, 7, and 8. While the specific immune response may differ, they still both exhibit strong activation of the cellular and humoral immune responses (Ferreira et al. 2018). The manufacturing of the 17D vaccine requires the use of embryonated chicken eggs according to the WHO recommendation. However, in the 1960s, there were incidents where avian leucosis virus contaminated embryonated chicken eggs used for the 17D vaccine manufacturing (Klug et al. 2016). On behalf of the WHO, the Robert Koch Institute established an avian leucosis virus-free

version of the 17D-204 vaccine, leading to the YFV 17D vaccines being used today. The 17D-204 vaccine is used in most countries, with the 17D-204-WHO vaccine only being used in a few countries, such as Australia, Germany, and Russia. The 17DD vaccine is mainly used in Brazil (Barrett 1997).

While the 17D vaccines are still considered safe, some adverse effects have been reported. Although rare, severe adverse reactions occur in 1 per 250,000-500,000 vaccinations (Barrett and Teuwen 2009, Ferreira et al. 2018). There are two adverse effects: vaccine-associated neurotropic disease (YEL-AND) and vaccine-associated viscerotropic disease (YEL-AVD). Vaccine-associated neurotropic disease, previously named postvaccine encephalitis, typically occurs 2-30 days post-vaccination. Symptoms include neurological dysfunction and seizures, which can lead to death, but the case fatality rate is less than 5%. Some risk factors for YEL-AND include age, sex, and a history of thymus disease with a thymectomy. YEL-AVD, previously named febrile multiple organ system failure or post-vaccination multiple organ system failures, occurs in patients who have received either the 17D-204 or 17DD vaccine for the first time. Symptoms usually occur 2-5 days post-vaccination, including liver, renal, and respiratory failure. The first reported case was from a Brazilian woman vaccinated in 1975, although the phenomenon was not reported in the literature until 2001 (Barrett and Teuwen 2009, Monath 2010). There have only been about 60 cases of YEL-AVD ever reported out of over 800 million doses of 17D vaccines administered in human medical history, but 35 of them resulted in the death of the patient (Ferreira et al. 2018). Although vaccine-associated adverse effects have been reported occasionally, the YFV 17D vaccine has shown to be one of the most potent and successful LAVs ever developed (Barrett and Teuwen 2009).

Another very successful empirically derived LAV is the JEV SA14-14-2 LAV. The SA14-14-2 strain is among the many attempts to attenuate the virulence phenotype of the wild-type JEV SA14 strain. The empirical attenuation process of the SA14 strain began with one mouse brain passage, one hundred passages in primary hamster kidney (PHK) cells, and three rounds of plaque purification to select the SA14-12-1-7 strain (Yu 2013). The attenuated phenotype of the SA14-12-1-7 strain was unstable. Another attenuated SA14-5-3 strain was derived from six mouse passages and six rounds of plaque purifications of the SA14-12-1-7 strain (Davis et al. 2021). The clinical trial in 400,000 children proved that the SA14-5-3 strain has low immunogenicity. This led to the five serial passages of the SA14-5-3 strain in suckling mice, followed by two rounds of plaque purification in PHK cells. The resulting SA14-14-2 strain is equally attenuated as the SA14-5-3 strain but is more immunogenic in mice, guinea pigs, and pigs. Although the immunization regime usually involves two doses, clinical trials have shown that one single immunization of SA14-14-2 LAV is sufficient to elicit long-lasting immunity (Tandan et al. 2007, Yu 2010). Since its initial licensure in 1988 in China, the SA14-14-2 vaccine has proven highly effective at reducing the number of clinical cases of the disease yearly due to its ability to provide effective cross-neutralization between the five genotypes of JEV (Erra et al. 2013). Additionally, the SA14-14-2 strain also lost the ability to be transmitted by mosquitoes (Araujo et al. 2020, Davis et al. 2021).

The wild-type JEV SA14 strain and the vaccine SA14-14-2 strain differ by 57 mutations in the nucleotide sequence and 23 substitutions in the amino acid sequence (Table 1-2). Because the mutations are distributed across all of the genes, it was believed that multigenic mutations are also responsible for the attenuated phenotype of the SA14-14-2 strain (Yun et al. 2016). However, the E protein mutations were proven to have the utmost importance for the attenuation process of the SA14-14-2 strain and lead to the complete attenuation of mouse neurovirulence phenotype,

one of the important markers for the safety of the SA14-14-2 LAV (Gromowski et al. 2015b). The mutations in the E protein have been individually examined using infectious clones and mouse models, revealing the putative attenuation mechanism(s) of the SA14-14-2 strain (Wang et al. 2017).

Table 1-2: Comparison of the complete genome sequences of JEV SA14 and SA14-14-2

Protein	Position		Nucleotide Changes		Amino Acid Changes		Amino Acid Substitution
	Genome	Polyprotein	SA14	SA14-14-2	SA14	SA14-14-2	
5'NTR	39		U	A			
C	292	66	U	C	L	S	L66S
prM	538	148	G	A	G	D	G21D
E	1061	322	U	C	–	–	–
	1296	401	C	U	L	F	L107F
	1389	432	G	A	E	K	E138K
	1503	470	A	G	I	V	I176V
	1506	471	A	G	T	A	T177A
	708	538	A	G	E	G	E244G
	1769	558	G	U	Q	H	Q264H
	1813	573	A	U	K	M	K279M
	1921	609	C	U	A	V	A315V
	2293	733	A	G	K	R	K439R
	2441	782	G	A	–	–	–
NS1	2691	866	C	A	–	–	–
	2843	916	U	C	–	–	–
	3181	1029	G	A	G	D	G235D
	3351	1086	G	A	G	S	G292S
	3493	1133	G	U	R	M	R339M
	3528	1145	G	C	D	H	D351H
NS2A	3539	1148	U	A	N	K	N2K
	3599	1168	G	A	–	–	–
	3652	1186	C	U	A	V	A40V
	3677	1194	C	U	–	–	–
	3776	1227	C	U	–	–	–
	3801	1236	C	U	–	–	–
	4013	1306	A	G	–	–	–
	4106	1337	A	G	–	–	–
NS2B	4403	1436	G	U	E	D	E63D

Protein	Position		Nucleotide Changes		Amino Acid Changes		Amino Acid Substitution
	Genome	Polyprotein	SA14	SA14-14-2	SA14	SA14-14-2	
NS2B	4408	1438	A	G	D	G	D65G
NS3	4782	1563	A	G	M	V	M59V
	4921	1609	C	G	A	G	A105G
	4922	1609	U	C	—	—	—
	6008	1971	C	U	—	—	—
	6425	2110	A	G	—	—	—
NS4A	6638	2181	U	C	—	—	—
	6728	2211	G	A	—	—	—
NS4B	6944	2283	A	G	—	—	—
	7121	2342	C	U	—	—	—
	7193	2366	C	U	—	—	—
	7227	2378	A	G	I	V	I106V
NS5	7736	2547	C	U	—	—	—
	8099	2668	C	U	—	—	—
	8276	2727	C	U	—	—	—
	8394	2767	C	U	—	—	—
	8832	2913	C	U	H	Y	H386Y
	8882	2929	A	U	—	—	—
	8891	2932	C	U	—	—	—
	9593	3166	G	U	Q	H	Q639H
	9688	3198	U	C	V	A	V671A
	9695	3200	G	A	—	—	—
	9818	3241	C	U	—	—	—
	9917	3274	A	U	—	—	—
	10046	3317	G	A	—	—	—
	10139	3348	C	U	—	—	—
	10217	3374	U	C	—	—	—
3'UTR	10428		U	C			

For example, the E-E138K mutation led to the complete attenuation of the neuroinvasive and neurovirulence phenotype of JEV. One explanation is the location of the E-E138 residues within the structure of the E protein homodimer. Surrounding the hole structures of the E protein are residues that exhibit binding activity with glycosaminoglycans, a class of putative residues for flaviviruses, and the E-E138K mutation residues. The affinity of JEV to glycosaminoglycans can interfere with the receptor binding of JEV. The attenuated phenotype of the SA14-14-2 strain can also be linked to the impairment of the membrane fusion process. Specifically, the E-L107F mutation is part of the flavivirus-conserved residues within the fusion loop structure located at the tip of EDII (Chambers et al. 1999, Zhao et al. 2005, Yang et al. 2014). Engineering of the E-L107F mutation in the E protein further attenuated the neurovirulence phenotype of the ChimeriVax-WN1 candidate LAV and created the Chimerivax-WN2 candidate LAV proven to be safe and immunogenic in clinical trials (NIH 2016). However, it is important to note that 1) ChimeriVax-WN candidate LAVs were constructed on the backbone of YFV 17D LAV, which contains other attenuating mutations in the NS proteins, and 2) the E-L107F mutation alone cannot lead to the stable attenuated phenotype in either WNV or DENV. So, there are no broadly effective attenuating mutations in the fusion loop for developing candidate flavivirus LAVs.

The SA14-14-2 vaccine exhibits some adverse effects, although overall meta-analysis concluded that the SA14-14-2 vaccine is safe (Eckels et al. 1984, Liu et al. 1997). A wide range of symptoms have been reported after SA14-14-2 vaccination, but the majority show a range of mild to moderate symptoms, of which fever is the most common. These adverse effects occur in less than 3% of vaccinations (Schiøler et al. 2007, Satchidanandam 2020). Some more severe but rare symptoms include anaphylaxis, idiopathic thrombocytopenic purpura, measles-like rash, and vomiting (Li et al. 2014, Sanchayan et al. 2016).

Due to the safety and immunogenicity safety profile, there are currently ten endemic countries that utilize the SA14-14-2 vaccine to help control the spread of the virus, including Cambodia, China, India, Indonesia, Lao People's Democratic Republic, Malaysia, Myanmar, Nepal, Republic of Korea, Sri Lanka, and Thailand (WHO 2023a). Case-control studies from Nepal and India have shown that the SA14-14-2 vaccine elicits rapid onset of neutralizing antibody protection with 90% seroconversion followed by a five-year efficacy of 96% after a single dose of the vaccine (Tandan et al. 2007, Kumar et al. 2009, Halstead and Thomas 2010). The SA14-14-2 strain was also used to manufacture the IXIARIO-inactivated JEV vaccine. This inactivated vaccine, also known as JEspect®, is licensed by the U.S. Food and Drug Administration (FDA) in the U.S. and by the European Medicines Agency in Europe, targeting the travelers' market (FDA 2019, European Medicines Agency 2023).

While the empirically derived LAVs have played an essential role in controlling YFV and JEV, the empirical attenuation process is ineffective for most flaviviruses and cannot meet the demand for rapid vaccine development in response to the emergence and re-emergence of flaviviruses. The YF 17 LAV and the JE SA14-14-2 LAV are compatible with modern cell culture technologies and can be manufactured on a large scale. However, in the 21st century, there is a growing interest in the rational design of candidate LAVs based on the knowledge of genetic determinants for the virulence phenotype of flaviviruses and immunogenicity. Rationally designed recombinant candidate flavivirus LAVs use WHO-approved vaccine manufacturing substrates, which can support large-scale production to meet the demands of outbreak responses.

B. Chimeric vaccines

Although the JE SA14-14-2 LAV is an effective vaccine, the Chengdu Institute of Biological Products manufacturing facility did not complete the WHO prequalification. It became

eligible for the global market in 2013 (Vannice et al. 2021). The chimeric JE LAV was constructed using the ChimeriVax® platform and modern cell culture technologies. The ChimeriVax®-JE vaccine involves the insertion of the prM and E genes of the SA14-14-2 strain into the backbone of the YFV 17D strain (Konishi et al. 1998, Chambers et al. 1999, Arroyo et al. 2001). Non-human primates vaccinated with the ChimeriVax®-JE vaccine survived the challenge with the wild-type JEV SA14 strain, suggesting its ability to protect against natural infections (Monath et al. 1999). Regarding the spread of the recombinant virus through mosquitoes, it was shown that ChimeriVax®-JE could replicate in mosquitoes but failed to disseminate and transmit the virus (Bhatt et al. 2000, Guy et al. 2010a). The YFV 17D vaccine was used as a backbone because of the YFV 17D RNA-dependent RNA polymerase. The YFV 17D RNA-dependent RNA polymerase has been shown to have low mutation rates compared to other RNA polymerases, such as the SP6 RNA polymerase, resulting in high genetic stability (Xie et al. 1998, Pugachev et al. 2004).

The ChimeriVax®-JE vaccine was evaluated by clinical trials in Korea, Thailand, Vietnam, and the Philippines and is licensed as IMOJEV in at least seven JEV-endemic countries. There were some concerns during human testing with possible reversion to the virulent viruses, the spread of recombinants in nature, and the risk of viscerotropism due to its YFV origins (Appaiahgari and Vрати 2010). The ChimeriVax®-JE vaccine was approved for human use in Australia in 2012 and since then has been approved in other countries such as Cambodia, Malaysia, Brunei, Hong Kong, Indonesia, the Philippines, Singapore, South Korea, and Thailand (Chokeyhaibulkit et al. 2015). The ChimeriVax® technology has been used as a basis for the construction of other chimeric LAVs, including WNV and DENV.

The application of the ChimeriVax® technology to DENV LAV development produced chimeric YFV-DENV tetravalent dengue vaccine (CYD-TDV), which was later licensed and marketed as Dengvaxia® LAV by Sanofi Pasteur. The CYD-TDV vaccine is made up of four recombinant live attenuated chimeric vaccines that are based on the YFV 17D backbone. The prM and E genes of 17D were removed, and the DENV prM and E proteins were added. This recombination was done four times to create four chimeric cDNAs that have structural proteins from one of the four DENV serotypes: DENV-1 Thai strain PUO-359/TVP-1140, DENV-2 Thai strain PUO-218, DENV-3 Thai strain PaH881/88, and DENV-4 Indonesian strain TVP-980 (Guirakhoo et al. 2002, Guy et al. 2010b, Morrison et al. 2010).

The CYD-TDV vaccine was less neurovirulent than YFV 17D LAV and exhibited reduced infectivity in *Ae. aegypti* and *Ae. albopictus* (Guirakhoo et al. 2002, Higgs et al. 2006). The CYD-TDV vaccine is administered in one primary and then two booster doses at 6 and 12 months. Human vaccine trials showed that CYD-TDV was safe and immunogenic in dengue naïve adults. There are some discrepancies between the efficacy of the various viruses of the vaccine according to the DENV serotypes. However, despite that variation, the vaccine demonstrated strong neutralizing antibody responses for all four serotypes (Sabchareon et al. 2012, Capeding et al. 2014). The CYD-TDV vaccine was first licensed in Mexico in 2015 but is now licensed in 20 countries (Torresi et al. 2017, WHO 2018). However, Dengvaxia may increase the risk of hospitalization and severe dengue in immunologically naïve individuals following natural infection (Halstead 2018, Sridhar et al. 2018). The increased risk of severe disease led to the recommendation of two vaccination strategies. The first strategy was “mass vaccination of a population seroprevalence without screening,” and it would have overall benefits in areas where predicted transmission was high. The second recommendation was “pre-vaccination screening,”

maximizing the benefit of seropositive individuals while avoiding harm to identified seronegative individuals due to the risk of adverse effects (Wilder-Smith et al. 2019). This is reflected by the licensure of Dengvaxia® in the U.S. The FDA took the recommendation of pre-screening. It approved the use of Dengvaxia to individuals 9 through 16 years of age with laboratory-confirmed previous dengue infection and living in endemic areas (FDA 2023).

The second licensed vaccine is the QDENGGA® vaccine (Takeda Vaccines Inc.), based on the tetravalent formation of chimeric DENV (DENVax). This vaccine was only recently licensed in December of 2022 in Europe for individuals 6-45 years of age. The backbone of the QDENGGA® LAV is the DENV-2 PDK53 strain. Briefly, the DENV-2 16681 strain was serially passaged 53 times to generate the attenuated PDK53 strain (Bhamarapavati et al. 1987). The infectious clone-derived DENV-2 PDK53 strain was engineered to contain the structural genes prM and E from DENV-1 (16007 strain), DENV-3 (16562 strain), and DENV-4 (1036 strain). The resulting tetravalent LAV has a two-dose regimen: one primary vaccination and one booster vaccination at three months (Huang et al. 2000, Osorio et al. 2011, Torresi et al. 2017, Takeda 2022).

The vaccine has been shown to induce both humoral and cellular immune responses as well as neutralizing antibody responses, indicating that the vaccine can be protective against dengue infection (Henriques et al. 2013). Phase III trials demonstrated that the vaccine was effective against systematic dengue in children and adolescents and reduced their risk of experiencing sequential episodes of symptomatic dengue (Rivera et al. 2022, Sáez-Llorens et al. 2023). Although DENVax® has shown to be effective, it remains to be monitored after the licensure and deployment in the field (Tricou et al. 2019, de Silva and White 2023).

In addition to Dengvaxia® and QDENGGA®, a tetravalent DENV LAV, initially developed by the National Institute of Allergy and Infectious Diseases (NIAID) Laboratory of Viral Diseases,

has also been demonstrated to be a promising candidate vaccine. The 30-nucleotide deletion in the 3'UTR was first created to attenuate the virulence and mosquito competence phenotype of the DENV-4 Dominica/81 strain (McArthur et al. 2008). The same attenuation strategy was expanded to the DENV-1 Western Pacific strain and DENV-3 Sleman/78 strain. The DENV-3 virus was produced by introducing a 30 and 31-nucleotide deletion at the 3'UTR. Due to the inability of the 30-nucleotide deletion in the 3'UTR to attenuate the virulence phenotype of DENV-2 Tonga/74 strain, the DENV-2 component of the tetravalent candidate LAV consists of the DENV-2/DENV-4 chimera by inserting the prM and genes of DENV-2 Tonga/74 strain into the backbone of DENV-4 Dominica/81 strain. This tetravalent candidate DENV LAV is undergoing clinical trials as the Merck V181 candidate LAV and the Butantan-DV candidate LAV. Currently, the Butantan-DV vaccine, which contains the lyophilized tetravalent attenuated DENV strains, is in Phase III clinical trials and is showing to have a high ability to generate an immune response against DENV (Durbin et al. 2011, Kallas et al. 2020, Butantan-Institute 2022).

Another primary target for flavivirus vaccine development is WNV. There are two candidate WN LAVs evaluated in clinical trials. The ChimeriVax®-WN02 candidate LAV uses the backbone of the YFV 17D strain and differs from the ChimeriVax®-WN01 candidate LAV by three amino acids, including the E-L107F, E-A316V, and E-K440R mutations. The three mutations were implicated for the attenuating effect in the JEV SA14-14-2 strain and were selected to attenuate the mouse neurovirulence phenotype of the ChimeriVax®-WN01 candidate LAV (Arroyo et al. 2004, Davis et al. 2021). The Phase II clinical trials showed that the vaccine was highly immunogenic and tolerated even in elderly patients (Dayan et al. 2012, Dayan et al. 2013). However, the licensure will require the ChimeriVax®-WN02 candidate LAV to complete the phase III clinical trials. Another human WNV vaccine is a LAV based on the DENV-4 backbone

called WN/DENV-4-3'Δ30. This vaccine was developed by the NIAID Laboratory of Viral Pathogens. A phase I clinical trial showed the WN/DENV-4-3'Δ30 candidate LAV is less immunogenic and requires a two-dose regimen to achieve a 90-95% seroconversion rate (Durbin et al. 2013, NIH 2016, Pierce et al. 2017, Lei et al. 2018).

C. Inactivated vaccines

Several inactivated flavivirus vaccines, including the inactivated JE, TBE, and KFD vaccines, are licensed for human and veterinary use. The development of inactivated JE vaccines preceded the development of JE LAVs. The first licensed inactivated JE vaccine was prepared from a formalin-inactivated mouse brain and marketed as the Biken® vaccine in Japan in the 1950s (Satchidanandam 2020). Similarly, the formalin-inactivated JE vaccine prepared with the Beijing-1 strain was implemented for pediatric immunization in Taiwan (Defraites et al. 1999). Manufacturing of inactivated JE vaccines using mouse brains has ceased in the 21st century. Currently, licensed inactivated JE vaccines are prepared from Vero cell culture (Satchidanandam 2020).

One vaccine licensed for use in the U.S., Australia, and Europe is the IC51 vaccine, marketed as Ixiaro®. This vaccine is a purified, formalin-inactivated form of the JEV SA14-14-2 strain. It was developed at the Walter Reed Army Institute of Research using the SA14-14-2 strain passaged eight times in primary dog kidney cells, cultivated in Vero cells, and then formulated with 0.1% aluminum hydroxide adjuvant. The vaccine is administered in two doses, and it has been shown to have a good immunogenicity profile in a short-term setting but requires boosters as the long-term protection only lasts about six years (Halstead and Thomas 2010, Amicizia et al. 2019, Kling et al. 2020). Another JEV strain for inactivated JE vaccines is the wild-type Beijing-1 strain, which was found superior in eliciting neutralizing antibody responses than the prototype

Nakayama strain in a field study (Ku et al. 1994). At least three licensed inactivated JE vaccines have been derived from the Beijing-1 strain propagated in Vero cells, including the JEBIKV®, ENCEVAC®, and TC-JEV® vaccines (Hegde and Gore 2017). The three inactivated vaccines are implemented in the pediatric immunization programs in Japan and South Korea (WHO 2023c, WHO 2023d).

Inactivated vaccines play a vital role in the control of TBEV in Europe. There are five different inactivated TBE vaccines licensed in Europe and Russia. The FSME-Immun® (Pfizer) and the Encepur® (GlaxoSmithKline) vaccines are both prepared using the European subtype of TBEV (Barrett et al. 2003). The FSME-Immun® vaccine contains the Neudoerfl strain and was first licensed in 1976 for people residing in endemic or high-risk areas. In 2021, the TicoVac® vaccine, analogous to the FSME-Immun® vaccine, received FDA approval for use in individuals at one year of age or older, targeting healthcare and laboratory workers, travelers, military personnel, and dependents in the U.S. (Ehrlich et al. 2003, Pfizer 2023). The Encepur® vaccine was prepared from the Karlsruhe (K23) strain (Amicizia et al. 2013). The FSME-Immun® and Encepur® vaccines have adult and pediatric formulations that differ in antigen concentrations. Three inactivated TBEV vaccines manufactured in the Russian Federation are based on the Far Eastern subtype. The Chumakov Federal Scientific Center for Research and Development of Immune and Biological Products of the Russian Academy of Sciences produces the TBE Moscow vaccine and the Tick-E-Vac® vaccine using the Sofjin strain. The EnceVir® vaccine (Microgen) is prepared from the 205 strain (Amicizia et al. 2013).

All five vaccines require at least three doses on the conventional vaccine schedule, followed by a booster every three years. Immunization with the inactivated TBE vaccine prepared from the European subtype elicits neutralizing antibody responses effective for the Far Eastern and

Siberian subtypes (Orlinger et al. 2011, McAuley et al. 2017). Deploying inactivated vaccines led to a significant decrease in TBE mortality in Austria. Before the introduction of the annual vaccination in 1981, about 600 cases of TBE were seen every year in Austria. By the 2000s, TBE cases significantly dropped, with only 41 cases in 1999 and 60 cases in 2000. To date, over 90% of Austrians have received at least one dose of the vaccine, and at least half of the population is reported to receive routine boosters (Demicheli et al. 2009).

Kyasanur forest disease virus is another tick-borne virus with an inactivated vaccine for use in India; however, controlling KFDV has not been very successful. The first KFDV vaccine (RSSEV) was a formalin-inactivated vaccine produced by the Walter Reed Army Institute of Research at the request of the Indian Council of Medical Research in the 1950s. However, RSSEV later proved not to prevent KFD. Subsequence attempts led to a formalin-inactivated chick embryo fibroblast vaccine in the 1990s (Dandawate et al. 1980, Holbrook 2012). The vaccine has been shown to elicit significant protective effects and was licensed in India (Dandawate et al. 1994). Optimal vaccination response included two doses over one month, followed by a first booster 6-9 months later, then annual boosters every five years. The disease incidence should have dropped 62% after two doses and 83% after the first booster. With no reported cases of human-to-human transmission, all susceptible populations needed to be vaccinated. However, of the 35,500 targeted population, only 32% received the first dose, and 13.2% received two doses. Further poor administration of the first booster and annual boosters, 4.9% and 0.5%, respectively, led to insufficient vaccine coverage. The vaccination plan did not consider suitable times for receiving the vaccines, maintaining an adequate stock, or raising awareness of the vaccines (Kasabi et al. 2013, Oliveira et al. 2019).

Although no human WN vaccine is available, inactivated WN vaccines have become one of the four core vaccines recommended by the American Association of Equine Practitioners (AAEP 2022). There are three inactivated veterinary WN vaccines licensed in the U.S., including the WN Innovator® (Zoetis), Vetera® WNV (Boehringer), and Prestige®WNV (Merck Animal Health) vaccines. Both the WN Innovator® and Vetera® WNV vaccines consist of inactivated WNV virion, whereas the Prestige® WNV vaccine contain the inactivated ChimeriVax-WN01 vaccine virus (Tesh et al. 2002, Chaintoutis et al. 2015). All three inactivated vaccines require annual boosters in adult horses to maintain the level of neutralizing antibodies. A live chimeric virus combines the WNV prM/E into a canarypox backbone, marketed as the Recombitek™ Equine WNV vaccine (Pfizer Animal Health) (Grosenbaugh et al. 2004). Although there is no clear evidence that canarypox-based vaccines can replicate in horses, some studies have shown that canarypox-based WNV vaccines elicit cell-mediated and humoral immune responses (Garch et al. 2008, Kaiser and Barrett 2019).

In contrast to using inactivated vaccines to prevent WN equine encephalitis, there is limited progress in developing a human inactivated WN vaccine. Neither the formalin-inactivated candidate WN vaccine nor the hydrogen peroxide-inactivated candidate WN vaccine showed sufficient immunogenicity with less than 50% seroconversion at 15 days after a second dose of the vaccine (NIH 2019, Gould et al. 2023). Other technologies have also been applied to the development of candidate WN vaccines. For example, the WN-80 candidate vaccine is a recombinant subunit vaccine based on the *Drosophila* S2 cell expression system. This subunit vaccine includes a truncated E protein at the C-terminus, leaving only 80% of the native E protein (80E). The truncation removes the membrane anchor portion of the protein, allowing the E protein to be secreted into the extracellular medium (Lieberman et al. 2007). The WN-80 vaccine has

shown to be highly effective in generating a neutralizing antibody response in geese and rhesus monkeys, but little research has been done on its effect on humans. The phase I clinical trials occurred in 2009, but the data have never been published (Jarvi et al. 2008, Lieberman et al. 2009, NIH 2009). The WNV prM-E DNA vaccine produced by Vical and NIAID is a single-plasmid, recombinant DNA vaccine consisting of a closed, circular DNA plasmid vector incorporating a cytomegalovirus promotor along with the WNV prM and E proteins downstream from a modified JEV signal sequence. The phase I clinical trials showed the vaccine to be safe and well-tolerated with no significant adverse effects, along with neutralizing antibody responses shown to be protective in studies with horses (Martin et al. 2009, Amanna and Slifka 2014, NIAID 2015).

Many groups continue to investigate DENV vaccines, and some have looked at alternative methods to produce a vaccine, including subunit and DNA vaccines. The subunit vaccine made by Hawaii Biotech Inc. and Merck is called DEN1-80E. This vaccine includes a truncated DENV E protein containing only 80% of the N-terminal E protein. This modification removes the transmembrane sequence of the protein, creating an E protein that has improved secretion, purification, and immunogenicity. However, the vaccine is still in phase I clinical trials (Torresi et al. 2017). The US Naval Medical Research Center developed the nucleic acid vaccine called D1ME100 vaccine. The vaccine includes a monovalent DNA plasmid with prM and E genes of the DENV-1 virus. This allows for the production of prM and 92% of the E protein, introducing antigenic targets that initiate the development of neutralizing antibodies. To make a vaccine that was effective against all four serotypes, a tetravalent DNA vaccine was developed called Vaxfectin®. This vaccine has been through Phase I trials and has significantly improved immunogenicity (Torresi et al. 2017, Danko et al. 2018). The Walter Reed Army Institute of Research and GlaxoSmithKline have also developed purified inactivated vaccine (PIV) candidates

for DENV. Their version for DENV-1 has shown in clinical trials to be safe and immunogenic, with a consistent induction of antigen-specific T helper cells that can produce neutralizing antibodies. Recent studies have shown the development of a PIV for DENV-2 that has shown promising results in mice. The Walter Reed Army Institute of Research aims to use these vaccines to create a tetravalent DENV PIV that could be used for a broad age range and health status (Martinez et al. 2015, Friberg et al. 2020, Wu et al. 2022).

While inactivated vaccines have been successfully used in the control programs for human JE, human TBE, and equine WN encephalitis, low immunogenicity requires multiple doses for immune protection. Multi-dose regimens increase immunization costs and often become a limiting factor for marketing inactivated vaccines. For example, the inactivated YF candidate vaccines developed in Brazil and Russia require at least two doses. They are better suited for the immunization of travelers than implementing routine immunization programs or outbreak responses (Hayes 2010). The more cost-effective mRNA vaccine technology program to deliver the structural proteins as immunogens may offer a potential solution for the high cost associated with multiple diseases. For example, a ZIKV mRNA vaccine using nucleoside-modified ZIKV prM and E mRNA in lipid nanoparticles has been shown to elicit neutralizing antibodies in rhesus macaques for up to 12 weeks post-immunization; however, it has not yet been proven in clinical trials (Pardi et al. 2017).

D. Rational design of live attenuated vaccines

Only the empirically derived YF 17D and JE SA14-14-2 LAVs can elicit protective immune responses with one immunization dose among the licensed and candidate flavivirus vaccines. However, the empirical attenuation process used to develop the YF 17D and JE SA14-14-2 LAVs often cannot reach the balance of attenuation and immunogenicity in many pathogenic

flaviviruses (Eckels et al. 1984, McKee Jr et al. 1987). Critically, the empirically derived LAVs often do not have a defined molecular basis of attenuation, which regulators expect in the 21st century. Such expectations will be accomplished by the rational design of candidate LAVs produced using the infectious clone technology to encode attenuating mutations. The pattern of mutations in naturally occurring flavivirus attenuated isolates and the licensed LAVs suggests that a safe level of attenuation in flaviviruses requires multigenic mutations (Davis et al. 2007, McElroy et al. 2008). The flavivirus RNA-dependent RNA polymerase is naturally error-prone, so multigenic mutations will ensure the attenuated phenotype does not revert to the wild-type sequence (Arroyo et al. 2004, Kaiser et al. 2017). The most effective approach to rationally designing candidate LAVs will involve common attenuating mutations effective for a broad range of flaviviruses. Most of the broadly effective attenuating mutations have been identified in the flavivirus-shared motifs that encode the common structure-function of NS proteins, including the NS1, NS4B, and NS5 proteins.

One practical approach to engineer attenuating mutations in the NS1 protein is to target the glycosylation sites. Flaviviruses in the JEV serocomplex have three asparagine residues that act as N-linked glycosylation sites in the NS1 protein. In contrast, the NS1 protein of other flaviviruses has two N-linked glycosylation sites. Studies have shown that the glycosylation sites on NS1 are essential residues for the structure and function of the protein. Interference with the glycosylation of NS1 protein was first shown to attenuate the neurovirulence phenotype of TBEV/DENV-4 chimera (Crabtree et al. 2005). The attenuating effect was then demonstrated in two mosquito-borne flaviviruses using the model system of the YFV 17D strain and DENV-2 16681 strain. Further, the multi-site mutations that eliminate all glycosylation sites in the E and NS1 proteins fully attenuated the neurovirulence and neuroinvasive phenotype of WNV in mice (Whiteman et

al. 2011). Mutations of the glycosylation sites in the NS1 protein impair virion morphogenesis, causing the accumulation of the E protein in the ER. One crucial observation is that the fully attenuated WNV mutants lacking the E and NS1 glycosylation sites can still elicit neutralizing antibody responses, suggesting that these broadly effective attenuating mutations are unlikely to compromise the immunogenicity of WNV (Whiteman et al. 2010).

The discovery of attenuating mutations in the NS4B protein involved sequencing the naturally occurring mutations in NS4B and analyzing the mutations by site-directed mutagenesis guided by the predicted structure (Davis et al. 2007). The consensus sequence of the Bird1153 isolates from Texas compared to the NY99 strain identified a combination of mutations in the nonstructural proteins and 3' UTR, including the NS4B mutation E-249G (Davis et al. 2007). Specifically, the mutation E249G showed to reduce RNA synthesis in host cells, contributing to a decrease in virulence and promoting immunogenicity (Botha et al. 2008). Site-directed mutagenesis of the NS4B protein guided by the predicted structure identified two domains for the engineering of attenuating mutations. The NS4B-P38G mutation in the N-terminal domain can lead to the temperature-sensitive phenotype in cell culture and attenuate the mouse neuroinvasive phenotype of WNV (Wicker et al. 2012). Amino acid 35 to amino acid 60 of the NS4B protein resembles the structure of an immunomodulatory tyrosine inhibitory motif, which can antagonize the type-I interferon signaling (Isakov 1997). However, the NS4B-P38G mutation alone is unstable and must be accompanied by the compensatory NS4B-T116I and NS3-N480H mutations (Wicker et al. 2012).

In comparison with the NS4B-P38G mutation, the site-directed mutagenesis of the four flavivirus conserved cysteine residues in the NS4B protein identify the single NS4B-C102S mutation, located in the central hydrophobic transmembrane domain, can attenuate both the

neuroinvasive and neurovirulence phenotype of WNV (Wicker et al. 2006). Mutations in the equivalent regions of NS4B protein have also been shown to exhibit attenuating effects in DENV-4, YFV, and JEV (Pletnev et al. 2002, McElroy et al. 2006a). Such knowledge was then applied to the rational design of candidate ZIKV LAVs, showing that the equivalent NS4B-C100S mutation can produce a fully attenuated and immunogenic mutant based on the FSS130025 strain (Wicker et al. 2006, Li et al. 2019).

One attenuation process that can potentially be effective for multiple flaviviruses is to target the structure-function of the methyltransferase domain encoded in the NS5 protein. Engineering of attenuating mutations in the methyltransferase domain of the NS5 protein was first undertaken in the model system of DENV-2. The comparable NS5 mutations E216A in DENV-1 and E217A in DENV-2 have also been shown to elicit strong immune responses, with E217A even showing reduced vector infectivity in *Ae. aegypti* mosquitoes (Züst et al. 2013). The double NS5-K61A and NS5-E217A amino acid mutations were shown to fully attenuate the virulence phenotype of the DENV-2 TSV01 strain in immunocompromised AG129 mice. The attenuating effect was proven in all four serotypes of DENV using mutations at the equivalent DENV-1, -3, and -4 residues (Züst et al. 2013). However, the same double mutations were unstable in WNV as the NS5-K61A and NS5-E218A double mutants reverted to the wild-type sequence (Kaiser et al. 2019a). Intriguingly, the single NS5-K61A and NS5-E218A mutations still exhibit an attenuating effect on the neuroinvasive phenotype of WNV, suggesting that 1) single mutations in the NS5 methyltransferase domain have the potential to support the rational design of candidate WNV LAVs and 2) the potential difference in the structure-function of NS5 protein may affect the stability of attenuating mutations across different flaviviruses. While both mutations individually showed promise as attenuating mutations, the WNV NS5-E218A mutation showed the most

promise. Previous research has shown the WNV NS5-E218A mutation has attenuation potential in other flaviviruses, suggesting it could be a broadly attenuating flavivirus mutation (Kaiser et al. 2019a).

One common attenuating mutation in the structural protein is the YFV M-L36F mutation encoded in the 17D vaccine strain. The M-L36F mutation can significantly attenuate the mosquito infection phenotype of the wild-type Asibi strain in *Ae. aegypti*. The equivalent JEV M-I36F and WNV M-I36F mutations can also attenuate the neurovirulence phenotype of JEV in mice (Basset et al. 2020). The mutation attenuated the JEV phenotype *in vivo*, resulting in the production of neutralizing antibodies (de Wispelaere et al. 2016). Despite the role of the major structural protein in the flavivirus virion, there have been few broadly effective attenuating mutations identified in the E protein. To date, attenuating mutations in the E protein have been mainly described in two regions: the flavivirus-conserved fusion loop in EDII and the upper lateral surface of EDIII. While the E-L107F mutation in the fusion loop was shown to be important for the attenuated phenotype of JEV SA14-14-2 strain, engineering of equivalent mutations in either WNV or DENV-2 did not produce a stable attenuated phenotype and often can lead to reversion or compensatory mutations (Zhang et al. 2006, Huang et al. 2010, Yang et al. 2017). Few of the attenuating mutations in the EDIII are located at flavivirus-conserved residues (Lee and Lobigs 2008). Additionally, the utility of attenuating mutations in the upper lateral surface of EDIII remains unclear, as the YFV E-T380R mutation was reported to increase the infectivity in *Ae. aegypti*, raising concern over the potential transmission of vaccine viruses (Huang et al. 2014).

1.4 Structure-function of the E protein and the rational design of candidate live-attenuated vaccines

Fundamental knowledge of the structure-function of the E protein has a unique importance in flavivirus vaccine development. The flavivirus E protein is a major structural protein in the virion and plays a significant role in various stages of the viral life cycle. Firstly, the E protein is a class II fusion protein that mediates the receptor binding and membrane fusion of the flavivirus virion with the host cell (Vaney et al. 2022). The virus-cell membrane fusion process acts as the flavivirus-conserved mechanism of viral entry. The cell entry process is crucial for flavivirus pathogenesis, making the E protein a significant component in developing an attenuation strategy for all flaviviruses. The E protein is also the principal target of neutralizing antibodies, the most reliable correlate of protection against flaviviruses (Teoh et al. 2012, Lu et al. 2019). A detailed understanding of the structure-function of the E protein involved in the membrane fusion process and antibody-mediated neutralization can support the rational design of candidate LAVs that reach the balance of safety and immunogenicity. The E protein exhibits up to 40% amino acid sequence homology between flaviviruses (Hung et al. 2004). Significantly, detailed knowledge of flavivirus-conserved residues involved in the membrane fusion mechanism and antibody-mediated neutralization provides a common strategy for rationalizing candidate LAVs for a broad range of flaviviruses.

A. Structure-function of the E protein involved in the flavivirus-host cell membrane fusion process

Enveloped viruses enter the host cell via the membrane fusion process. One or multiple structural proteins catalyze the fusion between viral and cellular membranes to release the viral genome into the cytoplasm. There are three classes of fusion proteins, each involving a distinct

membrane fusion mechanism to expose and insert the fusion loop structure into the host cell membrane. Class I fusion proteins include influenza HA protein and coronavirus spike protein. Before membrane fusion, class I fusion proteins are oriented in a homotrimeric arrangement. Each monomer is a single-chain precursor protein (HA0) folded in half and connected by two disulfide bonds to become HA1 and HA2 (Boonstra et al. 2018, Lousa and Soares 2021). Binding to a cellular receptor on the cell membrane inducing viral uptake by endocytosis, followed by a massive irreversible conformational change of each monomer, is triggered by the acidic pH of the endosome. The conformational change releases the disulfide bonds HA1 dissociates from HA2, exposing the N-terminal fusion peptide on each monomer inserted into the cellular membrane. The glycoprotein ends move toward each other, bringing cellular and viral membranes together until they are fused to make a fusion pore (Boonstra et al. 2018, Lousa and Soares 2021).

Class III fusion proteins include vesicular stomatitis virus glycoprotein and herpes simplex virus gpB protein. Class III proteins are oriented in a homotrimeric formation that spikes from the virion surface, like class I fusion proteins. Binding to the cellular ligand (likely proteoglycans due to the mechanism evolving to low pH environments) initiates a conformational change that extends the glycoprotein subunit with the fusion loop towards the target membrane. After extension and reformation of a trimeric formation, the protein collapses, so the two membranes come together. What is unique about the class III fusion proteins compared to the class I or class II is that the conformational change of extension and trimerization is reversible. However, hemifusion of the viral and cellular membrane and fusion pore formation is irreversible (Harrison 2015, Kim et al. 2017).

All arbovirus envelope proteins are classified as class II fusion proteins. The flavivirus E protein's primary function is to act as a class II fusion protein and fuse the viral and cellular

membranes to allow the viral RNA into the cell cytoplasm (Figure 1-18). In a neutral pH outside the cell, the E protein exists in a dimerized formation parallel to the virion surface (Figure 1-19). During this moment, the cd loop or the “fusion loop” at the end of the EDII domain is concealed and protected against the virion surface. Once the virion is exposed to a target cell, the EDIII domain will bind to the cellular receptor. Binding to the cell receptor eventually causes the cell to endocytose the virus through clathrin-mediated endocytosis, where the virion ends up in an endosome (Hackett and Cherry 2018). The acidic environment of the endosome initiates the first movement of the conformational change of the E protein.

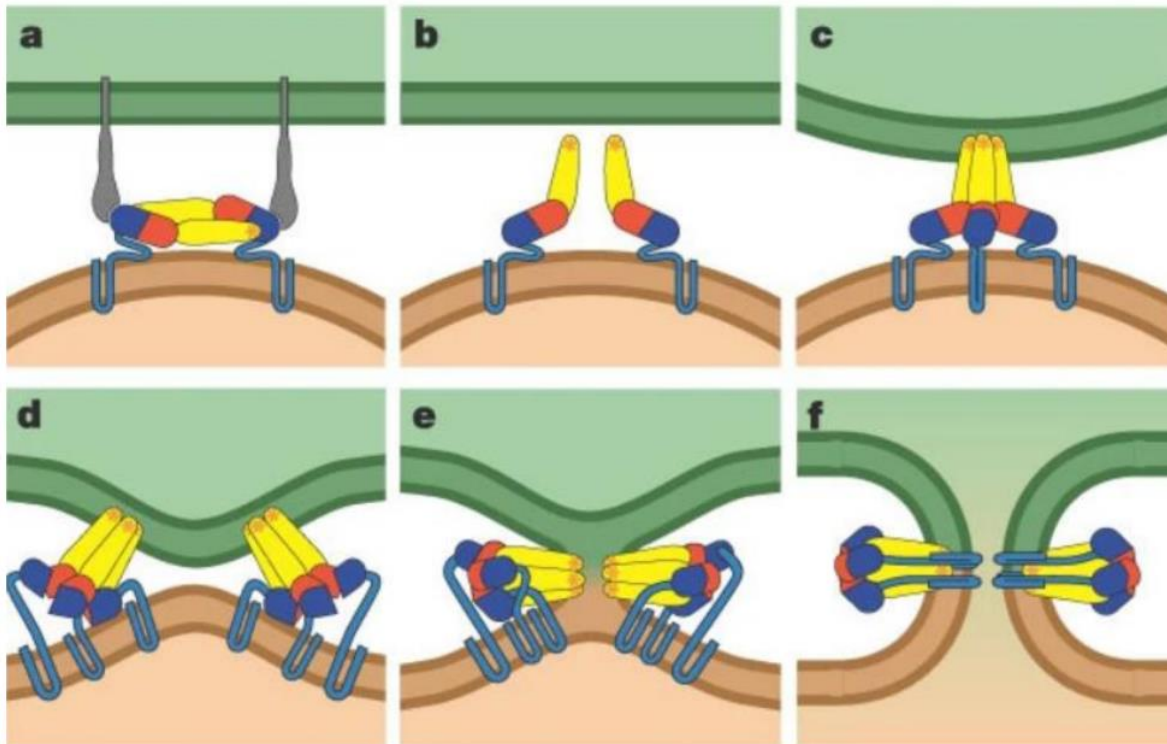


Figure 1-18: Fusion of the viral membrane with the cellular membrane by the flavivirus class

II fusion protein

(a) E protein binds to the cell receptor and starts internalization into the endosome. (b) The reduced pH in the endosome causes the first conformational change at the EDI-EDII hinge region and exposes the fusion loop to the cellular membrane. (c) The fusion loop inserts itself into the cell membrane, initiating the formation of a trimer. (d) The second conformational change initiates with the EDIII domain shifting and rotating back towards the fusion loop to create trimer contacts. (e) Creation of more trimer contacts along the EDII domain that leads to hemifusion. (f) Formation of the lipidic fusion pore. Image taken from *Nature*, Volume 427, Y Modis et al., "Structure of the dengue virus envelope protein after membrane fusion." 313-319. (2004); with permission from Springer Nature.

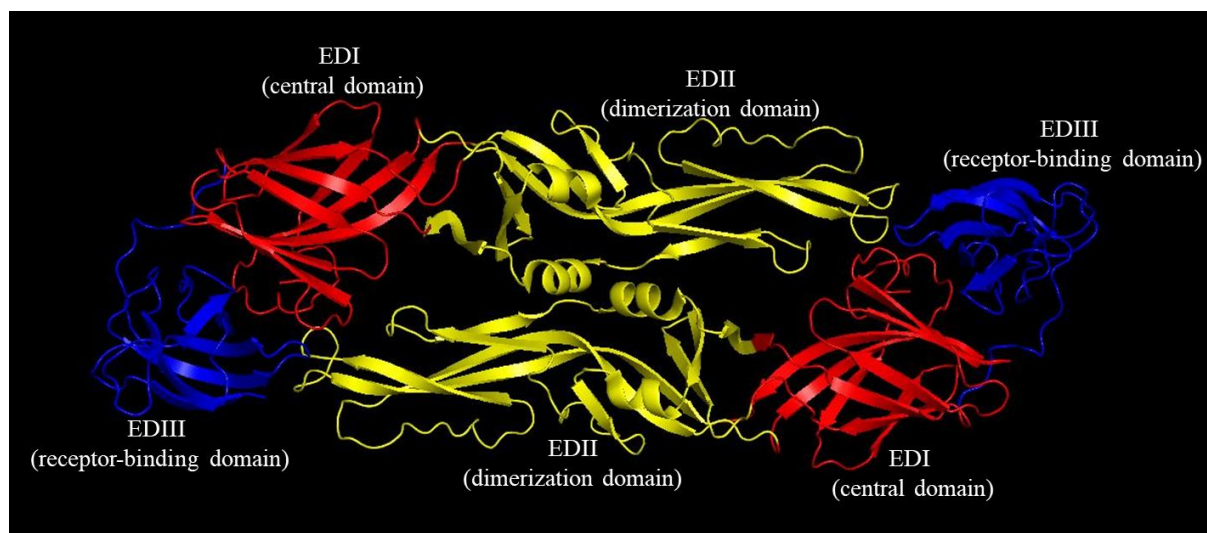


Figure 1-19: The crystal structure of the flavivirus E protein dimer

The crystal structure of DENV-2 E protein dimer (PDB ID: 1OAN). The domains are labeled red (EDI), yellow (EDII), and blue (EDIII). The image was generated in PyMOL.

The first movement involves the EDII domain undergoing a 30° rotation using the structurally central EDI domain as a central anchorage point. The E protein region responsible for the EDII domain's movement is the interdomain region between EDI and EDII. The four motifs that encode for this region are known as the EDI-EDII hinge region due to the molecular hinge-like motion responsible for the exposure of the fusion loop to the cellular membrane. The EDI-EDII hinge region is functionally vital as mutation to the region can cause defects to the membrane fusion process and alter the ability of the virus to enter a cell (Hurrelbrink and McMinn 2003). Within the EDI-EDII hinge region is a pocket of hydrophobic amino acids that is a hinge point for the fusion-activating conformation change near the k1 β -hairpin. A shift in the k1 β -hairpin opens the hydrophobic pocket, acting like a “greased hinge” and allowing the EDII domain to hinge away from its dimer partner and project the fusion loop at its tip away from the virion surface and toward the cellular membrane. The dissociation process into monomers is irreversible after exposure to

an acidic pH environment (Allison et al. 1995, Allison et al. 2001, Kuhn et al. 2002, Modis et al. 2003).

The movement of EDII exposes the fusion loop to the extracellular space before associating with the cellular lipid membrane, catalyzing an irreversible E protein trimer configuration formed at low pH (Stiasny et al. 2002). As the EDI-EDII hinge region moves to produce an elongated intermediate structure, the EDIII domain undergoes the most significant displacement in the dimer-to-trimer transition. The EDIII domain shifts 70° toward the gravity center of the E protein and 39 Å closer to the fusion loop. The region responsible for this movement is the single motif between EDI and EDIII, known as the EDI-EDIII linker. This small interdomain linker, ten residues long, inserts itself into the EDI domain as a short β strain between the strands A_0 and C_0 . As a result of this arrangement, the C-terminal region of A_0 peels away from C_0 . It switches to the other β -sheet, creating the surface for an annular trimer contact with the two different A_0 strands within the trimer. This connection stabilizes the trimers into an irreversible conformation that can exhibit membrane fusion activity (Modis et al. 2004).

The formation of the contacts between the three E proteins eventually spreads from the fusion loops at the trimer tip in EDII to the base of EDI. Once complete, the EDIII domain shifts and rotates, folding the trimer toward the fusion loop. The EDI-EDIII linker allows for rotation of the individual EDIII, allowing for the spontaneous symmetry-breaking movement required. The movement occurs until an exchange of β -strands locks the final trimer interaction of EDI and the final folded-back position of EDIII. The free energy released by this refolding can drive the viral and cellular membranes toward each other (Russell et al. 2001).

The “hemifusion stalk” formation is an essential intermediate in the membrane fusion process. Hemifusion can occur at any point during the conformation change of folding back the

trimer formation. Still, it has to be completed and fully form the fusion pore before the transmembrane segments of the E protein trimer reach their final positions around the periphery of the fusion loops. While the hemifusion stalk will open the narrow fusion pores, migration of the transmembrane segments will prevent the pore from closing (Razinkov et al. 1999). In the final stage, the stems are docked along the surface of EDII with the fusion loop, while the transmembrane anchors are now next to each other in a fused membrane (Bressanelli et al. 2004).

The conformational change of the flavivirus E protein from dimer in parallel to the virion surface to trimer inserted into the host cell has been investigated in detail using two model systems, DENV-2 and TBEV (Bressanelli et al. 2004, Zhang et al. 2012). The X-ray crystal structures of the pre-fusion and post-fusion conformations of the respective flavivirus E proteins show the dimer-to-trimer transition involves the rearrangement of three domains (Figure 1-20). Specifically, the interdomain movements of the E protein involve the relocation of EDII and EDIII about the structurally central EDI. The EDI-EDII interdomain movement facilitates the homodimer into two monomers and orients the EDII towards the endosomal membrane. Dissociation of the E protein dimer releases the fusion loop from the protected position created by a neighboring monomer (Modis et al. 2004). Three copies of E protein monomers are assembled into an elongated trimeric intermediate structure, which inserts the fusion loop into the host cell membrane and establishes the physical interaction required for the virus-cell membrane fusion. However, the elongated trimeric intermediate structure is metastatically unstable. The relative movement of EDIII against EDI forms a compact E protein trimer that exhibits low free energy. Therefore, the conformational change of the E protein from dimer in the virion to trimer inserted into the host cell is an irreversible process. The end product is a fusion pore structure formed with the virus and cell membranes and

inserted with the E protein trimer. The fusion pore then acts as a tunnel to release the viral genome into the cytoplasm (Modis et al. 2004).

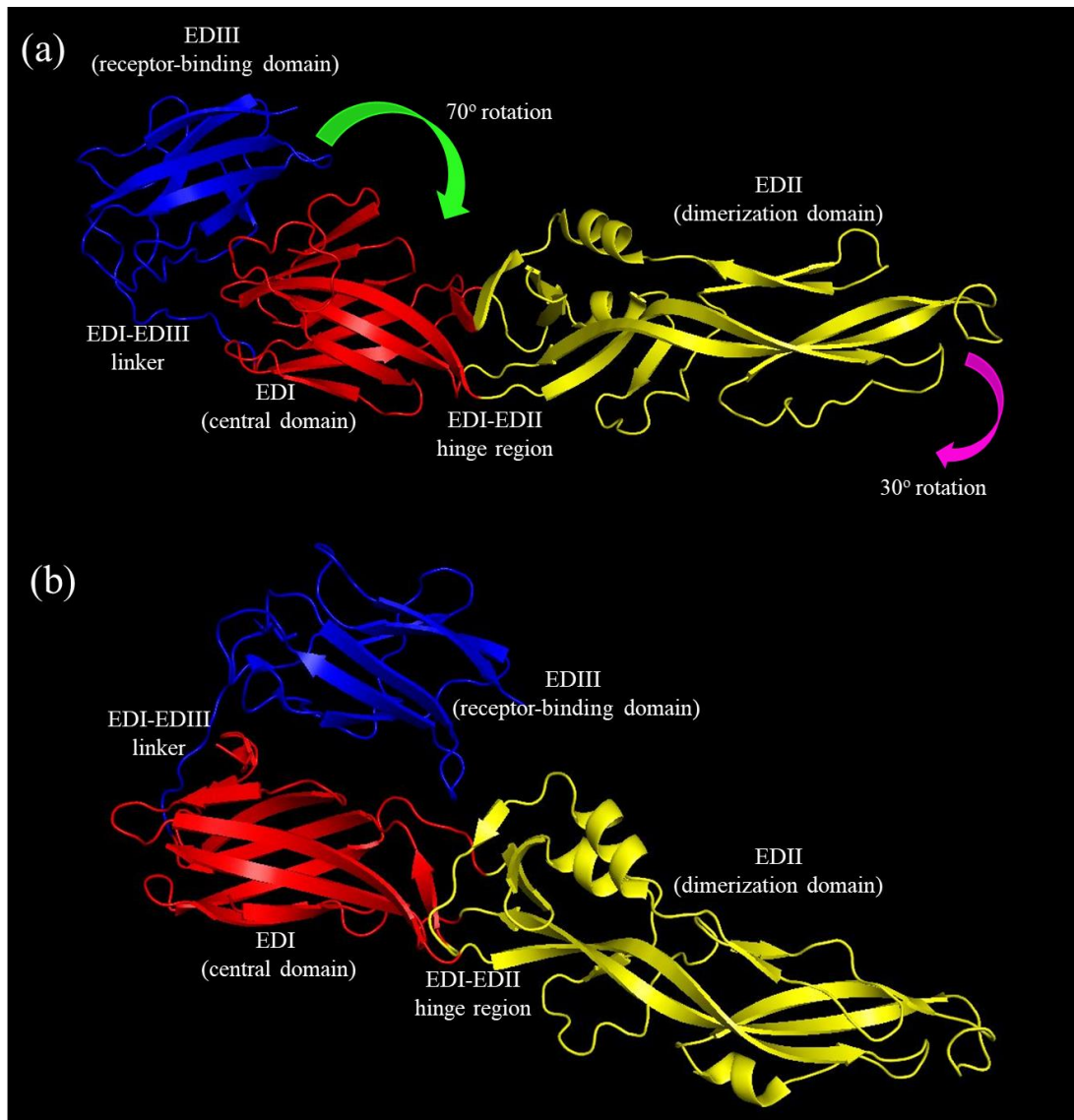


Figure 1-20: Crystal structure of the conformational change of the DENV-2 E protein monomer

Domains are labeled red (EDI), yellow (EDII), and blue (EDIII). (a) The conformation of the E protein before membrane fusion occurs in a neutral pH environment (PDB ID: 1OAN). (b) The conformation after membrane fusion is complete in an acidic pH environment (PDB ID: 1OK8). The image was generated in PyMOL.

Specifically, the EDI-EDII interdomain movement is controlled by the four motifs exhibiting the hinge effect at the domain interface, hence, the EDI-EDII hinge region. The four motifs of the EDI-EDII hinge region are encoded by discontinuous sequences, including motif #1 (amino acid 50-54), motif #2 (amino acid 130-135), motif #3 (amino acid 191-201), and motif #4 (amino acid 269-281) in WNV (Kanai et al. 2006). The EDI-EDII hinge region shows amino acid sequence homology between different flaviviruses. Even though the four motifs of the EDI-EDII hinge region are encoded in discontinuous sequences, the eight hydrophobic amino acid residues will interact with each other in the folded E protein to create the hydrophobic pocket that is essential to the “hinge” movement of the conformation change that marks the beginning of the trimeric formation. The EDI-EDIII interdomain movement is controlled by the single motif (amino acids 295-304) in WNV that links the respective domains, hence the EDI-EDIII linker. The amino acid sequence of the EDI-EDIII linker shows a high degree of sequence homology. The flavivirus conserved residues in the EDI-EDII hinge region, and the EDI-EDIII linker constitute the common motif in the membrane fusion process (Modis et al. 2004, Kanai et al. 2006). Mutations of the flavivirus conserved residues in the respective interdomain regions can potentially impair the common structure-function of the E protein required for viral entry and have the potential to attenuate the virulence phenotype of a broad range of flaviviruses. Broadly, the relative movement of EDII and EDIII about the EDI is conserved across all class II fusion proteins.

Therefore, investigating the flavivirus conserved structure-function of the E protein involved in the membrane fusion process will establish a common attenuation strategy and provide a model system to elucidate the membrane fusion mechanism of different arboviruses. Such information may ultimately provide the molecular basis for developing a broadly effective attenuation process for arboviruses, including emerging alphaviruses and bunyaviruses.

B. Flavivirus conserved E protein residues and antibody-mediated neutralization

Mutations suited for the rational design of candidate LAVs for flaviviruses (or any virus) should not compromise immunogenicity. The E protein is the major target for neutralizing antibodies. Furthermore, the EDI-EDII hinge region and the EDI-EDIII linker contain residues that are part of the epitopes recognized by human-neutralizing antibodies. Residues in the EDI-EDII hinge region often comprise the type-specific neutralizing epitopes encompassing multiple domains of the E protein. For example, the potently neutralizing monoclonal antibody (mAb) CR4354 isolated from a recovered patient binds to the interface of two neighboring E protein homodimers, which are part of the herringbone pattern (Figure 1-21) (Kaufmann et al. 2010).

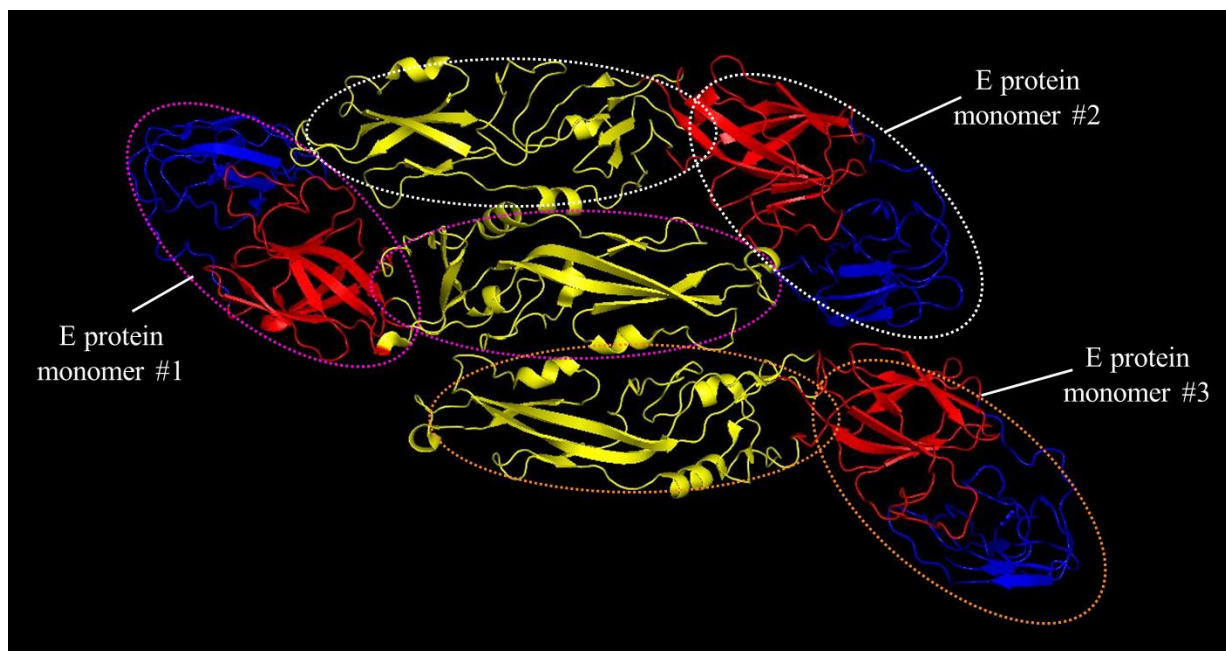


Figure 1-21: Herringbone pattern of JEV E protein dimers on the virion surface (PDB ID: 3J27)

Domains are labeled red (EDI), yellow (EDII), and blue (EDIII). There are three copies of the E protein monomer. Two copies of the E protein monomer arranged in the antiparallel direction from the E protein homodimer are labeled “E protein monomer #1 and #2.” For ease of viewing, only one copy of the E protein monomer in the neighboring E protein homodimer is labeled “E protein monomer #3.” Each E protein monomer is depicted with two dashed circles (white, magenta, and orange) to show the herringbone pattern. The image was generated in PyMOL.

The binding site includes the EDI-EDII hinge region of the first homodimer and the EDIII of the neighboring homodimer. One conformational neutralizing epitope shared between wild-type YFV and the 17D vaccine strain involves residues in motif #1 of the EDI-EDII hinge region (Lu et al. 2019). Similarly, motifs #1 and #4 of the EDI-EDII hinge region are the conformational epitopes of the potentially neutralizing DENV-1-specific human monoclonal antibody (Table 1-3) (Teoh et al. 2012).

Table 1-3: Fab HM14c10 epitope on DENV-1 E protein

Bold protein residues indicate residues common in both epitopes.

Fab	Envelope protein molecule in the asymmetric unit	Envelope protein domain	Envelope protein residues
HM14c10 (I)	A	I	T51 , L135, K136, G159 , T160 , T165 , P166 , Q167 , E172 , I173 , T275
	A	II	N52 , G274
	B	III	K310 , E384, K385
HM14c10 (II)	B	I	T51 , Q131, Y132, G159 , T160 , T165 , P166 , Q167 , E172 , I173 , L175, T275
	B	II	N52 , G274
	C	III	L308, K310

Residues in the EDI-EDIII linker in the structure-function of the E protein are also part of multiple conformational neutralizing epitopes and have been best investigated in DENV. Two quaternary neutralizing epitopes in the DENV-2 E protein involve residues in the EDI-EDIII linker. The E-S300 residue at the C-terminus of the EDI-EDIII linker is crucial for the antigenic structure recognized by human anti-DENV-2 monoclonal antibody 2D22. Moreover, the other human anti-DENV-2 monoclonal antibody, 3F9, recognizes the E-L294 residue at the N-terminus of the EDI-EDIII linker (Gallichotte et al. 2018). Similarly, the N-terminus of the EDI-EDIII linker is also crucial for the human neutralizing antibody response raised against DENV-3, as demonstrated with the binding and neutralization of nine human monoclonal antibodies isolated from patients recovered from natural infection (Young et al. 2020). Type-specific neutralizing epitopes are crucial for neutralizing antibody responses and vaccine protection (Rosenzweig et al. 1963, Mason et al. 1972, Henein et al. 2021).

In summary, investigating mutations of flavivirus conserved residues in the EDI-EDII hinge region and the EDI-EDIII linker in animal models will provide the knowledge critical for the attenuation and immunogenicity of candidate flavivirus LAVs. If successful, such mutations can potentially achieve a superior breadth of attenuating effect compared to mutations in the E protein discovered to date. Despite the significant effort, very few published mutations in the E protein have shown utility for vaccine development across a broad range of flaviviruses. Based on the E-L107F mutation in the E protein of the JEV 14-14-2 strain, several site-directed mutagenesis studies were undertaken to engineer broadly effective attenuating mutations among the flavivirus conserved residues in the fusion loop. Although the attenuated phenotype is translatable to DENV-2 and WNV, mutations of the flavivirus-conserved residues in the fusion loop are unstable and can revert to the wild-type sequence. Therefore, mutations of the flavivirus conserved residues in the fusion loop may not be suited for the rational design of LAVs for a broad range of flaviviruses. Multiple site-directed mutagenesis studies have also targeted residues within the upper lateral surface of EDIII, the receptor binding region for all flaviviruses. While the attenuated phenotype was observed in the neuroinvasive disease mouse model for several mosquito-borne encephalitic flaviviruses, e.g., JEV, WNV, and the immunocompromised mouse model for YFV, it is unclear if mutations in the EDIII can be applied to the rational design of candidate flavivirus LAVs, as the YFV E-T380R mutation was shown to enhance the mosquito competence phenotype in *Ae. aegypti*.

1.5 Central hypothesis, model systems, and rationale of this project

A. Central hypothesis

Compared to mutations within the fusion loop and EDIII identified from empirically derived LAVs or serially passaged mutants, our approach targets the flavivirus conserved residues

in the EDI-EDII hinge region and EDI-EDIII linker. Our approach is supported by the structural biology and biophysics of the flavivirus E protein and has the potential to provide a defined molecular basis for attenuation. *The central hypothesis of this project is that mutations of the flavivirus-conserved residues in the EDI-EDII hinge region and the EDI-EDIII linker will reach the balance between attenuation and immunogenicity in mice.* We used two model systems to test this hypothesis: WNV and YFV.

B. Model system #1: West Nile virus

We selected WNV as our model system to represent neurotropic flaviviruses. Importantly, a two-plasmid reverse genetics system of the WNV NY99 strain is available to support engineering mutations in the E protein. The NY99 strain is the first isolate of WNV following its emergence in North America. Both the biological isolate and recombinant virus of WNV NY99 have been extensively characterized in several mouse models to support the investigation of attenuating effect of each mutation.

Both outbred and inbred mice are susceptible to the challenge of WNV via various routes of inoculation. The intraperitoneal (IP) and intracerebral (IC) challenges of outbred Swiss mice led to lethal neurotropic disease, reflecting the mouse neuroinvasive and neurovirulence of WNV, respectively. Outbred Swiss mice are susceptible to both the IP and IC challenge with the WNV NY99 strain, each of which exhibits a 50% lethal dose (LD₅₀) lower than 0.5 plaque forming unit (PFU) (Beasley et al. 2002). The low LD₅₀ is essential for selecting attenuating mutations by supporting high-dose challenges, e.g., 1000 LD₅₀ or greater. Another frequently used neuroinvasive and neurovirulence mouse model for WNV is inbred C57BL/6 mice. The subcutaneous challenge of C57BL/6 mice with WNV NY99 strain can lead to uniform lethality at or above 100 PFU (Diamond et al. 2003, Sitati and Diamond 2006). We chose the neuroinvasive

disease model of outbred Swiss mice to investigate the attenuation process of WNV due to the number of published studies using this model, including the fully attenuated E-NS1 deglycosylated, NS4B, NS5 mutants (Crabtree et al. 2005, McElroy et al. 2006a, Davis et al. 2007, Whiteman et al. 2011, Wicker et al. 2012, Züst et al. 2013, Kaiser et al. 2019a).

Additionally, an outbred mouse strain is more economical and will support the evaluation of each mutant with a sample size that reaches the statistical power accepted by the field (Whiteman et al. 2010, Wicker et al. 2012, Kaiser et al. 2019a). As a proof-of-concept, we demonstrated that mutations can attenuate the mouse neuroinvasive phenotype of WNV in outbred Swiss mice first. The hypothesis could then be tested in the mouse viscerotropic disease model of wild-type YFV due to the cost and logistical challenge of obtaining the immunocompromised AG129 mice (see section 1.5.C).

Broadly, WNV is an important encephalitic flavivirus in the U.S. and many other countries across six major continents. In the U.S. alone, the healthcare cost for hospitalized WN cases and individuals with permanent sequelae is estimated to exceed 56 million dollars annually. There is growing interest in revisiting the development of an effective WN vaccine (Barrett 2014, Gould et al. 2023). Our rationale is that the mutations proven to attenuate the mouse neuroinvasive phenotype of WNV will not only advance our understanding of the structure-function of the E protein but also provide the knowledge to support the rational design of candidate WN LAVs.

C. Model system #2: yellow fever virus

We chose the wild-type YFV Asibi strain as a model system to represent viscerotropic flaviviruses. Few flaviviruses can consistently produce viscerotropic diseases in mice. Our team has access to the reverse genetics system of the YFV Asibi strain. It has collaborated on the wild-type YFV viscerotropic disease mouse model using immunocompromised AG129 mice.

It is essential to distinguish the viscerotropic and neurotropic disease mouse models for YFV. Both wild-type YFV strains and the 17D vaccine strain can cause neurotropic disease in outbred and inbred mice following the IC challenge. However, YFV does not exhibit neurotropism, and the neurotropic disease mouse model does not recapitulate human YF. At least two genotypes of wild-type YFV strains have been shown to cause viscerotropic disease in immunocompromised mouse strains that lack type-I and type-II interferon receptors (Meier et al. 2009). The AG129 mice have previously been shown to succumb to the IP challenge with wild-type YFV Asibi strain at 1,000 PFU, making it an ideal model to investigate the attenuating effect of mutations in the E protein. Our rationale is that the attenuating mutations identified in the wild-type YFV Asibi strain will show that mutations of flavivirus conserved residues in the interdomain regions of the E protein can produce attenuated phenotype translatable between at least two flaviviruses. Notably, the mutations can potentially be applied to the rational design of candidate second-generation recombinant YF LAVs, which can potentially be compatible with modern cell culture technologies.

1.6 Specific aims of this project

This project selected eight flavivirus-conserved residues in the EDI-EDII hinge region and eight flavivirus-conserved residues in the EDI-EDIII linker for site-directed mutagenesis. As a proof-of-concept, we chose WNV and YFV as model systems to demonstrate that flavivirus-conserved amino acids within the respective interdomain regions are crucial for the structure-function of the E protein and the virulence phenotype in mice in three independent but related specific aims. The rationale is that mutations that demonstrate the attenuating effect on WNV and/or YFV can be translated to support the rational design of other candidate flavivirus LAVs.

Specific Aim #1: Determine the attenuating effect of mutations of the flavivirus-conserved hydrophobic residues in the EDI-EDII hinge region using the WNV neuroinvasive disease mouse model.

In specific aim #1, we focused on examining amino acid substitutions of the eight flavivirus conserved hydrophobic amino acids in the EDI-EDII hinge region of WNV about the structure-function of the E protein involved in the membrane fusion process and the mouse neuroinvasive phenotype, using a cDNA infectious clone of the NY99 strain (WNV NY99ic) (Kinney et al. 2006). The eight flavivirus conserved residues assessed for this study are the E-A54, E-I130, E-I135, E-I196, E-Y201, E-A269, E-V272, and E-L281 residues. Few published studies have comprehensively analyzed the eight flavivirus-conserved hydrophobic amino acids in the EDI-EDII hinge region, and even fewer have demonstrated that mutations of these residues can attenuate the virulence phenotype of flaviviruses in vertebrate hosts (Butrapet et al. 2011). ***Our working hypothesis is that amino acid substitutions of at least the flavivirus-conserved hydrophobic residues in the EDI-EDII hinge region will impair the structure-function of the E protein and consequently attenuate the neuroinvasive mouse phenotype of WNV.*** As such, we have undertaken the site-directed mutagenesis of each flavivirus conserved hydrophobic residues in the EDI-EDII hinge region. We have selected the single mutations that exhibit attenuating effects in the neuroinvasive disease mouse model. Further, we evaluated 19 amino acid substitutions of the selected residues to identify mutations that can balance full attenuation and immunogenicity.

Specific Aim #2: Investigate the attenuating effect of mutation of the flavivirus conserved residues of the EDI-EDIII linker using the WNV neuroinvasive disease mouse model.

In specific aim #2, we target the eight flavivirus conserved residues in the EDI-EDIII linker for the engineering of mutations that can impair the structure-function of the E protein and attenuate the mouse neuroinvasive phenotype of WNV. The eight flavivirus conserved amino acids in the EDI-EDIII linker include the WNV E-L295, E-L297, E-K298, E-G299, E-T301, E-Y302, E-V304, and E-C305 residues. Only one single publication examined the structural and functional importance of the DENV-2 E-G296, E-S298, and E-Y299 residues, which are equivalent to WNV E-G299, E-T301, and E-Y302 residues, respectively. Further, the attenuating effect of each mutation remains unknown (de Wispelaere and Yang 2012). As such, ***our working hypothesis is that amino acid substitutions of at least one flavivirus-conserved residue will fully attenuate the mouse neuroinvasive phenotype of WNV NY99ic.*** To test this hypothesis, we undertook alanine screening of the flavivirus-conserved E-L295, E-L297, E-K298, E-G299, E-T301, E-Y302, E-V304, and E-C305 residues to select putative residues for engineering of attenuating mutations, followed by engineering of alternative amino acid substitutions of the selected E protein residues and evaluating the attenuating effect of selected mutations.

Specific Aim #3: Investigate the translation of attenuating effect between WNV and YFV through site-directed mutagenesis of flavivirus-conserved hydrophobic residues in the EDI-EDII hinge region.

In specific aim #3, we undertook site-directed mutagenesis of the flavivirus conserved YFV E-A54, E-I130, E-V130, E-V188, E-Y194, E-A261, E-V264, and E-L276 residues to demonstrate that mutations of flavivirus conserved hydrophobic residues in the EDI-EDII hinge region can attenuate the virulence phenotype of multiple flaviviruses in mice. As a proof of concept, we chose the wild-type YFV viscerotropic disease model in the immunocompromised AG129 mice to determine the attenuating effect of the respective mutations (Meier et al. 2009). ***Our working***

hypothesis is that at least one single mutation of the flavivirus-conserved hydrophobic amino acids will attenuate the virulence phenotype of the wild-type YFV Asibi strain rescued from a cDNA infectious clone. We recovered the single YFV mutants containing amino acid substitutions of flavivirus-conserved residues in the EDI-EDII hinge region, followed by identifying mutations that have attenuating effects in the mouse viscerotropic disease model.

Chapter 2 - Materials and Methods

The research described in this dissertation utilized the reverse genetics system of WNV and YFV to introduce single-site mutations into the E protein gene, followed by the characterization of mutants in cell culture and mouse models. Section 2.1 contains information on the *Escherichia coli* competent cells used for manipulation of the plasmids encoding the complementary DNA (cDNA) of WNV NY99 strain and YFV Asibi strain, as well as the eukaryotic cell lines used for recovery and characterization of WNV. Section 2.2 provides information on the molecular cloning procedures used to modify the WNV and YFV E gene sequence, followed by the recovery of the recombinant wild-type parental strain and individual mutants. Section 2.3 describes the characterization of the WNV and YFV mutants in cell culture. Mouse models used to compare the virulence phenotype of the WNV mutants and YFV mutants with the respective parental wild-type strains are described in Section 2.4. All statistical methods are summarized in Section 2.5. Section 2.6 describes the molecular visualization software used for creating the protein models used in this dissertation.

The WNV EDI-EDII hinge region mutants E-A54S, E-I130N, E-I135N, E-I196N, E-Y201P, E-A269S, E-V272T, and E-L281N described in Section 2.2.A, were originally published in Ashley Bilyeu's K-State Master's thesis and further analyzed in this dissertation. The procedures used in Ashley Bilyeu's K-State Master's thesis include the plaque assay described in Section 2.3.B and the mouse neuroinvasive disease model in Section 2.4.A and 2.4.C (Bilyeu 2023).

2.1 Cell lines and competent cells

A. Bacterial competent cells

E. coli strain XL1-Blue was obtained from Agilent Technologies. The XL1-Blue was recommended for the manipulation of the WNV cDNA infection clone consisting of the pWN-AB and pWN-CG plasmids, following previously published procedures (Beasley et al. 2005, Kinney et al. 2006). Stocks were made from 16-hour overnight cultures in 2x YT media (1.6% tryptone, 1% yeast extract, 0.5% NaCl) and were preserved in 10% glycerol at -80°C. The competent cells were propagated in 2X YT broth at 37°C and 300 r.p.m. Competent cells transformed with WNV plasmids were propagated in 2X YT broth with ampicillin at the appropriate concentration. Details of the transformation and cloning procedures are listed in Section 2.2. The *E. coli* strain MC1061 was obtained from Dr. Ilya Frolov's laboratory. The MC1061 strain was the *E. coli* strain that was originally used in the construction of the YFV 17D cDNA infectious clone (pANCR-FLYF17Dx), allowing for stable maintenance of the full-length YFV genome (Brednebeek et al. 2003). The same plasmid was modified to encode the genome sequence of the wild-type YFV Asibi strain, giving rise to the pANCR-FLYFAsibi infectious clone. Competent cells for chemical transformation were propagated in 2X YT broth at 37°C and 300 r.p.m. Competent cells transformed with YFV full-length infectious clones were propagated in a mixture of 90% terrific broth (1.2% tryptone, 2.4% yeast extract, 0.4% glycerol) and 10% terrific broth salt solution (0.17 M KH₂PO₄ and 0.72 M K₂HPO₄), supplemented with ampicillin at appropriate concentrations.

B. Mammalian cells

Two mammalian cell lines were used in this dissertation work: BHK-21 cells derived from baby hamster (*Mesocricetus auratus*) kidney cells and African green monkey (*Cercopithecus aethiops*) Vero cells. Both cell lines were maintained in minimum essential media (MEM) α

supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, and MEM vitamin solution at 37°C with 5% CO₂.

C. Mosquito cells

The mosquito cell line C6/36 cells were derived from the larva of Asian tiger mosquitoes (*Ae. albopictus*). These cells were maintained in media containing an equal volume of high-glucose Dulbecco's Modified Eagle Medium and Mitsuhashi and Maramorosch insect media supplement in addition to 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine at 28°C.

2.2. Reverse genetics systems and molecular cloning procedures

A. West Nile virus cDNA infectious clone and PCR-site-directed mutagenesis

The cDNA infectious clone of WNV NY99 strain used to engineer mutations by site-directed mutagenesis was previously described in published studies (Kinney et al. 2006, Beasley et al. 2005) and was provided to us by Dr. Claire Huang's laboratory at the Centers for Disease Control and Prevention in Fort Collins, CO. The WNV genome was encoded in two plasmids, utilizing a derivative of the plasmid pBRUC-139S. The pWN-AB plasmid encodes a Φ 2.5 T7 promoter preceding the WNV genomic region between nucleotides 1 to 2495, which encodes the 5' untranslated region (UTR), the structural proteins (C, prM, and E), and the first 30 bases of non-structural protein 1 (NS1). The pWN-CG plasmid contains the WNV genomic region between nucleotides 2495 to 11029, which encodes the remainder of NS1 starting from the *NgoMIV* restriction enzyme cut site at nucleotide 2495, NS2A, NS2B, NS3, NS4A, NS4B, NS5, and the 3' UTR. Ligation of the pWN-AB and pWN-CG plasmids will constitute the whole WNV genome. All plasmids were low-copy plasmids propagated using XL1-Blue *E. coli* cells in 2X YT broth supplemented with ampicillin at 50 µg/mL (Figure 2-1).

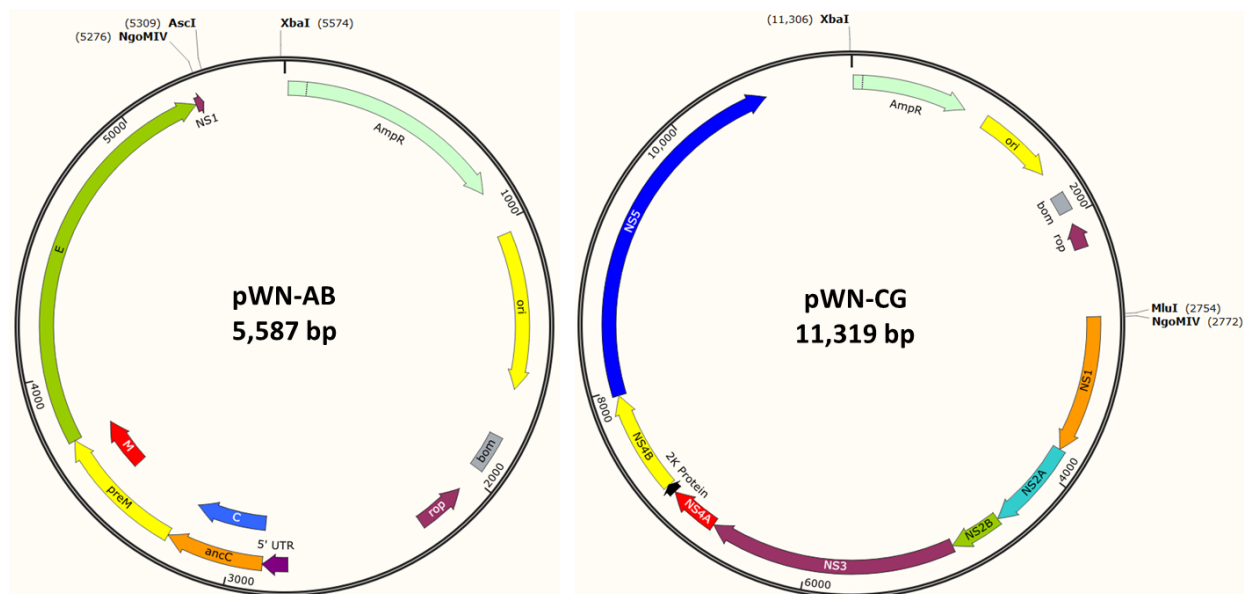


Figure 2-1: Plasmid maps of pWN-AB and pWN-CG

Image was generated from SnapGene molecular biology software.

All amino acid substitutions were engineered within two interdomain regions of the E protein. Polymerase chain reaction (PCR) site-directed mutagenesis was undertaken to introduce mutations into these two regions using the InFusion cloning method (Takara). Specifically, we engineered amino acid substitutions of 1) the eight flavivirus-conserved hydrophobic residues in the EDI-EDII hinge region and 2) the eight flavivirus-conserved residues in the EDI-EDIII linker (Table 2-1).

Table 2-1: Amino acid sequences of the flavivirus EDI-EDII hinge regions and EDI-EDIII linker among different flaviviruses

The properties of amino acids are shown in green (hydrophobic), pink (polar), red (negatively charged), blue (positively charged), and black (unique). The numbering corresponds to the amino acid positions in the WNV E protein. Underlines and bolded residues are highly conserved residues. GenBank access numbers: WNV-AF253491, YFV-AY640589, JEV-M18370, SLEV-AY289618 DENV-1-KM204119, DENV-2-AF038403, DENV-3-M93130, DENV-4-AY947539, ZIKV-MW143022, TBEV-U27495, and POWV- L06436.

	EDI EDII Hinge Region				EDI-EDIII
	Motif #1	Motif #2	Motif #3	Motif #4	Linker
	(51-54)	(130-135)	(191-201)	(269-281)	(295-305)
WNV	ANLA	ILKENI	EPRSGIDTNA-Y	AIPVEFS---SNTVKL	LQLKGTTYGVC
JEV	SQLA	IQPENI	EPRSGINTEA-F	AIVVEYS---S-SVKL	LALKGTTYGMC
YFV	DGPA	VDQTKI	QVQTAVDFGNSY	AMRVTKDNTDNNLYKL	LTLLKGTSYKMC
DENV-1	TNPA	VQYENL	SPRTGLDFNE-M	ATEIQTS---GT-TTI	LTLLKGM SYVMC
DENV-2	KQPA	VQPENL	SPRTGLDFNE-M	ATEIQMS---SG-NLL	LQLKGM SYSMC
DENV-3	TQLA	VQHENL	SPRTGLDFNE-M	ATEIQTS---GG-TSI	LELLKGM SYAMC
DENV-4	KEVA	VQIENL	EPRSGIDFNE-M	ATEVDSG---DG-NHM	LRIKGM SYTMC
ZIKV	SNMA	IQPENL	EPRTGLDFSD-L	ALEAEMD---GAKGRL	LRLKGV SYSLC
TBEV	ENPA	YDANKI	RVASGVDLAQTV	VPVAHIE---GTKYHL	LKMKGLTYTMC
POWV	ESPA	YDSTKI	KVASGIDVAQTV	VPLASVE---GQKYHL	LKLKGTTYSMC

The respective hydrophobic residues in the EDI-EDII hinge region are present in four different motifs and were each replaced with an amino acid with a structurally similar side chain but a lower hydrophobicity (Table 2-2). We undertook the alanine screening of the six flavivirus conserved amino acids in the single motif of the EDI-EDIII linker to select residues that can tolerate alanine substitutions, followed by the engineering of alternative amino acid substitutions

at the respective flavivirus conserved residues. Residues crucial for the structure-function of the E protein were identified based on the characterization of infectivity in cell culture and the virulence phenotype in the outbred Swiss mice neuroinvasive disease model (Beasley et al. 2002, Beasley et al. 2005)

Table 2-2: Hydrophobicity of the amino acids

The amino acids are listed from the most hydrophobic (phenylalanine at a hydrophobicity index of 100) to the least (aspartic acid at a hydrophobicity index of -55).

Amino Acid	Amino Acid Abbreviations		Hydrophobicity Index	Biochemical Properties
Phenylalanine	Phe	F	100	Aromatic
Isoleucine	Ile	I	99	Aliphatic
Tryptophan	Trp	W	97	Aromatic
Leucine	Leu	L	97	Aliphatic
Valine	Val	V	76	Aliphatic
Methionine	Met	M	74	Aliphatic
Tyrosine	Tyr	Y	63	Aromatic
Cysteine	Cys	C	49	Polar
Alanine	Ala	A	41	Aliphatic
Threonine	Thr	T	13	Polar
Histidine	His	H	8	Basic
Glycine	Gly	G	0	Unique
Serine	Ser	S	-5	Polar
Glutamine	Gln	Q	-10	Polar
Arginine	Arg	R	-14	Basic
Lysine	Lys	K	-23	Basic
Asparagine	Asn	N	-28	Polar
Glutamic Acid	Glu	E	-31	Acidic
Proline	Pro	P	-46	Unique
Aspartic Acid	Asp	D	-55	Acidic

There are two alanine residues present in the EDI-EDII hinge region of WNV E protein, i.e., the E-A54 and E-A269 residues (hydrophobic index: 41), which were changed to serine (hydrophobic index: -5) by the G1126U and G1771U nucleotide substitutions, respectively. The

E-I130, E-I135, and E-I196 residues (hydrophobic index: 99) and E-L281 residue (hydrophobic index: 97) were replaced with asparagine (hydrophobic index: -28). The codons encoding the E-I130, E-I135, and E-I196 residues were each engineered with a single U→A transversion in the second nucleotide of the codon, i.e., genome positions 1355, 1370, and 1553, respectively. The E-L281N mutation required three nucleotide changes in the codon, including U1807A, U1808A, and G1809C. The E-V272 residue (hydrophobic index: 76) was mutated to threonine (hydrophobic index: 13) by two nucleotide substitutions at the first and second bases in the codon: G1780A and U1781C. Tyrosine has the lowest hydrophobicity among all amino acids that contain an aromatic ring in the side chain. Therefore, the E-Y201 residue (hydrophobic index: 63) was changed to proline, which maintains the ring structure in the side chain and exhibits lower hydrophilicity at physiological pH (hydrophobic index -46) (Monera et al. 1995, Kovacs et al. 2006). The E-Y201P mutation involved the change of the first and second positions in the codon, including U1567C and A1568C (Table 2-3).

Table 2-3: Mutations of the eight conserved hydrophobic residues in the WNV EDI-EDII hinge region.

This table was modified from a table originally published in Ashley Bilyeu's K-State Master's thesis (Bilyeu 2023).

Location	Amino acid substitution	Nucleotide substitutions in the genome		
	in the E protein	1st codon	2nd codon	3rd codon
Motif #1	A54S	G1126U		
Motif #2	I130N		U1355A	
	I135N		U1370A	
Motif #3	I196N		U1553A	
	Y201P	U1567C	A1568C	
Motif #4	A269S	G1771U		
	V272T	G1870A	U1871C	
	L281N	U1807A	U1808A	G1809C

Further, 19 alternative amino acid substitutions of the E-A54 and E-Y201 residues were evaluated due to the attenuating effect of the E-A54S and E-Y201P mutations (Tables 2-4 and 2.5). The single-site E-A54I, E-A54E, E-Y201C, and E-Y201D mutants were selected to investigate the neuroinvasive phenotype in outbred Swiss mice, following the characterization in cell culture. Specifically, the E-A54 residue was replaced with isoleucine and glutamate by introducing the C1127A and G1126A+C1127U nucleotide changes, respectively. The E-Y201 residue was replaced with cysteine and aspartic acid by introducing the A1568C+C1569U and U1567G nucleotide changes, respectively. In addition, we also undertook codon optimization of the E-Y201P mutation based on the codon bias of WNV, creating a three-nucleotide substitution of U1567C, A1568C, and C1569A. This configuration of the nucleotides of the codon is present in 64% of all proline residues found in the WNV genome.

Table 2-4: Mutations of the E-A54 residues in the WNV EDI-EDII hinge region for optimization of attenuation.

Location	Amino acid substitution in the E protein	Nucleotide substitutions in the genome position		
		1st codon	2nd codon	3rd codon
Motif #1	A54C	G1126U	C1127G	A1128U
	A54D		C1127A	A1128U
	A54E		C1127A	
	A54F	G1126U	C1127U	A1128U
	A54G		C1127G	
	A54H	G1126C	C1127A	A1128U
	A54I	G1126A	C1127U	
	A54K	G1126A	C1127A	
	A54L	G1126C	C1127U	
	A54M	G1126A	C1127U	A1128G
	A54N	G1126A	C1127A	A1128U
	A54P	G1126C		A1128U
	A54Q	G1126C	C1127A	
	A54R	G1126A	C1127G	
	A54T	G1126A		
	A54V		C1127U	
	A54W	G1126U	C1127G	A1128G
	A54Y	G1126U	C1127A	A1128U

Table 2-5: Mutations of the E-Y201 residues in the WNV EDI-EDII hinge region for optimization of attenuation.

Location	Amino acid substitution in the E protein	Nucleotide substitutions in the genome position		
		1st codon	2nd codon	3rd codon
Motif #3	Y201A	U1567G	A1568C	
	Y201C		A1568G	C1569U
	Y201D	U1567G	A1568C	
	Y201E	U1567G		C1569A
	Y201F		A1568U	C1569U
	Y201G	U1567G	A1568G	
	Y201H	U1567C		
	Y201I	U1567A	A1568U	
	Y201K	U1567A		C1569G
	Y201L		A1568U	C1569G
	Y201M	U1567A	A1568U	C1569G
	Y201N	U1567A		
	Y201Q	U1567C		C1569G
	Y201R	U1567C	A1568G	
	Y201S		A1568C	
	Y201T	U1567A	A1568C	
	Y201V	U1567G	A1568U	
	Y201W		A1568G	C1569G
	Y201P	U1567C	A1568C	C1569A
	(Optimized)			

We have also undertaken alanine screening of eight flavivirus-conserved residues in the WNV EDI-EDIII linker, including E-L295, E-L297, E-K298, E-G299, E-T301, E-Y302, E-V304, and E-C305 residues (Table 2-6). Due to the comparatively small size of the glycine and alanine

amino acids, the E-G299 residue was also replaced with proline to induce a more profound phenotypic change.

Table 2-6: Mutations of the eight conserved hydrophobic residues in the WNV EDI-EDIII linker.

Location	Amino acid substitution in the E protein	Nucleotide substitutions in the genome position		
		1st codon	2nd codon	3rd codon
Motif #1	L295A	U1849G	U1850C	
	L297A	C1855G	U1856C	
	K298A	A1858G	A1859C	
	G299A		G1862C	
	G299P	G1861C	G1862C	
	T301A	A1867G		
	Y302A	U1870G	A1871C	
	V304A		U1877C	
	C305A	U1879G	G1880C	

The E-L295 and E-V304 residues were further substituted with 18 alternative amino acid substitutions due to the reduced infectivity and retention of the nucleotide substitutions encoding the E-L295A and E-V304A mutations (Tables 2-7 and 2.8). The E-L295 residue was further analyzed when replaced with phenylalanine, serine, threonine, and valine by introducing the G1851U, U1850C+G1851C, U1849A+U1850C, and U1849G+G1851U nucleotide changes respectively. The E-V304 residue was further analyzed when replaced with asparagine by introducing the G1876A+U1877A nucleotide changes.

Table 2-7: Mutations of the E-L295 residues in the WNV EDI-EDIII linker for optimization of attenuation.

Location	Amino acid substitution in the E protein	Nucleotide substitutions in the genome position		
		1st codon	2nd codon	3rd codon
Motif #1	L295C		U1850G	G1851C
	L295D	U1849G	U1850A	G1851C
	L295E	U1849G	U1850A	
	L295F			G1851C
	L295G	U1849G	U1850G	
	L295H	U1849C	U1850A	G1851C
	L295I	U1849A		G1851U
	L295K	U1849A	U1850A	
	L295M	U1849A		
	L295N	U1849A	U1850A	G1851C
	L295P	U1849C	U1850C	
	L295Q	U1849C	U1850A	
	L295R	U1849A	U1850G	
	L295S		U1850C	G1851C
	L295T	U1849A	U1850C	
	L295V	E-L295		G1851U
	L295W		U1850G	
	L295Y		U1850A	G1851C

Table 2-8: Mutations of the E-V304 residues in the WNV EDI-EDIII linker for optimization of attenuation.

Location	Amino acid substitution in the E protein	Nucleotide substitutions in the genome position		
		1st codon	2nd codon	3rd codon
Motif #1	V304C	G1876U	U1877G	
	V304D		U1877A	C1878U
	V304E		U1877A	C1878G
	V304F	G1876U		
	V304G		U1877G	C1878G
	V304H	G1876C	U1877A	
	V304I	G1876A		
	V304K	G1876A	U1877A	C1878G
	V304L	G1876C		C1878G
	V304M	G1876A		C1878G
	V304N	G1876A	U1877A	
	V304P	G1876C	U1877C	
	V304Q	G1876C	U1877A	C1878G
	V304R	G1876C	U1877G	
	V304S	G1876U	U1877C	
	V304T	G1876A	U1877C	
	V304W	G1876U	U1877G	C1878G
	V304Y	G1876U	U1877A	
	V304C	G1876U	U1877G	

The mutations were first introduced into the pWN-AB plasmid by PCR site-directed mutagenesis with Phusion Flash high-fidelity PCR Master Mix, followed by removal of methylated DNA template using *DpnI* digestion at 37°C for 60 minutes (Thermo Scientific). Each PCR reaction contained a set of overlapping primers unique to each mutation (Table 2-9). The

PCR reactions themselves were comprised of a total of 50 μL , consisting of 25 μL of 2X Phusion Flash high-fidelity PCR Master Mix, 1 μL of plasmid template, 1.5 μL of 10 μM sense and antisense primers, and 21 μL of nuclease-free water. The cycling parameters of the reaction were denaturation at 98°C for 30 seconds, 25 cycles of denaturation at 98°C for 10 seconds, primer annealing at primer-specified temperatures for 30 seconds, and elongation at 72°C for 3 minutes, and finally, incubation at 72°C for 10 minutes. After digestion with 1 μL of *DpnI* to degrade any methylated template plasmid present in the reaction, the digested amplicons were separated on a 1% agarose gel, followed by guanidine thiocyanate purification using the Zymoclean Gel DNA Recovery kit (Zymo Research) or the PCR Cleanup and Gel Extraction Kit (Takara).

Table 2-9: Overlapping primer sets for site-directed mutagenesis of WNV mutants

Primer Name	Forward Primer (5' to 3')	Reverse Primer (3' to 5')
WNV E A54C	GCGGCCAACCTGTGTGAGGTCC	AATAACTGCGGACCTCACACAGGTTGGCC
WNV E A54D	GCGGCCAACCTGGATGAGGTCC	ATAACTGCGGACCTCATCCAGGTTGGC
WNV E A54E	CGGCCAACCTGGAAGAGGTCCG	TAACTGCGGACCTCTTCAGGTTGGC
WNV E A54F	GCGGCCAACCTGTTTGAGGTCCGC	GCAATAACTGCGGACCTCAAACAGGTTGGC
WNV E A54G	CGGCCAACCTGGGAGAGGTCC	TAACTGCGGACCTCTCCCAGGTTGG
WNV E A54H	CGGCCAACCTGCATGAGGTCCG	ATAACTGCGGACCTCATGCAGGTTGGC
WNV E A54I	AGGCGGCCAACCTGATAGAGGTCC	TAACTGCGGACCTCTATCAGGTTGGCCG
WNV E A54K	GCGGCCAACCTGAAAGAGGTCCG	TAACTGCGGACCTCTTTCAGGTTGGCC
WNV E A54L	GCGGCCAACCTGCTAGAGGTCC	TAACTGCGGACCTCTAGCAGGTTGGC
WNV E A54M	GGCGGCCAACCTGATGGAGGTCC	ATAACTGCGGACCTCCATCAGGTTGGCCG
WNV E A54N	GGCGGCCAACCTGAATGAGGTCC	CAATAACTGCGGACCTCATTAGGTTGGCC
WNV E A54P	GCGGCCAACCTGCCAGAGGT	AACTGCGGACCTCTGGCAGGTTGGCC
WNV E A54Q	GCGGCCAACCTGCAAGAGGTCC	AACTGCGGACCTCTTGCAGGTTGGC
WNV E A54R	GGCGGCCAACCTGAGAGAGGTCC	TAACTGCGGACCTCTCTCAGGTTGGCC
WNV E A54S	CCAACCTGTCAGAGGTCCGCAGTTATTG	GCGGACCTCTGACAGGTTGG
WNV E A54T	GGCGGCCAACCTGACAGAGGTC	AACTGCGGACCTCTGTGAGGTTGGC
WNV E A54V	GCGGCCAACCTGGTAGAGGTCC	TAACTGCGGACCTCTACCAGGTTGGC
WNV E A54W	CGGCCAACCTGTGGGAGGTCC	AATAACTGCGGACCTCCCACAGGTTGGC
WNV E A54Y	GCGGCCAACCTGTATGAGGTCCG	ATAACTGCGGACCTCATACAGGTTGGCC
WNV E I130N	AATAGGAAGAACCAACTTGAAAGAG	TATTCTCTTTCAAGTTGGTTCTTCC
WNV E I135N	CTTGAAAGAGAATAACAAGTACGAAGT	CTTGAAAGAGAATAACAAGTACGAAGT
WNV E I196N	ACCACGGTCAGGGAATGACACCAAT	TGCATTGGTGTCTTCCCTGACCG
WNV E Y201A	ATTGACACCAATGCAGCCTACGTGATG	CAGTCATCACGTAGGCTGCATTGGT
WNV E Y201C	TTGACACCAATGCATGTTACGTGATGA	CAACAGTCATCACGTAAATGCATTGGT
WNV E Y201D	GATTGACACCAATGCAGACTACGTGATG	CAGTCATCACGTAGTCTGCATTGGTG
WNV E Y201E	ATTGACACCAATGCAGAGTACGTGATGA	AACAGTCATCACGTACTCTGCATTGGT
WNV E Y201F	TTGACACCAATGCATTTTACGTGATGA	AACAGTCATCACGTAAAATGCATTGGT
WNV E Y201G	ATTGACACCAATGCAGGCTACGTGATG	ACAGTCATCACGTAGCCTGCATTGGTG
WNV E Y201H	GGGATTGACACCAATGCACACTACGTGATGA	CCAACAGTCATCACGTAGTGTGCATTGGTGTCAA
WNV E Y201I	GGGATTGACACCAATGCACACTACGTGATGA	CCAACAGTCATCACGTAGTGTGCATTGGTGTCAA
WNV E Y201K	GATTGACACCAATGCAAAGTACGTGATGA	AACAGTCATCACGTACTTTGCATTGGTG
WNV E Y201L	TTGACACCAATGCATTGTACGTGATGA	AACAGTCATCACGTACAATGCATTGGT

Primer Name	Forward Primer (5' to 3')	Reverse Primer (3' to 5')
WNV E Y201M	GATTGACACCAATGCAATGTACGTGATGA	AACAGTCATCACGTACATTGCATTGGTG
WNV E Y201N	GGATTGACACCAATGCAAACTACGTGAT	ACAGTCATCACGTAGTTTGCATTGGTG
WNV E Y201P	TGACACCAATGCACCCTACGTGATG	CAGTCATCACGTAGGGTGCATTGGTG
WNV E Y201Q	ATTGACACCAATGCACAGTACGTGATG	CAGTCATCACGTACTGTGCATTGGTG
WNV E Y201R	GGATTGACACCAATGCACGCTACGTGATGA	AACAGTCATCACGTAGCGTGCATTGGTGT
WNV E Y201S	TTGACACCAATGCATCCTACGTGATG	CAGTCATCACGTAGGATGCATTGGT
WNV E Y201T	ATTGACACCAATGCAACCTACGTGATG	CAGTCATCACGTAGGTTGCATTGGT
WNV E Y201V	ATTGACACCAATGCAGTCTACGTGATG	GTCATCACGTAGACTGCATTGGTGT
WNV E Y201W	GGGATTGACACCAATGCATGGTACGTGATGACTG	TCCAACAGTCATCACGTACCATGCATTGGTGTCA
WNV E A269S	AAGCTTTGGCTGGATCCATTCTCTGT	TTCCACAGGAATGGATCCAGCCAAA
WNV E V272T	TTGGCTGGAGCCATTCTACGGAATTTTCAAGC	GTGTTGCTTGAAAATTCCTAGGAATGGCTCCAG
WNV E L281N	GCAACACTGTCAAGAACACGTCGGGTC	TCAAATGACCCGACGTGTTCTTGACAGTG
WNV E L295A	GATGGAAAAAGCGCAGCTGAAG	CTTCAGCTGCGCTTTTCCATC
WNV E L295C	TGAAGATGGAAAAATGCCAGCTGAAGG	TTCCCTTCAGCTGGCATTITTTCCAT
WNV E L295D	GTGAAGATGGAAAAAGACCAGCTGAAGG	GTTCCCTTCAGCTGGTCTTTTCCAT
WNV E L295E	AGTGAAGATGGAAAAAGAGCAGCTGAAGG	TTCCCTTCAGCTGCTCTTTTCCATC
WNV E L295F	TGAAGATGGAAAAATTTTCAAGCTGAAGGG	TGTTCCCTTCAGCTGAAATTTTCCA
WNV E L295G	GTGAAGATGGAAAAAGGGCAGCTGAAG	CCCTTCAGCTGCCCTTTTCCAT
WNV E L295H	GTGAAGATGGAAAAACACCAGCTGAAGG	GTTCCCTTCAGCTGGTGTITTTCCAT
WNV E L295I	GTGAAGATGGAAAAAATTCAGCTGAAGG	GTTCCCTTCAGCTGAATTTTTCATC
WNV E L295K	GTGAAGATGGAAAAAAGCAGCTGAAG	TCCCTTCAGCTGCTTTTTTCCAT
WNV E L295M	GTGAAGATGGAAAAAATGCAGCTGAA	CCCTTCAGCTGCATTITTTCCAT
WNV E L295N	GTGAAGATGGAAAAAACCAGCTGAAGG	GTTCCCTTCAGCTGGTITTTTCCAT
WNV E L295P	GTGAAGATGGAAAAACCGCAGCTGAAGG	GTTCCCTTCAGCTGCGGTTTTTCCATC
WNV E L295Q	AGTGAAGATGGAAAAACAGCAGCTGAAG	TTCCCTTCAGCTGCTGTITTTCCAT
WNV E L295R	GTGAAGATGGAAAAAAGGCAGCTGAAGG	GTTCCCTTCAGCTGCCTTTTTTCCATC
WNV E L295S	TGAAGATGGAAAAATCCCAGCTGAAGG	GTTCCCTTCAGCTGGGATTTTCCA
WNV E L295T	GTGAAGATGGAAAAACGCAGCTGAAGG	GTTCCCTTCAGCTGCGTTTTTCCATC
WNV E L295V	GTGAAGATGGAAAAAGTTCAGCTGAAGG	GTTCCCTTCAGCTGAACTITTTCCAT
WNV E L295W	GTGAAGATGGAAAAATGGCAGCTGAAGG	GTTCCCTTCAGCTGCCATTTTCCAT
WNV E L295Y	TGAAGATGGAAAAATACCAGCTGAAGG	GTTCCCTTCAGCTGGTATTTTCCA
WNV E L297A	AAAATTGCAGGCGAAGGGAACA	TGTTCCCTTCGCTGCAATTTT
WNV E K298A	TTGCAGCTGGCGGGAACAAC	GTTGTTCCCGCCAGCTGCAA
WNV E G299A	CAGCTGAAGGCAACAACCTATGG	CCATAGGTTGTTGCCTTCAGCTG

Primer Name	Forward Primer (5' to 3')	Reverse Primer (3' to 5')
WNV E G299P	GCAGCTGAAGCCAACACCTAT	ATAGGTTGTTGGCTTCAGCTGC
WNV E T301A	GAAGGGAACAGCCTATGGCG	CGCCATAGGCTGTTCCCTTC
WNV E Y302A	GGAACAACCGCTGGCGTCTGTT	AACAGACGCCAGCGGTTGTTCC
WNV E V304A	CCTATGGCGCCTGTTCAAAGG	CCTTTGAACAGGCGCCATAGG
WNV E V304C	GGAACAACCTATGGCTGCTGTTCAAAG	AAGCCTTTGAACAGCAGCCATAGG
WNV E V304D	GAACAACCTATGGCGATTGTTCAAAGG	GAAAGCCTTTGAACAATCGCCATAGGT
WNV E V304E	GAACAACCTATGGCGAGTGTCAAAGG	AAAGCCTTTGAACACTCGCCATAGG
WNV E V304F	GGAACAACCTATGGCTTCTGTTCAAAG	GCCTTTGAACAGAAGCCATAGGT
WNV E V304G	GAACAACCTATGGCGGTGTTCAAAGG	AAAGCCTTTGAACACCCGCCATAGG
WNV E V304H	GGAACAACCTATGGCCACTGTTCAAAG	AAAGCCTTTGAACAGTGGCCATAGG
WNV E V304I	GGAACAACCTATGGCATCTGTTCAA	AAGCCTTTGAACAGATGCCATAGG
WNV E V304K	GGAACAACCTATGGCAAGTGTCAAAGG	GAAAGCCTTTGAACACTTGCCATAGGTT
WNV E V304L	GGAACAACCTATGGCCTGTGTTCAAAGG	GAAAGCCTTTGAACACAGGCCATAGGTT
WNV E V304M	GGAACAACCTATGGCATGTGTTCAAAGG	GAAAGCCTTTGAACACATGCCATAGGTT
WNV E V304N	GGAACAACCTATGGCAACTGTTCAAAG	AAAGCCTTTGAACAGTTGCCATAGG
WNV E V304P	GGAACAACCTATGGCCCCTGTTCAAAG	AAAGCCTTTGAACAGGGGCCATAGG
WNV E V304Q	GGAACAACCTATGGCCAGTGTCAAAGG	GAAAGCCTTTGAACACTGGCCATAGGTT
WNV E V304R	GGAACAACCTATGGCCGCTGTTCAAAG	AAAGCCTTTGAACAGCGGCCATAGG
WNV E V304S	GGAACAACCTATGGCTCCTGTTCAAAG	AAAGCCTTTGAACAGGAGCCATAGG
WNV E V304T	GGAACAACCTATGGCACCTGTTCAAAG	AAAGCCTTTGAACAGGTGCCATAGG
WNV E V304W	GGAACAACCTATGGCTGGTGTCAAAGG	GAAAGCCTTTGAACACCAGCCATAGGTT
WNV E V304Y	GGAACAACCTATGGCTACTGTTCAAAG	AAAGCCTTTGAACAGTAGCCATAGG
WNV E C305A	CTATGGCGTCGTTCAAAGGC	GCCTTTGAAGCGACGCCATAG

Re-combination of two ends of the linearized PCR product was conducted using InFusion cloning. Upon digestion with the vaccinia DNA exonuclease present in the InFusion Enzyme Mix from Takara at 42°C for 20 minutes, the overlapping ends of the cDNA were recombined to create a circular plasmid (Figure 2-2). The purified DNA fragments contain complementary ends, joined together by brief chilling on ice. The resulting circular form of DNA was then heat-shock transformed into XL1-Blue cells pre-chilled in BF2 buffer (0.1M MOPS, 0.05M CaCl₂, and 0.01M

RbCl at a pH of 6.5). Individual colonies were selected on 2X YT agar plates supplemented with 2000x concentration of ampicillin, and propagation of the plasmids occurred in 10 ml 2X YT broth supplemented with ampicillin followed by the miniprep purification method. The mutations were confirmed by Sanger sequencing.

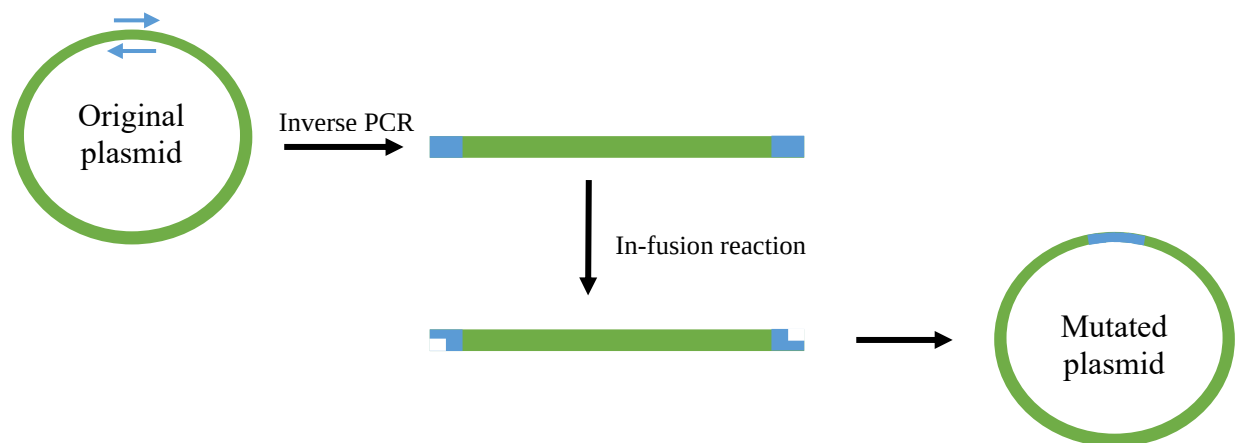


Figure 2-2: In-fusion cloning reaction to insert single amino acid mutation

Image was generated in PowerPoint.

The pWN-AB and pWN-CG plasmids were combined to create a full-length WNV genome using the following procedures (Figure 2-3). Ten μg of the mutated pWN-AB plasmid was digested with *AscI* (New England Biolabs) and *NgoMIV* (New England Biolabs), followed by the treatment with Quick calf intestinal alkaline phosphatase (CIP) (New England Biolabs). Approximately 30 μg of the pWN-CG plasmid was first digested with *MluI-HF* (New England Biolabs) and treated with Quick CIP. The digested pWN-AB and pWN-CG plasmids were purified using the Nucleospin® column. The purified pWN-CG plasmid was subsequently digested with *NgoMIV*, followed by an additional round of purification. The digested pWN-AB and pWN-CG plasmids were ligated with T4 DNA ligase at room temperature for two hours, followed by inactivation at 70°C for 15 minutes. The ligated fragment was digested with *XbaI* (New England Biolabs) and

proteinase K, then purified using the phenol-chloroform extraction and ethanol precipitation method.

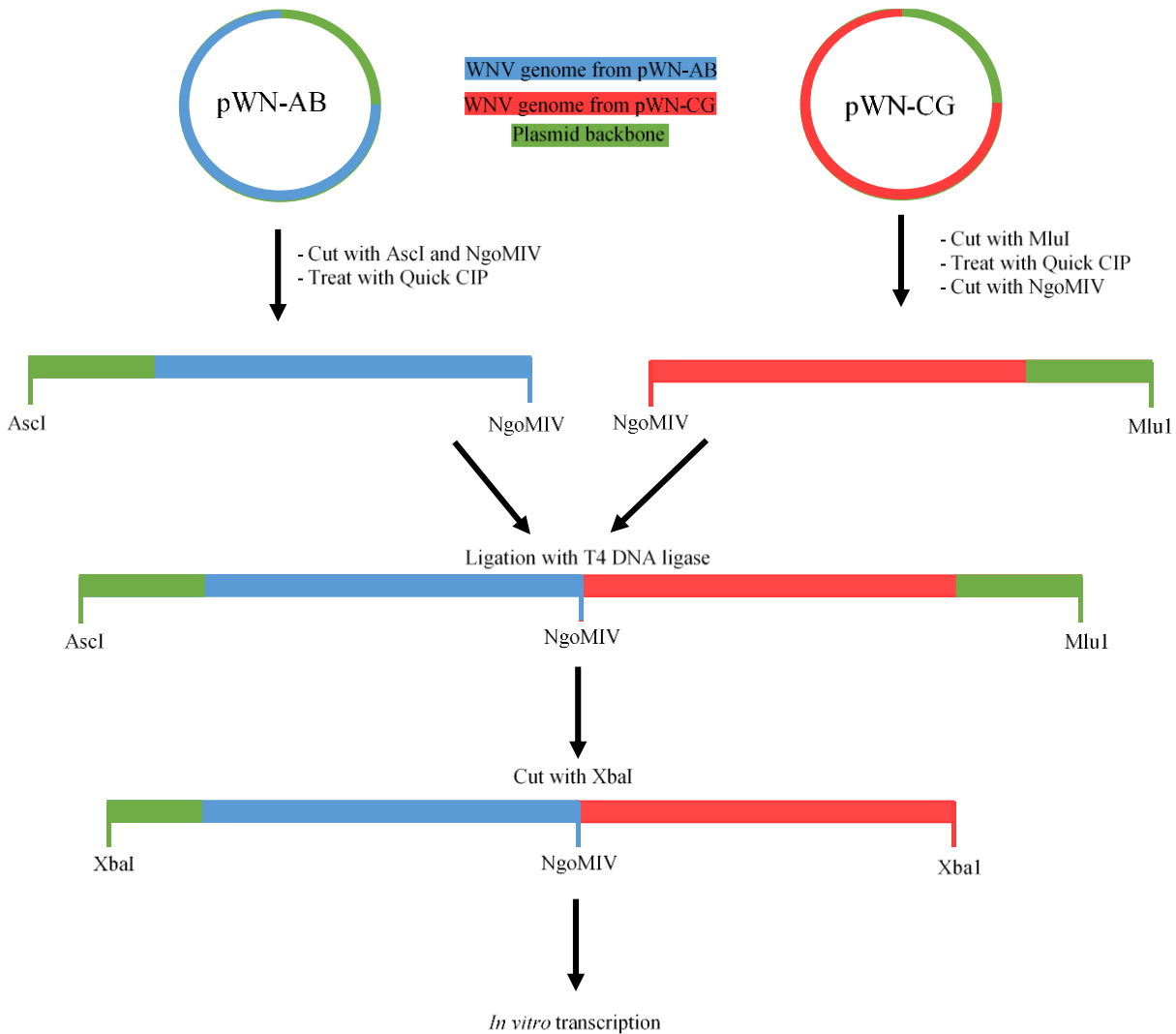


Figure 2-3: Molecular cloning strategy for combining WNV infectious clone plasmids pWN-AB and pWN-CG.

Image was generated in PowerPoint.

The viral genome was produced by *in vitro* transcription with a G(5')ppp(5')A RNA cap structure analog (New England Biolabs) using the Ampliscribe T7 High Yield Transcription kit (Lucigen) at 37°C for one hour. Transfection of RNA transcript was conducted by electroporation

(Huang et al. 2010). Briefly, 10 μ L of RNA transcripts were transfected into four million Vero cells suspended in 400 μ L of ice-cold Dulbecco's phosphate buffer saline without Mg^{2+}/Ca^{2+} using the BioRad GenePulser® system with one pulse of single wave condition at 225 volts for 25 msec in a 4 mm cuvette. Electroporated cells were allowed to recover on ice for 15 minutes and seeded in a T75 flask with pre-warmed media. The virus was harvested four days after transfection when cytopathic effects became apparent in 70-80% of cells. All *in vitro* and *in vivo* experiments were performed using virus stocks harvested from transfected Vero cells without additional passage, except for sequence analysis of the genetic stability of the viruses.

B. Yellow fever virus cDNA infectious clone and PCR site-directed mutants

The cDNA infectious clones of the YFV Asibi strain were established in previously published studies (Bredenbeek et al. 2003, McElroy et al. 2005). All YFV plasmids were selected based on the ampicillin resistance (Figure 2-4). The subcloned plasmid, known as pGEM® YFV-Asibi-prM+E, is based on the pGEM® T Easy system and includes part of the YFV genome from nucleotide 473 to 2970. This plasmid includes the last nine nucleotides of the capsid, all of prM and E, and the first 518 bases of NS1. The pGEM plasmids were propagated in 2X YT broth supplemented by ampicillin at 100 μ g/ml. The full-length cDNA infectious clones based on the low-copy number pANCR system were propagated in terrific broth supplemented by ampicillin at 50 μ g/ml.

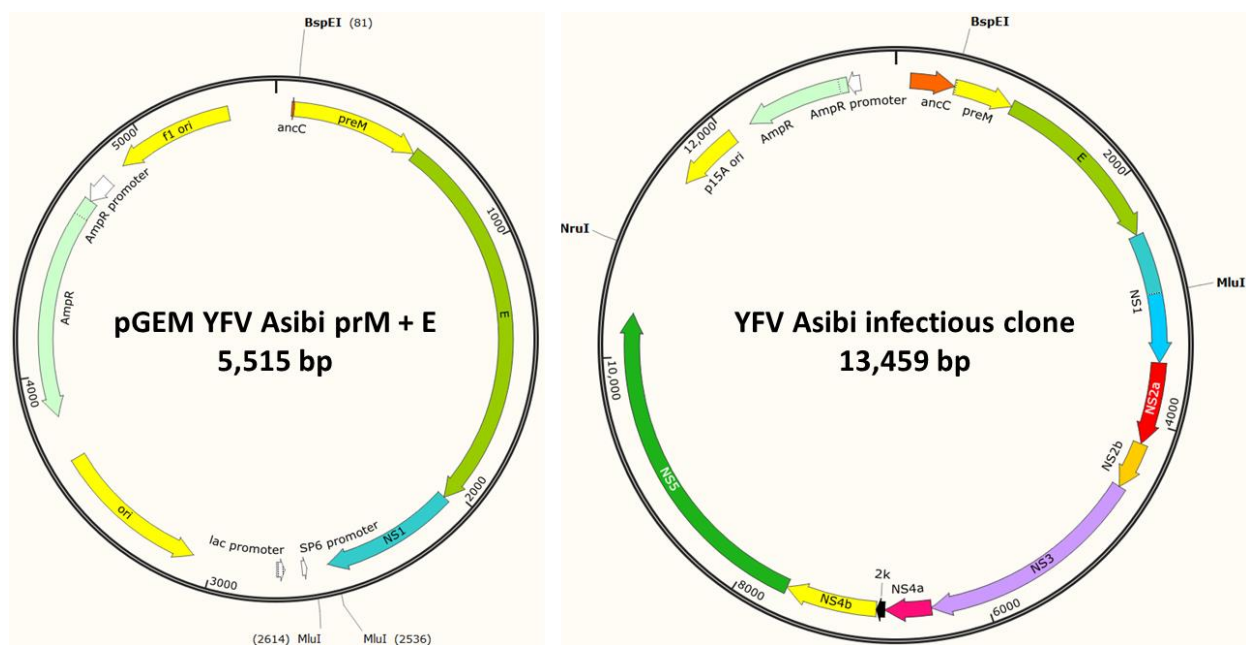


Figure 2-4: Plasmid maps of the YFV subcloned plasmid and the YFV infectious clone

Image was generated in SnapGene molecular biology software.

The wild-type YFV Asibi strain is a model system to determine whether or not mutations of flavivirus conserved hydrophobic EDI-EDII hinge region mutations utilized in the WNV model. Eight amino acid residues were identified in YFV to be highly conserved across flaviviruses and comparable to WNV mutations from specific aim #1. All eight residues were replaced with amino acids with decreased hydrophobicity but similar biochemical structures.

The E-A54S and E-A261S residues (hydrophobic index: 41) were changed to serine (hydrophobic index: -5) by the G1133T and G1754T nucleotide substitutions, respectively. The E-V130, E-V188, and E-V264 residues (hydrophobic index: 76) were mutated to threonine (hydrophobic index: 13). This was achieved by the same two nucleotide changes for all three mutations: a G→A substitution at the first nucleotide of the codon and a U→C substitution at the second nucleotide of the codon. The nucleotide substitutions were at genome position 1361 and 1362 for E-V130T, 1535 and 1536 for E-V188T, and 1763 and 1764 for E-V264T. The E-I135

(hydrophobic index: 99), E-L273, and E-L276 (hydrophobic index: 97) were replaced with asparagine (hydrophobic index: -28). The E-I135N mutation required a single nucleotide change in the codon: U1377A. The E-L273N mutations were introduced with the C1790A and U1791A nucleotide substitutions. The E-L276N mutation required three nucleotide changes in the codon, including C1799A, U1800A, and A1801U (Table 2-10).

Table 2-10: Mutations of the eight conserved hydrophobic residues in the YFV EDI-EDII hinge region.

Location	Amino acid substitution in the E protein	Nucleotide substitutions in the genome position		
		1st codon	2nd codon	3rd codon
Motif #1	A54S	G1133T		
Motif #2	V130T	G1361A	U1362C	
	I135N		U1377A	
Motif #3	V188T	G1535A	U1536C	
Motif #4	A261S	G1754T		
	V264T	G1763A	U1764C	
	L273N	C1790A	U1791A	
	L276N	C1799A	U1800A	A1801U

A molecular cloning strategy using a shuttle plasmid was designed for the mutation of the YFV infectious clone (Figure 2-5). The mutations were first introduced into the pGEM® YFV-Asibi-prM+E plasmid by polymerase chain reaction (PCR) site-directed mutagenesis with Phusion Flash high-fidelity PCR Master Mix (Thermo Scientific). Each PCR reaction contained a set of overlapping primers unique to each mutation (Table 2-11). The general reaction setup and cycling parameters were identical to the setup described in section 2.2.A. The amplicons were digested with the *DpnI* restriction endonuclease at 37°C for 60 minutes to remove the template with the

methyalted wildtype sequence. The PCR product was circularized with the InFusion cloning method (Takara) and was transformed into MC1061 cells. Propagation of the plasmids occurred in 10 ml 2X YT broth supplemented with ampicillin, followed by the alkaline-lysis miniprep purification method. The mutations were confirmed by Sanger sequencing.

Table 2-11: Overlapping primer sets for site-directed mutagenesis of YFV mutants

Primer Name	Forward Primer (5' to 3')	Reverse Primer (3' to 5')
YFV E A54S	GCCATTGATGGACCTTCTGAGGCG	CTTTCCTCGCCTCAGAAGGTCCATCA
YFV E V130T	GAGTTTGTGTTGAGACTGATCAGACCA	TGGTCTGATCAGTCTCAAACAACTC
YFV E I135N	GATCAGACCAAAAATCAGTATGTCATCAG	CTGATGACATACTGATTTTTGGTCTGATC
YFV E V188T	AGGTGCAAACTGCGACGGACTTTG	CTGTTACCAAAGTCCGTCGCAGTTTGC
YFV E A261S	CTTACCGGCTCAATGAGGGT	ACCCTCATTGAGCCGGTAAG
YFV E V264T	GCGCAATGAGGACTACAAAGGAC	GTCCTTTGTAGTCCTCATTGCGC
YFV E L273N	ATGACAACAACAATTACAAACTACATGG	CCATGTAGTTTGTAAATTGTTGTTGTCAT
YFV E L276N	ACCTTTACAAAATCATGGTGGACAT	CATGTCCACCATGATTTTTGTAAAGGT

The mutated pGEM® T Easy-Asibi plasmids were digested with the *BspEI* (New England Biolabs) and *MluI* (New England Biolabs) at 37°C for 60 minutes to generate the insert for cloning into the corresponding position in the cDNA infectious clones of the Asibi strain. The Asibi infectious clone was also digested with *BspEI* and *MluI* at 37°C for 60 minutes before purifying the plasmid fragments using phenol-chloroform extraction and ethanol precipitation. Before the ligation reaction, the digested subcloned plasmids and infectious clone fragments were separated by electrophoresis with 0.8% agarose gels. The DNA fragments were excised from the agarose gels and purified with the Zymoclean Gel DNA Recovery kit (Zymo Research) or PCR cleanup and gel extraction kit (Takara). The inserts and vectors were quantified by O.D. 260 and ligated together at the molar ratio of 9:1 at 4 °C overnight with T4 DNA ligase to generate the full-length

cDNA infectious clones of YFV mutants before being transformed into MC1061 cells. The mutants were selected by propagating individual colonies in 10ml 2X YT broth, purified by the miniprep purification method, and confirmed by Sanger sequencing. The full-length mutants were transformed into MC1061 cells and propagated in 250 ml terrific broth containing ampicillin at 50 µg/ml. The plasmids were purified by alkaline lysis (Nucleobond® Xtra Maxi kit, Takara).

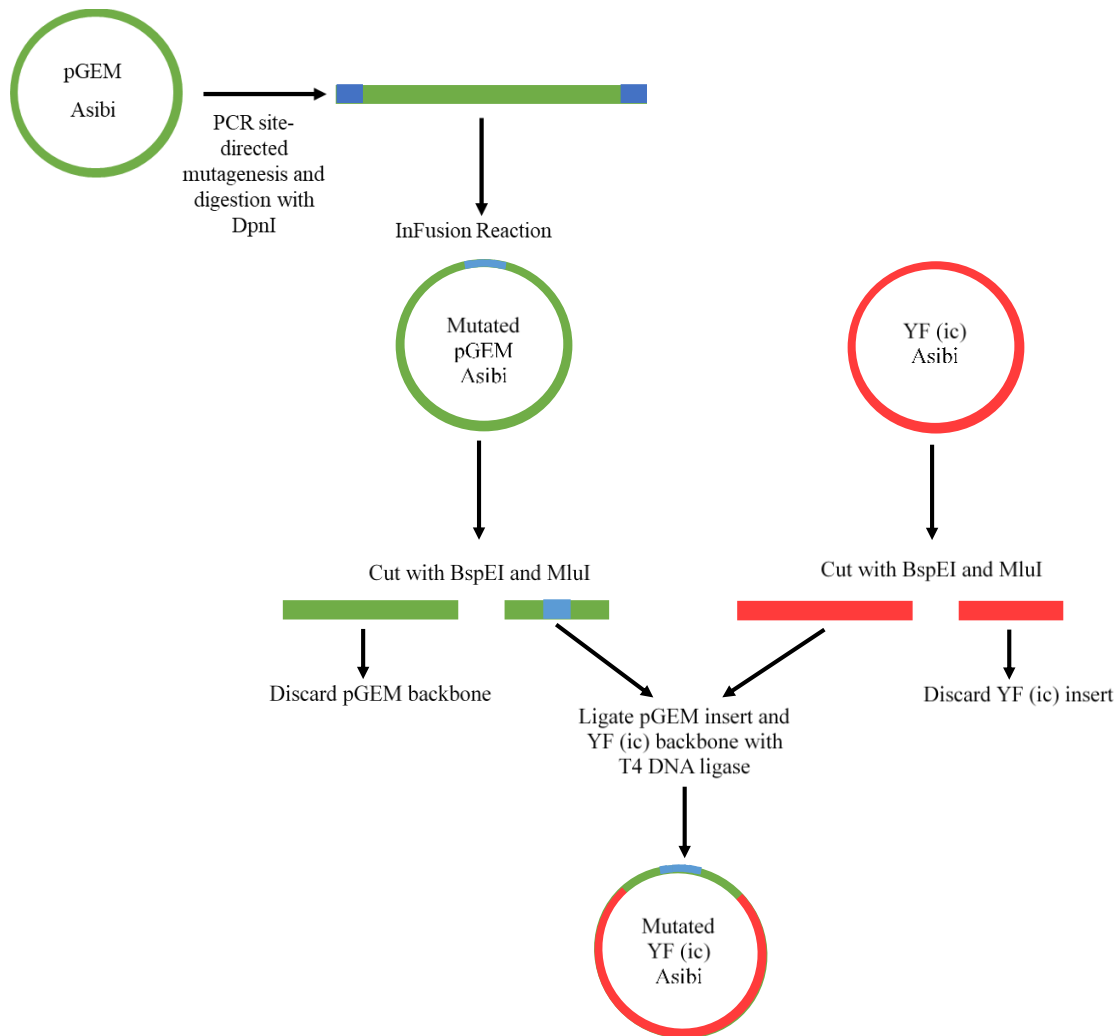


Figure 2-5: Molecular cloning strategy for combining inserting the mutated YFV fragment from pGEM plasmids into the YFV infectious clone

Image was generated in PowerPoint.

C. Procedures for the recovery of recombinant WNV and YFV mutants

The transformation of all WNV and YFV cDNA infectious clones and subcloned plasmids was performed by the permeabilization of the bacterial cells MC1061 and XL1-Blue treated with calcium chloride (CaCl₂) and heat-shock transformation.

The overnight starting culture of both MC1061 and XL1-Blue cells was made by inoculating ~5 µL of the glycerol stock from the -80°C freezer into 5 ml of 2X YT broth. The cultures were then propagated at 37°C for 16 hours at 300 r.p.m. The optimal log phase culture was prepared by transferring 1 mL of the overnight culture into 50 mL of 2X YT broth, which was then incubated in a shaking incubator at 37°C for 1 hour at 300 r.p.m. The cells were chilled on ice for 10 minutes before being placed in a centrifuge and were pelleted down at 4,000 r.p.m. for 10 minutes at 4°C. The cells were resuspended in 10 mL ice-cold BF1 buffer (0.01M MOPS and 0.01M rubidium chloride (RbCl) at a pH of 7.0) and centrifuged again at 4,000 r.p.m. for 10 minutes at 4°C. The pellet was gently resuspended in 10 mL ice-cold BF2 buffer (0.1M MOPS, 0.05M CaCl₂, and 0.01M RbCl at a pH of 6.5) and placed on ice for at least 20 minutes.

During this time, the plasmid DNA was prepared for transformation. For the transformation of WNV pWN-AB and YFV pGEM® T Easy-Asibi plasmids that underwent PCR site-directed mutagenesis, InFusion cloning was performed. The reaction was set up with 2 µL of 5X InFusion Enzyme Mix, 80-120 ng of inverse PCR product, and enough nuclease-free water to fill the reaction to 10 µL. The reaction was incubated at 50°C for 20 minutes before being chilled on ice before transformation. For transformation of the ligation of the YFV mutated fragment from pGEM® T Easy-Asibi into the YFV infectious clone backbone, the two fragments were ligated together at the molar ratio of 9:1. The reaction contained 1 µL of 10X T4 ligation buffer, 1 µL of T4 ligase, and enough nuclease-free water to fill the reaction to 10 µL after addition of the cDNA

fragments. The reaction was incubated in a water bath at 4°C for 16 hours overnight and heat-inactivated at 70°C for 15 minutes before being chilled on ice before transformation.

The competent cells were pelleted down at 4,000 r.p.m. for 10 minutes at 4°C and resuspended in 220 µL of BF2 buffer per transformation reaction for up to 8 reactions. Cells for each transformation were aliquoted into pre-chilled 15 ml conical tubes and mixed with 3 µL of dimethyl sulfoxide (DMSO) and 5 µL of plasmid DNA reactions from either the InFusion reaction or the ligation before being chilled on ice for 30 minutes. The mixture of competent cells and plasmids reactions were then heat-shocked at 43.5°C for 45 seconds. The cells could recover on ice for 5 minutes before being gently resuspended in 3 ml of 2X YT broth. The cells were incubated at 37°C for 1 hour at 300 r.p.m. Transformed bacteria were centrifuged at 4,000 r.p.m. for 10 minutes and plated on 2X YT agar supplemented with ampicillin at 50 µg/ml for pWN-AB and YFV infectious clone and 100 µg/ml for pGEM® T Easy-Asibi.

The plates were incubated overnight at 37°C until individual colonies could be recognized. The individual colonies were suspended in 10 mL of 2X YT broth and the appropriate ampicillin concentration for all cDNA plasmids. The colonies were then incubated at 37°C for 16 hours at 300 r.p.m. before miniprep purification. Large-scale cultures for maxiprep purification were made in 250 mL cultures. During the day, individual colonies were suspended in 10 mL of media for 6-8 hours at 37°C and 300 r.p.m. before 1 mL of culture was transferred to a new 250 mL culture that was incubated at 37°C for 16 hours overnight at 300 r.p.m. All cultures were grown in 2X YT broth except for large-scale maxiprep cultures for the YFV infectious clone, which were grown in terrific broth.

D. Purification of plasmids

Two alkaline lysis methods of plasmid purification were used in this project. The miniprep purification was performed with overnight cultures from transformed *E. coli* cells in 10 mL of 2X YT broth. Plasmids from these preps were used for Sanger sequencing verification. Depending on the plasmid, the larger maxiprep stocks were performed with 250 mL overnight cultures in either 2X YT or terrific broth. These stocks were used to prepare the plasmids for *in vitro* transcription and electroporation.

The overnight minipreps were performed using the ZymoPURE® Plasmid Miniprep Kit (Zymo Research) with slightly modified procedures. The overnight cultures were centrifuged at 4,000 r.p.m for 10 minutes. The bacterial pellet was resuspended in 250 μ L of the red resuspension buffer (P1) with RNase and lysed with 250 μ L of the blue lysis buffer (P2). The reaction was gently inverted 4-6 times before sitting at room temperature for 2-3 minutes. After lysis, 250 μ L of the yellow neutralization buffer (P3) was added before inverting 4-6 times to mix the solution. The reaction was left at room temperature for 5 minutes to allow the reaction to neutralize completely, and the color had changed entirely to yellow; the tubes were centrifuged at 13,000 r.p.m for 10 minutes. In a new tube, the supernatant from the lysis reaction was combined with 275 μ L of Binding Buffer added for every 600 μ L of lysed reaction. The mixture was then transferred to a spin column and centrifuged at 13,000 r.p.m for 1 minute. The flow through was discarded, and 800 μ L of Wash 1 was added to the column before centrifuging at 13,000 r.p.m for 1 minute. The flow through was discarded, and 800 μ L of Wash 2 was added to the column before centrifuging at 13,000 r.p.m for 1 minute. The flow through was discarded, and 200 μ L of Wash 2 was added to the column before centrifuging at 13,000 r.p.m for 1 minute. After discarding the flow through, the column was centrifuged again at 13,000 r.p.m for 1 minute to remove an excess wash solution

from the column. The column was transferred to a new 1.5 mL Eppendorf tube, and 50 μ L of nuclease-free water was added. The column was centrifuged at 13,000 r.p.m for 1 minute before the column was discarded, and the water flow through with plasmid was stored in the -20°C freezer.

The Nucleobond® Xtra Maxi kit (Takara) was used to purify plasmids with modified procedures. The 250 mL bacterial culture was centrifuged at 4,000 r.p.m. for 10 minutes at 4°C. The pellet was then resuspended in 24 mL of Resuspension Buffer (RES) before being lysed with 24 mL of Lysis Buffer (LYS). The solution was inverted several times to mix the solutions and allowed to sit at room temperature for 3-4 minutes. After lysis, 24 mL of Neutralization Buffer (NEU) was added, and the solution was inverted several times gently until the dark blue color of the LYS buffer became a clear, light-yellow liquid. Centrifuge the lysed solution 4,000 r.p.m. for 10 minutes at 4°C. While the solution was pelleted, the gravity column filters were set up and secured before 25 mL of Equilibration Buffer (EQU) was used to wet the column before the lysed solution was added.

Additionally, 50 mL conical tubes were obtained, and a thin piece of autoclaved gauze was secured over the tube's opening with paper clips. After centrifugation, the lysed solution was transferred and filtered through the autoclaved gauze and into the 50 mL conical tube to remove the cell debris that could clog the gravity column filter. The lysed solution was transferred to the gravity filter column, and the flow through was discarded. Once the column stopped dripping liquid, 15 mL of EQU buffer was added, and the flow-through was discarded.

The fabric filter was discarded once the column stopped dripping, leaving only the silica column behind. Twenty-five mL of Wash Buffer (WASH) was added to the column, and the flow-through was discarded. After the column had stopped dripping, a 50 mL conical tube with 10.5

mL of isopropanol inside was placed under the column. Fifteen mL of Elution Buffer (ELU) was added to the column, and the flow through was collected in the 50 mL conical tube. After the column stopped dripping, the solution in the 50 mL conical tube was vortexed briefly to make and precipitated plasmid dissolve in the solution. The solution was pelleted by centrifugation at 13.000 r.p.m for 30 minutes at 4°C. The plasmid pellet was washed twice with 70% ethanol and centrifuged at 13.000 r.p.m for 30 minutes at 4°C. After the second wash, the ethanol was removed from the tube, leaving only the pellet behind. The pellet was air-dried for 20-30 minutes before resuspending in 100 µL of nuclease-free water. The plasmid solution was stored in the -20°C freezer.

E. Ligation and linearization of WNV two-plasmid reverse genetics system

The WNV infectious clone system required the deconstruction and reassembly of the two plasmids pWN-AB and pWN-CG to obtain an entire linear genome of WNV NY99 that could be used for *in vitro* transcription.

The two WNV plasmids were initially cut with restriction enzymes and exposed to Quick CIP, which nonspecifically catalyzes the DNA and RNA's dephosphorylated 5' and 3' ends. The pWN-AB plasmid was cut with *Asc1* and *NgoMIV* and exposed to Quick CIP simultaneously. This reaction cuts the plasmid at the end of the WNV NS1 sequence and linearizes the plasmid, so the plasmid backbone comes before the WNV genome. The addition of Quick CIP in this reaction ensured the ends of the DNA were dephosphorylated and could not accidentally reattach to any partially digested DNA that remained in the solution; this reaction was 50 µL, including 10 µg of pWN-AB plasmid, 5 µL of 10X CutSmart Buffer, 5 µL of *Asc1*, 5 µL of *NgoMIV*, 5 µL of Quick CIP, and enough nuclease-free water to full to 50 µL. The pWN-CG plasmid was only cut with one restriction enzyme, *Mlu1*, initially to linearize the plasmid and was exposed to Quick CIP to

dephosphorylate the ends of the DNA. The pWN-CG reaction was also 50 μ L, including 30 μ g of pWN-CG plasmid, 5 μ L of 10X CutSmart Buffer, 5 μ L of *Mlu*1, 5 μ L of Quick CIP, and enough nuclease-free water to fill to 50 μ L. Both reactions were incubated at 37°C for 1.5 hours before being purified with the PCR cleanup and gel extraction kit (Takara).

After purification, the pWN-CG plasmid was digested again with the restriction enzyme *Ngo*MIV, which will generate a complementary restriction site on the 5' end of the linearized pWN-CG plasmid and allow ligation with the 3' end of the pWN-AB plasmid. Notably, the digestion with the *Ngo*MIV created a phosphate group, which enables the formation of a phosphodiester bond with the last nucleotide on the 3' end of pWN-AB digested with *Ngo*MIV. The setup for this reaction included the entire volume of the initial pWN-CG reaction, 10 μ L of 10X CutSmart Buffer, 5 μ L of *Ngo*MIV, and enough nuclease-free water to fill the reaction to 100 μ L. The reaction was incubated at 37°C for 1.5 hours before being purified with the PCR cleanup and gel extraction kit (Takara).

The pWN-AB and pWN-CG plasmids were ligated at a 1:2 molar ratio. This reaction would create a linear DNA molecule containing the full-length WNV genome. This reaction included 2 μ g of pWN-AB plasmid cut with *Asc*1 and *Ngo*MIV, 8 μ g of pWN-CG plasmid cut with *Mlu*1 and *Ngo*MIV, 20 μ L of 10X T4 ligase buffer, 3 μ L of T4 ligase, and enough nuclease-free water to fill the reaction to 200 μ L. The reaction was incubated at room temperature for 2 hours, followed by inactivation of T4 DNA ligase at 70°C for 15 minutes. The DNA template was then digested with the restriction enzyme *Xba*1 to 1) create a run-off site for the T7 DNA-dependent RNA polymerase used for the *in vitro* transcription reaction and 2) to remove the duplicated T7 promoter sequence at the 3' end of the DNA molecular. The remaining single T7 promoter drives *in vitro* transcription of the full-length WNV genome. This reaction included 40

μL of 10X CutSmart Buffer, 6 μL of *Xba*1, 200 μL of the WNV ligation reaction, and 154 μL of nuclease-free water, then incubated at 37°C for 1.5 hours. The nuclease digestion was terminated by adding 5 μL of proteinase K and incubation at 37°C for 1-2 hours.

To prepare the DNA template for *in vitro* transcription, the protease K digestion reaction of the DNA plasmid was purified using phenol-chloroform extraction and ethanol precipitation method. The 400 μL reaction was mixed with 400 μL of phenol:chloroform:isoamylalcohol 25:24:1 solution (Life Technologies) by vortexing at 3,000 r.p.m. for 15 seconds until the solution became completely cloudy. The solution was centrifuged at 13,000 r.p.m. for 5 minutes to separate the aqueous and phenol solutions. The aqueous layer was transferred to a clean tube, and any excess phenol was removed by adding 400 μL of chloroform:isoamylalcohol 24:1 (Amresco). The solution was vortexed at 3,000 r.p.m. for 15 seconds and then at 13,000 r.p.m. for 5 minutes. The aqueous layer was precipitated with 880 μL of 100% ethanol and 40 μL of 3M sodium acetate pH 4.2 at -80°C for at least 20 minutes and pelleted at 13,000 r.p.m. for 30 minutes at room temperature. The DNA pellet was collected and washed once with 400 μL of 70% ethanol solution by centrifugation at 13,000 r.p.m. for 5 minutes. All of the solution was removed from the tube, and the pellet was air-dried for 20-30 minutes to remove any remaining ethanol. The pellet was resuspended in 100 μL nuclease-free water and subjected to *in vitro* transcription.

F. Linearization of YFV infectious clone

Following Sanger sequencing confirmation of the mutant YFV infectious clone, linearization was performed to generate a linear form of the plasmid to provide the termination site for the SP6 DNA-dependent RNA polymerase. The digestions were then purified using phenol-chloroform purification and ethanol precipitation methods. The digestion was performed with 5 μg of YFV cDNA infectious clone plasmid, 4 μL of 10X CutSmart Buffer, 1 μL of *Nru*1, and

enough nuclease-free water to fill the reaction to 40 μ L. The reaction was incubated at 37°C for 1 hour.

Purification with phenol-chloroform purification and ethanol precipitation was conducted in the same manner as will the WNV infectious clone but with slight modifications. Initially, 60 μ L of nuclease-free water was added to the restriction enzyme digestion reaction and mixed with 100 μ L of phenol:chloroform:isoamylalcohol 25:24:1 solution by vortexing. After centrifugation, at 13,000 r.p.m. for 5 minutes, the aqueous layer was transferred to a new tube and mixed with 100 μ L of chloroform:isoamylalcohol 24:1 by vortexing. After centrifugation, at 13,000 r.p.m. for 5 minutes, the aqueous layer was transferred to a new tube and mixed with 220 μ L of 100% ethanol and 10 μ L of 3M sodium acetate pH 4.2. The plasmid DNA was stored at -80°C for at least 20 minutes, followed by centrifugation at 13,000 r.p.m. for 30 minutes at ambient temperature. The pellet was collected and washed with 400 μ L of 70% ethanol solution by centrifugation at 13,000 r.p.m. for 5 minutes. All of the solution was removed from the tube, and the pellet was air-dried for 20-30 minutes to remove any remaining ethanol. The pellet was resuspended in 12 μ L nuclease-free water and subjected to *in vitro* transcription.

G. *In vitro* transcription

The *in vitro* transcription of WNV cDNA infectious clones described in the study is regulated by the T7 promoter sequence before the viral genome sequence. It is used to generate the positive-sense viral RNA transcript that acts as a template for viral protein synthesis and replication following the transfection of Vero cells. All reactions for WNV *in vitro* transcription were performed using the Ampliscribe T7 High Yield Transcription Kit (Lucigen) with modifications to the procedure. The reactions were performed by combining 300 ng of the purified WNV infectious clone plasmid, 9 μ L capping NTP-1/5A (25% of 100 mM CTP, 25% of 100 mM

GTP, 25% of 100 mM UTP, 5% of 100 mM ATP, and 20% nuclease-free water), 3 μ L of 100 mM dithiothreitol (DTT), 3 μ L of T7 enzyme solution, 10 μ L of 10X Reaction Buffer, 2.25 μ L of G(5')ppp(5')A RNA cap structure analog (New England Biolabs), and enough water to fill the reaction to 30 μ L. It is important to note that a cap analogy with an adenosine nucleotide cap is used due to a Φ 2.5 T7 promoter. The reaction was incubated at 37°C for 1 hour before being aliquoted into three separate 10 μ L aliquots.

The *in vitro* transcription of YFV cDNA infectious clones described in the study is regulated by the SP6 promoter sequence before the viral genome sequence. All the YFV *in vitro* transcription reactions were performed and modified from the procedures of the mMESSAGE mMACHINE® SP6 Transcription kit (Life Technologies). The reactions were assembled with 9 μ L of linear YFV infectious clone plasmid, 3 μ L of 20 mM GTP, 2.1 μ L of Enzyme Mix, 15 μ L of 2X Cap/NTP, and 3 μ L of 10X Reaction Buffer and then incubated at 37°C for 1 hour. After incubation, the reactions were aliquoted into three separate 10 μ L aliquots.

H. Rescue of recombinant WNV and YFV

Rescue of the recombinant WNV and YFV was undertaken via transfection of Vero cells with RNA transcripts of the respective viruses. Due to the current manufacturing guidelines for recombinant LAVs, Vero cells were used to recover the WNV and YFV mutants (Guy et al. 2010a). Approximately three million Vero cells were seeded in tissue-culture-treated T150 flasks two days before electroporation to ensure the cultures could reach 80-90% confluence. Each flask was rinsed with 20 mL of Dulbecco's phosphate buffer saline (DPBS) without Ca^{2+} or Mg^{2+} (Cellgro) and treated with 3 mL of 0.25% trypsin-EDTA (Life technologies). The cells were incubated at 37°C for 4 minutes to allow for the cells to detach from the surface of The flask, followed by suspension with 7 mL of MEM- α media supplemented with 10% FBS. The cells were

centrifuged at 1,200 r.p.m. for 5 minutes at 4°C. The cells were resuspended in 10 mL of pre-chilled DPBS and briefly vortexed before being centrifuged at 1,200 r.p.m. for 5 minutes at 4°C. Three washes were performed with pre-chilled DPBS to remove dead cells that can release ribonucleases.

After the final centrifugation, each pellet was resuspended in 400 µL of pre-chilled DPBS before being mixed with 10 µL of the viral RNA transcript. The mixture was immediately transferred to a pre-chilled 4 mm electroporation cuvette (Fisher Scientific) and electroporated using the BioRad GenePulser® system. The electroporation was performed using a single pulse at 140 volts for 25 msec. The cuvette was then placed on ice for 2 minutes to recover before being rinsed with 800 µL of pre-warmed MEM- α media three times. After each wash, the fluid in the cuvette was transferred to the T75 flask containing 7.5 mL of fresh MEM- α media.

During the wash steps and before the final centrifugation in ice-cold DPBS, the total number of cells in each flask was determined using the Countess3 Automated Cell Counter (ThermoFisher Scientific). Approximately 100 µL of the resuspended cells were sampled and mixed with 100 µL of 0.5% Trypan Blue-PBS solution (ThermoFisher Scientific). After the final centrifugation, the cell pellet was resuspended in enough ice-cold DPBS to have a concentration of 1,000,000 cells/mL. Four hundred µL of the resuspended cells were mixed with 10 µL of the viral RNA transcript. The mixture was immediately transferred to a pre-chilled 4 mm electroporation cuvette (BioRad) and electroporated using the BioRad GenePulser® system. The electroporation was performed using a single pulse at 225 volts for 25 msec. The cuvette was then placed on ice for 15 minutes to recover before being rinsed with 800 µL of pre-warmed MEM- α media three times. After each wash, the fluid in the cuvette was transferred to the T75 flask containing 7.5 mL of fresh MEM- α media.

Transfected Vero cells were maintained at 37°C with 5% CO₂. All WNV mutants were cultured in BSL-2 for four days. All YFV mutants were propagated in BSL-3 for seven days. The incubation periods are based on when cytopathic effects become apparent in 70-80% of cells.

2.3 Characterization of WNV and YFV mutants in cell culture

A. Nucleotide sequencing analysis

Confirmation of the presence of the selectively engineered amino acid substitutions was first confirmed by Sanger sequencing. Next-generation sequencing (NGS) methods further analyzed each viral RNA population.

Sanger sequencing and NGS used total RNA extract from 250 µL of virus stock, using the Direct-zol RNA Miniprep Plus kit (Zymo Research) with slightly modified procedures. Each 250 µL sample of virus stock was mixed with 750 µL of TRIzol LS Reagent (ThermoFisher) and 1 mL of 100% ethanol. The mixture was transferred to a spin column and centrifuged at 13,000 r.p.m. for 1 minute. The flow through was discarded, and 400 µL of RNA Wash Buffer was added to the column before centrifuging at 13,000 r.p.m. for 1 minute. The flow-through was discarded, and 80 µL of a DNase solution (75 µL of DNase Digestion Buffer and 5 µL of DNase) was added to each column. The DNase solution in the column was incubated at room temperature for 15 minutes. Next, 400 µL of RNA PreWash Buffer was added to the column and centrifuged at 13,000 r.p.m. for 1 minute. The flow through was discarded, and 400 µL of RNA PreWash Buffer was added to the column before centrifuging at 13,000 r.p.m. for 1 minute. The flow through was discarded, and 700 µL of RNA Wash Buffer was added to the column before centrifuging at 13,000 r.p.m. for 1 minute. The flow-through was discarded, the column was transferred to a new 1.5 mL Eppendorf tube, and 40 µL of nuclease-free water was added. The column was centrifuged at 13,000 r.p.m.

for 1 minute before the column was discarded, and the water flow through with the RNA was stored in the -80°C freezer.

Sanger sequencing was undertaken to determine the consensus sequence of each electroporated virus (P0) of WNV and YFV and up to five passages (P5) of WNV. Oligonucleotide fragments that corresponded to parts of prM and E genes of WNV (Figure 2-6) or YFV (Figure 2-7) were used to amplify the RNA via RT-PCR the mutated regions of the viruses to create a cDNA fragment from the viral RNA that could be sequenced as previously described. (Beasley et al. 2003).

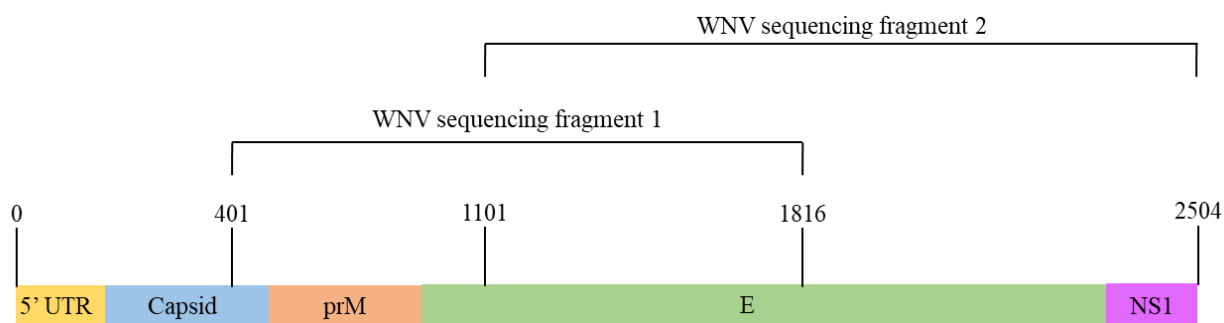


Figure 2-6: Sanger sequencing cDNA fragments for verification of WNV mutations

The image represents the full 5'UTR, capsid, prM, and E proteins of the YFV genome and the first part of NS1. The numbering is based on the WNV NY99 sequence. The image was generated in PowerPoint.

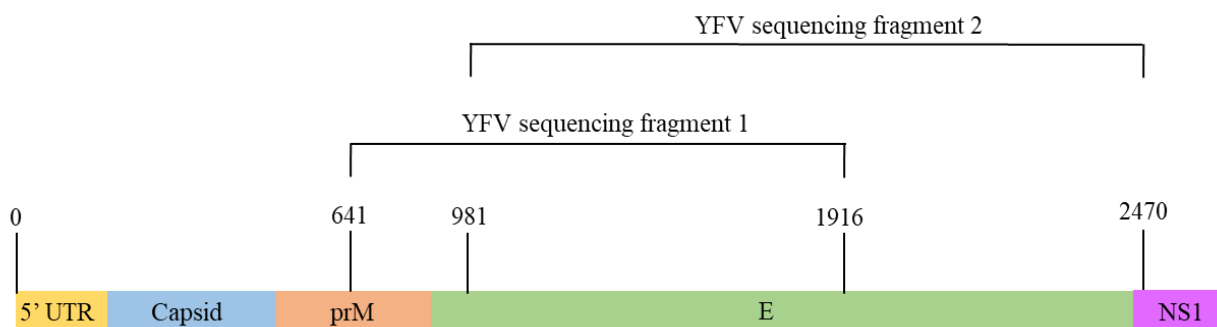


Figure 2-7: Sanger sequencing cDNA fragments for verification of YFV mutations

The image represents the full 5'UTR, capsid, prM, and E proteins of the YFV genome and the first part of NS1. The numbering is based on the YFV Asibi sequence. The image was generated in PowerPoint.

The RT-PCR reaction utilized the PrimeScript® One-Step RT-PCR Kit Ver.2 (Takara). Each reaction consisted of 2 µL of PrimeScript® 1 step Enzyme Mix, 25 µL of 2X 1 step buffer, 2 µL of 10 µM forward primer, 2 µL of 10 µM reverse primer, 15 µL of nuclease-free water, and 4 µL of viral RNA template. The cDNA fragment was produced by activating the enzymes at 50°C for 30 minutes, denaturization at 94°C for 2 minutes, 30 cycles of denaturization at 94°C for 30 seconds, and primer annealing at 50°C for 30 seconds, and elongation at 72°C for 1 minute, before finally cooling the reaction at 4°C for 5 minutes. The resulting cDNA was isolated by 1% agarose gel electrophoresis and purified by the Zymoclean Gel DNA Recovery kit (Zymo Research) or PCR cleanup and gel extraction kit (Takara). The Sanger sequencing samples were sent to Genewiz and Plasmidsaurus, and the results were compared to mutated plasmid maps created in the SnapGene software to confirm the mutations had remained.

Further, NGS was performed by Dr. Alan Barrett's laboratory at the University of Texas Medical Branch to investigate the RNA population of the P0 virus or passage one (P1) stocks of the WNV NY99ic strain and the respective mutants rescued in two independent replicates. The

NGS libraries were constructed using random hexamer primers and sequenced on the NextSeq550 platform using a paired-end 75nt base protocol (Carpio et al. 2023). The sequencing files from each virus were randomly down-sampled to 3500 reads per nucleotide position to determine the consensus sequence, identify single nucleotide variants (SNVs) that exceed 1% frequency, and calculate Shannon Entropy using previously published methods (Kaiser et al. 2019a, Kaiser et al. 2019b).

B. Determination of infectivity and multiplication kinetics

Two methods were utilized to determine the viral infectivity of the WNV and YFV mutants: plaque assay and 50% tissue culture infectious dose method (TCID₅₀).

The plaque assays were performed to determine the infectivity of the P0 and P1 viruses by determining the genome copy-to-plaque-forming units (PFU) ratios. These ratios allow us to measure the number of viral particles in each sample that can complete a full infectious cycle. Plates with 24 wells were seeded with 8×10^4 cells per well and incubated at 37°C for 1 day. Fifty μ L of 10-fold dilutions of the virus, going from dilutions 10^0 to 10^{-7} , were inoculated into the pre-seeded 24-well plates before being incubated at 37°C for 1 hour. Every 15 minutes of the hour incubation, the plates were shaken to ensure an even spread of the virus over the cells and maximum absorption of the virus. The plates were maintained in cell culture media supplemented with 1.5% carboxymethylcellulose and maintained at 37°C with 5% CO₂.

For WNV plates, the plaque assays were incubated for 5 days before the plates were fixed for 30 minutes with 10% formol saline and then stained with 1% crystal violet solution (1% crystal violet, 40% methanol, 60% distilled water). For YFV plates, the plaque assays were incubated for 7 days. Each well was washed twice with 1 mL of DPBS. After the second wash, 100 μ L of 12 mM of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were added to each

well. The plates were incubated for 1 hour at 37°C. All liquid was removed from the wells and the number of plaques was counted using a light box.

The multiplication kinetics of WNV and YFV mutants in Vero and BHK-21 cells were determined by TCID₅₀. One hundred µL of culture supernatant was collected from the respective cell lines infected at a multiplicity of infection of 0.1 every 24 hours and was inoculated into 96-well microtiter plates in duplicate and 10-fold serially diluted from 10⁰ to 10⁻¹⁰. Following dilution, 100 µL of Vero cells (2*10⁶ cells/ml) were added into each well. The plates were then incubated at 37°C for 7 days. Samples of 100 µL were collected each day before replacing the missing media with 100 µL of fresh media.

Cultures of Vero cells infected with WNV were stained with 0.5% amido black solution containing 35% of isopropyl alcohol and 10% of glacial acetic acid, and their titers were calculated using the Muench-Reed method. Cultures of Vero cells infected with YFV were inactivated with 200 µL per well of acetone (75% ice-cold acetone and 25% phosphate-buffered saline (PBS)) for 15 minutes. The solution in the wells was then discarded, and the plates were dried to prepare them for indirect immunofluorescent assay. After drying, the plates were placed at 37°C for 5 minutes to warm the plate. The primary antibody (MA93 mouse anti-YFV hyperimmune serum) was diluted 200-fold in PBS. Forty µL of the diluted primary antibody was added to each well and then incubated at 37°C for 40 minutes. The plates were then washed twice with 200 µL of PBS per well. The secondary antibody (Alexa Fluor 488 goat anti-mouse IgG (H+L)) was diluted 200-fold in PBS supplemented with 0.005% Evans blue counter stain. Forty µL of the diluted secondary antibody was added to each well and then incubated at 37°C for 40 minutes. Each well was then washed twice with 200 µL of PBS and once with 200 µL of deionized water. The fluid was removed by gentle decanting, followed by adding one drop of mounting media consisting of

glycerol-PBS 9:1 mixture supplemented with anti-fading agent 1,4-diazobicyclo-(2,2,2-octane) at 25 mg/ml pH 8.6. Infectivity was then scored using an Olympus XP71 fluorescent microscope.

C. Genome-PFU ratio analysis

To eliminate the background RNA inoculum introduced by transfection, the genome-to-PFU ratios of the respective mutants were determined by infecting Vero cells with P0 stocks. Approximately 2×10^5 cells were seeded to each well of a 6-well plate. The virus stocks were diluted to 2×10^4 PFU/mL in 250 μ L. The plates were incubated at 37°C for 60 minutes to allow virus absorption into the cells, followed by removing the inoculum and two washes with DPBS before adding 4 mL of fresh culture media into each well. The plates infected with WNV and YFV were then incubated at 37°C for 3 and 5 days, respectively. Supernatant from each well was stored at -80°C. The timing of harvesting the virus was established according to the highest infectivity achieved by the wild-type parental strain of the respective viruses.

One aliquot of each sample was titrated by plaque assay as described in section 2.3.B. The total RNA was extracted from a separate aliquot using the Direct-zol RNA Miniprep Plus kit (Zymo Research) described in section 2.3.A. Viral RNA in each sample was analyzed by quantitative RT-PCR using the pan-flavivirus mFU1 and CFD2 primer set and a virus-specific probe conjugated with 6-carboxyfluorescein at the 5' terminus (WNV-specific probe: 5'-TGCGTGAAGTTGGCACCCGGCCT-3'; YFV-specific probe: 5'-TCAGAGACCTGGCTGCAATGGATGGT-3') (Chao et al. 2007). Each reaction included 2 μ L of RNA extract that was amplified with 5 μ L of 2x iTaq universal probe master mix, 0.5 μ L of 10 μ M mFU1, 0.5 μ L of 10 μ M CFD2, 0.2 μ L of 10 μ M TaqMan WNV probe, 0.25 μ L of iScript reverse transcriptase, and 1.55 μ L of nuclease-free water. The reactions were incubated under the following parameters: reverse transcription at 50°C for 20 minutes, hot-start activation at 95°C for

3 minutes, and 45 cycles of denaturation at 95°C for 15 seconds and annealing and amplification at 48°C for 3 minutes with continuous fluorescence detection. The quantity of viral RNA in each sample was calculated using the standard curve generated from serially diluted RNA templates derived from the *in vitro* transcription of the respective viral genomes. A Ct value of 33 was used as the cut-off for detecting the viral genome (Lyons et al. 2018).

D. Serial passaging of WNV mutants

Serial passage of WNV mutants in Vero cells was undertaken to determine the stability of the engineered amino acid substitutions. The P0 stock of the respective mutants and the wild-type parental virus was inoculated into a monolayer of Vero cells (2×10^5 cells, moi=0.1) in triplicate, adsorbed at 37°C for 60 minutes, washed twice with DPBS, replenished with 4 ml of fresh media, and incubated for three days and harvested to generate three biological replicates of the P1 stock. The procedure was repeated up to passage 5 (P5).

E. Hemagglutination activity of WNV mutants

Stocks of each WNV mutant were prepared in C6/36 cells and infected at a moi of 0.001, resulting in P1 virus stocks. These stocks were then used for hemagglutination (HA) assay. Gander erythrocytes (Innovative Research) were used for all HA assays, following the protocol as previously described (Clarke and Casals 1958). Each mutant and WNV NY99ic wild type were serially diluted in a 2-fold dilution, and their optimal pH for HA activity was determined between pH 5.8 and pH 7.4 in 0.2 pH increments. Nine pH solutions were created for the HA assay. They involved a combination of borate saline (0.5M borate, 1.5M NaCl, and 1M NaOH at a pH of 9), disodium phosphate solution (0.15M NaCl and 0.2M disodium phosphate (Na_2HPO_4) at a pH of 4-5), and sodium dihydrogen phosphate solution (0.15M NaCl and 0.2M sodium dihydrogen

phosphate (NaH_2PO_4) at a pH of 8.5-9.5. The quantities required for each of the three solutions in order to create the proper pH solution are listed in Table 2-12.

Table 2-12: Buffer quantities for simulated hemagglutination in various pH environments

Final pH	Volume (mL) of 0.15M NaCl and 0.2M Na_2HPO_4	Volume (mL) of 0.15M NaCl and 0.2M NaH_2PO_4	Volume (mL) of 0.5M borate saline
5.8	3.0	97.0	100
6.0	12.5	87.5	100
6.2	22.0	78.0	100
6.4	32.0	68.0	100
6.6	45.0	55.0	100
6.8	55.0	45.0	100
7.0	64.0	36.0	100
7.2	72.0	28.0	100
7.4	79.0	21.0	100

In 96-well plates, each WNV mutant was 2-fold serially diluted between 2 and 4096-fold. Each dilution was performed at the nine borate pH solutions constructed using Table 2-12. Fifty μL borate pH solution was added to each well. Then 50 μL of C6/36 grown virus was added to the first well and underwent a 2-fold dilution. The 5% gander erythrocytes were diluted 10-fold, followed by adding 50 μL of gander erythrocytes to each well. The plates were then covered and incubated at 37°C for up to 60 minutes, depending on the development of hemagglutination activity. The HA titers were calculated based on the reciprocal of the highest dilution leading to complete agglutination. The optimal pH for the HA activity of each WNV mutant was determined by the pH yielding the highest HA titer.

2.4 Virulence phenotype of WNV and YFV mutants in mice

A. WNV neuroinvasive disease mouse model

Groups of four-week-old outbred Swiss Webster mice (Charles River) were used to investigate the neuroinvasive phenotype of WNV mutants. Previous studies have shown 3- to 4-week-old Swiss Webster are an effective animal model for WNV studies. The outbred Swiss Webster mice are susceptible to WNV NY99ic, and they will succumb to neuroinvasive disease after being challenged intraperitoneally with a lethal dose (LD_{50}) of 0.5 PFU (Beasley et al. 2002, Beasley et al. 2005). Therefore, a high dose of infectious wild-type WNV (1000 LD_{50}) was chosen to determine the attenuating effect the WNV E protein mutants had on the neuroinvasive phenotype. The WNV mutants or the wild-type WNV NY99ic were each injected into groups of five male and five female mice via the intraperitoneal (IP) route and monitored for 14 days (Figure 2-8). Animals were euthanized after 20% or greater weight loss compared to the starting weight reached or the onset of neurotropic diseases such as paralysis. Brain samples were collected from all mice after euthanizing, and blood samples were collected via cardiac stick from all mice that survived to the end of the 14-day study. All animal experiments were conducted in compliance with the *National Institute of Health Guide for the Care and Use of Laboratory Animals* and approved by the Institutional Animal Care and Use Committee within Kansas State University under protocol number 4322.

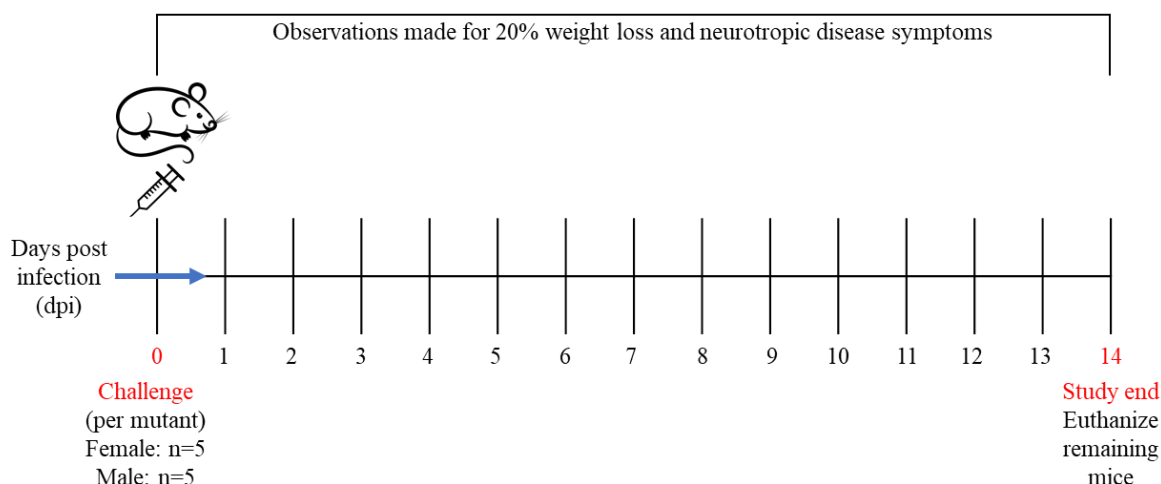


Figure 2-8: Mouse study design for outbred Swiss Webster mice intraperitoneally injected with WNV

Image used with permission from Ashley Bilyeu's 2023 Master's thesis (Bilyeu 2023).

B. Wild-type YFV viscerotropic disease mouse model

Due to the logistical difficulties and high cost of acquiring immunocompromised AG129 (IFN $\alpha/\beta/\gamma$ R^{-/-}) mice (Marshall Bioresources), all work related to the wild-type YFV viscerotropic disease mouse model was undertaken by scientists at Bioqual, Inc. Briefly, groups of 6-week-old AG129 mice were bred at Bioqual Inc. and used to investigate the virulence phenotype of YFV mutants. Previous studies have shown that AG129 mice are highly susceptible to wild-type YFV, succumbing within 6-7 days, making them an effective model for YFV studies. Additionally, AG129 mice develop a viscerotropic disease similar to human YF instead of the encephalitis signs typically observed in mice (Meier et al. 2009). A high dose of infectious wild-type YFV was chosen to determine the attenuating effect of YFV E protein mutants on the viscerotropic phenotype. The YFV mutants and the wild-type YFV Asibi strain were each injected into groups of four male and four female mice at 1,000 PFU via the intraperitoneal (IP) route and monitored for 21 days (Figure 2-9). Animals were euthanized after 20% or greater weight loss compared to

the starting weight reached or the onset of viscerotropic disease. Blood samples were collected via cardiac sticks from all mice after being euthanized.

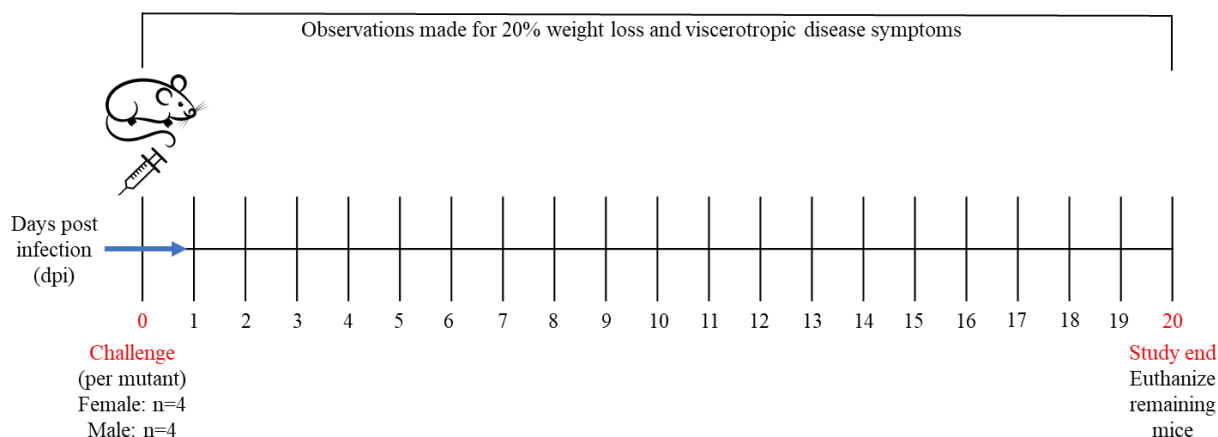


Figure 2-9: Mouse study design for immunocompromised AG129 mice intraperitoneally injected with YFV

The image was generated in PowerPoint.

All experiments at Bioqual Inc. complied with the *National Institute of Health Guide for the Care and Use of Laboratory Animals* and approved by the KSU Institutional Animal Care and Use Committee (Protocol number: 21-49).

C. Analysis of mouse tissues

The brain of each mouse was collected following euthanasia to quantify tissue viral load. Each brain sample was resuspended in 1 mL of 1x L-15 media supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, and 10% tryptose phosphate buffer and stored at -80°C. The tissue was homogenized using a Qiagen TissueLyzer II set to a frequency of 26 Hz for 4 minutes to collect the viral RNA from the brain tissue. After homogenization, the samples were pelleted down by centrifugation at 13,000 r.p.m. for 10 minutes.

RNA from the homogenized tissue supernatant was extracted using the Direct-zol RNA miniprep plus columns (Zymo Research). The viral RNA was also used to confirm the virus did

not revert to the virulent wild-type sequence using the Sanger sequence. Briefly, the RNA extract was reverse transcribed to cDNA fragments and amplified by the E gene-specific primers using the PrimeScript® One-Step RT-PCR Kit Ver.2 (Takara). Each amplicon was separated by agarose gel electrophoresis, extracted using the PCR cleanup and gel extraction kit (Takara), and submitted for Sanger sequencing as conducted in section 2.3.A. The results were compared to mutated plasmid maps created in the SnapGene software to confirm the mutations had remained. The remainder of the RNA was used to quantify the tissue viral load using quantitative RT-PCR using the same procedure depicted in section 2.3.D. As previously described, any Ct value above 33 was considered negative detection (Lyons et al. 2018).

Serum samples were collected from the surviving mice at 14 dpi to determine serum-neutralizing antibody activity by 50% plaque reduction neutralization test (PRNT₅₀) as previously described (Roehrig et al. 2008). The serum samples were heat-inactivated at 56°C for 30 minutes and 2-fold serially diluted. The dilutions were mixed with approximately 100 PFU of WNV NY99 and incubated at 37°C for 1 hour before being inoculated into 24-well plates seeded the day before with 2×10^5 cells/well. At 5 dpi, the plates were fixed for 30 minutes with 10% formol saline and then stained with 1% crystal violet solution. The PRNT₅₀ titer was calculated by the reciprocal of the highest dilution that created a 50% reduction in plaque number compared to the virus control.

2.5 Statistical analysis

This project was generated with continuous and categorical data. Infectivity, genome-PFU ratio, and mouse brain viral load of WNV mutants were compared against WNV NY99ic using one-way analysis of variance (ANOVA). Multiplication kinetics of WNV mutants in Vero and BHK-21 cells and infectivity of serially passaged WNV mutants were analyzed using two-way ANOVA. A pairwise comparison between each mutant and the parental wild-type WNV NY99ic

control was performed using Dunnett's test. Survival outcomes of mice challenged with WNV mutants were compared using the Kaplan-Meier survival curve. All statistical analysis was performed using the Prism 9 program (GraphPad Software).

2.6 Generation of 3D protein models with PyMOL

All crystal structure images used in this dissertation were generated using the open-source molecular visualization software PyMOL. The base structure for every image was obtained from the Protein Data Bank (PDB), a database of three-dimensional structures of large biological molecules such as proteins and nucleic acids. Every crystal structure image this dissertation uses is annotated with the source PDB molecule, i.e., PDB ID: 1OAN. The PyMOL software allows for easy identification of important domains and residues of particular proteins, including altering the amino acid sequence to depict different single-site mutations. However, PyMOL is not a protein folding simulation despite altering the amino acid sequence. Changing the amino acids does not change the location of individual α -helices and β -sheets. Therefore, each image is an estimated structure based on the source PDB crystal structure.

Chapter 3 - Engineering of Attenuating Mutations in the WNV EDI-EDII Hinge Region

The results presented in this chapter involved multiple collaborative projects. The WNV NY99 infectious clone was obtained from Dr. Claire Huang's laboratory at the Centers for Disease Control and Prevention in Fort Collins, CO. Modifications of this clone and analysis of the mutations in the WNV EDI-EDII hinge region *in vitro* were performed with the assistance of Ashley Bilyeu and Adrienne Pohl in Dr. Vanlandingham's laboratory at Kansas State University. The plaque assay infectivity titers of the eight mutations tested in Section 3.2.A were originally published in Ashley Bilyeu's 2023 Master's thesis and further analyzed in this dissertation (Bilyeu 2023). The next-generation sequencing in Section 3.2.E was performed by Dr. Alan Barrett's laboratory at the University of Texas Medical Branch and further analyzed by Bailey Maloney in Dr. Vanlandingham's laboratory at Kansas State University. The *in vivo* mouse study of the eight mutations tested in Section 3.2.F was originally published in Ashley Bilyeu's K-State Master's thesis and further analyzed in this dissertation (Bilyeu 2023). The mouse studies and analyses in Section 3.2.I were performed in collaboration with several members of Dr. Vanlandingham's laboratory, with the exception of the PRNT₅₀ titers of the E-A54E and E-A54I mutants in Section 3.2.I, which Dr. Claire Huang's laboratory performed. Specific credits to each individual who contributed to or conducted each experiment are summarized in Table 3-1.

Table 3-1: Acknowledgements for collaborative projects in Chapter 3

Collaborators from Kansas State University: Natalia Costa Ball – NCB, Ashley N Bilyeu – ANB, Bailey E Maloney – BEM, Emily K Mantlo – EKM, So Lee Park – SLP, Adrienne E Pohl – AEP, Danielle R D Saunders – DRS, and Alexis D. Schneider – ADS

Collaborators from the University of Texas Medical Branch: Alan D T Barrett – ADB and Kassandra L Carpio – KLC

Collaborator from the Centers for Disease Control and Prevention: Claire Y H Huang – CYH

Key Experiments	Relevant Figures and Tables	Collaborators
Modification and recovery of WNV mutants	Table 3-6	BEM, ANB, CYH
	Table 3-12	BEM, ANB, AEP, CYH
	Table 3-13	BEM, AEP, CYH
Characterization of WNV mutants in cell culture	Figure 3-10	BEM, ANB
	Figure 3-12	ADB, KLC
	Tables 3-8, 3-9	
	Figure 3-18	BEM, ANB, AEP
	Figure 3-21	BEM, AEP
Characterization of WNV mutants in mouse neuroinvasive disease model	Figure 3-13	BEM, ANB, SLP, DRS
	Tables 3-10, 3-11	
	Figure 3-15	ANB
	Figures 3-23, 3-26	BEM, NCB, AEP, SLP
	Tables 3-14, 3-15, 3-16, 3-17	
	Figure 3-25	CYH
	Figure 3-28	BEM, NCB, AEP, EKM, ADS
	Tables 3-18, 3-19	

3.1 Introduction

A. Configuration of the EDI-EDII hinge region

The EDI-EDII hinge region is crucial for the conformational change of the E protein from dimer in the virion to trimer inserted into the host cell because the hinge effect at the EDI-EDII domain interface controls the formation of the E protein trimer (Figures 3-1 and 3-2).

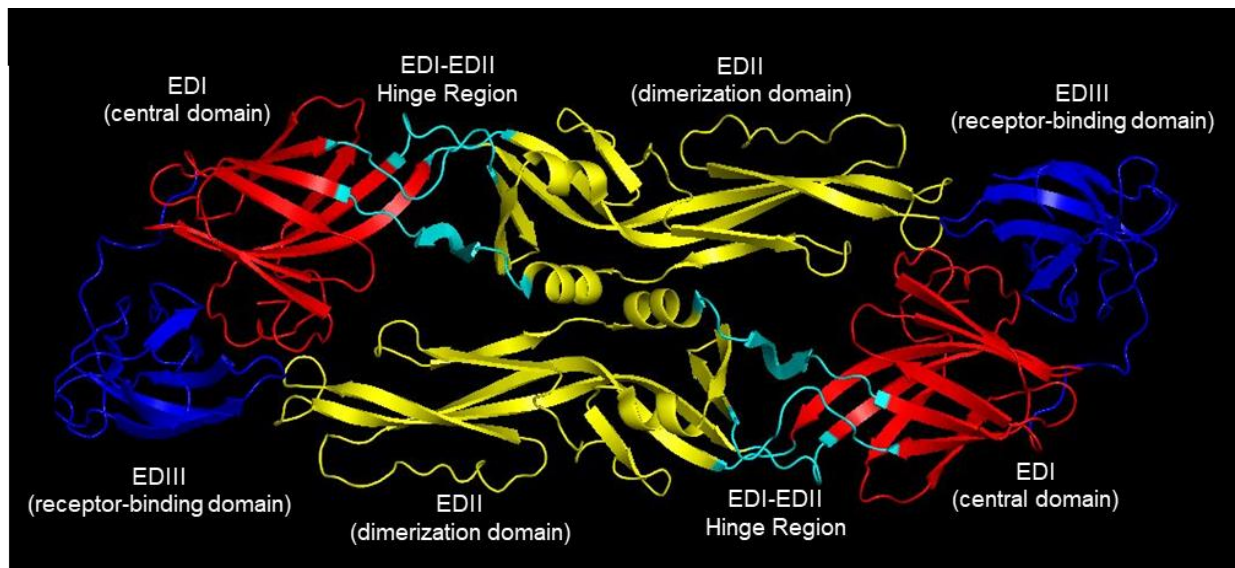


Figure 3-1: The crystal structure of the flavivirus E protein dimer of DENV-2 with EDI-EDII hinge region (PDB ID: 1OAN)

The three domains are red for EDI, yellow for EDII, and blue for EDIII. The EDI-EDII hinge region is labeled with cyan. The image was generated in PyMOL.

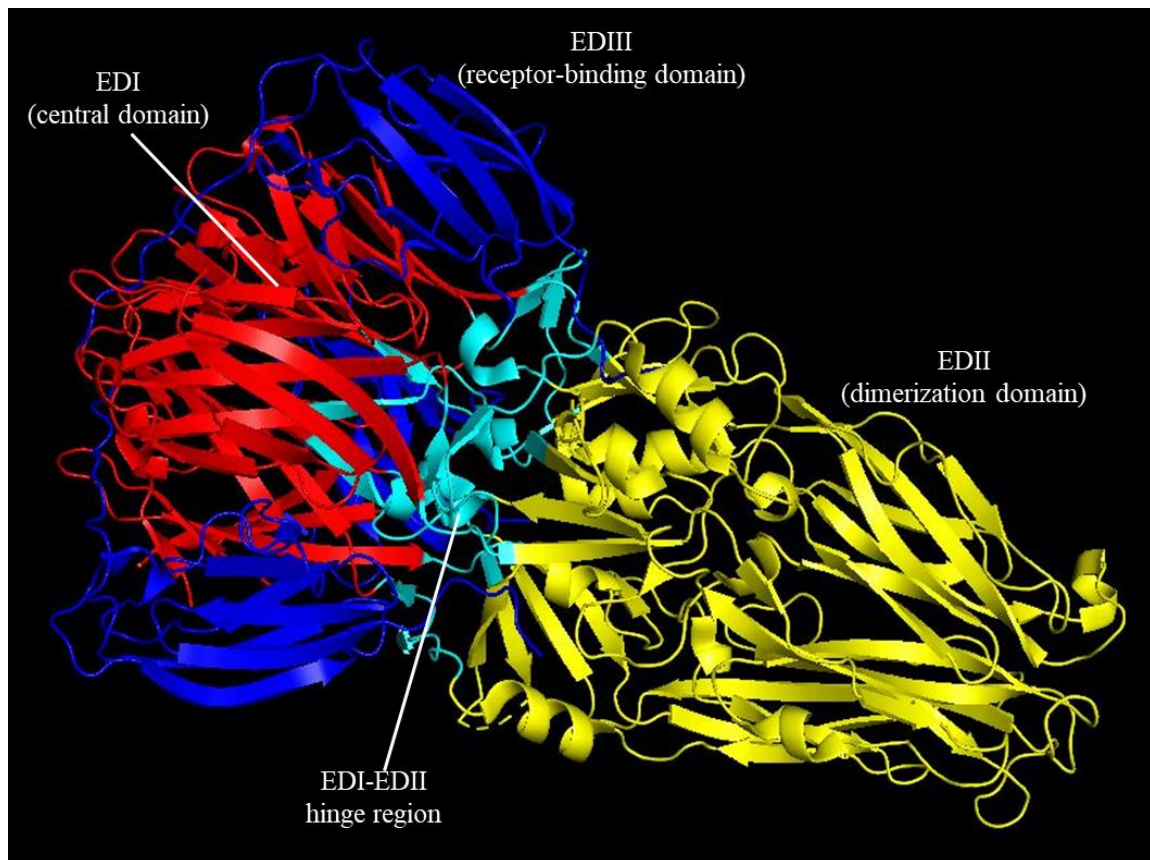


Figure 3-2: The crystal structure of a flavivirus E protein trimer of TBEV in the post-fusion conformation after transition from a dimer to a trimer (PDB ID: 1URZ)

The three domains are red for EDI, yellow for EDII, and blue for EDIII. The EDI-EDII hinge region is labeled with cyan. The only available crystal structure of the flavivirus E protein in the trimer formation is for TBEV. All other crystal structures from PDB are monomers in post-fusion conformation. The image was generated in PyMOL.

The EDI-EDII hinge region consists of four motifs encoded by a discontinuous sequence. Comparison of the DENV-2 E protein dimer and trimer suggests the EDI-EDII hinge region connects the A₀C₀D₀E₀F₀ and B₀I₀G₀H₀ β-sheets of EDI to the "gfeah" β-sheet at the base of EDII. The dimer-to-trimer transition of the E protein rearranges the relative orientation of the "gfeah" β-sheet of EDII about the structurally central EDI. Specifically, the A₀C₀D₀E₀F₀ and B₀I₀G₀H₀ β-

sheets are integral to the molecular architecture of EDI (Figure 3-3) (Zhang et al. 2012). Both β -sheets are configured almost parallel, with one facing the extracellular space, away from the virion, and one facing the viral membrane. The $A_0C_0D_0E_0F_0$ β -sheet faces externally toward the extracellular space. It contains four β -strands $C_0D_0E_0F_0$ with a short A_0 β -strand parallel to the C_0 β -strand. The $G_0H_0I_0B_0$ β -sheet faces toward the viral membrane (Kuhn et al. 2002, Zhang et al. 2004).

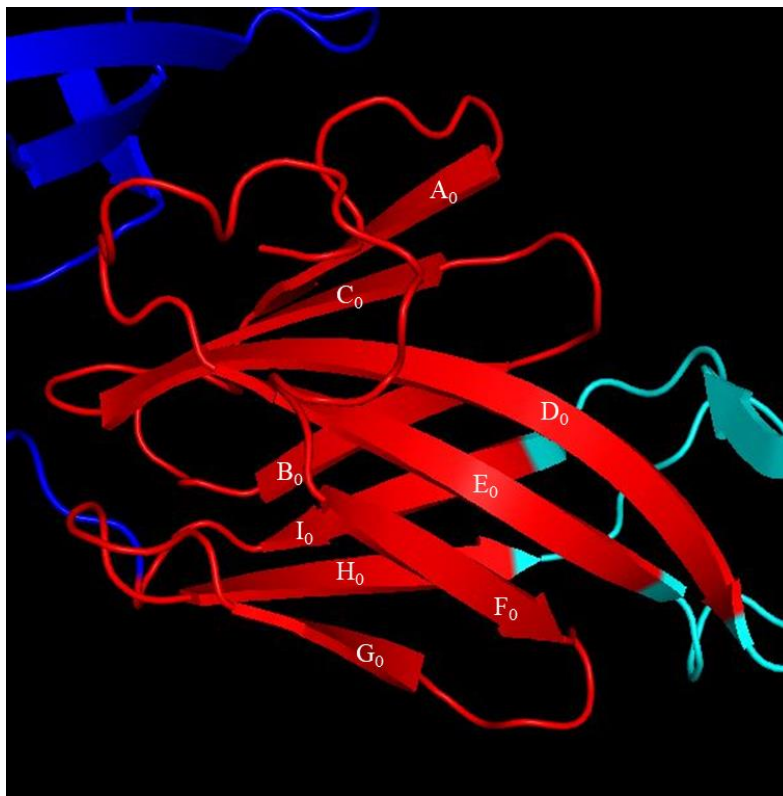


Figure 3-3: Configuration of the EDI β -sheets in DENV- 2 E protein (PDB ID: 1OAN)

Image was generated in PyMOL.

The new orientation of EDII facilitates the dissociation of the E protein dimer into two copies of monomers. Every two copies of E monomers can be assembled to form the E protein dimer. Notably, the orientation of the “gfeah” β -sheet will expose the fusion loop located at the tip of the “bcd” β -sheet within EDII from the protected position in the dimeric conformation (Figure

3-4). Therefore, the EDI-EDII hinge region plays an essential role in the molecular basis of the conformational change of the E protein from dimer in the virion to trimer inserted into the host cell (Rey et al. 1995, Allison et al. 2001).

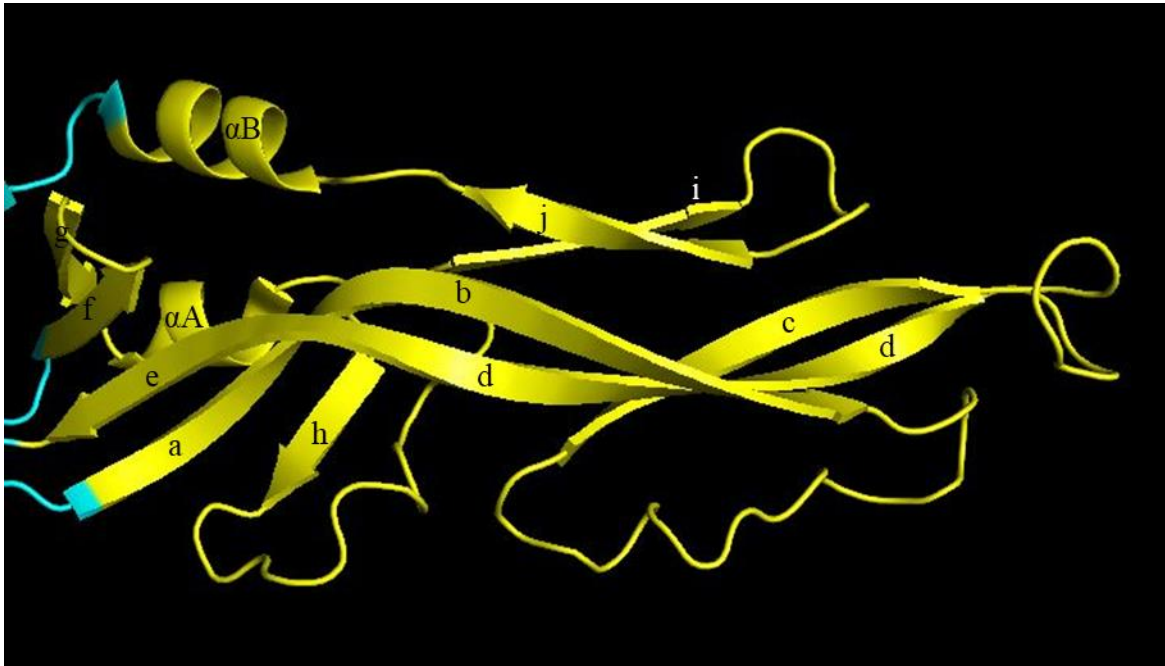


Figure 3-4: Configuration of the EDII β -sheets and α -helices in DENV-2 E protein (PDB ID: 1OAN)

Image was generated in PyMOL.

The EDI-EDII hinge region comprises two β -sheets that move during the conformational change of the E protein from a dimer to a trimer (Figure 3-5). The first is the gfeah β -sheet, comprised of β -strands that include parts of the EDI-EDII hinge region and the EDII domain. The second β -sheet is the kID₀ β -sheets which is comprised of two β -strands in the EDI-EDII hinge region and one β -strand in the EDI domain (Modis et al. 2003).

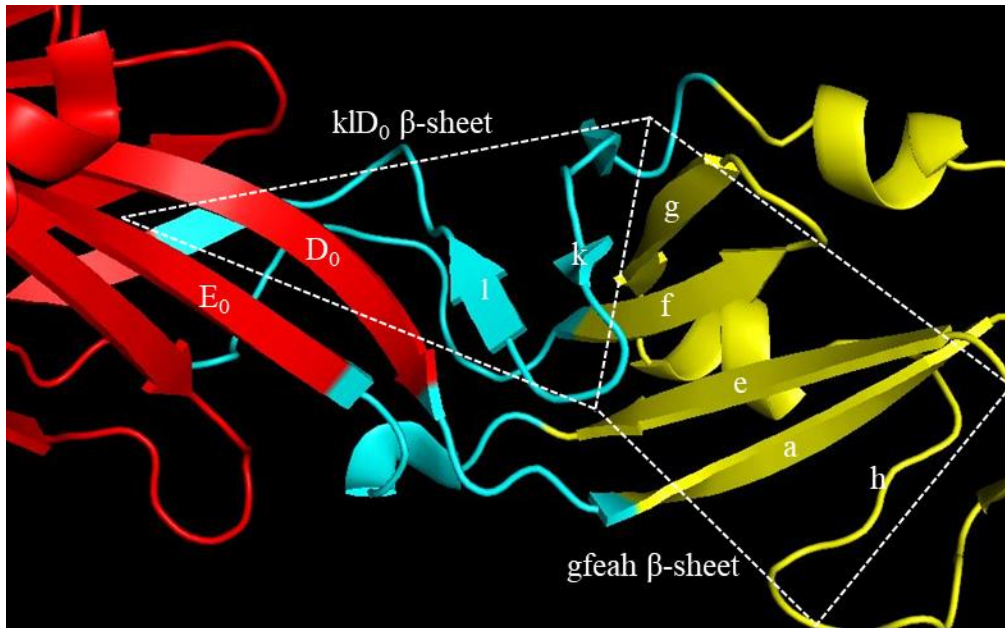


Figure 3-5: The relative location of the kID₀ and the gfeah β -sheets in the crystal structure of the WNV E protein monomer in the pre-fusion structural conformation (PBD ID: 2HG0)

EDI is labeled in red, EDII in yellow, and the EDI-EDII hinge region is labeled in cyan. Two β -sheets comprise the EDI-EDII hinge region: kID₀ (left) and the gfeah (right) β -sheets. The image was generated in PyMOL.

During the conformational change of the E protein, the gfeah β -sheet rotates along the axle of the fg hairpin to change the orientation of EDII and expose the fusion loop (Bressanelli et al. 2004). The rotation of the gfeah β -sheet triggers the rearrangement of the three β -strands in the kID₀ β -sheet, resulting in the formation of hydrogen bonds between l and D₀ β -strands in the E protein trimer (Modis et al. 2004). Additionally, the k β -strand moves away from the l β -strand, resulting in a change from being parallel to each other to being perpendicular (Figure 3-6).



Figure 3-6: Conformational change of the klD₀ and the gfeah β -sheets in the post-fusion crystal structure of the EDI-EDII hinge region in the DENV-2 E protein (PBD ID: 1OK8)

EDI is labeled in red, EDII in yellow, and the EDI-EDII hinge region is labeled in cyan. The dotted white lines depict the conformational change of the kl hairpin from two β -strands parallel to each other to being situated perpendicular. The green arrow shows the gfeah β -sheet's rotation about the fg hairpin's axis. The image was generated in PyMOL.

B. Attenuation of the flavivirus EDI-EDII hinge region

Previous research has examined single-site mutations in the EDI-EDII hinge region that exhibit the attenuated phenotype. For example, the E-S277I mutation was previously shown to fully attenuate the neuroinvasive phenotype of Murray Valley encephalitis virus (MVEV) in Swiss Webster mice (Hurrelbrink and McMinn 2001). While the MVEV E-S277I mutant exhibited a loss

of HA activity, this is indicative of interference with the membrane fusion process. The MVEV E-S277 resides in the 4th motif of the EDI-EDII hinge region and is part of the Ser-Ser-Ser/Asn-Thr consensus sequence, which is conserved in the JEV serocomplex. The Ser-Ser-Ser/Asn-Thr consensus sequence resides in the hydrophilic β -turn that links the k and l β -sheets of the kl hairpin and therefore provides the first evidence that the molecular hinge region can serve as a determinant of neurovirulence (Hurrelbrink and McMinn 2001).

In a study by Leslie Goo, another EDI-EDII hinge region mutation in WNV attenuated the neurovirulence phenotype in mice (Goo et al. 2017). The WNV E-T198F mutant resides in the 3rd motif of the EDI-EDII hinge region, and it exhibited an increased exposure of the fusion loop, resulting in increased sensitivity to neutralizing antibodies. The E-T198F mutant changed the conformational flexibility of the E protein, which had no substantial impairment of WNV growth kinetics *in vitro*. However, testing in mice revealed a reduction in viremia early after infection, before WNV-specific antibodies become detectable, suggesting the early suppression of infection led to the attenuation of the neuroinvasive phenotype in mice. Testing of mutations in the equivalent residues in DENV-1 (E-F193T) and ZIKV (E-F198T) revealed similarly increased accessibility to the fusion loop for neutralizing antibodies DENV-1, but not in ZIKV (Goo et al. 2017). This study showed that producing an attenuated phenotype through mutation of the EDI-EDII hinge region could apply to more than one flavivirus. However, these mutations are located at virus-specific residues. The MVEV E-S277I and WNV E-T198F mutants are located at residues that share some amino acid homology within their serocomplexes but are not conserved across all serocomplexes. Therefore, it is unclear whether these mutations benefit vaccine development. Ideal mutations in these regions for the development of candidate flavivirus vaccines would be in residues that are conserved among all the serocomplexes of flaviviruses.

C. Amino acid sequence homology of four motifs that constitute the EDI-EDII hinge region

This dissertation looks at eight flavivirus-conserved hydrophobic residues in the EDI-EDII hinge region of WNV to probe the importance of the hydrophobic pocket for the structure-function of the E protein involved in the membrane fusion process. Further, mutations at these residues that can attenuate the mouse neuroinvasive phenotype without compromising the immunogenicity will apply to the rational design of candidate WN LAVs. Each of these eight residues resides within the four molecular hinge peptides, loosely packed structures that connect the EDI and EDII domains of the flavivirus E protein (Modis et al. 2004, Kanai et al. 2006). The E-A54S mutation resides within the 1st motif of the EDI-EDII hinge region between the D₀ β -strand of EDI and the “a” β -strand of EDII. The first motif of the EDI-EDII hinge region consists of four residues, each exhibiting different levels of amino acid sequence plasticity (Table 3-2). The first two amino acids equivalent to WNV E-A51 and E-N52 residues are highly variable. The only serocomplex where conservation of the sequence of these two residues was observed was in the TBEV serocomplex, which encodes the E-E51 and E-N52 residues. The amino acid equivalents to WNV E-L53 and E-A54 residues showed a conserved amino acid sequence within each serocomplex: JEV serocomplex (E-L53 and E-A54), YFV serocomplex (E-P53 and E-A54), DENV serocomplex (E-P53 and E-A54), and TBEV serocomplex (E-P53 and E-A54). Therefore, we selected the WNV E-A54 residue for the site-directed mutagenesis analysis.

Table 3-2: Sequence of the 1st motif of the EDI-EDII hinge region between flaviviruses

The properties of amino acids are shown in green (hydrophobic), pink (polar), red (negatively charged), blue (positively charged), and black (unique). The numbering corresponds to the amino acid positions in the WNV E protein. Highly conserved hydrophobic amino acids are labeled with bold text and underlined. The numbering of amino acids in the motif is based on the WNV NY99 sequence.

Serocomplex	Virus	Strain	Sequence of 1 st Motif (E 51-54)
JEV	WNV	NY99	ANL <u>A</u>
	JEV	SA14	SQ <u>L</u> <u>A</u>
	JEV	SA14-14-2	SQ <u>L</u> <u>A</u>
	KNV	MRM61C	ANL <u>A</u>
	SLEV	MS1-7	TEL <u>A</u>
YFV	YFV	Asibi	DGP <u>A</u>
	YFV	17D	DRP <u>A</u>
DENV	DENV-1	Hawaii	TNP <u>A</u>
	DENV-2	New Guinea C	KQP <u>A</u>
	DENV-3	H87	TQ <u>L</u> <u>A</u>
	DENV-4	H241	KEV <u>A</u>
TBEV	TBEV	Neudoerfl	ENP <u>A</u>
	OHFV	Bogluvska	ENP <u>A</u>

The 2nd motif of the EDI-EDII hinge region resides between the E₀ strand of EDI and the e β -strand of EDII (Table 3-3). This motif exhibits a large amount of sequence plasticity except for the amino acids at the amino- and carboxyl-terminus. The three residues (130, 133, and 135 in WNV E protein) contain conserved residues across different flaviviruses. The amino acid equivalents to the WNV E-I130 residue exhibit hydrophobic biochemical properties, including isoleucine in the JEV serocomplex, valine in the YFV and DENV serocomplexes, and tyrosine in

the TBEV serocomplex. Similarly, the amino acid equivalents to the WNV E-I135 residue exhibit hydrophobic biochemical properties, including isoleucine in the JEV, YFV, and TBEV serocomplexes and leucine in the DENV serocomplex. To a lesser extent, the amino acid equivalents to the WNV E-E133 residue also showed to have conserved biochemical properties within the different serocomplexes, with glutamic acid in the JEV and DENV serocomplexes and polar threonine and asparagine in the YFV and TBEV serocomplexes, respectively. Therefore, we selected the WNV E-I130 and E-I135 residues for site-directed mutagenesis based on the flavivirus-conserved hydrophobic biochemical properties of the equivalent residues and the high conservation for specific amino acids.

Table 3-3: Sequence of the 2nd motif of the EDI-EDII hinge region between flaviviruses

The properties of amino acids are shown in green (hydrophobic), pink (polar), red (negatively charged), blue (positively charged), and black (unique). The numbering corresponds to the amino acid positions in the WNV E protein. Highly conserved hydrophobic amino acids are labeled with bold text and underlined. The numbering of amino acids in the motif is based on the WNV NY99 sequence.

Serocomplex	Virus	Strain	Sequence of 2 nd Motif (E 130-135)
JEV	WNV	NY99	<u>IL</u> <u>KEN</u> <u>I</u>
	JEV	SA14	<u>I</u> <u>Q</u> <u>PEN</u> <u>I</u>
	JEV	SA14-14-2	<u>I</u> <u>Q</u> <u>PEN</u> <u>I</u>
	KNV	MRM61C	<u>IL</u> <u>KEN</u> <u>I</u>
	SLEV	MS1-7	<u>IL</u> <u>REN</u> <u>I</u>
YFV	YFV	Asibi	<u>VD</u> <u>Q</u> <u>TK</u> <u>I</u>
	YFV	17D	<u>VD</u> <u>Q</u> <u>TK</u> <u>I</u>
DENV	DENV-1	Hawaii	<u>V</u> <u>Q</u> <u>YEN</u> <u>L</u>
	DENV-2	New Guinea C	<u>V</u> <u>Q</u> <u>PEN</u> <u>L</u>
	DENV-3	H87	<u>V</u> <u>Q</u> <u>HEN</u> <u>L</u>
	DENV-4	H241	<u>V</u> <u>Q</u> <u>IEN</u> <u>L</u>
TBEV	TBEV	Neudoerfl	<u>Y</u> <u>D</u> <u>ANK</u> <u>I</u>
	OHFV	Bogluovska	<u>Y</u> <u>D</u> <u>ANK</u> <u>I</u>

The 3rd motif of the EDI-EDII hinge region resides between the H₀ β -strand of EDI and the f β -strand of EDII. Most of the residues in this motif exhibit less sequence plasticity than some residues in the 1st and 2nd motifs, with three residues (194, 196, and 201 in WNV E protein) containing residues with conserved biochemical properties across different flaviviruses (Table 3-4). The amino acid equivalents in the WNV E-I196 residue exhibit conservation of hydrophobic amino acids, including a mixture of leucine and isoleucine in the JEV and DENV serocomplexes

and valine in the YFV and TBEV serocomplexes. Similarly, the amino acid equivalents to the WNV E-Y201 residue exhibit hydrophobic biochemical properties, including tyrosine and phenylalanine in the JEV and YFV serocomplexes, methionine in the DENV serocomplex, and valine in the TBEV complex. Alternatively, while still maintaining conserved biochemical properties, the WNV E-194 residue showed conserved polar amino acids with serine for the JEV and TBEV serocomplexes, threonine for the YFV serocomplex, and a combination of serine and threonine for the DENV serocomplex. Therefore, we selected the WNV E-I196 and E-Y201 residues for site-directed mutagenesis based on the flavivirus highly conserved hydrophobic biochemical properties of the equivalent residues.

Table 3-4: Sequence of the 3rd motif of the EDI-EDII hinge region between flaviviruses

The properties of amino acids are shown in green (hydrophobic), pink (polar), red (negatively charged), blue (positively charged), and black (unique). The numbering corresponds to the amino acid positions in the WNV E protein. Highly conserved hydrophobic amino acids are labeled with bold text and underlined. The numbering of amino acids in the motif is based on the WNV NY99 sequence.

Serocomplex	Virus	Strain	Sequence of 3 rd Motif (E 191-201)
JEV	WNV	NY99	EPRSG <u>I</u> DTNA- <u>Y</u>
	JEV	SA14	EPRSG <u>L</u> NTEA- <u>F</u>
	JEV	SA14-14-2	EPRSG <u>L</u> NTEA- <u>F</u>
	KNV	MRM61C	EPRSG <u>I</u> DTNA- <u>Y</u>
	SLEV	MS1-7	EARS <u>G</u> INTED- <u>Y</u>
YFV	YFV	Asibi	QVQT <u>A</u> <u>V</u> DFGNS <u>Y</u>
	YFV	17D	QVQT <u>A</u> <u>V</u> DFGNS <u>Y</u>
DENV	DENV-1	Hawaii	SPRT <u>G</u> <u>L</u> DFNE- <u>M</u>
	DENV-2	New Guinea C	SPRT <u>G</u> <u>L</u> DFNE- <u>M</u>
	DENV-3	H87	SPRT <u>G</u> <u>L</u> DFNE- <u>M</u>
	DENV-4	H241	EPRSG <u>I</u> DFNE- <u>M</u>
TBEV	TBEV	Neudoerfl	RVAS <u>G</u> <u>V</u> DLAQT <u>V</u>
	OHFV	Bogluvska	RVAS <u>G</u> <u>V</u> DLAQT <u>V</u>

The 4th motif of the EDI-EDII hinge region is the longest motif of the four and shows the greatest sequence and structure diversity. This motif resides between the B α -helix of EDII and the I₀ β -strand of EDI. Within that region is another β -sheet known as the kl hairpin. The 4th motif consists of 12 to 13 residues for most flaviviruses except for YFV, which consists of 16 residues (Table 3-5). This motif exhibits a large amount of sequence plasticity except for three amino acid residues: the WNV E-A269, E-V272, and E-L281 residues. All three residues exhibit conserved

hydrophobic biochemical properties across all the serocomplexes. Specifically, the WNV E-A269 residue and its equivalent residues are the most highly conserved residues, with all of the serocomplexes exhibiting an alanine except for the TBEV serocomplex, which has a mixture of alanine and valine. The WNV E-V272 and E-L281 residues exhibit more hydrophobic amino acids between the different serocomplexes than the WNV E-A269 residue. The equivalent residues to the WNV E-V272 residue have a valine in the YFV serocomplex, alanine in the TBEV serocomplex, a mixture of valine and alanine in the JEV serocomplex, and a mixture of isoleucine and valine in the DENV serocomplex. The amino acid equivalents to the WNV E-L281 residue conserve the amino acid leucine across the JEV, YFV, and TBEV serocomplexes but have a mixture of leucine, isoleucine, and methionine in the DENV serocomplex. Therefore, we selected all three WNV residues (E-A269, E-V272, and E-L281) for site-directed mutagenesis based on the highly conserved hydrophobic biochemical properties of the equivalent residues and the high conservation of specific amino acids.

Table 3-5: Sequence of the 4th motif of the EDI-EDII hinge region between flaviviruses

The properties of amino acids are shown in green (hydrophobic), pink (polar), red (negatively charged), blue (positively charged), and black (unique). The numbering corresponds to the amino acid positions in the WNV E protein. Highly conserved hydrophobic amino acids are labeled with bold text and underlined. The numbering of amino acids in the motif is based on the WNV NY99 sequence.

Serocomplex	Virus	Strain	Sequence of 4 th Motif (E 269-281)
JEV	WNV	NY99	<u>A</u> <u>I</u> P <u>V</u> <u>E</u> FSSN---TV <u>K</u> <u>L</u>
	JEV	SA14	<u>A</u> <u>I</u> V <u>V</u> <u>E</u> YSS----SV <u>K</u> <u>L</u>
	JEV	SA14-14-2	<u>A</u> <u>I</u> V <u>V</u> <u>E</u> YSS----SV <u>M</u> <u>L</u>
	KNV	MRM61C	<u>A</u> <u>I</u> P <u>V</u> <u>E</u> FSSN---TV <u>K</u> <u>L</u>
	SLEV	MS1-7	<u>A</u> <u>I</u> P <u>A</u> TVSSS---TL <u>T</u> <u>L</u>
YFV	YFV	Asibi	<u>A</u> MR <u>V</u> TKDTNDNNLY <u>K</u> <u>L</u>
	YFV	17D	<u>A</u> MR <u>V</u> TKDTNDNNLY <u>K</u> <u>L</u>
DENV	DENV-1	Hawaii	<u>A</u> TE <u>I</u> QTSG----TT <u>T</u> <u>I</u>
	DENV-2	New Guinea C	<u>A</u> TE <u>I</u> QMSS----GN <u>L</u> <u>L</u>
	DENV-3	H87	<u>A</u> TE <u>I</u> QTSG----GT <u>S</u> <u>I</u>
	DENV-4	H241	<u>A</u> TE <u>V</u> DSGD----GN <u>H</u> <u>M</u>
TBEV	TBEV	Neudoerfl	<u>V</u> PV <u>A</u> HIEG---TKY <u>H</u> <u>L</u>
	OHFV	Bogluvska	<u>A</u> PL <u>A</u> HIEG---TKY <u>H</u> <u>L</u>

Each WNV mutant resides at integral locations within the EDI-EDII hinge region (Figure 3-7). Three of the mutations reside directly next to the gfeah β -sheet of the EDII domain, with the E-A54S mutant next to the “a” β -strand, the E-I130N mutant next to the e β -strand, and the E-Y201P mutant next to the f β -strand. One mutant resides directly next to the C₀D₀E₀F₀ β -sheet of EDI on the opposite side of the EDI-EDII hinge region than the gfeah β -sheet. The E-I135N mutant resides next to the E₀ β -strand. While most of the highly conserved hydrophobic residues of the 1st

through 3rd motifs reside at the very edges of the EDI-EDII hinge region, one mutant in the 3rd motif resides in the middle of the motif, directly between the H₀ β -strand of EDI and f β -strand of EDII: the E-I196N mutant. The final three mutants, E-A269S, E-V272T, and E-L281N, reside within the kl hairpin. Together, these eight residues interact to form the hydrophobic pockets that allow the E protein to undergo a hinge-like conformational change.

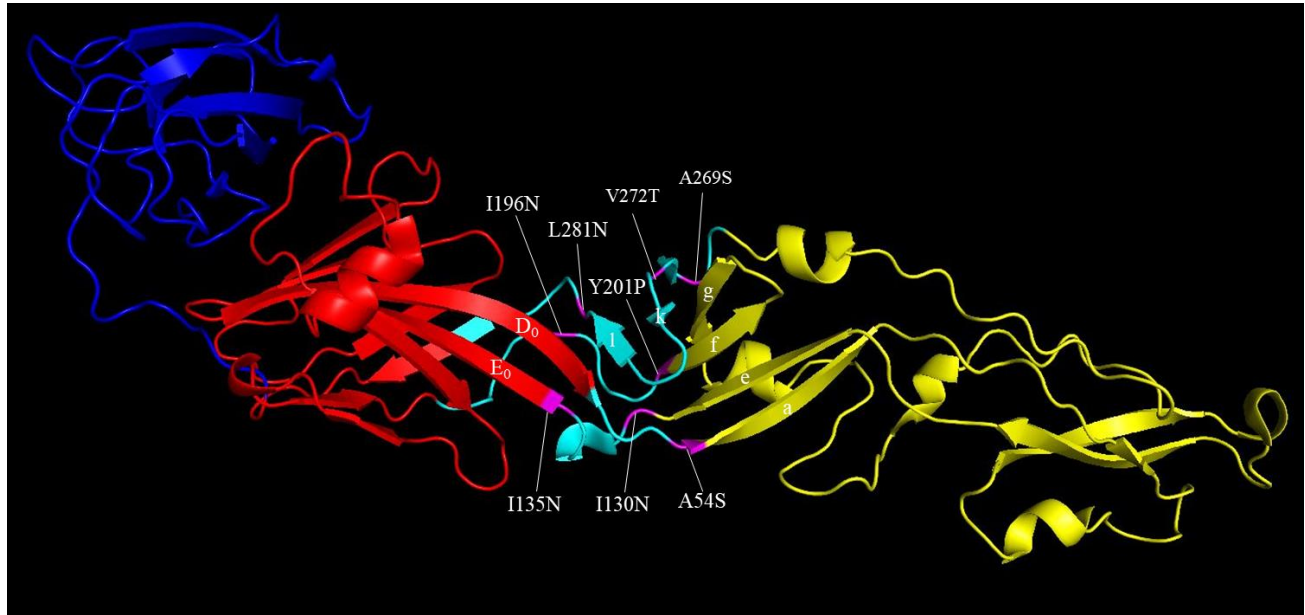


Figure 3-7: Locations of the eight flavivirus conserved hydrophobic residues in the EDI-EDII hinge region of the WNV E protein monomer

Each location of the eight flavivirus conserved residues in the EDI-EDII hinge region is based on the pre-fusion conformations of the WNV E protein shown in magenta (PDB ID:2HG0). The E protein monomer is colored red (EDI), yellow (EDII), blue (EDIII), and cyan (EDI-EDII hinge region). The image was generated in PyMOL.

In summary, the EDI-EDII hinge region has been shown to control the neuroinvasive phenotype of different flaviviruses in mice. Attenuating mutations found previously are virus-specific and have little application in vaccine development for other flaviviruses. Our goal is to identify residues and attenuating mutations that could affect a broad range of flaviviruses and

eventually be used to design candidate LAVs. For this study, we engineered mutations of the eight flavivirus conserved residues in the EDI-EDII hinge region to probe the hydrophobic pocket's importance for the E protein's structure and function. The virulence phenotype of each mutant in mice was tested to determine whether or not these mutations can support the rational design of candidate WNV LAVs.

3.2 Results

A. Recovery and infectivity of WNV EDI-EDII mutants

Each of the eight flavivirus conserved amino acids in the EDI-EDII hinge region was mutated using site-directed mutagenesis of the WNV NY99 infectious clone plasmid, including the E-A54S, E-I130N, E-I135N, E-I196N, E-Y201P, E-A269S, E-V272T, and E-L281N residues. These eight mutations and the data in Section 3.2.A and 3.2.F were originally published in Ashley Bilyeu's K-State Master's thesis and further analyzed in this dissertation (Bilyeu 2023). Each mutant was rescued by transfection of Vero cells with the viral genome derived from *in vitro* transcription. Plaque assay performed using culture supernatant collected four days after transfection showed the eight single-site mutants could be categorized into three phenotypes: viable and stable, lethal, and reversion phenotypes (Table 3-6).

Table 3-6: Viability and stability of WNV EDI-EDII mutants

The WNV NY99ic plasmids were obtained from Dr. Claire Huang's laboratory. Modifications and recovery of WNV EDI-EDII hinge region mutants were conducted by Bailey Maloney and Ashley Bilyeu.

Location	Mutation	Viability	Compensatory Mutations
Motif #1	A54S	Viable	None detected
Motif #2	I130N	Lethal	n/a
	I135N	Lethal	n/a
Motif #3	I196N	Lethal	n/a
	Y201P	Viable	None detected
	A269S	Viable	None detected
Motif #4	V272T	Viable	None detected
	L281N	Viable	Reversion

The single E-I130N, E-I135N, and E-I196N mutants are lethal to WNV NY99ic, as observed with three attempts of transfection. None of the biological replicates of the E-I130N, E-I135N, and E-I196N mutations produced viable viruses detectable by plaque assay (limit of detection 10 PFU/ml). Despite the lack of infectivity, the cell culture supernatant from each transfection experiment contained viral RNA detectable by reverse transcription-polymerase chain reaction (RT-PCR). RNA extracts of the E-I130N and E-I135N mutants retained the nucleotide substitutions in the inoculum. It is unclear whether or not viral replication occurred.

Interestingly, two repeats of transfection with RNA transcript encoding the E-I196N mutant genome produced the WNV NY99ic sequence, indicating the E-I196N mutant multiplied at a low level and quickly reverted to the wild-type sequence. The E-L281N mutant that encoded the change of all three codons produced viable viruses. However, Sanger sequencing revealed the

E-L281N mutant reverted to the wild-type sequence in all three independent transfections, suggesting that the E-L281 residue is crucial for the structure-function of the WNV E protein. In contrast, amino acid substitutions of the E-A54, E-Y201, E-A269, and E-V272 residues were tolerated. The E-A269S and E-V272T mutants each retained the engineered nucleotide substitutions in all of the biological replicates but had no demonstrable difference in the infectivity in comparison with the wild-type NY99ic virus (exceeding $8\log_{10}\text{PFU/ml}$) (Figure 3-8).

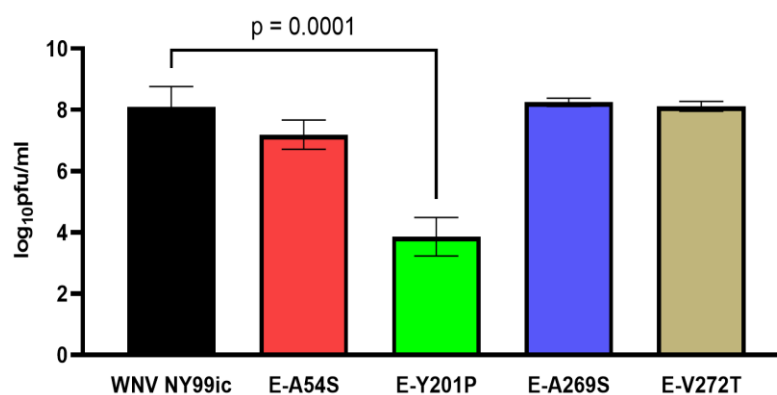


Figure 3-8: Infectivity of WNV mutants containing single mutations in the EDI-EDII hinge region

Infectivity of WNV NY99ic and triplicate isolates of the four single mutants, following transfection of Vero cells. Infectivity between WNV NY99ic and each mutant was compared using one-way ANOVA followed by Dunnett's pairwise comparison. The graph was originally published in Ashley Bilyeu's 2023 Master's thesis (Bilyeu 2023). Recovery of mutant viruses and plaque assays was conducted by Bailey Maloney.

The E-Y201P mutation on the third motif of the EDI-EDII hinge region showed a 5400-fold reduction in infectivity compared with the WNV NY99ic ($p=0.0001$) (Figure 3-8). The average titer of the E-A54S mutant was approximately $7.5\log_{10}\text{PFU/ml} \pm 0.5$ and 2.5-fold lower compared to the WNV NY99ic ($p=0.43$). Neither the E-Y201P nor the E-A54S mutation

developed any reversion to the wild-type sequence in the consensus sequence determined by Sanger sequencing.

Overall, the results demonstrated that six of the eight flavivirus conserved hydrophobic amino acids in the EDI-EDII hinge region are important for the structure-function of WNV E protein, including the E-A54, E-I130, E-I135, E-I196, E-Y201, and E-L281 residues. The importance of the E-I130, E-I196, E-Y201, and E-L281 residues has not been previously reported (Butrapet et al. 2011).

B. Reduction in multiplication kinetics of WNV E-A54 and E-Y201 mutants in Vero and BHK-21 cells

Further, we hypothesized that mutations impair the E protein's structure-function will reduce the multiplication kinetics of WNV-NY99ic in cell culture. The E-A54S, E-Y201P, E-A269S, and E-V272T mutants were each inoculated onto a monolayer of Vero cells or BHK-21 cells (moi: 0.1), followed by daily sampling for up to 7 dpi. As seen in Figure 3-9, the infectivity of WNV-NY99ic in Vero cells can be discerned in the linear (0-2 dpi) and plateau phases (3-7 dpi) with peak titers at approximately $8.5 \log_{10} \text{TCID}_{50}/\text{ml}$ (3-4 dpi). The infectivity of WNV-NY99ic in BHK-21 cells can be discerned in the linear (0-5 dpi) and plateau phases (6-7 dpi) with peak titers at approximately $8.5 \log_{10} \text{TCID}_{50}/\text{ml}$ (5-6 dpi). The E-A54S and E-Y201P mutants exhibited reduced infectivity compared to the wild-type NY99ic.

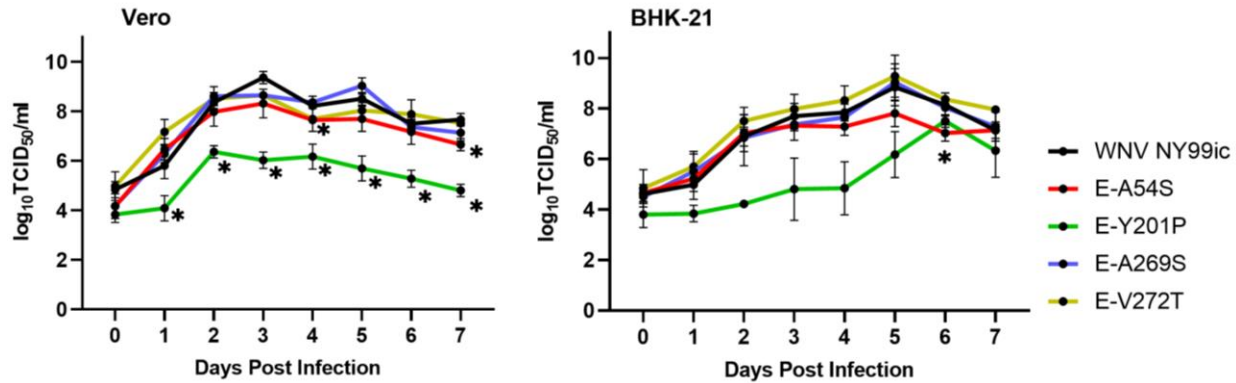


Figure 3-9: Growth curves of WNV mutants over 7 days in Vero and BHK-21 cells

Average titers were calculated in Vero and BHK-21 cells infected at a moi of 0.1. Data points from duplicate replicates represent the mean viral titer, and error bars represent the standard deviation of three biological replicates. The asterisk sign indicates the significant difference ($p < 0.05$) detected by two-way ANOVA followed by Dunnett's pairwise comparison at a specific time. The image was generated in GraphPad. Growth curves were conducted by Bailey Maloney.

The multiplication kinetics of the E-Y201P mutant in Vero cells was reduced by up to 1,000-fold compared to WNV NY99ic between 1 and 7 dpi ($0.0005 \leq p \leq 0.047$) and only reached about 6 $\log_{10}\text{TCID}_{50}/\text{ml}$ compared to the 8-9 $\log_{10}\text{TCID}_{50}/\text{ml}$ of WNV NY99ic. The infectivity of the E-Y201P mutant did not differ significantly from WNV NY99ic in BHK-21 cells ($0.08 \leq p \leq 0.65$). The general trend showed the reduced infectivity of the E-Y201P by over 13-fold between 2 and 5 dpi, averaging about 5 $\log_{10}\text{TCID}_{50}/\text{ml}$. The multiplication kinetics of the E-A54S mutant, when compared to the wild-type WNV NY99ic, exhibited no demonstrable differences in both Vero and BHK-21 cells before 3 dpi during the initial log phase of infection. However, the infectivity of the E-A54S mutant differed from WNV NY99ic in Vero cells during the plateau phase of infection, reaching statistical significance at 4 and 7 dpi with a 3.7-fold ($p = 0.0001$) and 10-fold ($p = 0.02$) reduction in infectivity, respectively.

Similarly, the infectivity of the E-A54S mutant was approximately 13-fold lower than WNV NY99ic in BHK-21 cells during the plateau phase of infection, leading to a significant reduction in infectivity at 6 dpi ($p=0.04$). In both Vero and BHK-21 cells, the growth of the E-A54S mutant averaged about $7.5\log_{10}\text{TCID}_{50}/\text{ml}$ during the plateau phase of infection. The E-A54S and E-Y201P mutants exhibited reduced infectivity compared to NY99ic, resembling the attenuated WNV mutants previously reported (Wicker et al. 2006, Whiteman et al. 2010, Whiteman et al. 2011). The comparable results of the E-A54S and E-Y201P mutants in both BHK-21 and Vero cells indicate that the reduction in multiplication kinetics is not a cell-type-specific event. The E-A269S and E-V272T mutants showed comparable multiplication kinetics to WNV NY99ic, exceeding $8\log_{10}\text{TCID}_{50}/\text{ml}$ in Vero cells between 3 and 7 dpi and BHK-21 cells between 5 and 7 dpi.

C. Single E-A54S and E-Y201P mutations impair WNV infection in cells

We compared the genome-PFU ratio or the particle-PFU ratio of each mutant with WNV-NY99ic to investigate whether or not reduced infectivity is due to the release of noninfectious virus particles into the cell culture supernatant (Figure 3-10). Viral titers and RNA extract from the P1 virus harvested from Vero cells at four dpi were compared to establish the genome-PFU ratio. The E-A54S and E-Y201P mutants exhibited significant increases in genome-PFU ratios compared to WNV-NY99ic. In contrast, there is no demonstrable difference in the genome-PFU ratio of the E-A269S mutant or the E-V272T mutant compared to WNV-NY99ic.

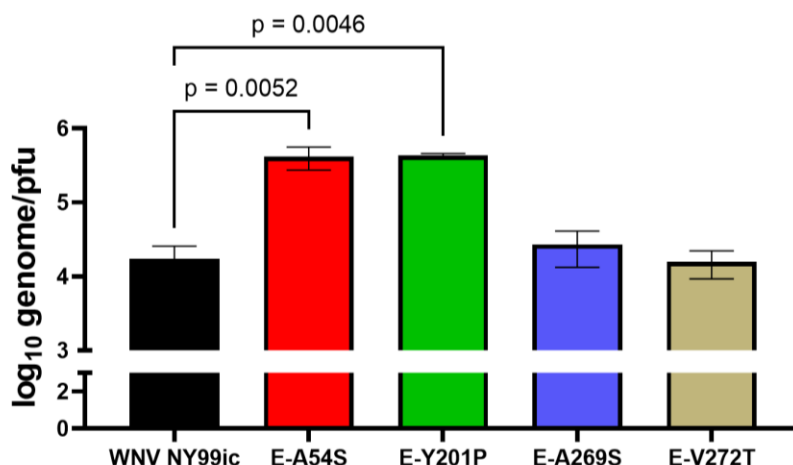


Figure 3-10: PFU-genome ratio of WNV EDI-EDII hinge region mutants

Genome-PFU ratios of WNV NY99ic and the triplicate replicates of the four mutants were grown in culture supernatants of Vero cells infected at a moi of 0.1 and harvested at 3 dpi. Data points represent the average genome copy-to-PFU ratios, and error bars represent the standard deviation of two biological replicates prepared from Vero cells infected with WNV NY99ic or each mutant at moi of 0.1. Comparison of genome copy-to-PFU ratio was performed using one-way ANOVA followed by Dunnett's pairwise comparison. The image was generated in GraphPad. The PFU-genome ratios were conducted by Bailey Maloney and Ashley Bilyeu.

The genome-PFU ratio of the E-A54S and E-Y201P mutants was 25-fold ($p=0.0052$) and 28-fold ($p=0.0046$) higher than WNV NY99ic, respectively, with ratios averaging 320,000. This result indicates that the E-A54 and E-Y201P mutants can each be assembled and secreted in cell culture to produce virions that can act as immunogens. However, many of the virions are not infectious. In contrast, the genome-PFU ratios of the E-A269S mutant nor the E-V272T mutant remained at approximately 10,000.

Since the EDI-EDII interdomain movement initiates the formation of the E protein trimer, which in turn leads to the hemagglutination (HA) activity of flaviviruses, we examined the HA

titer of each WNV mutant at a range of pH between pH 5.8 and pH 7.4. The hemagglutination activity of flaviviruses is due to the binding and membrane fusion with the erythrocytes mediated by the E protein, including WNV. The membrane fusion process takes place due to the free energy supplied by the protonation of endosomes. Therefore, we hypothesize that reducing the optimal pH for hemagglutination activity will show that mutations of the flavivirus-conserved hydrophobic amino acids in the EDI-EDII hinge region can interfere with membrane fusion activity. The WNV NY99ic exhibited the highest HA titer at neutral pH of 7.0 - 7.2, which was also the optimal pH for the HA activity of the E-A54S, E-A269S, and E-V272T mutants (Table 3-7). In comparison, the E-Y201P mutation exhibited a noticeable reduction in the optimal pH for the HA activity to pH 6.6-6.8. Collectively, both the E-A54S and E-Y201P mutations interfere with the formation of infectious viruses, and the E-Y201 residue is crucial for the conformational change of the E protein from dimer to trimer as shown by alteration of the optimum pH for HA activity

Table 3-7: Optimal pH for the HA activity of WNV NY99ic and each single mutant

The geometric mean HA titer of WNV NY99ic and triplicate replicates of each mutant were determined by two biological replicates of HA test at gradient pH between pH 5.75 and pH 7.4 using 0.5% gander erythrocytes. The hemagglutination assays were conducted by Bailey Maloney.

Virus	Optimal pH for HA activity
WNV NY99ic	7.0-7.2
E-A54S	7.2
E-Y201P	6.6-6.8
E-A269S	6.8-7.2
E-V272T	7.0
EL281N	6.8-7.2

D. Serial passage of E-A54S and E-Y201P mutants in Vero cells

The E-A54S, E-Y201P, E-A269S, and E-V272T mutants were each serially passaged in Vero cells at a moi of 0.1 for five passages. Cell culture supernatant was harvested at 3 dpi of each passage during the beginning of the plateau phase of the multiplication curve of WNV-NY99ic in Vero cells (Figure 3-11).

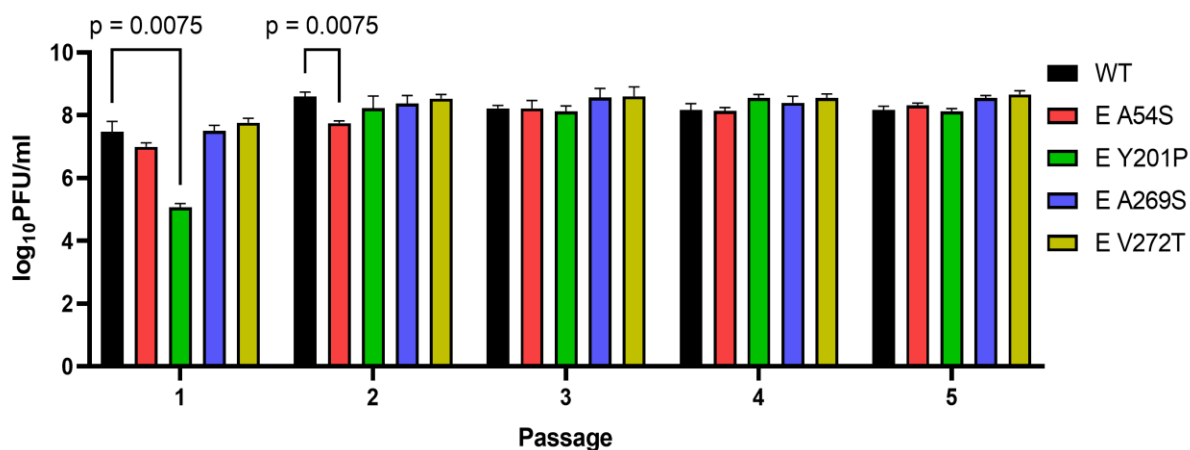


Figure 3-11: Serially passaged WNV EDI-EDII hinge region mutants over 5 passages

Data points represent the average titers and error bars represent the standard deviation of three biological replicates prepared from Vero cells infected with WNV NY99ic or each mutant at moi of 0.1. Infectivity at each passage was compared by two-way ANOVA and Dunnett's pairwise comparison. The image was generated in GraphPad. The serial passaging experiments were conducted by Bailey Maloney.

Comparing the reduced infectivity observed in passages 0 (P0) and 1 (P1), the infectivity of the E-Y201P mutant became comparable to virus titers to WNV NY99ic after the second passage in Vero cells. A compensatory mutation occurred at nucleotide position 1568, where the engineered cysteine mutation transitioned to uracil. This nucleotide change resulted in the loss of the proline mutation and a leucine substitution in the consensus sequence of the 201 residues of

the WNV E protein. The leucine mutation at the E-Y201 residue indicated that the hydrophobic residue is important to the structure and function of the E protein as the leucine mutation in P2 resulted in a large hydrophobic amino acid was preferred over proline, which is lower in hydrophobicity. In contrast, the E-A54S mutation was retained after five passages despite exhibiting similar reductions in infectivity to the E-Y201P mutation. Neither the E-A269S nor the E-V272T mutant reverted to the wild-type sequence or accumulated compensatory mutations after five passages in Vero cells.

E. Next-generation sequencing of WNV mutants

To investigate the stability of the RNA population of the mutants, we undertook next-generation sequencing (NGS) of WNV NY99ic and the four single mutants that retained the respective nucleotide substitutions engineered by PCR site-directed mutagenesis in the consensus sequence. All of the NGS experiments were performed by Dr. Alan Barrett's laboratory at the University of Texas Medical Branch and further analyzed in the Vanlandingham laboratory. The NGS analysis included virus stocks in two different passages: the P0 stocks from transfected Vero cells and the P1 stock from one single passage in Vero cells (moi: 0.1). Two replicates of WNV NY99ic, and each of the mutants was rescued independently to obtain confirmatory data between different biological isolates. One additional mutant was tested as an internal control. The E-L281N mutation was an EDI-EDII mutation with three individual nucleotide mutations that were confirmed to all revert to the wild-type consensus sequence. In contrast to the retention of the single E-A54S, E-Y201P, E-A269S, and E-V272T amino acid mutations in the consensus sequence in the P0 stocks, there was evidence of selective pressure within the E-L281N mutant viral RNA population. The P0 stock of the E-L281N mutant showed to have no detectable single nucleotide variants (SNVs) encoding for the asparagine (N) mutation (AAC) at the E-L281

residue, and the consensus sequence of all three nucleotides became identical to the wild-type WNV NY99ic (AAC to GUU).

We compare the SNVs identified in the E-A54S, E-Y201P, E-A269S, and E-V272T mutants and WNV NY99ic stocks, using the 1% cutoff accepted to the field (Carpio et al. 2023) (Figure 3-12 and Tables 3-8 and 3.9). Two biological isolates of the WNV NY99ic showed comparable levels of SNVs in their P0 and P1 viruses, with 12 SNVs ranging from 1.0 to 34.6% in the P0 virus and 15 SNVs from 1.1 to 38.9% in the P1 virus. These data are comparable to the SNVs exhibited by the E-L281N mutant that reverted to the wild-type sequence (P0: 14 SNVs from 1.0 to 34.7%, P1: 13 SNVs from 1.0 to 36.5%). The four EDI-EDII hinge region mutants that did not revert to the wild-type sequence exhibited similar NGS results to the wild-type: E-A54S (P0: 7 SNVs from 1.0 to 25.2%, P1: 5 SNVs from 1.0 to 21.0%), E-Y201P (P0: 31 SNVs from 1.1 to 4.0%, P1: 32 SNVs from 1.1 to 9.6%), E-A269S (P0: 28 SNVs from 1.0 to 9.9%, P1: 28 SNVs from 1.1 to 9.3%), and E-V272T (P0: 24 SNVs from 1.0 to 14.4%, P1: 24 SNVs from 1.2 to 16.0%). None of the SNVs identified in the four mutants were detected at positions of nucleotides involved in the mutations. Most SNVs detected in the P0 stocks were present in the P1 stocks of WNV NY99ic and the respective mutants (Table 3-9). Since the SNVs are comparable between the two passages of the virus and between the mutants and wild-type viruses, this indicates that the mutations will not change the RNA population of WNV significantly.

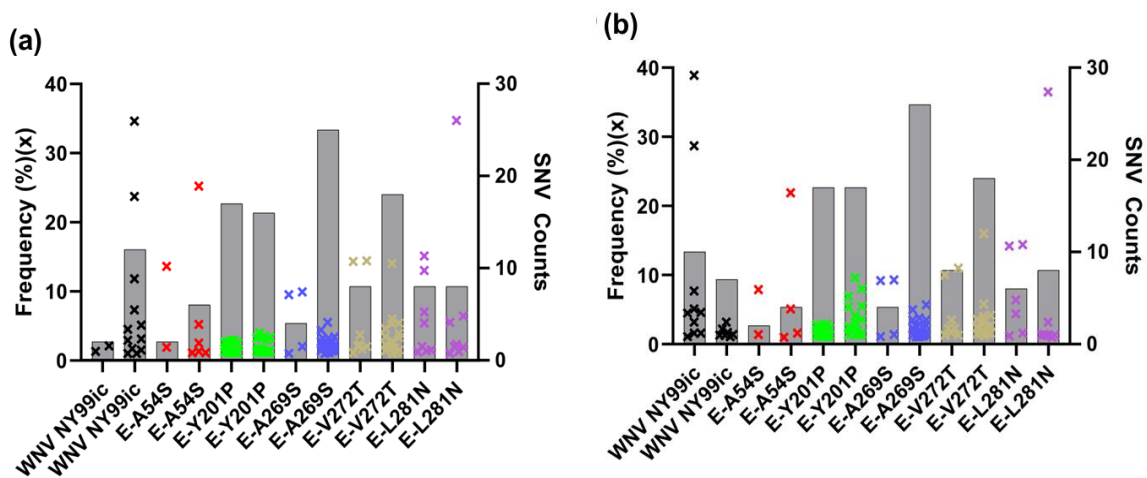


Figure 3-12: Single nucleotide variants of recovered P0 and P1 WNV mutants

The SNV profiles of each virus's two P0 stocks and two P1 stocks are summarized in (a) and (b), respectively. Each × represents an SNV above 1% of the RNA population in the left y-axis. Grey bars denote the SNV count of each virus on the right y-axis. The image was generated in GraphPad. The next generation sequencing was performed by Kassandra Carpio in Dr. Alan Barrett's laboratory.

Table 3-8: Summary of SNVs >1% identified in the P0 stocks of WNV NY99ic and the respective mutants

The WNV NY99ic and the WNV mutants were tested in two replicates. Each SNV denotes the location in the WNV genome, the original nucleic acid base, the mutated nucleic acid base, the protein the base is located in, the compensatory mutation (if applicable), and the frequency of occurrence. The next generation sequencing was performed by Kassandra Carpio in Dr. Alan Barrett's laboratory.

Virus	Replicate	Genomic Position	Original Base	Variant Base	Genome Region	Residue Change	Frequency (%)
WNV NY99ic	1	5166	G	A	NS3	-	2.1
		10888	U	A	3'UTR	-	1.3
	2	414	A	C	Capsid	-	1.5
		491	A	U	prM	K9M	23.7
		546	A	G	prM	-	34.6
		808	A	G	prM	T115A	3.1
		2421	U	G	E	F485L	1.0
		3061	C	G	NS1	L198V	7.3
		3786	A	C	NS2A	-	5.1
		5166	G	A	NS3	-	2.9
		6203	G	A	NS3	R531K	1.0
		8738	A	U	NS5	Q353L	11.8
		9072	C	U	NS5	-	4.5
		10888	U	A	3'UTR	-	1.7
E-A54S	1	5166	G	A	NS3	-	1.9
		8077	U	C	NS5	F133L	13.6
	2	2732	U	C	NS1	V88A	1.3

Virus	Replicate	Genomic Position	Original Base	Variant Base	Genome Region	Residue Change	Frequency (%)
E-A54S	2	3612	A	G	NS2A	-	1.1
		4483	A	G	NS2B	N89D	25.2
		4690	A	U	NS3	T27S	1.1
		5166	G	A	NS3	-	2.5
		5997	C	U	NS3	-	5.2
E-Y201P	1	546	A	G	prM	-	1.2
		866	A	U	prM	Y134F	1.2
		1920	A	U	E	-	1.1
		1968	A	G	E	-	1.3
		2313	A	C	E	-	2.9
		3477	A	G	NS1	-	1.9
		4920	C	U	NS3	-	2.6
		4950	A	C	NS3	-	1.5
		5166	G	A	NS3	-	1.4
		5369	A	G	NS3	N253T	1.7
		5917	A	G	NS3	T436A	1.3
		6203	G	A	NS3	R531K	1.1
		6634	U	C	NS4A	-	1.1
		8352	A	C	NS5	-	1.1
		10370	C	U	NS5	T897I	2.5
		10421	A	C	3'UTR	-	2.9
		10913	A	C	3'UTR	-	2.6
	2	831	A	G	prM	-	2.9
		1684	A	G	E	M240V	1.3
		2679	A	G	NS1	-	1.2
		2745	A	G	NS1	-	3.5
		3205	A	G	NS1	T246A	3.2
		4641	A	G	NS3	-	4.0

Virus	Replicate	Genomic Position	Original Base	Variant Base	Genome Region	Residue Change	Frequency (%)
E-Y201P	2	5166	G	A	NS3	-	1.7
		5812	G	A	NS3	D401N	1.3
		6117	A	U	NS3	-	1.1
		6203	G	A	NS3	R531K	1.3
		7662	A	G	NS4B	-	3.2
		7777	A	U	NS5	I33F	1.2
		7794	A	C	NS5	-	1.2
		8361	C	A	NS5	-	1.6
		8889	A	G	NS5	-	1.4
		10769	A	G	3'UTR	-	3.3
E-A269S	1	1774	A	C	E	I270L	9.9
		2982	C	U	NS1	-	9.5
		5166	G	A	NS3	-	2.0
		9126	A	G	NS5	-	1.0
	2	406	A	G	Capsid	K104E	3.2
		683	C	U	prM	A73V	1.7
		710	C	A	prM	T82N	1.8
		1162	A	G	E	S66G	2.3
		2234	A	G	E	D423G	2.7
		2313	A	C	E	-	2.2
		2679	A	G	NS1	-	1.1
		4396	A	C	NS2B	I60L	3.2
		4614	A	C	NS3	-	1.5
		4965	A	G	NS3	-	1.9
		5166	G	A	NS3	-	2.2
		5250	A	G	NS3	I213M	1.4
		5346	A	U	NS3	-	1.2
		5571	A	C	NS3	-	3.4

Virus	Replicate	Genomic Position	Original Base	Variant Base	Genome Region	Residue Change	Frequency (%)
E-A269S	2	6912	A	G	2K	-	1.7
		6996	C	U	NS4B	-	5.5
		7707	A	G	NS5	-	2.4
		8523	C	A	NS5	N281K	1.4
		9637	U	A	NS5	F653I	2.0
		10306	A	C	NS5	-	2.7
		10408	C	U	3'UTR	-	2.1
		10650	A	G	3'UTR	-	1.5
		10740	A	C	3'UTR	-	1.0
		10750	C	U	3'UTR	-	1.4
		10778	A	G	3'UTR	-	4.4
E-V272T	1	217	A	C	Capsid	K41Q	14.3
		1907	C	U	E	T314I	1.6
		2052	U	C	E	-	2.3
		2581	A	G	NS1	T38A	14.4
		4238	U	C	NS2B	V7A	3.7
		5166	G	A	NS3	-	2.1
		7626	U	C	NS4B	-	2.0
		10888	U	A	3'UTR	-	1.1
	2	106	A	G	Capsid	K4E	1.4
		287	C	A	Capsid	A64E	3.9
		913	A	C	prM	M150L	5.3
		1311	A	C	E	-	1.9
		1800	U	C	E	-	2.3
		2427	A	C	E	-	1.8
		3593	C	G	NS2A	L24V	4.6
		3677	A	G	NS2A	Y51C	6.0
		4497	A	C	NS2B	-	5.4

Virus	Replicate	Genomic Position	Original Base	Variant Base	Genome Region	Residue Change	Frequency (%)
E-V272T	2	5166	G	A	NS3	-	1.8
		6263	U	C	NS3	V551A	1.1
		7420	A	C	NS4B	M169L	14.0
		8164	A	G	NS5	I162V	3.4
		9628	A	U	NS5	T650S	1.0
		9660	C	A	NS5	-	1.5
		9861	A	G	NS5	-	2.8
		10350	A	C	NS5	-	3.3
		10888	U	A	3'UTR	-	1.8
E-L281N	1	4292	A	C	NS2B	D25A	1.2
		4481	U	C	NS2B	M88T	5.4
		4719	A	U	NS3	-	15.1
		5166	G	A	NS3	-	2.0
		5325	A	G	NS3	-	13.0
		8457	C	U	NS5	-	1.4
		8769	G	A	NS5	-	7.0
		10888	U	A	3'UTR	-	1.5
	2	2222	C	U	E	A419V	2.3
		3961	A	C	NS2A	I146L	34.7
		3964	A	C	NS2A	T147P	6.4
		5166	G	A	NS3	-	1.9
		5938	A	G	NS3	I443V	2.0
		6646	U	C	NS4A	-	1.0
		9724	U	G	NS5	S682A	5.5
		10888	U	A	3'UTR	-	1.3

Table 3-9: Summary of SNVs >1% identified in the P1 stocks of WNV NY99ic and the respective mutants.

The WNV NY99ic and the WNV mutants were tested in two replicates. Each SNV denotes the location in the WNV genome, the original nucleic acid base, the mutated nucleic acid base, the protein the base is located in, the compensatory mutation (if applicable), and the frequency of occurrence. The next generation sequencing was performed by Kassandra Carpio in Dr. Alan Barrett's laboratory.

Virus	Repeat	Genomic Position	Major Base	Variant Base	Gene Region	Residue Change	Frequency (%)
WNV NY99ic	1	414	A	C	Capsid	-	1.6
		491	A	U	prM	K9M	28.7
		546	A	G	prM	-	38.9
		808	A	G	prM	T115A	5.2
		3061	C	G	NS1	L198N	6.9
		3786	A	C	NS2A	-	4.7
		5116	G	A	NS3	-	1.5
		6203	G	A	NS3	R531K	1.1
		8738	A	U	NS5	Q353L	3.1
		9072	C	U	NS5	-	4.5
	2	638	C	G	prM	A58G	1.4
		1274	A	C	E	N103T	2.8
		1708	A	U	E	T248S	1.1
		1863	A	G	E	-	1.4
		5166	G	A	NS3	-	1.7
		6203	G	A	NS3	R531K	1.4
		9584	A	G	NS5	N103T	2.1
E-A54S	1	5166	G	A	NS3	-	1.4
		6203	G	A	NS3	R531K	1.0

Virus	Repeat	Genomic Position	Major Base	Variant Base	Gene Region	Residue Change	Frequency (%)
E-A54S	1	8077	U	C	NS5	F133L	7.9
	2	4483	A	G	NS2B	N89D	21.0
		5166	G	A	NS3	-	1.6
		5997	C	U	NS3	-	5.1
E-Y201P	1	866	A	U	prM	Y134F	1.3
		1568	C	A	E	P201H	2.9
		1920	A	U	E	-	1.4
		2145	C	A	E	-	1.2
		2313	A	C	E	-	2.9
		3477	A	G	NS1	-	2.4
		4920	C	U	NS3	-	2.7
		4950	A	C	NS3	-	1.3
		5166	G	A	NS3	-	1.7
		5369	A	G	NS3	N253S	1.2
		6203	G	A	NS3	R531K	1.1
		6321	A	C	NS3	-	1.1
		8815	A	G	NS5	T379A	1.1
		10370	C	U	NS5	T897I	2.4
		10421	A	C	3'UTR	-	1.9
		10833	A	G	3'UTR	-	2.4
		10913	A	C	3'UTR	-	2.3
	2	831	A	G	prM	-	2.5
		1568	C	U	E	P201L	9.6
		1568	C	A	E	P201H	8.0
		1684	A	G	E	M240V	6.9
		2679	A	G	NS1	-	1.4
		2745	A	G	NS1	-	3.2
		2858	A	G	NS1	N130S	5.5

Virus	Repeat	Genomic Position	Major Base	Variant Base	Gene Region	Residue Change	Frequency (%)
E-Y201P	2	2991	C	U	NS1	-	3.6
		3205	A	G	NS1	T246A	2.1
		4641	A	G	NS3	-	4.1
		5166	G	A	NS3	-	1.8
		5812	G	A	NS3	D401N	1.5
		7662	A	G	NS4B	-	5.4
		8356	U	A	NS5	S226T	1.4
		8361	C	A	NS5	-	1.3
		8889	A	G	NS5	-	2.0
		10769	A	G	3'UTR	-	2.3
E-A269S	1	1774	A	C	E	I270L	9.2
		2982	C	U	NS1	-	9.3
		5166	G	A	NS3	-	1.4
		6203	G	A	NS3	R531K	1.1
	2	406	A	G	Capsid	K104E	2.5
		683	C	U	prM	A73V	1.6
		1162	A	G	E	S66G	2.3
		1491	A	C	E	-	1.2
		2234	A	G	E	D423G	1.2
		2313	A	C	E	-	2.6
		2679	A	G	NS1	-	1.3
		4396	A	C	NS2B	I60L	3.5
		4614	A	C	NS3	-	1.1
		4965	A	G	NS3	-	3.5
		5166	G	A	NS3	-	1.4
		5250	A	G	NS3	I213M	1.3
		5346	A	U	NS3	-	1.1
		5571	A	C	NS3	-	3.8

Virus	Repeat	Genomic Position	Major Base	Variant Base	Gene Region	Residue Change	Frequency (%)
E-A269S	2	6203	G	A	NS3	R531K	1.5
		6912	A	G	2K	-	2.1
		6996	C	U	NS4B	-	5.0
		7707	A	G	SN5	-	1.8
		8523	C	A	NS5	N281K	1.8
		9637	U	A	NS5	F653I	2.9
		10306	A	C	NS5	-	2.7
		10369	A	C	NS5	T897P	1.4
		10408	C	U	3'UTR	-	1.6
		10650	A	G	3'UTR	-	1.3
		10740	A	C	3'UTR	-	1.1
		10778	A	G	3'UTR	-	5.7
E-V272T	1	217	A	C	Capsid	K41Q	11.0
		1907	C	U	E	T341I	1.7
		2052	U	C	E	-	2.3
		2581	A	G	NS1	T38A	10.0
		4238	U	C	NS2B	V7A	3.5
		5166	G	A	NS3	-	1.3
		6203	G	A	NS3	R531K	1.3
		7626	U	C	NS4B	-	2.3
		106	A	G	Capsid	K4E	1.9
	2	287	C	A	Capsid	A64E	4.2
		913	A	C	prM	M150L	5.8
		1311	A	C	E	-	2.2
		1800	U	C	E	-	3.2
		2427	A	C	E	-	1.5
		3595	C	G	NS2A	L24V	2.7
		3677	A	G	NS2A	Y51C	2.6

Virus	Repeat	Genomic Position	Major Base	Variant Base	Gene Region	Residue Change	Frequency (%)
E-V272T	2	4497	A	C	SN2B	-	4.1
		5166	G	A	NS3	-	1.6
		6203	G	A	NS3	R531K	1.2
		6263	U	C	NS3	V551A	1.6
		7420	A	C	NS4B	M169L	16.0
		8164	A	G	NS5	I162V	3.6
		9628	A	U	NS5	T650S	1.7
		9660	C	A	NS5	-	1.5
		9861	A	G	NS5	-	3.5
		10350	A	C	NS5	-	2.4
E-L281N	1	4481	U	C	NS2B	M88T	4.4
		4719	A	U	NS3	-	14.4
		5166	G	A	NS3	-	1.2
		5325	A	G	NS3	-	14.2
		6203	G	A	NS3	R531K	1.0
		8457	C	U	NS5	-	1.6
		8769	G	A	NS5	-	6.4
		2222	C	U	E	A419V	3.2
	2	3961	A	C	NS2A	I146L	36.5
		3964	A	C	NS2A	T147P	1.3
		5166	G	A	NS3	-	1.0
		5938	A	G	NS3	I443V	1.3
		6203	G	A	NS3	R531K	1.3
		6646	U	C	NS4A	-	1.4
		9724	U	G	NS5	S682A	1.3

Neither the E-Y201P nor the E-A54S mutant contained SNVs indicative of reversion to the wild-type sequence or any compensatory mutations in the P0 stocks. Therefore, using the P0

stocks to determine the attenuating effect of the E-A54S or E-Y201P mutations on the mouse neuroinvasive phenotype of WNV NY99ic is scientifically sound (see section 3.3.F). The E-Y201P mutation was engineered by changing the first and second nucleotides of the codons at positions 1567 and 1568. Interestingly, one of the two P1 stocks of the E-Y201P mutant contained two SNVs that resulted in a nonsynonymous mutation at the target residue. The C1568U mutation appeared in 9.6% of reads, and the C1568A transversion occurred in 8.0%. During the passage experiment from section 3.2.D, the second passage of the virus (P2) resulted in the C1568U mutation that gave rise to a leucine residue that became a consensus mutation. The C1568A transversion encoding a histidine residue was not selected for further passages. The selection of the C1568U mutation further confirms a hydrophobic amino acid in the E-Y201 residue is important for the structure-function of the E protein. Both the P0 and P1 stocks of the E-A269S mutant developed an SNV. The A1774C mutation was present in > 9% of reads, which encodes an E-I270L mutation in the viral RNA population, but the mutation is not present in the consensus sequence, at least for the P0 and P1 virus stocks. The E-I270L mutation is directly neighboring the E-A269S, making it a compensatory mutation due to the structural and functional change caused by the E-A269S mutation. So, in addition to the E-A54, E-I130, E-I135, E-I196, E-Y201, and E-L281 residues proved to have the structural and functional importance of the E protein, the E-A269 residue is also crucial for the hinge effect at the EDI-EDII domain interface.

F. Attenuation of the mouse neuroinvasive phenotype of WNV by E-A54S and E-Y201P mutants

The neuroinvasive phenotype of each mutant was compared with WNV NY99ic in four-week-old outbred Swiss mice that were intraperitoneally challenged with 500 PFU of WNV (Figure 3-13). Each mutant was tested in ten mice (five female and five male mice) to demonstrate

sex is not a biological variable that can affect the outcome. All data in section 3.2.F was originally published in Ashley Bilyeu's K-State Master's thesis and further analyzed in this dissertation (Bilyeu 2023). The E-A54P and E-Y201P mutations attenuated the mouse neuroinvasive phenotype of WNV NY99ic, while the E-A269S and E-V272T mutants showed uniform lethality.

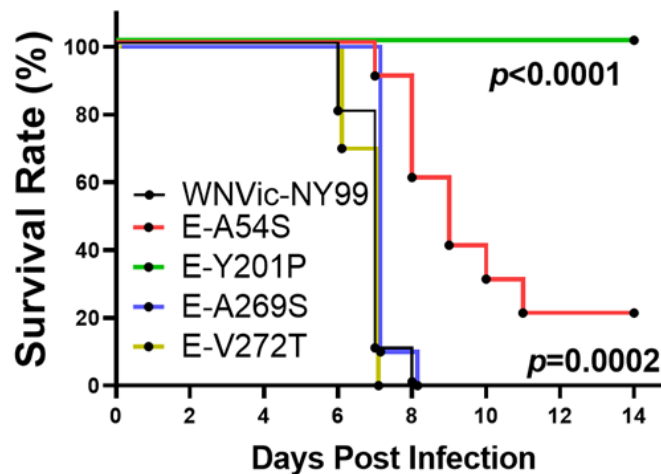


Figure 3-13: Attenuation of the neuroinvasive phenotype of WNV in four-week-old mice

Groups of five male and five female mice received the IP challenge with each WNV mutant or WNV NY99ic at 500 PFU. The survival of mice receiving the E-A54S, E-Y201P, E-A269S, E-V272T mutants, or the parental WNV-NY99ic is depicted. The Kaplan-Meier survival curve showed differences in survival outcomes and average survival time, and Dunnett's pairwise comparison followed one-way ANOVA. The image was generated in GraphPad. The mouse neuroinvasive disease study was conducted by Bailey Maloney, Ashley Bilyeu, So Lee Park, and Danielle Saunders.

Infection with the wild-type WNV NY99ic led to uniform lethality between 6 and 8 dpi. Significantly, no mortality was observed in mice challenged with the E-Y201P mutant ($p<0.0001$). Therefore, the E-Y201P mutation fully attenuated the neuroinvasive phenotype in mice challenged with 500 PFU. The E-A54S mutation exhibited a partially attenuated phenotype, as 20% of mice

survived up to the end of the study at 14 dpi and had an extended average survival time of 9.8 days compared to the 6.9 days for the wild-type mice ($p<0.01$) (Table 3-10). The average survival time was determined by the day post-infection when mortality was observed. The neuroinvasive phenotype of the E-A269S and E-V272T mutants was indistinguishable from wild-type WNV NY99ic and similarly caused 100% mortality between 6 and 8 dpi.

Table 3-10: Average survival times of mice infected with WNV EDI-EDII mutants

This table details the average survival time post-infection for each group of inoculated mice and the standard deviation seen within the respective group. The average survival time of each group was calculated using 14 days as the end point of the study. The table was originally published in Ashley Bilyeu's 2023 Master's thesis (Bilyeu 2023). The mouse neuroinvasive disease study was conducted by Bailey Maloney, Ashley Bilyeu, So Lee Park, and Danielle Saunders.

Group	Average Survival Time (days)	Standard Deviation
WNV NY99 (wt)	6.9	0.6
E-A54S	9.8	2.5
E-Y201P	>14	n/a ¹
E-A269S	7.1	0.3
E-V272T	6.7	0.5

An important indicator of the onset of West Nile neurotropic disease in mice, sometimes before the start of symptoms, is weight loss. The weights of each of the mice were measured during the morning of each day of the study (Table 3-11). The wild-type mice showed an increase in weight until 5 to 6 dpi, during which WNV likely began to multiply and disseminate from the peritoneal cavity. After 5 to 6 dpi, the weights began to decrease, and symptoms started to show.

¹ No endpoint data was retrieved in this mouse group so no standard deviation could be calculated.

By the morning of 8 dpi, the mice had all died. The female mice in the wild-type group lost weight initially following inoculation, potentially due to the configuration of the ventilated cages that can be challenging for young female mice to access water. However, weight gain within this group of females was consistent after the cages' reconfiguration until 5 dpi, when two began to show weight loss. Similar to the males, all females had begun to show weight loss by 6 dpi. The mice challenged with the E-Y201P mutant steady increases in weight throughout the study, with weight gains of 65 to 108% of their initial weight at 0 dpi. The E-Y201P group was the only group of mice that did not experience weight loss after 5 dpi, similar to the wild-type mice, and they were the only group where the peak weight of all ten mice was the same as their final weight. The E-A54S-challenged mice exhibited weight loss, with the males losing weight at 5 to 6 dpi and 7 to 9 dpi. The one male that survived began to gain weight again at 9 dpi, and the one female that survived began to gain weight again at 12 dpi, potentially showing a recovery from the WNV infection. The mice challenged with E-A269S and E-V272T exhibited similar weight loss to the wild-type, with initial weight loss starting at 6 to 7 dpi.

Table 3-11: Individual weights for mice infected with WNV EDI-EDII mutants

The weight of each mouse was measured in grams every morning of the study at about 8:30 am. The starting weight is the mouse's weight on the morning of 0 dpi before challenging the mice with WNV. The peak weight is the heaviest weight each mouse attained during the study. The final weight is the mouse's weight the morning the mice were euthanized or the morning of the end of the study at 14 dpi. “M” represents the male mice, and the notation “F” represents the female mice. Grey coloring represents a mouse that was euthanized or found dead prior to the end of the study, and white represents a mouse that survived to 14 dpi. This table was modified from a table originally published in Ashley Bilyeu’s 2023 Master’s thesis (Bilyeu 2023). The mouse neuroinvasive disease study was conducted by Bailey Maloney, Ashley Bilyeu, So Lee Park, and Danielle Saunders.

Group	Mouse (Male)	Starting Weight (g)	Peak Weight (g)	Final Weight (g)	Mouse (Female)	Starting Weight (g)	Peak Weight (g)	Final Weight (g)
WNV NY99 (wt)	1M	15.9	21.2	18.2	1F	9.3	11.9	10.3
	2M	12.9	17.55	14.5	2F	11	14.9	10.9
	3M	14.2	20.0	16.5	3F	9.3	12.1	10.0
	4M	15.5	21.3	17.7	4F	9.3	11.1	9.0
	5M	20.5	26.5	21.5	5F	8.5	10.2	8.7
A54S	1M	18.0	25.2	22.3	1F	10.7	14.6	12.4
	2M	16.0	23.2	21.0	2F	10.3	16.1	14.8
	3M	15.7	28.7	29.7	3F	10.5	12.9	11.2
	4M	18.3	25.3	20.4	4F	11.8	14.1	13.8
	5M	14.6	22.5	17.0	5F	12.2	14.2	11.7
Y201P	1M	14.6	30.3	30.3	1F	6.6	17.2	17.2
	2M	16.2	29.4	29.4	2F	8.5	21.8	21.8
	3M	15.4	29.0	29.0	3F	9.3	19.6	19.6

Group	Mouse (Male)	Starting Weight (g)	Peak Weight (g)	Final Weight (g)	Mouse (Female)	Starting Weight (g)	Peak Weight (g)	Final Weight (g)
Y201P	4M	15.1	28.6	28.6	4F	10.8	23	23
	5M	15.6	25.7	25.7	5F	10.1	19.8	19.8
A269S	1M	15.0	30.4	17.0	1F	11.3	15.6	15.5
	2M	16.2	22.6	19.8	2F	7.5	10.3	9.5
	3M	17.0	24.2	20.7	3F	9.0	12.4	10.2
	4M	15.1	21.6	19.2	4F	8.8	10.9	8.7
	5M	14.6	20.4	16.0	5F	7.7	9.9	8.2
V272T	1M	15.7	21.9	19.9	1F	7.6	11.2	9.3
	2M	18.0	25.6	21.0	2F	9.3	12.4	10.4
	3M	14.8	19.8	19.8	3F	9.7	15.2	13.3
	4M	14.9	16.3	16.3	4F	8.9	14.1	11.5
	5M	14.0	15.7	15.7	5F	11.6	17.4	14.5

Disease symptoms were initially observed in the wild-type WNV NY99ic mice at 6 dpi. The first signs included hunched spinal posture, inhibited motor movements, ruffled fur, a lack of escape attempts from human handling, and quiescence. Symptoms progressed quickly to show signs of neurological disease such as leaning to one side, depression, uncoordinated movement, paralysis, and one observed seizure case in one of the E-A269S challenged mice. In addition to a lack of weight loss, all E-Y201P mice exhibited no signs of disease during the study. The lack of symptoms of WNV neurotropic disease provides evidence that the E-Y201P mutation can fully attenuate the neuroinvasive phenotype of WNV in mice. The E-A54S mice started showing signs of disease at 7 dpi. The one male mouse that survived showed no signs of disease at any point in the study. The one female mouse that survived showed minor signs of disease from 8 to 14 dpi, such as ruffled fur and a slightly hunched posture but remained bright, alert, and responsive the

entire study. The mice challenged with E-A269S and E-V272T exhibited a similar onset of disease symptoms compared to the wild-type, at 6 dpi, before quickly showing neurological signs of disease.

As no mortality was observed in mice challenged with the E-Y201P mutant, there was no viral RNA above the limit of detection in the brains collected at day 14 dpi (Figure 3-14), suggesting that the E-Y201P mutant can fully attenuate the neuroinvasive phenotype of WNV in outbred Swiss mice up to 500 PFU. Although eighty percent of mice succumbed to the neuroinvasive disease caused by the E-A54S mutant, none of the surviving mice showed detectable brain viral load at 14 dpi. Further, there was a 14-fold reduction of brain viral load in mice receiving the IP challenge with the E-A54S mutant and developed lethal neuroinvasive disease (6.5×10^{12} genome copies/gram of tissue) in comparison with wild-type WNV NY99ic (9.3×10^{13} genome copies/gram of tissue) ($p < 0.01$). Although the E-A269S and E-V272T mutants caused 100% mortality, viral RNA load was significantly reduced in mouse brains collected between 6 to 8 dpi. Because the mouse brains were infected with WNV NY99ic, the reduced brain viral loads suggest that both the E-A269S mutation and the E-V272T mutation still exhibit attenuating effects on the mouse neuroinvasive phenotype of WNV. However, the attenuation level is insufficient to prevent the onset of lethal disease. Specifically, mice challenged with the E-A269S mutant and the E-V272T mutants exhibited 5-fold (1.7×10^{13} genome copies/gram of tissue) ($p = 0.002$) and 4-fold reduction (2.2×10^{13} genome copies/gram of tissue) ($p = 0.004$) in brain viral load in comparison with WNV NY99ic, respectively.

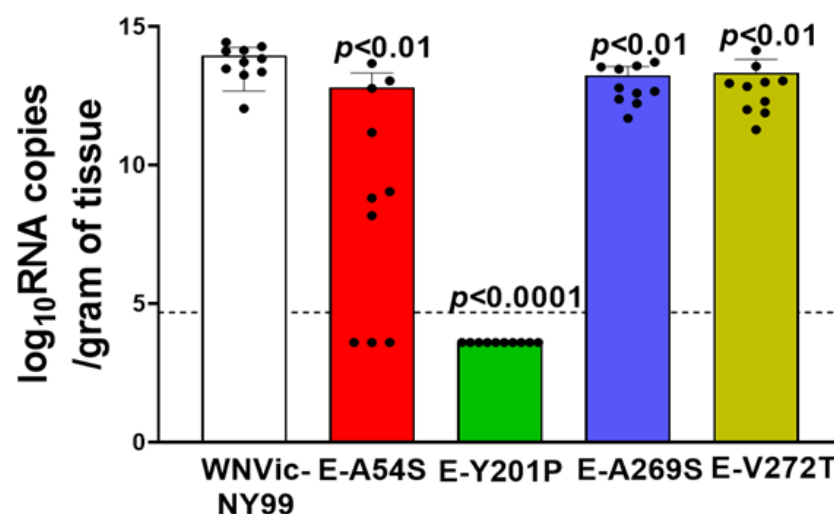


Figure 3-14: Viral RNA loads in mice infected with WNV EDI-EDII hinge region mutants

Brain viral load of mice challenged with WNV NY99ic or the respective singles was compared by one-way ANOVA and Dunnett's pairwise comparison. The dashed line indicates the limit of detection. The graph was originally published in Ashley Bilyeu's 2023 Master's thesis (Bilyeu 2023). The RT-qPCR was conducted by Bailey Maloney.

The E gene was also amplified from the total mouse brain RNA extract using RT-PCR and gene-specific primers. The consensus sequence of the E-A54S, E-A269S, and E-V272T mutants demonstrated that none of the three mutants reverted to the wild-type sequence.

We undertook PRNT₅₀ to determine the immunogenicity of the fully attenuated E-Y201P mutant and the partially attenuated E-A54S mutant, using serum samples collected at 14 dpi, i.e., the study's endpoint. Both the E-Y201P and E-A54S mutants elicited neutralizing antibody responses in surviving mice. Nine mice (90%, 9/10) that survived the IP challenge with the E-Y201P mutant showed 1:10 or greater serum PRNT₅₀ titer with a geometric mean of serum PRNT₅₀ titer of 43.2 (Figure 3-15). Similarly, both mice that survived the IP challenge with the E-A54S mutant also had a detectable serum-neutralizing activity with a geometric mean of serum

PRNT₅₀ titer of 160. Although the sample size for the E-A54S mutants is small, the fully attenuated E-Y201P mutant exhibited lower immunogenicity than the partially attenuated E-A54S mutant, presumably due to the different levels of viral replication.

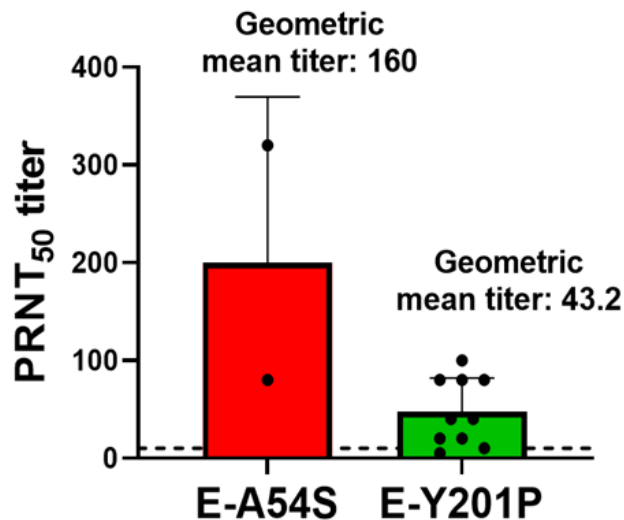


Figure 3-15: Serum-neutralizing antibodies in mice receiving the IP challenge with the E-A54S or E-Y201P mutant.

PRNT₅₀ titers were determined using the biological isolates of the WNV NY99 strain. The dashed line indicates 1:10 serum PRNT₅₀ titer. The image was generated in GraphPad. The PRNT₅₀ assays were conducted by Ashley Bilyeu.

As a proof-of-concept, the fully attenuated E-Y201P mutant and the partially attenuated E-A54S mutant show at least two flavivirus-conserved hydrophobic amino acids in the EDI-EDII hinge region of WNV can be targets for the engineering of attenuating mutations. The infectivity of the E-Y201P mutant is low and may be insufficient for manufacturing candidate WN LAVs. The E-A54S mutation cannot fully attenuate the neuroinvasive phenotype of WNV in mice. Nevertheless, both the E-A54 and E-Y201 residues are worthwhile investigating for engineering mutations that can potentially reach the balance of attenuation and immunogenicity. As such, two independent approaches were evaluated to optimize the E-A54 and E-Y201 mutants. Firstly, we

evaluated all 19 alternative amino acid substitutions of the E-A54 and E-Y201 residues, followed by selecting two single E-A54 mutants and two E-Y201 mutants to investigate the attenuating effect on the mouse neuroinvasive phenotype of WNV NY99ic. Secondly, we also engineered the E-Y201P mutant based on the codon preference of WNV to increase the infectivity of the E-Y201P mutant in Vero cells.

G. Recovery and infectivity of WNV E-A54 and E-Y201 optimized mutants

In addition to the E-A54S and E-Y201P mutations, engineering of all 18 alternative amino acid substitutions of the respective residues produced the single-site mutants summarized in Tables 3-12 and 3-13.

Table 3-12: Viability and stability of WNV E-A54 mutants

The WNV NY99ic plasmids were obtained from Dr. Claire Huang's laboratory. Modifications and recovery of WNV E-A54 mutants were conducted by Bailey Maloney, Ashley Bilyeu, and Adrienne Pohl.

Location	Mutation	Viability	Compensatory Mutations
Motif #1	A54C	Viable	None detected
	A54D	Viable	None detected
	A54E	Viable	None detected
	A54F	Lethal	n/a
	A54G	Viable	None detected
	A54H	Viable	None detected
	A54I	Viable	None detected
	A54K	Viable	None detected
	A54L	Viable	Reversion
	A54M	Viable	None detected
	A54N	Viable	None detected
	A54P	Viable	None detected
	A54Q	Viable	None detected
	A54R	Viable	None detected
	A54T	Viable	None detected
	A54V	Viable	Reversion
	A54W	Lethal	n/a
	A54Y	Lethal	n/a

Table 3-13: Viability and stability of WNV E-Y201 mutants

The WNV NY99ic plasmids were obtained from Dr. Claire Huang's laboratory. Modifications and recovery of WNV E-A54 mutants were conducted by Bailey Maloney and Adrienne Pohl.

Location	Mutation	Viability	Compensatory Mutations
Motif #3	Y201A	Viable	Reversion
	Y201C	Viable	None detected
	Y201D	Viable	None detected
	Y201E	Viable	None detected
	Y201F	Viable	None detected
	Y201G	Lethal	n/a
	Y201H	Viable	None detected
	Y201I	Viable	None detected
	Y201K	Viable	None detected
	Y201L	Viable	None detected
	Y201M	Viable	None detected
	Y201N	Viable	None detected
	Y201P (optimized)	Viable	None detected
	Y201Q	Viable	None detected
	Y201R	Viable	None detected
	Y201S	Viable	Reversion
	Y201T	Lethal	n/a
	Y201V	Lethal	n/a
	Y201W	Lethal	n/a

Surprisingly, 13 amino acid substitutions of the E-A54 residue were tolerated, albeit the E-A54 residue is strictly conserved across all flaviviruses (Table 3-12). The E-A54 mutants can be categorized into two different groups. The first group includes twelve amino acid substitutions of

the E-A54 residue that significantly reduced the infectivity of WNV NY99ic in Vero cells transfected with the viral genome, including the E-A54C, E-A54D, E-A54E, E-A54G, E-A54H, E-A54I, E-A54K, E-A54M, E-A54N, E-A54P, E-A54Q, E-A54R, and E-A54T mutations. The E-A54P mutation had no demonstrable impact on the infectivity of WNV NY99ic. In contrast, the second group of mutants produced non-viable phenotypes. The E-A54G, E-A54L, and E-A54V mutants are unstable, and the E-A54F, E-A54W, and E-A54Y mutations precluded the recovery of viable viruses. The E-A54L mutation involves two codon changes, the G1126C nucleotide substitution in the first codon and the C1127U nucleotide substitution in the second codon. In one of the three independent rescues of the E-A54L mutants, the C1127U nucleotide substitution reverted to the wild-type sequence, resulting in the CCA codon encoding proline.

The E-A54V mutation is due to the C1127U nucleotide change in the second codon. Two independent transfection experiments with the RNA transcript encoding the E-A54V mutant resulted in the loss of engineered nucleotide substitution. The compensatory proline mutation resulted in two mutations: G1126C and U1127C. The change in the first nucleotide was new to the sequence, and the change in the second was a reversion to the wild-type sequence. The E-A54G mutation occurred at the second nucleotide, resulting in a U1127G mutation. The E-A54G mutation resulted in a mixed population of viruses. One of two biological replicates demonstrated a lethal phenotype, while the other replicates retained the engineered amino acid substitution. Consistent with the attenuated phenotype of the E-A54S mutant, the alternative amino acid substitutions led to the reduced infectivity, reversion to the wild-type sequence, and lethal phenotype collectively reflect that the flavivirus conserved E-A54 residue is crucial for the structure-function of the E protein.

We can further distinguish the eleven E-A54 mutations based on the reduced infectivity level compared to WNV NY99ic ($8.5\log_{10}\text{PFU/ml}$) (Figure 3-16). The E-A54M mutation reduced the infectivity of WNV NY99ic by 99-fold. Infectivity of the E-A54C, E-A54I, E-A54K, and E-A54Q mutants was approximately at $4\log_{10}\text{PFU/ml}$, which can be translated into a 2730-, 14750-, 6210-, and 4820-fold reduction, respectively, in comparison with the WNV NY99ic. The E-A54D, E-A54E, and E-A54T mutants had average titers of $3\log_{10}\text{PFU/ml}$, and the E-A54H, E-A54N, and E-A54R mutants had average titers of $2\log_{10}\text{PFU/ml}$.

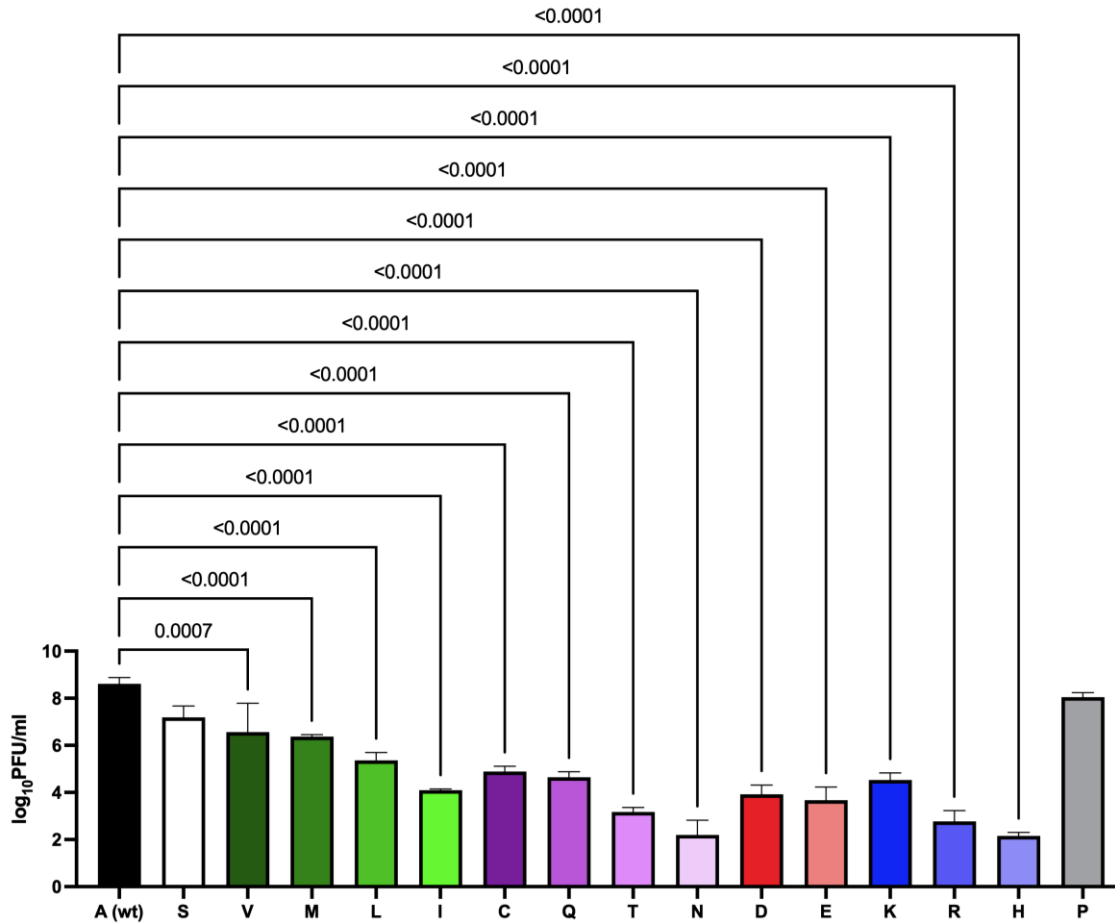


Figure 3-16: Infectivity of WNV E-A54 mutants in Vero cells

Infectivity of WNV NY99ic and the E-A54 mutations, following transfection of Vero cells. Infectivity between WNV NY99ic and three biological replicates of each mutant collected at 3 dpi was compared using one-way ANOVA followed by Dunnett's pairwise comparison. Each mutant is denoted in the graph with the letter associated with the single-site mutation. The image was generated in GraphPad. Recovery of mutant viruses and plaque assays were conducted by Bailey Maloney.

Further, we selected the E-A54 mutants that reached infectivity greater than 2log₁₀PFU/ml in transfected Vero cells to determine the replication kinetics in Vero cells (moi: 0.1) because the low infectivity will be challenging for vaccine manufacturing (Figure 3-17). For the simplicity of

comparison, the replication kinetics in Vero cells was determined for up to 3 dpi when infectivity of WNV NY99ic reached the plateau phase. As anticipated, the E-A54P mutant resembled the multiplication kinetics of WNV NY99ic between 1 and 3 dpi. The E-A54I ($p=0.03$) and E-A54M ($p=0.04$) mutants failed to multiply to high infectivity at 3 dpi, compared with WNV NY99ic. Substituting the E-A54 residue with the acidic aspartic acid ($p=0.0009$) and glutamic acid ($p=0.001$) led to a significant reduction of infectivity at 2 dpi. Similarly, the E-A54K, E-A54C, E-A54Q, and E-A54T mutants also exhibited varying but significant levels of reduction in multiplication kinetics in comparison with WNV NY99ic between 1 and 3 dpi ($p<0.05$).

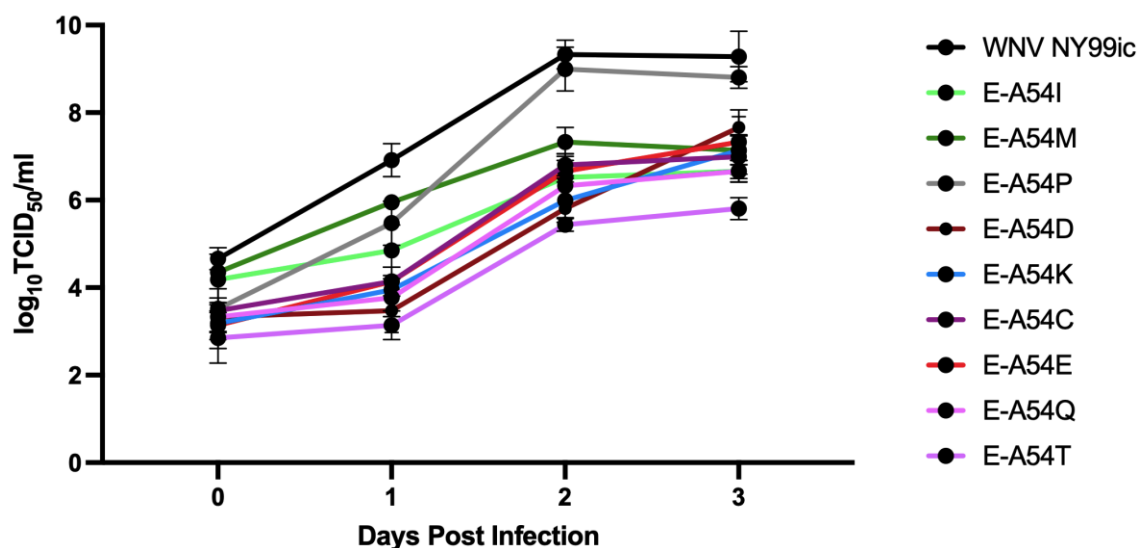


Figure 3-17: Multiplication kinetics of the E-A54 mutants in Vero cells between 0 and 3 dpi
Average titers of three replicates calculated from virus harvested from Vero cells infected at moi of 0.1. Data points represent the mean viral titer, and error bars represent the standard deviation of three biological replicates. The image was generated in GraphPad. Growth curves were conducted by were conducted by Bailey Maloney.

In addition to the multiplication kinetics in Vero cells, we determined the genome-PFU ratio of the E-A54 mutants, as the attenuated E-A54S and E-Y201P mutants both exhibited significantly higher genome-PFU ratios in comparison with WNV NY99ic (Figure 3-18). Seven

E-A54 mutations were shown to significantly increase the genome-PFU ratio of WNV NY99ic, including the E-A54C, E-A54D, E-A54E, E-A54I, E-A54K, E-A54Q, and E-A54T mutations. Notably, two acidic amino acid substitutions, i.e., the E-A54E and E-A54D mutations, showed a 2.7-fold increase in the genome-PFU ratio ($p<0.0001$). The increase in genome-PFU ratio can also be caused by replacing the E-A54 residue with the basic lysine (1.6-fold increase, $p=0.0406$). The E-A54C, E-A54Q, and E-A54T mutations increased the genome-PFU ratio of WNV NY99ic by 1.7 ($p=0.004$), 1.6 ($p=0.0063$), and 2.2 ($p<0.0001$) fold, respectively. Intriguingly, the genome-PFU ratio of WNV NY99ic could also be significantly elevated by one hydrophobic amino acid substitution, i.e., the E-A54I mutation (1.9-fold, $p=0.0006$). The remaining mutations, E-A54L, E-A54M, E-A54P, and E-A54V, showed no significant differences in their genome-PFU ratios compared to wild-type viruses. We then used three criteria to select the E-A54I and E-A54E mutants to evaluate the attenuation of the mouse neuroinvasive phenotype of WNV NY99ic, including 1) the reduced infectivity in transfected Vero cells, 2) the impairment of multiplication kinetics in infected Vero cells, and 3) the increased genome-PFU ratios in infected Vero cells.

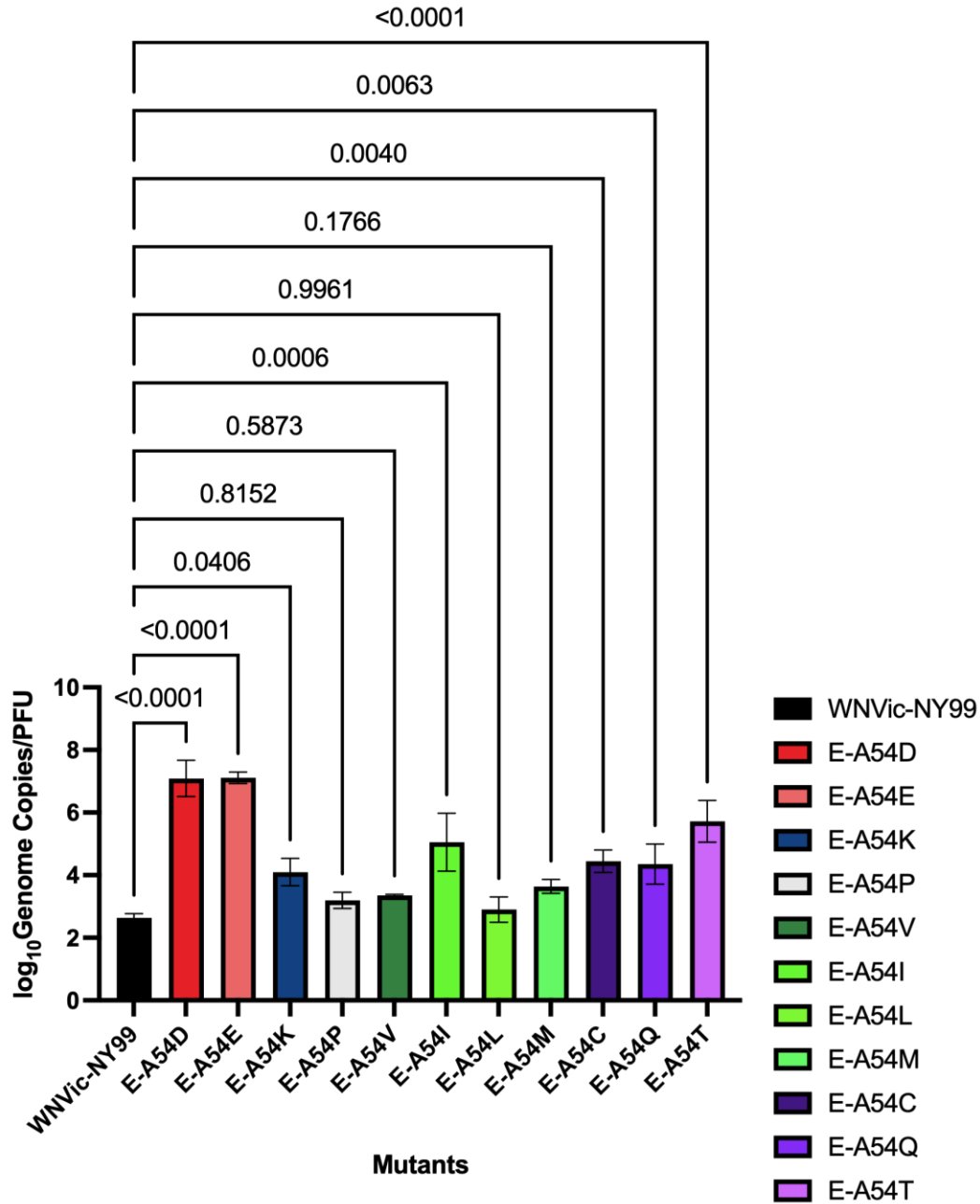


Figure 3-18: PFU-genome ratio of WNV E-A54 mutants

PFU-genome ratios of WNV NY99ic and the eleven mutants were grown in the culture supernatants of Vero cells infected at a moi of 0.1. Data points represent the average genome copy-to-PFU ratios, and error bars represent the standard deviation of two biological replicates prepared from Vero cells infected with WNV NY99ic or each mutant at moi of 0.1 and collected at

3 dpi. Comparison of genome copy-to-PFU ratio was performed using one-way ANOVA followed by Dunnett's pairwise comparison. The image was generated in GraphPad. The PFU-genome ratios were conducted by Bailey Maloney, Ashley Bilyeu, and Adrienne Pohl.

Transfection of Vero cells with RNA transcripts encoding the 18 alternative amino acid substitutions of the E-Y201 residue rescued 13 single E-Y201 mutants retaining the engineered nucleotide substitutions. The mutations E-Y201F, E-Y201H, E-Y201I, E-Y201L, E-Y201M, E-Y201N, and E-Y201R resembled the infectivity of WNV NY99ic, reaching approximately 7-8log₁₀PFU/ml (Figure 3-19). Four mutants exhibited the lethal phenotype, including the E-Y201G, E-Y201T, E-Y201V, and E-Y201W mutations (Table 3-13). The E-Y201A and E-Y201S mutations were unstable. Only one rescue of the E-Y201A mutant that encodes U1567G and A1568C nucleotide substitutions produced infectious viruses, which were proven to revert to the wild-type sequence. Similarly, only two transfection experiments of Vero cells with RNA transcripts encoding the E-Y201S mutation produced viable viruses, and one biological replicate retained the engineered amino acid substitution.

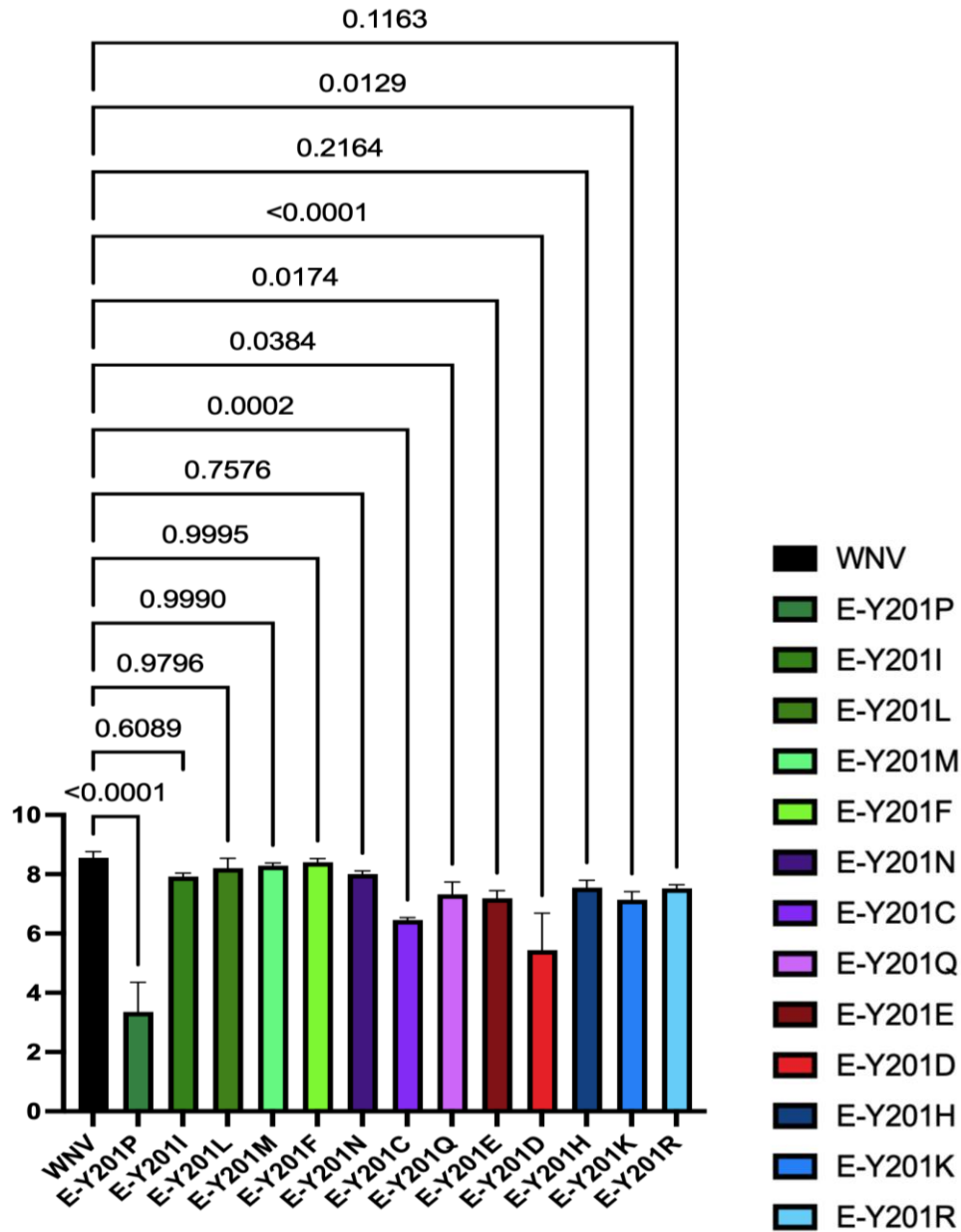


Figure 3-19: Infectivity of WNV E-Y201 mutants in Vero cells

Infectivity of WNV NY99ic and two replicates of the E-Y201 mutations, following transfection of Vero cells. Infectivity between WNV NY99ic and three biological replicates of each mutant collected at 3 dpi was compared using one-way ANOVA followed by Dunnett's pairwise comparison. The image was generated in GraphPad. Recovery of mutant viruses and plaque assays were conducted by Bailey Maloney.

Six E-Y201 mutants showed significantly reduced titers compared to WNV NY99ic, including 19-fold reduction caused by the E-Y201E mutation ($p=0.0174$), 22-fold reduction by the E-Y201K mutation ($p=0.0129$), and 11-fold reduction by the E-Y201Q mutations ($p=0.0384$). However, the most profound reduction of infectivity was observed in the E-Y201C and E-Y201D mutants. In comparison with WNV NY99ic, the E-Y201C and E-Y201D mutants had the largest reduction in infectivity, corresponding to 114-fold ($p=0.0002$) and 337-fold ($p<0.0001$) reduction, respectively. The multiplication kinetics and genome-PFU ratios in Vero cells (moi: 0.1) further justify the selection of the E-Y201C and E-Y201D mutants for evaluation in the neuroinvasive disease mouse model. Briefly, both the E-Y201C and E-Y201D mutants exhibited multiplication kinetics resembling the E-Y201P mutant (Figure 3-20). The genome-PFU ratio of the E-Y201C and E-Y201D mutants was also increased by 1.4 and 1.8-fold compared with WNV NY99ic, albeit with the lack of statistical significance (Figure 3-21).

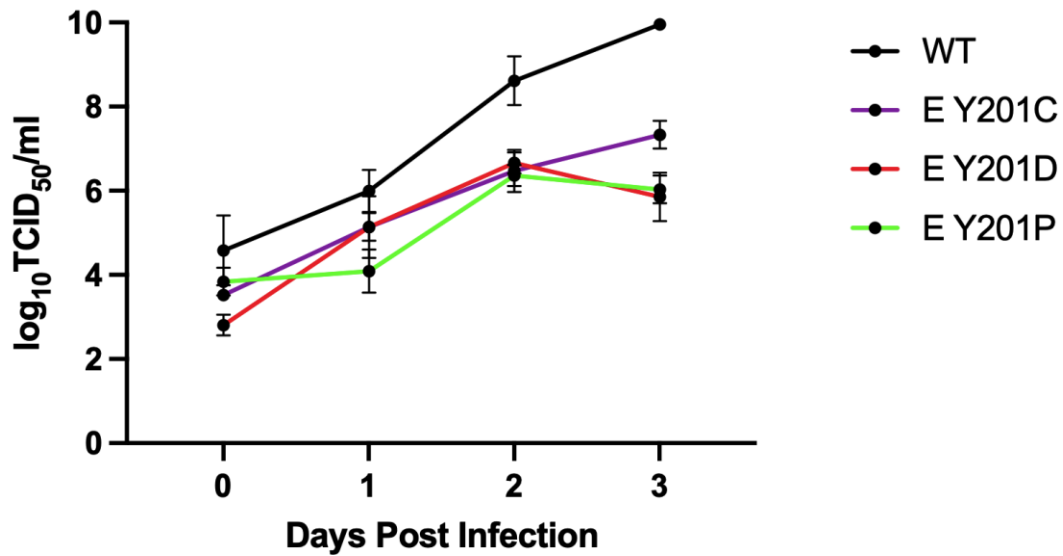


Figure 3-20: Reduced multiplication kinetics of the E-Y201C and E-Y201D mutants in Vero cells between 0 and 3 dpi.

Average titers were calculated in Vero cells infected at moi of 0.1. Data points represent the mean viral titer, and error bars represent the standard deviation of three biological replicates. The image was generated in GraphPad. Growth curves were conducted by Bailey Maloney,

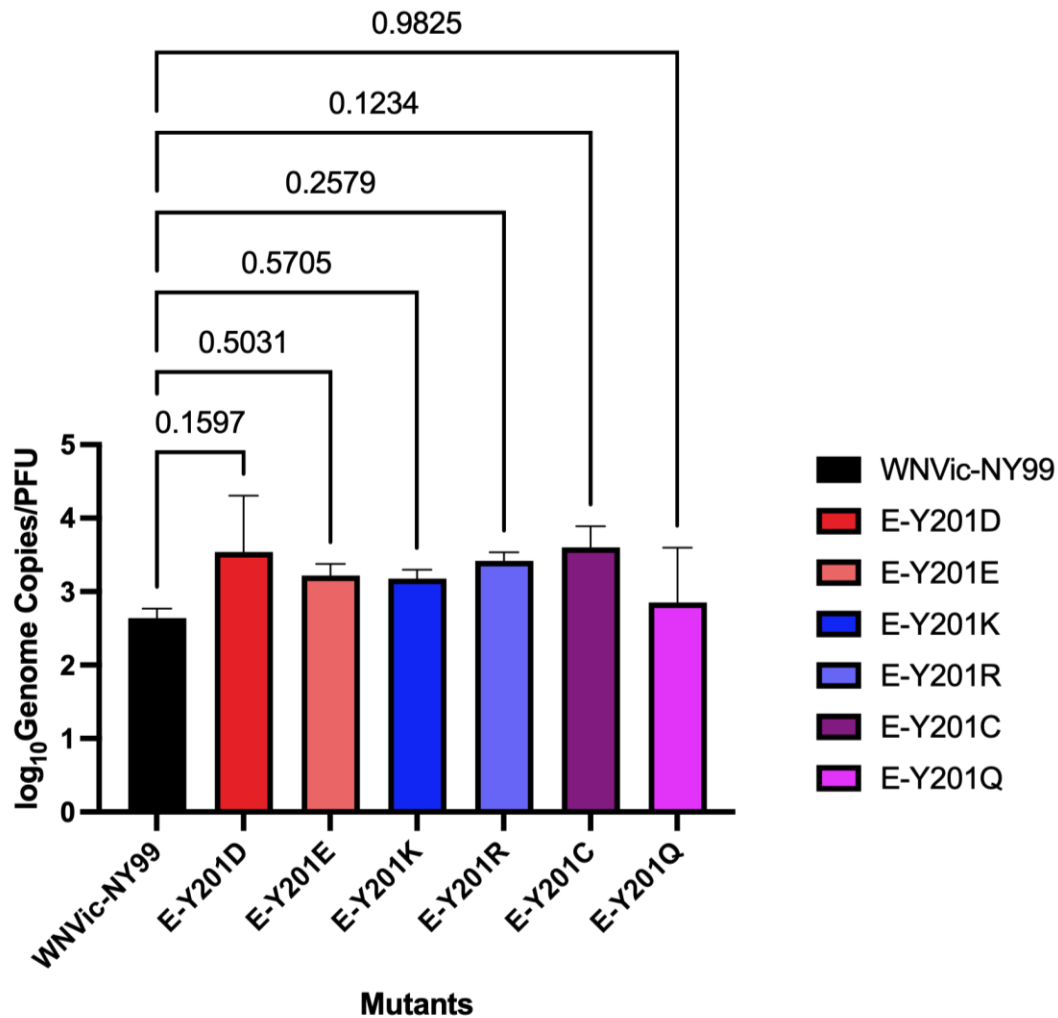


Figure 3-21: PFU-genome ratio of WNV E-Y201 mutants

Genome-PFU ratios of WNV NY99ic and the six mutants were grown in the culture supernatants of Vero cells infected at a moi of 0.1. Data points represent the average genome copy-to-PFU ratios, and error bars represent the standard deviation. Comparison of genome copy-to-PFU ratio was performed using one-way ANOVA followed by Dunnett's pairwise comparison. The image was generated in GraphPad. PFU-genome ratios were conducted by Bailey Maloney and Adrienne Pohl. The PFU-genome ratios were conducted by Bailey Maloney and Adrienne Pohl.

Of the E-Y201 mutants tested, none exhibited genome-PFU ratios significantly different from the wild-type virus. The E-Y201C, E-Y201D, E-Y201E, E-Y201K, and E-Y201R mutants

had ratios between $3-4\log_{10}\text{genome-PFU}$ while the E-Y201Q mutant showed the closest ratio to the wild-type at $2.85\log_{10}\text{genom-PFU}\pm 0.74$. Two of these mutants stood out as having visibly higher genome-PFU ratios even if they were not significantly different: E-Y201C ($p=0.1234$) and E-Y201D ($p=0.1597$).

H. Engineering of the E-Y201P mutation based on the codon bias of WNV

In addition to optimizing the amino acid substitution of the E-Y201 residue, we also hypothesized the E-Y201P mutant engineered according to the codon bias of WNV would increase infectivity in Vero cells. In Section 3.2.A, we initially utilized a proline at the E-Y201 residue using the nucleotide substitutions U1567C and A1568C, resulting in the CCC codon (Figure 3-22). To create a mutant containing the proline codon with the greatest codon bias for WNV, an additional nucleotide mutation was introduced at C1569A, resulting in a CCA codon. The optimized CCA codon increased the infectivity of the E-Y201P mutant by 4.1-fold compared with the E-Y201P mutant using the CCC codon. Reassuringly, the E-Y201P mutation encoded by the CCA codon retained the engineered amino acid substitution in the consensus sequence determined by Sanger sequencing. Therefore, the codon-optimized E-Y201P mutant was further evaluated for attenuation of the mouse neuroinvasive phenotype of WNV NY99ic.

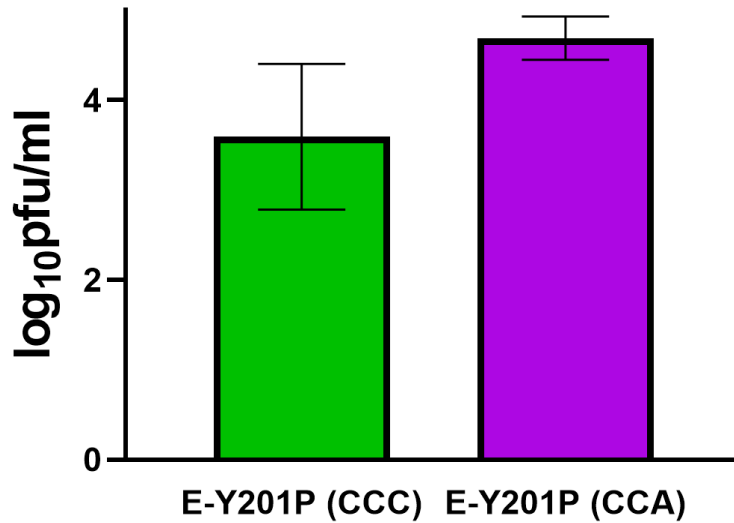


Figure 3-22: Infectivity comparison of WNV E-Y201P mutants

Infectivity of two replicates of the E-Y201P mutations following transfection of Vero cells. Green: E-Y201P mutation encoded by the CCC codon. Purple: E-Y201P mutation encoded by the CCA codon. The image was generated in GraphPad. Recovery of mutant viruses and plaque assays were conducted by Bailey Maloney.

I. Attenuation of the mouse neuroinvasive phenotype of optimized WNV E-A54 and E-Y201 mutants

The neuroinvasive phenotype of the E-A54I, E-A54E, E-Y201C, and E-Y201D mutants was each determined in groups of five male and five female four-week-old outbred Swiss mice, i.e., the same model system that demonstrated the attenuating effect of the E-A54S and E-Y201P mutations (Figure 3-23).

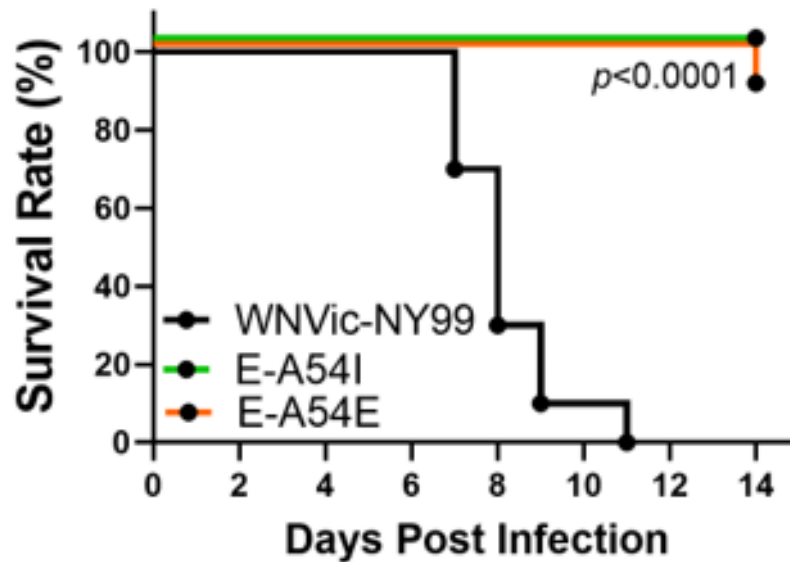


Figure 3-23: Attenuation of the neuroinvasive phenotype of WNV E-A54 mutants in four-week-old mice

Groups of five male and five female mice received the IP challenge with each WNV mutant or WNV NY99ic at 500 PFU. The Kaplan-Meier survival curve showed differences in survival outcomes and average survival time, and Dunnett's pairwise comparison followed one-way ANOVA. The image was generated in GraphPad. The mouse neuroinvasive disease study of E-A54 mutants was conducted by Bailey Maloney, Natalia Costa Ball, Adrienne Pohl, and So Lee Park.

Infection with the WNV NY99ic led to uniform lethality between 7 and 11 dpi. Significantly, no mortality was observed in mice challenged with the E-A54I mutant ($p < 0.0001$). Therefore, the E-A54I mutation can fully attenuate the neuroinvasive phenotype of WNV NY99ic in mice up to 500 PFU. The E-A54E mutant also exhibited a highly attenuated phenotype, as 90% of mice survived up to 14 days. Only one female mouse was euthanized during the final evening at 13 dpi. Therefore, mice challenged with the E-A54E mutant had an extended average survival time of 13.9 days ($p < 0.0001$) (Table 3-14).

Table 3-14: Comparison of average survival times between mice challenged with WNV NY99ic, the E-A54E mutant, and the E-A54I mutant

This table details the average survival time post-infection for each group of inoculated mice and the standard deviation seen within the respective group. The average survival time of each group was calculated using 14 days as the end point of the study. The mouse neuroinvasive disease study of E-A54 mutants was conducted by Bailey Maloney, Natalia Costa Ball, Adrienne Pohl, and So Lee Park.

Group	Average Survival Time (days)	Standard Deviation
WNV NY99 (wt)	8.2	1.5
E-A54E	13.9	0.3
E-A54I	>14	n/a ²

The lack of weight loss can also reflect the attenuating effect of the E-A54I and E-A54E mutations (Table 3-15). The mice receiving the IP challenge with WNV NY99ic began to exhibit weight loss at 5 to 6 dpi, followed by the onset of neurological symptoms and death. In contrast, no weight loss or sign of disease was observed in the mice challenged with the E-A54I mutant. Similar weight gain was observed in mice receiving the IP challenge with the E-A54E mutant. The only exception was the female mouse that began to experience hunched posture and ruffled fur at 11 dpi and reached the 20% weight loss criterion for euthanasia at 13 dpi.

² No endpoint data was retrieved in this mouse group so no standard deviation could be calculated.

Table 3-15: Comparison of weights for mice infected with WNV NY99ic, the E-A54E mutant, and the E-A54I mutant.

The weight of each mouse was measured in grams daily. The starting weight is the mouse's weight on the morning of 0 dpi before challenging the mice with WNV. The peak weight is the heaviest weight each mouse attained during the study. The final weight is the mouse's weight the morning the mice were euthanized or the morning of the end of the study at 14 dpi. “M” represents the male mice, and the notation “F” represents the female mice. Grey coloring represents a mouse that was euthanized or found dead prior to the end of the study, and white represents a mouse that survived to 14 dpi. The mouse neuroinvasive disease study of E-A54 mutants was conducted by Bailey Maloney, Natalia Costa Ball, Adrienne Pohl, and So Lee Park.

Group	Mouse (Male)	Starting Weight (g)	Peak Weight (g)	Final Weight (g)	Mouse (Female)	Starting Weight (g)	Peak Weight (g)	Final Weight (g)
WNV NY99 (wt)	1M	28.4	30.2	28.4	1F	22.2	23.4	19.7
	2M	29.5	30.6	27.7	2F	24.6	25.3	20.5
	3M	23.2	24.3	21.2	3F	22.7	24.3	20.0
	4M	30.2	31.2	26.7	4F	21	22.4	21.8
	5M	26.7	28.6	24.2	5F	24.6	25.6	22.6
A54E	1M	27	30.1	30.1	1F	23.4	27.1	26.4
	2M	29.6	32.8	32.8	2F	23.3	25.8	25.2
	3M	28.3	31.3	30.9	3F	23.3	25.7	25.7
	4M	30.5	35.3	35.3	4F	23.9	26.1	26.1
	5M	28.4	31.9	31.9	5F	21.3	22.2	16.1
A54I	1M	28.5	32.4	30.7	1F	23.7	26.5	26.5
	2M	27.3	31.0	31.0	2F	25.7	27.8	27.8
	3M	29.1	34.0	31.3	3F	24	26.3	26.3
	4M	23.8	26.8	26.8	4F	22.3	23.4	23.4
	5M	29.5	33.3	33.3	5F	21.2	25.3	25.3
	5M	29.5	29.8	28.8	5F	25.5	26.8	25.9

The lack of detectable viral RNA by RT-qPCR in the brain of mice challenged with the E-A54I mutant is consistent with its fully attenuated phenotype (Figure 3-24). Similarly, we did not detect viral RNA in the brain of mice receiving the IP challenge with the E-A54E mutant. However, amplicons were obtained from the brain of the only mouse that succumbed to the challenge with the E-A54E mutant by RT-PCR using the gene-specific primers targeting prM, E, and NS1 genes. The consensus sequence reverted to the wild-type sequence, explaining the signs of neurological disease between 10 and 13 dpi.

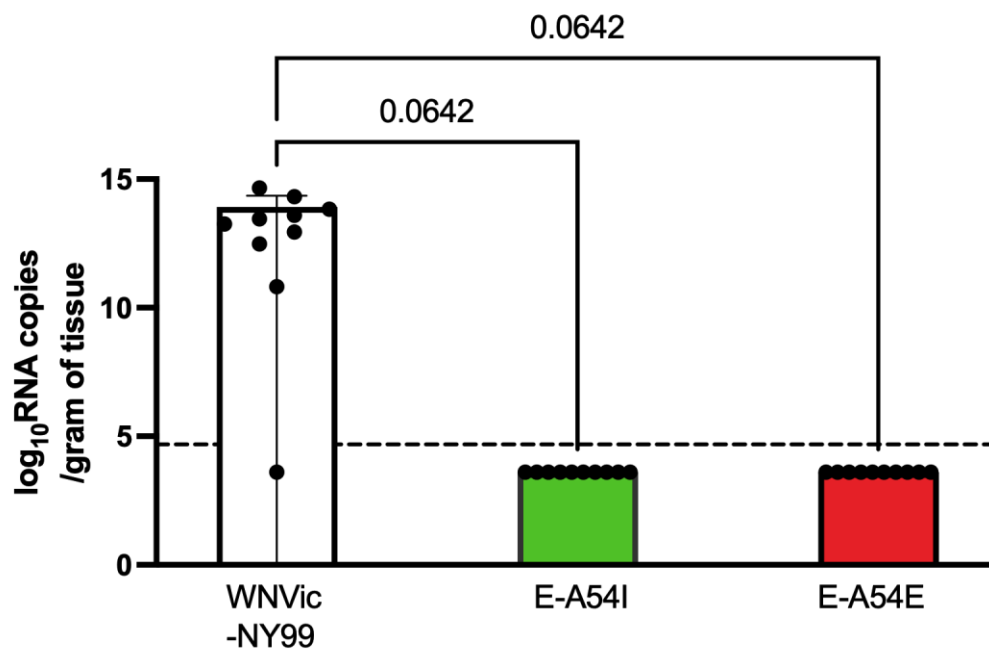


Figure 3-24: Comparison of brain viral loads in groups of mice infected with WNV NY99ic, the E-A54E mutant, and the E-A54I mutant.

Brain viral loads of mice challenged with WNV NY99ic or the E-A54E and E-A54I mutants were compared by one-way ANOVA and Dunnett's pairwise comparison. The dashed line indicates the limit of detection. The image was generated in GraphPad. The RT-qPCR was conducted by Bailey Maloney.

To prove the E-A54I and E-A54E mutations have utility for the rational design of candidate WN LAVs, we undertook PRNT₅₀ using serum samples collected from surviving mice at 14 dpi. All PRNT₅₀ assays for the E-A54E and E-A54I mutants were completed by Dr. Claire Huang's laboratory at the CDC. Both the E-A54E and E-A54I mutants can elicit neutralizing antibody responses in surviving mice, leading to a 100% seroconversion rate in the respective groups of mice based on 1:10 or greater serum PRNT₅₀ (Figure 3-25). Significantly, serum samples collected from 89% (8/9) and 70% (7/10) of mice receiving the E-A54E and E-A54I mutants, respectively, leading to the neutralization of more than 50% of the virus inoculum at 1:320 dilution. Therefore, the E-A54E and E-A54I mutants are highly immunogenic in 4-week-old outbred Swiss Webster mice.

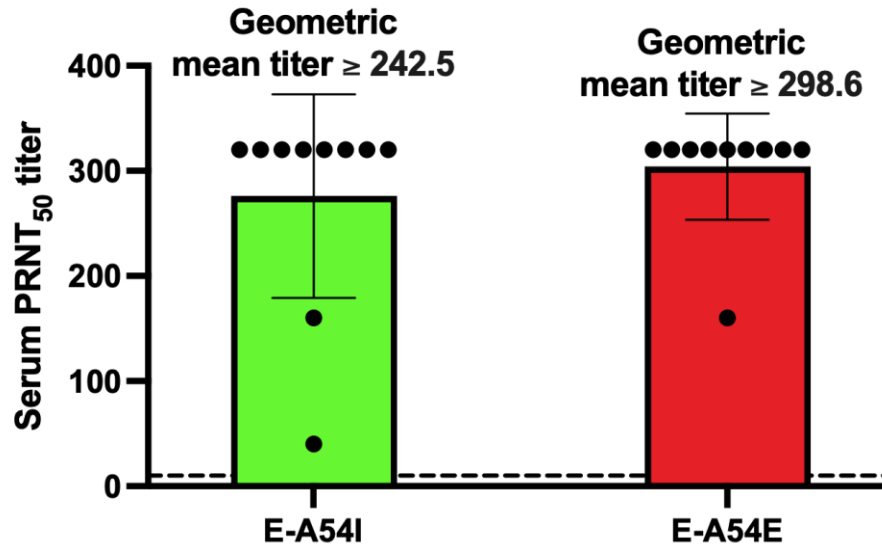


Figure 3-25: Comparison of serum neutralizing titers in mice that survived the IP challenge with the E-A54E and E-A54I mutants.

Serum PRNT₅₀ titer of mice surviving the challenge with the respective E-A54 mutants. Because 1:320 dilution did not reach the endpoint of 50% neutralizing in 89% (8/9) and 70% (7/10) mice that received the IP challenge with the E-A54E and E-A54I mutants, the geometric mean of PRNT₅₀ titers is denoted as equal or greater than 1:320. The dashed line indicates 1:10 serum PRNT₅₀ titer, representing the lowest antibody detection limit. The image was generated in GraphPad. The PRNT₅₀ assays were conducted by Dr. Claire Huang's laboratory.

We have also investigated the mouse neuroinvasive phenotype of the E-Y201C and E-Y201D mutants (Figure 3-26). In contrast to the uniform lethality induced by the IP challenge with WNV NY99ic, the E-Y201C and E-Y201D mutations both exhibited the attenuating effect on the mouse neuroinvasive phenotype of WNV NY99IC. Both the E-Y201C and E-Y201D mutants are partially attenuated, as observed with over 70% survival rate and significantly extended average survival times (E-Y201C mutant: 12.5 days, $p=0.0001$; E-Y201D mutant: 13.6 days, $p=0.0001$) in comparison with WNV NY99ic at 8.2 days (Table 3-16).

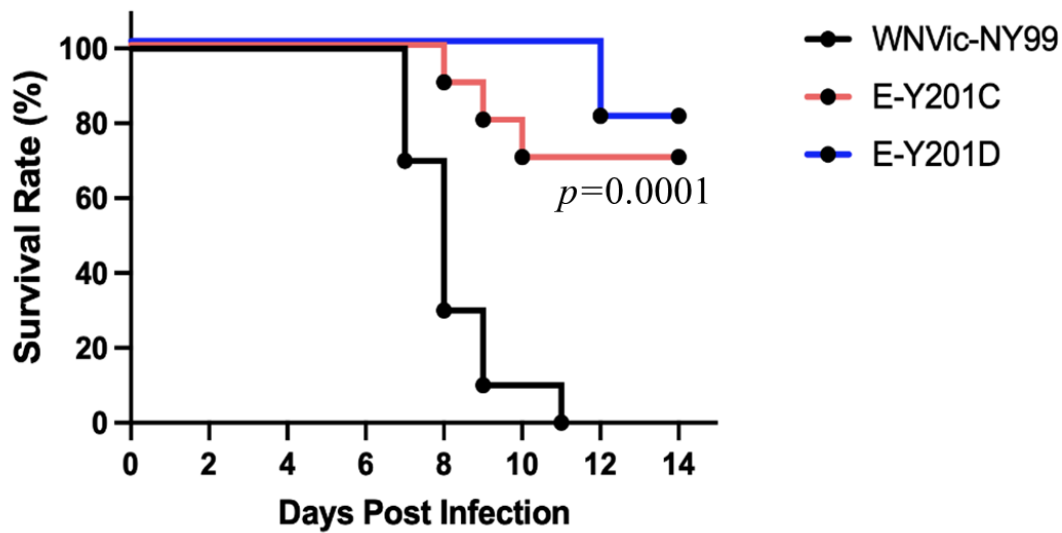


Figure 3-26: Survival curve of four-week-old outbred Swiss Webster mice receiving IP challenge with WNV NY99ic, the E-Y201C mutant, and the E-Y201D mutant.

Groups of five male mice and five female mice received the IP challenge with the respective mutants or WNV NY99ic at 500 PFU. The Kaplan-Meier survival curve showed differences in survival outcomes and average survival time, and Dunnett's pairwise comparison followed one-way ANOVA. The image was generated in GraphPad. The mouse neuroinvasive disease study of E-Y201 mutants was conducted by Bailey Maloney, Natalia Costa Ball, Adrienne Pohl, and So Lee Park.

Table 3-16: Comparison with average survival times of four-week-old outbred Swiss mice challenged with WNV NY99ic, the E-Y201C mutant, or the E-Y201D mutant

This table details the average survival time post-infection for each group of inoculated mice and the standard deviation seen within the respective group. The average survival time of each group was calculated using 14 days as the end point of the study. The mouse neuroinvasive disease study of E-Y201 mutants was conducted by Bailey Maloney, Natalia Costa Ball, Adrienne Pohl, and So Lee Park.

Group	Average Survival Time (days)	Standard Deviation
WNV NY99 (wt)	8.2	1.5
E-Y201C	12.5	2.7
E-Y201D	13.6	0.8

Intriguingly, the weight loss pattern was highly consistent with the outcome of the IP challenge with either the E-Y201C mutant or the E-Y201D mutant (Table 3-17). For example, the mice that succumbed to the neuroinvasive disease induced by the E-Y201C mutant (n=3) experienced weight similar to those challenged with WNV NY99ic, starting at 6 to 7 dpi. In contrast, the mice that survived the challenge with the E-Y201C mutant recovered from the initial loss (n=7), starting between 9 to 11 dpi. Similarly, 80% of mice challenged with the E-Y201D mutant all experienced gradual weight gain. Two male mice that succumbed to the neuroinvasive disease at 13 dpi exhibited weight loss resembling those challenged with WNV NY99ic.

Table 3-17: Weight change of mice infected with WNV NY99ic, the E-Y201C mutant, and the E-Y201D mutants

The weight of each mouse was measured in grams daily. The starting weight is the mouse's weight on the morning of 0 dpi, just before challenging the mice with WNV. The peak weight is the heaviest weight each mouse attained during the study. The final weight is the mouse's weight the morning the mice were euthanized or the morning of the end of the study at 14 dpi. "M" represents the male mice, and the notation "F" represents the female mice. Grey coloring represents a mouse that was euthanized or found dead prior to the end of the study, and white represents a mouse that survived to 14 dpi. The mouse neuroinvasive disease study of E-Y201 mutants was conducted by Bailey Maloney, Natalia Costa Ball, Adrienne Pohl, and So Lee Park.

Group	Mouse (Male)	Starting Weight (g)	Peak Weight (g)	Final Weight (g)	Mouse (Female)	Starting Weight (g)	Peak Weight (g)	Final Weight (g)
WNV NY99 (wt)	1M	28.4	30.2	28.4	1F	22.2	23.4	19.7
	2M	29.5	30.6	27.7	2F	24.6	25.3	20.5
	3M	23.2	24.3	21.2	3F	22.7	24.3	20.0
	4M	30.2	31.2	26.7	4F	21	22.4	21.8
	5M	26.7	28.6	24.2	5F	24.6	25.6	22.6
Y201C	1M	28.4	31.5	25.0	1F	25.1	29.6	29.6
	2M	23.9	25.5	19.9	2F	22.2	23.8	18.8
	3M	24.4	26.7	23.7	3F	23.7	25.4	24.9
	4M	29.2	31.9	31.9	4F	21.9	24.8	24.8
	5M	28.4	33.1	33.1	5F	21.6	24.3	24.1
Y201D	1M	26.2	29.4	29.4	1F	23.3	26.3	25.8
	2M	26.8	29.5	29.5	2F	22.7	27.0	27.0
	3M	27.3	30.4	24.7	3F	20.7	24.3	24.3
	4M	28.6	30.2	26.0	4F	24.2	26.9	26.9
	5M	26.1	28.4	28.4	5F	20.9	23.3	23.3

There was no difference in the timing for the onset of neuroinvasive disease in mice that succumbed to neuroinvasive disease induced by WNV NY99ic and the E-Y201C mutant. The mice challenged with E-Y201C began to show symptoms 8 dpi (n=3). Besides some weight loss, only the mice in the E-Y201C group that were euthanized or found dead exhibited symptoms. The onset of neurological disease in mice (n=2) challenged with the E-Y201D mutant was slightly delayed to 11 dpi. Nevertheless, both the E-Y201C and E-Y201D mutations exhibited a high attenuating effect because more than half of the mice in each group survived the challenge without developing signs of neurological disease.

In comparison with the average brain viral load of mice challenged with WNV NY99ic (1.38×10^{12} genome copies/gram of tissue), the average brain viral loads of mice receiving the E-Y201C mutant (6.90×10^{11} genome copies/gram of tissue) and E-Y201D mutant (3.45×10^9 genome copies/gram of tissue) were reduced by 2- ($p=0.1469$) and 400- ($p=0.0727$) fold, respectively (Figure 3-27).

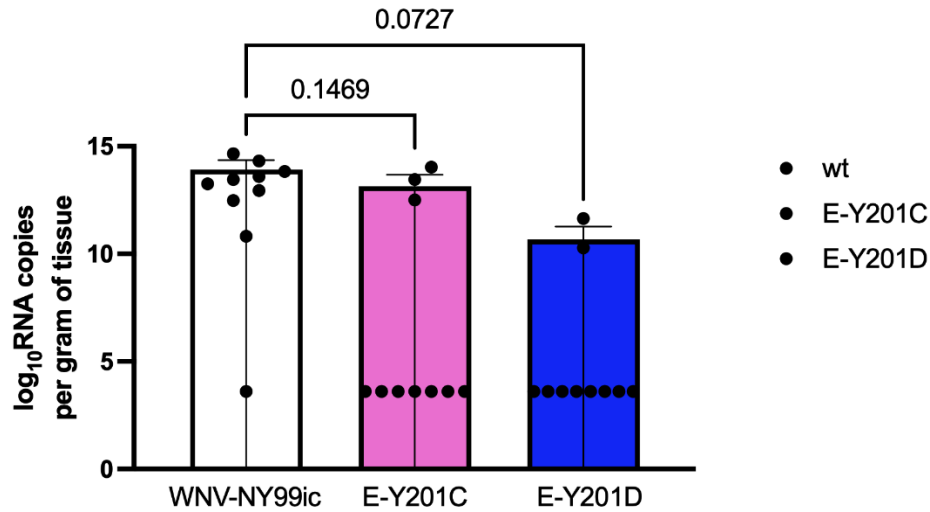


Figure 3-27: Comparison of brain viral load in mice infected with WNV NY99ic, the E-Y201C mutant, and the E-Y201 mutant.

Brain viral loads of mice challenged with WNV NY99ic or the E-Y201C and E-Y201D mutants were compared by one-way ANOVA and Dunnett's pairwise comparison. The image was generated in GraphPad. The RT-qPCR was conducted by Bailey Maloney.

Further, we have determined the sequence of viral RNA in the brain of each mouse succumbing to the challenge with the E-Y201C mutant or the E-Y201D mutant. None of the individual mice within each mutant group exhibited any reversion to the wild-type sequence.

In addition to the E-Y201C and E-Y201D mutations, we have also engineered the E-Y201P mutant based on the codon bias of WNV, followed by the investigation of attenuating effect at 500 PFU and 3300 PFU (Figure 3-28). The codon-optimized E-Y201P mutant showed an approximately 4.1-fold increase in infectivity compared to the E-Y201P mutant initially engineered with CCC codon in transfected Vero cells, supporting the investigation of attenuating effect at the higher dosage.

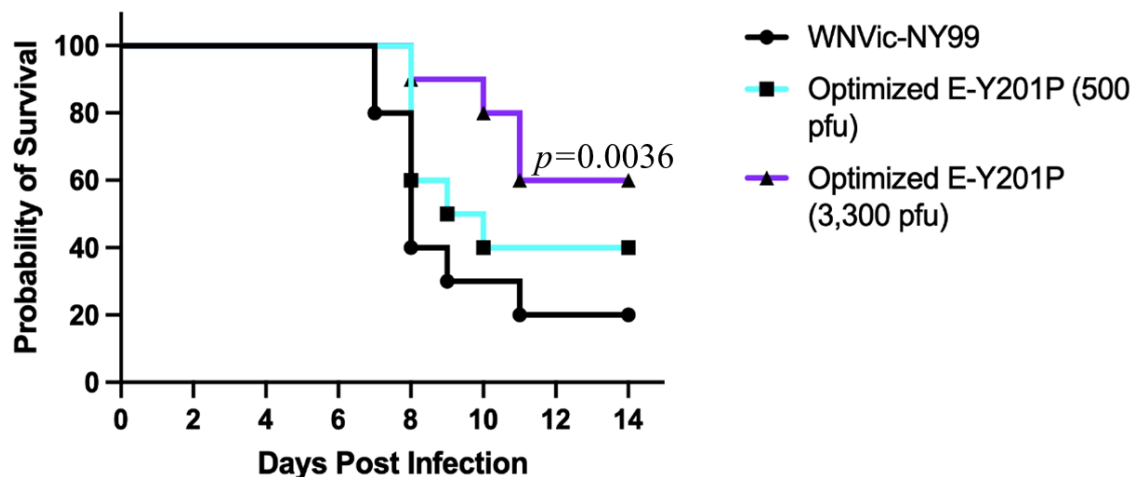


Figure 3-28: Survival curves of four-week-old outbred Swiss mice challenged with WNV NY99ic and the codon-optimized E-Y201P mutant

Groups of five male and five female mice received the IP challenge with the WNV NY99ic or codon-optimized WNV E-Y201P mutant at 500 PFU and 3300PFU. The Kaplan-Meier survival curve showed differences in survival outcomes and average survival time, and Dunnett's pairwise comparison followed one-way ANOVA. The image was generated in GraphPad. The mouse neuroinvasive disease study of codon-optimized E-Y201 mutants was conducted by Bailey Maloney, Natalia Costa Ball, Adrienne Pohl, Emily Mantlo, and Alexis Schneider.

Infection with the wild-type WNV NY99ic led to lethal neuroinvasive disease in most mice between 6 to 8 dpi except for one male mouse and one female mouse that survived to the end of the study, i.e., 80% mortality (Table 3-18). The optimized E-Y201P mutation was not fully attenuating, with 40% surviving in the 500 PFU group and 60% surviving in the 3300 PFU group. Both showed to be partially attenuating, with the average survival time at 10.7 days for the 500 PFU group ($p=0.1021$) and 12.4 days for the 3300 PFU group ($p=0.0036$).

Table 3-18: Comparison of average survival time between mice challenged with WNV NY99ic and the codon-optimized WNV E-Y201P mutant at 500 and 3,000 PFU

This table details the average survival time post-infection for each group of inoculated mice and the standard deviation seen within the respective group. The average survival time of each group was calculated using 14 days as the end point of the study. The mouse neuroinvasive disease study of codon-optimized E-Y201 mutants was conducted by Bailey Maloney, Natalia Costa Ball, Adrienne Pohl, Emily Mantlo, and Alexis Schneider.

Group	Average Survival Time (days)	Standard Deviation
WNV NY99 (wt)	8.4	2.9
Codon Optimized E-Y201P (500 PFU)	10.7	3.0
Codon Optimized E-Y201P (3300 PFU)	12.4	2.2

The majority of the mice receiving the IP challenge with WNV NY99ic experienced weight loss after 8 dpi, followed by the onset of neurological disease and mortality (Table 3-19). The two mice that survived the challenge with WNV NY99ic showed weight loss until 8 to 9 dpi, which may indicate active viral replication. The codon-optimized E-Y201P mutant is partially attenuated and causes lethal neuroinvasive disease at 500 and 3,300 PFU. Although there is no demonstrable difference in the level of weight loss between mice challenged with WNV NY99ic and the codon-optimized E-Y201P mutant, the mice that survived the challenge with the codon-optimized E-Y201P exhibited recovery of weight after initial loss starting between 9 to 11 dpi.

Table 3-19: Individual weights for mice infected with WNV NY99ic or the codon-optimized WNV E-Y201P mutants

The weight of each mouse was measured in grams daily. The starting weight is the mouse's weight on the morning of 0 dpi, just before challenging the mice with WNV. The peak weight is the heaviest weight each mouse attained during the study. The final weight is the mouse's weight the morning the mice were euthanized or the morning of the end of the study at 14 dpi. "M" represents the male mice, and the notation "F" represents the female mice. Grey coloring represents a mouse that was euthanized or found dead, and white represents a mouse that survived to 14 dpi. The mouse study of codon-optimized E-Y201 mutants was conducted by Bailey Maloney, Natalia Costa Ball, Adrienne Pohl, Emily Mantlo, and Alexis Schneider.

Group	Mouse (Male)	Starting Weight (g)	Peak Weight (g)	Final Weight (g)	Mouse (Female)	Starting Weight (g)	Peak Weight (g)	Final Weight (g)
WNV NY99 (wt)	1M	29.3	30.2	25.3	1F	31.2	31.6	29.5
	2M	30.1	31.8	24.3	2F	30.7	22.5	17.9
	3M	29.3	32.8	32.5	3F	21.1	22.9	20.1
	4M	30.3	30.3	28.9	4F	25.5	25.6	24.1
	5M	28.7	30.0	26.9	5F	27.1	28.1	24.5
Y201P Optimized 500 PFU	1M	26.0	29.9	29.9	1F	27.5	29.8	29.8
	2M	26.5	30.4	30.4	2F	28.2	29.5	25.1
	3M	27.9	28.6	24.5	3F	24.9	25.5	17.5
	4M	27.6	28.3	24.7	4F	21.7	23.3	21.3
	5M	26.9	29.0	24.7	5F	27.0	27.0	23.7
Y201P Optimized 3300 PFU	1M	25.3	28.5	28.5	1F	25.8	27.1	25.8
	2M	29.6	32.3	32.0	2F	27.6	27.6	22.3
	3M	29.3	31.4	30.6	3F	27.0	27.9	26.2
	4M	26.0	27.3	22.3	4F	27.9	28.7	20.7
	5M	29.5	29.8	28.8	5F	25.5	26.8	25.9

Both groups of mice challenged with the codon-optimized E-Y201P mutant exhibited a similar onset of neurological disease compared to those challenged with WNV NY99ic at 6 to 7 dpi. The mice that survived the codon-optimized E-Y201P groups exhibited signs of recovery and resolution of symptoms around 11 to 12 dpi.

3.3 Discussion

We have used WNV as a model system to demonstrate all eight flavivirus-conserved hydrophobic residues in the EDI-EDII hinge region are crucial for the structure-function of the E protein involved in the membrane fusion process. Our PCR site-directed mutagenesis comprehensively tested 19 alternative amino acid substitutions. It proved mutations of the E-A54 and E-Y201 residues could fully attenuate the mouse neuroinvasive phenotype of WNV without compromising immunogenicity.

Our conclusion on the other six eight flavivirus conserved hydrophobic amino acids can only be limited to the E-I130N, E-I135N, E-I196N, E-A269S, E-V272T, E-L281N mutations. The importance of the E-I130, E-I196, E-Y201, E-A269, and E-L281 residues has not been previously reported. Consistent with previously published studies, the E-A54 and E-I135 residues are crucial for the structure-function of the E protein in more than one flavivirus (Butrapet et al. 2011). Previously, the E-A54 and E-I135 residues have been shown to be important for the infectivity of DENV-2 (Butrapet et al. 2011). Evaluation of the E-A54 and E-I135 residues in WNV is a new extension of this observation since WNV is in a different genetic group of the flavivirus genus than DENV-2.

Three of the six amino acids constituting the 2nd motif are conserved between different flaviviruses, including amino acids equivalent to the WNV E-I130, E-E133, and E-I135 residues. The E-I130N and E-I135N mutations inhibited the recovery of viable viruses. Lysine mutation of

the flavivirus-conserved E-E133 residue is unstable in DENV-2 (Butrapet et al. 2011). The E-E138K mutation in the neighboring region reverted to the wild-type sequence in WNV NY99ic (Kaiser et al. 2019b). It becomes immediately apparent that there may be limited plasticity in the sequence of the second motif of the EDI-EDII hinge region. It is not coincidental the E-I196N mutant also produced a lethal phenotype. The 2nd motif and the N-terminus of the 3rd motif of the EDI-EDII hinge region are neighboring and have putative interactions (Christian et al. 2013). Therefore, the E-I130 and E-I135 residues in the 2nd motif and the E-I196 residue in the 3rd motif are located in a structurally and functionally important region that cannot tolerate the engineered mutations.

The E-A269 and the E-L281 residues reside within the 4th motif of the EDI-EDII hinge region. Both mutants produced the virus, but only the E-A269S mutant retained the engineered mutation. The differences in how the virus tolerates mutation to these two residues are likely due to their locations within the *kl* hairpin of the EDI-EDII hinge region. This structure is part of EDII and controls the stabilization of EDI-EDII interaction in the E protein trimer. The *kl* hairpin is two β -sheets located parallel to each other in the pre-membrane fusion structure of the E protein. After membrane fusion, the *k* β -strand moves away from the *l* β -strand until they reside perpendicular. Based on the crystal structure of the E protein, while the *k* β -strand moves during membrane fusion, the *l* β -strand continuously interacts with the *D₀* β sheet of the EDI domain (Modis et al. 2004). The E-A269 residue resides in the *k* β -strand that moves during membrane fusion. Therefore, the residue probably tolerates alternative mutations to compensate for the loss of alanine, as seen in the SNVs of the E-A269S mutation, where compensatory mutations occurred in the neighboring E-I270 residue. This hypothesis may be proven by continued serial passaging of the mutant to see if the compensatory mutation becomes a consensus mutation. The E-L281

residue resides at the end of the *I* β -strand, and mutation to this residue is not tolerated, as seen by the three-nucleotide reversion to the wild-type sequence. This lack of tolerance possibly indicates the E-L281 residue helps control or stabilize the interactions between the *I* and *D*₀ β -strands.

The mutations E-A54S and E-Y201P showed the most promise in producing an attenuated neuroinvasive phenotype. Both mutations are critical residues to the structure and function of the E protein because they reside within the “gfeah” β -sheet that controls the orientation of EDII during the EDI-EDII interdomain movement. Initially, in our *in vitro* studies, the E-A54S and E-Y201P mutations caused a significant reduction in the infectivity and multiplication kinetics of WNV, which is consistent with previously reported attenuated WNV mutants (Wicker et al. 2006, Whiteman et al. 2010, Whiteman et al. 2011). In addition to the reduced titers, the increased genome-PFU ratios suggest that both the E-A54S and E-Y201P mutations can impair the establishment of WNV infection in the host cell as viral RNA is being produced. However, it is not being translated to infectious virions at the same rate as the wild-type virus. Therefore, both mutants are likely to produce larger quantities of non-infectious particles that cannot infect healthy cells, which impedes disease progression.

Additionally, this indicates enough immunogens in cell culture to produce a serum-neutralizing antibody response. Reductions in infectivity of the E-Y201P mutant can be attributed to the interference with the conformational change of the E protein from dimer in the virion to trimer during the membrane fusion process. Based on the hemagglutination assay, the E-Y201P mutation exhibited lower optimal pH for HA activity, which suggests the E-Y201P mutant requires more free energy provided by protonation of the endosome to trigger the conformational change of the E protein from a dimer to a trimer. Future exploration into the HA activity of the highly

attenuated E-A54E and fully attenuated E-A54I mutants is worthwhile to investigate whether the mutations could impair the conformational change of the E protein just like the E-Y201P mutant.

Somewhat unanticipated is the E-A54 residue, which is strictly conserved across all flaviviruses, can tolerate at least 14 alternative amino acid substitutions. We have not only shown that the E-A54I mutation can fully attenuate the mouse neuroinvasive phenotype of WNV NY99ic but also shown at least three amino acid substitutions, i.e., isoleucine, glutamic acid, and serine, will not compromise immunogenicity. The same E-A54E mutation has also been demonstrated to impair the membrane fusion activity of the DENV-2 E protein (Butrapet et al. 2011, Kallas et al. 2020, Wei et al. 2022). Moreover, the neighboring E-G52 residue is a determinant for the neuroinvasive phenotype of YFV (Huang 2014). These observations warrant further investigation of the structural and functional importance of the first motif of the EDI-EDII hinge region and evaluation of mutations based on the utility for vaccine development.

Based on the specific mutations at the E-A54 residue, our research indicates that it is not the hydrophobicity that is important to the E-A54 residue but the size of the amino acid side chain that is crucial to the structure and function of the E protein. Isoleucine is a large amino acid that is more hydrophobic than the original alanine. The negatively charged glutamic acid substitution produced less attenuation than the isoleucine substitution, but glutamic acid still had a much higher attenuation level than serine. The other amino acid substitutions that showed decreased infectivity also exhibited this pattern of large amino acids with varying and indiscriminate levels of hydrophobicity, such as E-A54E (155 Å³), E-A54I (169 Å³), E-A54K (171 Å³), E-A54N (135 Å³), and E-A54Q (161 Å³). The original alanine mutation was only 92 Å³ (Chothia 1975).

The E-Y201 residue in the third motif of the EDI-EDII hinge region is the other flavivirus-conserved hydrophobic amino acid crucial for the structure-function of the E protein. This

conclusion agrees with the structural and functional importance of the WNV E-Y201 residue; the equivalent DENV-2 E-M196 residue is crucial for the membrane fusion process (de Wispelaere et al. 2018). At least three amino acid substitutions of the E-Y201 residue attenuate the WNV NY99ic virulence phenotype in the mouse neuroinvasive disease model. The E-Y201P mutation can attenuate the mouse neuroinvasive phenotype of WNV NY99ic in full, but the attenuation level varies between alternative codons for proline. Despite the identical amino acid sequence, only the E-Y201P mutant encoded by the CCC codon is fully attenuated. Although the E-Y201P mutant encoded by the optimized CCA codon showed to restore some infectivity in transfected Vero cells, the mutation only partially attenuated the virulence phenotype of WNV in mice.

These two amino acids are non-conservative large amino acids, with cysteine being 106 Å³ and aspartic acid being 125 Å³ (Chothia 1975). These two amino acids are very similar in size to proline at 129 Å³. All three amino acids are much smaller than the original tyrosine, which is 204 Å³. Further analysis of the other amino acid substitutions showed the E-Y201 residue did not tolerate small amino acids such as alanine (92 Å³), glycine (66 Å³), and serine (99 Å³). The E-Y201A, E-Y201G, and E-Y201S mutations all produced unstable phenotypes, with E-Y201A and E-Y201S exhibiting reversion to the wild-type sequence and E-Y201G producing a lethal phenotype.

Overall, our data indicate that the E-Y201 residue must be occupied by an amino acid with a large side chain. While the small amino acids are not tolerated, the larger the amino acid side chain becomes, the less effect it has on the attenuation of the neuroinvasive phenotype. Proline, cysteine, and aspartic acids are all smaller amino acids than tyrosine, allowing an attenuating effect. However, they are still large enough not to be detrimental to the virus, which is consistent with the equivalent residues in other flaviviruses. For example, the equivalent residue among the

DENV serocomplex members is methionine (171 Å³), tyrosine (204 Å³), or phenylalanine (203 Å³) among JEV serocomplex members, leucine (168 Å³) for ZIKV, and valine (142 Å³) among TBEV serocomplex members

Mutations at the E-A54 and E-Y201 residues attenuated the neuroinvasive phenotype fully. They were stable after analysis of the viral RNA found in the brains of mice that succumbed to the disease. Because the E-A54I and E-Y201P mutations can fully attenuate the mouse neuroinvasive phenotype of WNV NY99ic, we propose mutation of the flavivirus conserved hydrophobic residues in the EDI-EDII hinge region offers a more effective attenuation strategy than the previously described mutations in the fusion loop. This conclusion is of significance for the rational design of candidate LAVs as previously reported mutations in the EDI-EDII hinge region did not support the development of a flavivirus-common attenuation (Hurrelbrink and McMinn 2001, Goo et al. 2017, Kaiser et al. 2019b). The mutations in the fusion loop only partially attenuated the mouse neuroinvasive phenotype of WNV while reverting to the wild-type sequence in the brains of mice succumbing to neuroinvasive disease (Zhang et al. 2006). Future studies could be used to evaluate the other flavivirus-conserved hydrophobic residues in the EDI-EDII hinge region using either the process of alternative amino acid substitutions or codon optimization to identify other attenuating mutations.

Chapter 4 - Engineering of Attenuating Mutations in the WNV EDI-EDIII Linker

The results presented in this chapter involved multiple collaborative projects. The WNV NY99 infectious clone was obtained from Dr. Claire Huang's laboratory at the Centers for Disease Control and Prevention in Fort Collins, CO. Modifications of this clone and analysis of the mutations of the WNV EDI-EDIII linker *in vitro* were performed with the assistance of Adrienne Pohl in Dr. Vanlandingham's laboratory at Kansas State University. The *in vivo* mouse studies were performed in collaboration with several members of Dr. Vanlandingham's laboratory and the College of Veterinary Medicine at Kansas State University. Specific credits to each individual who contributed to or conducted each experiment are summarized in Table 4-1.

Table 4-1: Acknowledgements for collaborative projects in Chapter 4

Collaborators from Kansas State University: Natalia Costa Ball – NCB, Bailey E Maloney – BEM, Emily K Mantlo – EKM, Adrienne E Pohl – AEP, and Alexis D. Schneider – ADS

Collaborator from Centers for Disease Control and Prevention: Claire Y H Huang – CYH

Key Experiments	Relevant Figures and Tables	Collaborators
Modification and recovery of WNV mutants	Tables 4-3, 4-4, 4-5	BEM, AEP, CYH
Characterization of WNV mutants in mouse neuroinvasive disease model	Figures 4-9, 4-10 Tables 4-6, 4-7, 4-8, 4-9	BEM, NCB, AEP, ADS, EKM

4.1 Introduction

The second interdomain movement leading to the conformational change of the E protein from dimer in the virion to trimer inserted in the host cell is the relocation of EDIII towards the gravity center of the E protein. The EDI-EDIII interdomain movement creates the compact metastatically stable E protein post-fusion trimer, making the membrane fusion process an irreversible reaction. The EDI-EDIII interdomain is controlled by the single motif at the domain interface, hence, the name EDI-EDIII linker (Figures 4-1 and 4-2).

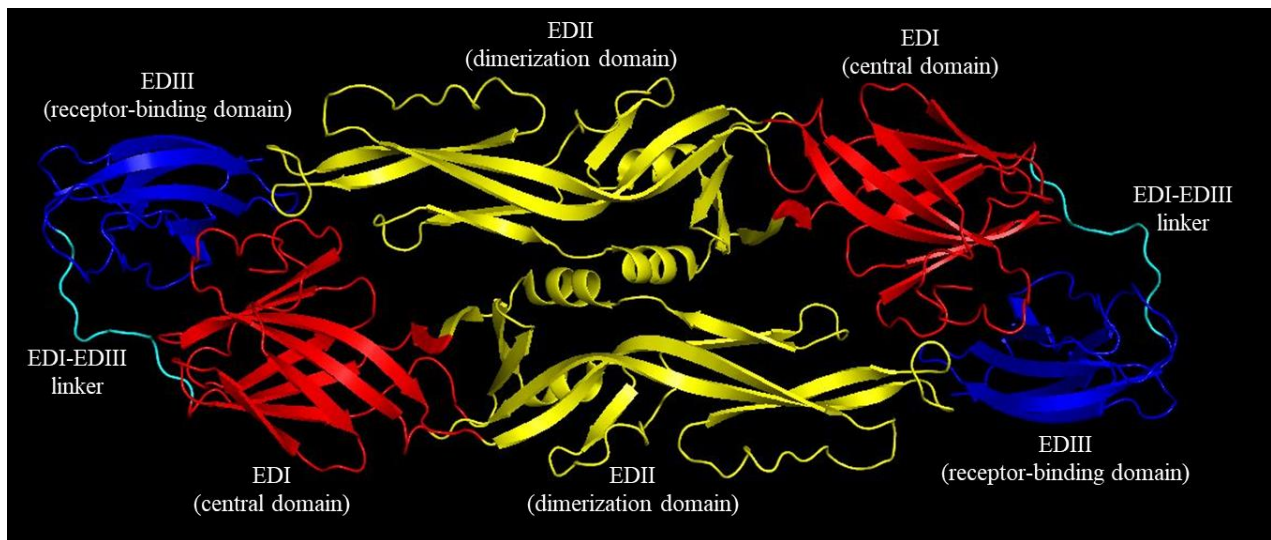


Figure 4-1: The crystal structure of the flavivirus E protein dimer of DENV-2 with EDI-EDIII linker before membrane fusion (PDB ID: 1OAN)

The three domains are red for EDI, yellow for EDII, and blue for EDIII. The EDI-EDIII linker is labeled in cyan. The image was generated in PyMOL.

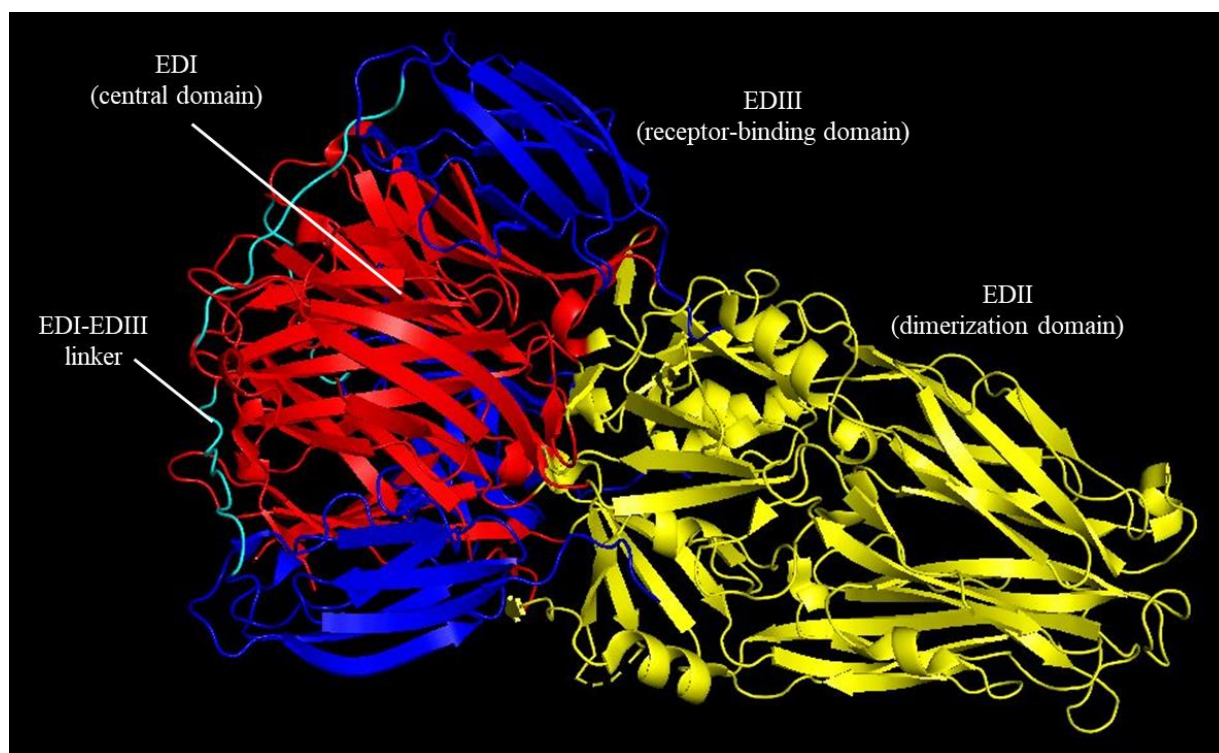


Figure 4-2: The crystal structure of a flavivirus E protein trimer of TBEV with EDI-EDIII linker after membrane fusion (PDB ID: 1URZ)

The three domains are red for EDI, yellow for EDII, and blue for EDIII. The EDI-EDIII linker is labeled in cyan. The only available crystal structure of the flavivirus E protein in the trimer formation is for TBEV. All other crystal structures from PDB are monomers in post-fusion conformation. The image was generated in PyMOL.

A. Location of the EDI-EDIII linker in the E protein

The EDI-EDIII domain interface consists of one single motif that connects the I₀ β -strand of the B₀I₀G₀H₀ β -sheet of EDI to the A β -strand of the ABDE β -sheet of EDIII (Figures 4-3 and 4-4).

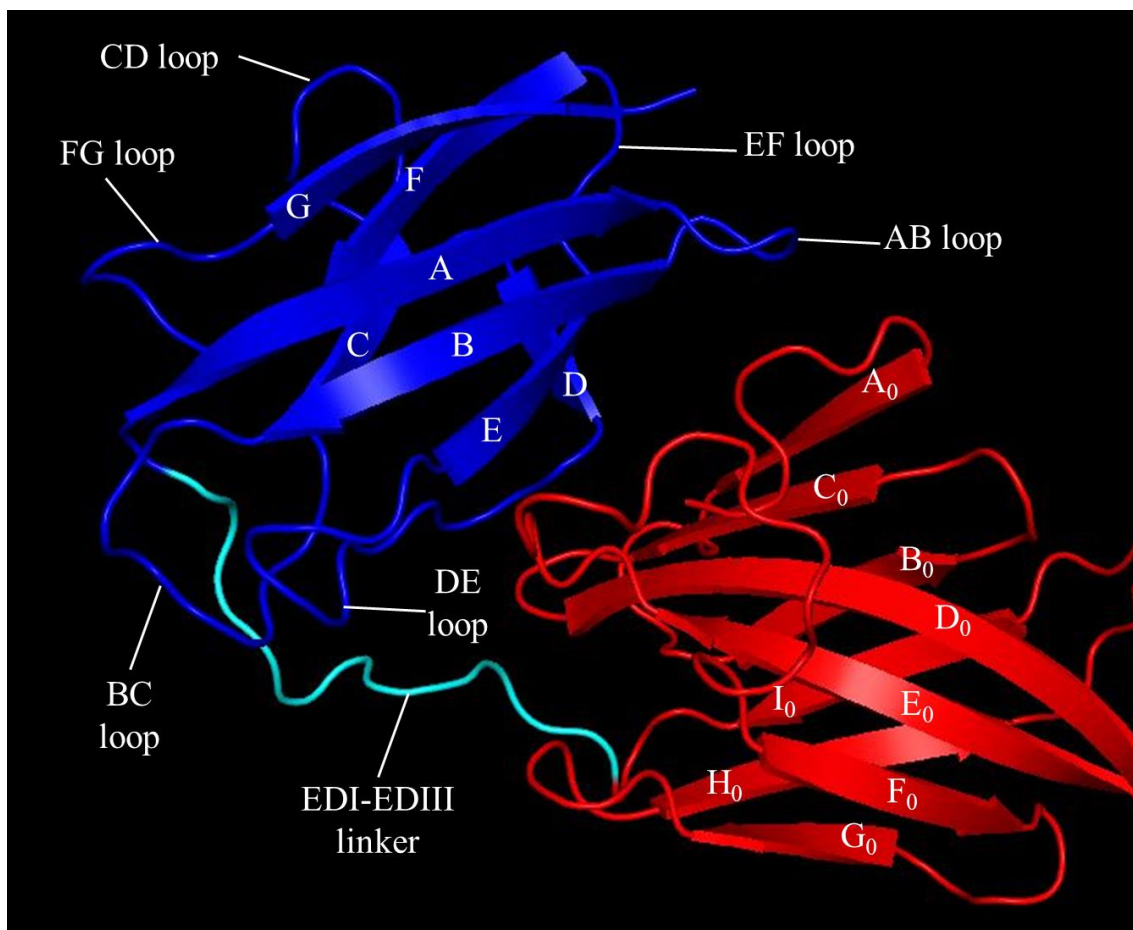


Figure 4-3: Relative location of the pre-fusion DENV-2 EDI-EDIII linker between the $A_0C_0D_0E_0F_0$ and $B_0I_0G_0H_0$ β -sheets of EDI and ABDE and CFG β -sheets of EDIII (PDB ID: 1OAN)

The three domains are red for EDI and blue for EDIII. The EDI-EDIII linker is labeled in cyan. The image was generated in PyMOL.

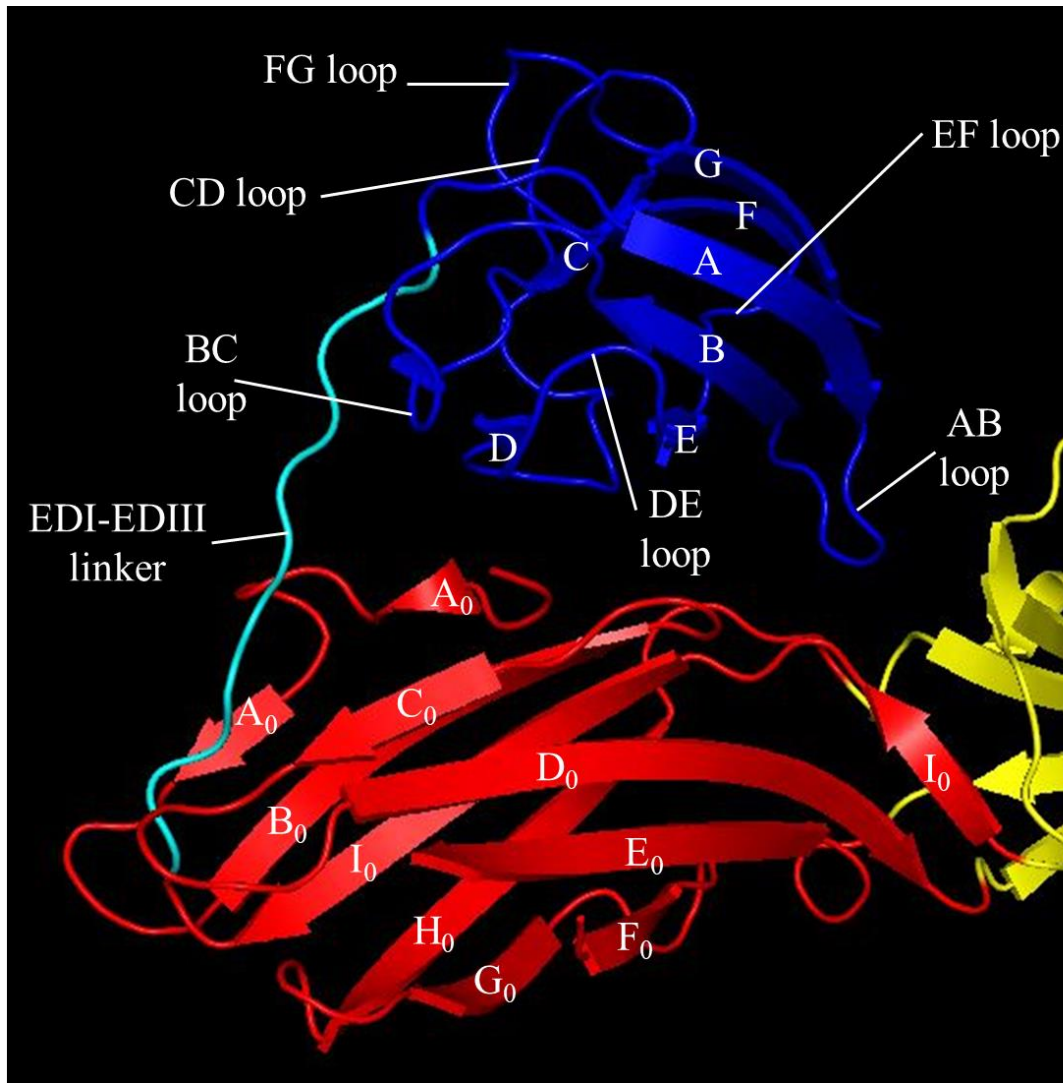


Figure 4-4: Relative location of the post-fusion DENV-2 EDI-EDIII linker between the $A_0C_0D_0E_0F_0$ and $B_0I_0G_0H_0$ β -sheets of EDI and ABDE and CFG β -sheets of EDIII (PDB ID: 1OK8)

The two domains are labeled in red for EDI and blue for EDIII. The EDI-EDIII linker is labeled in cyan. The image was generated in PyMOL.

Comparison of the crystal structure of DENV-2 E protein in prefusion and post-fusion conformations suggests that the dimer-to-trimer transition of the E protein involves a 70° rotation of EDIII towards the original position of EDII. The rotation of EDIII then puts its C terminus EDIII

39 Å closer to the fusion loop in DENV-2 (Modis et al. 2004). The disordered and extended structure of the EDI-EDIII linker presumably provides the flexibility of the EDI-EDIII interdomain movement. The EDI-EDIII linker is crucial for reorganizing the $A_0C_0D_0E_0F_0$ and $B_0I_0G_0H_0$ β -sheets of EDI. The rotation of EDIII inserts the N-terminus of the EDI-EDIII linker between the A_0 and C_0 β -strands of the $A_0C_0D_0E_0F_0$ β -sheet, separating the N-terminus from the C-terminus of the A_0 β -strand. The C-terminal region of the A_0 β -strand becomes part of the $B_0I_0G_0H_0$ β -sheet, thereby creating the compact E protein trimer (Figure 4-5).

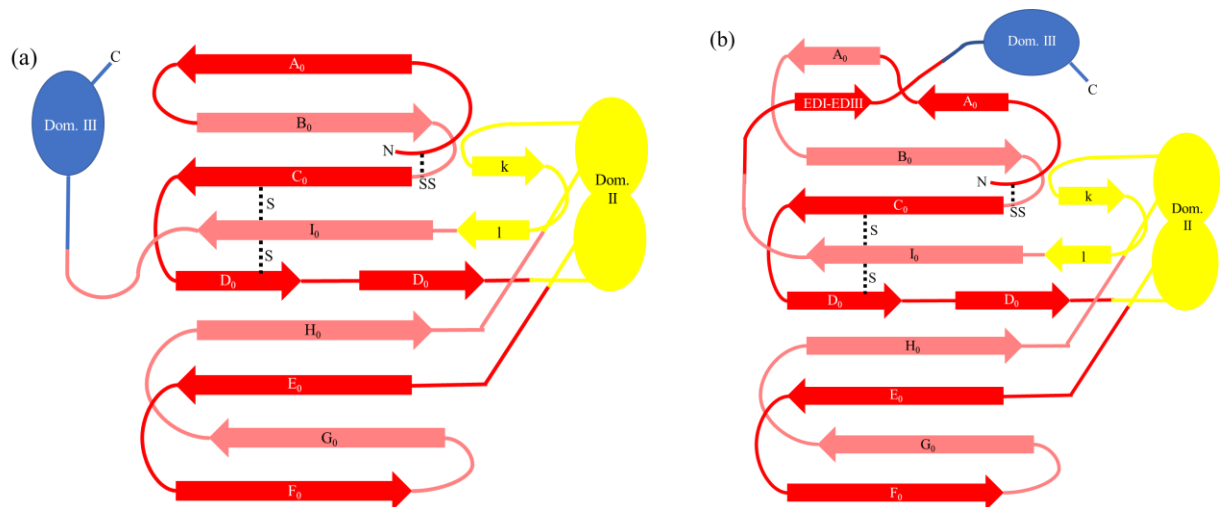


Figure 4-5: Stabilization movement of the EDI-EDIII linker during the formation of the E protein trimeric structure.

Yellow represents EDII, and blue represents EDIII. Red is the A₀C₀D₀E₀F₀ β-sheet, and pink is the B₀I₀G₀H₀ β-sheet of EDI. Black dashed lines are disulfide bonds. (a) Representation of the secondary structure of the E protein before the stabilization movement of the EDIII domain. (b) Secondary structure of the E protein after the EDIII domain's movement towards the E protein's gravity center and the insertion of the EDI-EDIII linker into the β-strands of EDI. This image was modified from an image taken from *Nature*, Volume 427, Y Modis et al., “Structure of the dengue virus envelope protein after membrane fusion.” 313-319., (2004), with permission from Springer Nature.

The EDI-EDIII linker is proximal to the upper lateral surface of EDIII, which consists of BC, DE, and FG loop structures between the ABDE and CFG β-sheets of the β barrel structure. (Hurrelbrink and McMinn 2003, Volk et al. 2009). B. The EDI-EDIII linker is crucial for the infectivity of multiple flaviviruses

B. The EDI-EDIII linker is crucial for the infectivity of multiple flaviviruses

The location of the EDI-EDIII linker in the E protein can be translated for the importance of flavivirus infectivity, as observed with YFV and DENV-2 (de Wispelaere and Yang 2012, Huang 2014). Sanger sequencing demonstrated that the E-M299I mutation in the EDI-EDIII linker is among the twenty consensus amino acid substitutions distinguishing the wild-type parental Asibi strain and the 17D vaccine strain (Hahn et al. 1987). This YFV E-M299 residue is located near the carboxyl terminus of the EDI-EDIII linker, close to EDIII (Hahn et al. 1987). While introducing the E-M299I mutation into the YFV Asibi strain produced infectious viruses, the YFV Asibi (ic) E-M299I mutant showed reduced infection prevalence compared with YFV Asibi (ic) in *Ae. aegypti* mosquitoes, following the engorgement of viremic blood meals at approximately $4 \log_{10} \text{TCID}_{50}/\text{ml}$. Reduced infectivity for orally infected *Ae. aegypti* is part of the attenuated phenotype of the YFV 17D vaccine strain, making the E-M299I mutation an attenuation determinant (Huang 2014).

Three flavivirus residues in the EDI-EDIII linker region have also been analyzed by site-directed mutagenesis performed with the cDNA infectious clone of DENV-2 NGS, including the E-G296, E-S298, and E-Y299 residues (de Wispelaere and Yang 2012). The DENV-2 E-G296 and E-Y299 residues are strictly conserved across flaviviruses. In contrast, the DENV-2 E-S298 residue (or its equivalent residues) is conserved across flaviviruses transmitted by *Aedes* species mosquitoes, and the equivalent residue in the E protein of *Culex* mosquito-borne and tick-borne flaviviruses contains a biochemically conserved threonine that also has a polar side chain (de Wispelaere and Yang 2012). These DENV-2 residues correspond with the WNV residues E-G299, E-T301, and E-Y302. The E-G296A, E-S298A, and E-Y299F mutations are lethal to DENV-2, but the compensatory E-Q400H mutation in the H1 perimembrane helix motif of EDIII can

facilitate the recovery of the E-Y299F mutant. The results serendipitously demonstrated that the E-Y299F mutation has putative importance in virion assembly and maturation because the E protein, a marker for the assembly and maturation of progeny virions, does not migrate past the endoplasmic reticulum of Huh7 hepatocytes transfected with full-length DENV-2 E-Y299F mutant genome. The YFV E-M299I mutant and the DENV-2 E-Y299F mutant showed the structural and functional importance of the EDI-EDIII linker may be broader than anticipated (de Wispelaere and Yang 2012). Nevertheless, how flavivirus-conserved residues in the EDI-EDIII linker contribute to the structure-function of the E protein and can be targeted for the engineering of attenuating mutations remain poorly understood.

C. Site-directed mutagenesis strategy of flavivirus conserved residues in the EDI-EDIII linker

Structural alignment of E protein between ten flaviviruses suggests the WNV E-L295, E-L297, E-K298, E-G299, E-T301, E-Y302, E-V304, and E-C305 residues are representative of the eight flavivirus conserved amino acids in the EDI-EDIII linker (Table 4-2). The I₀ β -strand of EDI precedes the WNV E-L295 residue in the EDI-EDIII linker, and the A β -strand of EDIII follows the WNV E-C305 residue. Most of the residues exhibit little to no sequence plasticity between different flaviviruses. We undertook site-directed mutagenesis of the respective amino acids in the EDI-EDIII linker. Four of the residues (298, 299, 302, and 305 of the WNV E protein) are strictly conserved across all known flaviviruses, including lysine represented by the WNV E-K298 residue, glycine represented by the WNV E-G299 residue, tyrosine represented by the WNV E-Y302 residue, and cysteine represented by the WNV E-C305 residue. It is known that amino acids equivalent to the WNV E-C305 residue form a disulfide bond with the WNV E-C336 residue of

EDIII (Nybakken et al. 2006). The WNV E-L295 residue exhibits highly conserved sequence homology except for one exception, i.e., the SLEV E-V295 residue.

Table 4-2: Amino acid sequence alignment of the EDI-EDIII linker between flaviviruses

Highly conserved amino acids are labeled with bold text and underlined. The properties of amino acids are shown in green (hydrophobic), pink (polar), red (negatively charged), blue (positively charged), and black (unique). The numbering of amino acids in the motif is based on the WNV NY99 sequence.

Serocomplex	Virus	Strain	Sequence of EDI-EDIII linker (E 295-304)
JEV	WNV	NY99	<u>L</u> <u>Q</u> <u>L</u> <u>K</u> <u>G</u> <u>T</u> <u>T</u> <u>Y</u> <u>G</u> <u>V</u> <u>C</u>
	JEV	SA14	<u>L</u> <u>A</u> <u>L</u> <u>K</u> <u>G</u> <u>T</u> <u>T</u> <u>Y</u> <u>G</u> <u>M</u> <u>C</u>
	JEV	SA14-14-2	<u>L</u> <u>A</u> <u>L</u> <u>K</u> <u>G</u> <u>T</u> <u>T</u> <u>Y</u> <u>G</u> <u>M</u> <u>C</u>
	KNV	MRM61C	<u>L</u> <u>Q</u> <u>L</u> <u>K</u> <u>G</u> <u>T</u> <u>T</u> <u>Y</u> <u>G</u> <u>V</u> <u>C</u>
	SLEV	MS1-7	<u>V</u> <u>K</u> <u>I</u> <u>K</u> <u>G</u> <u>T</u> <u>T</u> <u>Y</u> <u>G</u> <u>M</u> <u>C</u>
YFV	YFV	Asibi	<u>L</u> <u>T</u> <u>L</u> <u>K</u> <u>G</u> <u>T</u> <u>S</u> <u>Y</u> <u>K</u> <u>M</u> <u>C</u>
	YFV	17D	<u>L</u> <u>T</u> <u>L</u> <u>K</u> <u>G</u> <u>T</u> <u>S</u> <u>Y</u> <u>K</u> <u>I</u> <u>C</u>
DENV	DENV-1	Hawaii	<u>L</u> <u>T</u> <u>L</u> <u>K</u> <u>G</u> <u>M</u> <u>S</u> <u>Y</u> <u>V</u> <u>M</u> <u>C</u>
	DENV-2	New Guinea C	<u>L</u> <u>Q</u> <u>L</u> <u>K</u> <u>G</u> <u>M</u> <u>S</u> <u>Y</u> <u>S</u> <u>M</u> <u>C</u>
	DENV-3	H87	<u>L</u> <u>E</u> <u>L</u> <u>K</u> <u>G</u> <u>M</u> <u>S</u> <u>Y</u> <u>A</u> <u>M</u> <u>C</u>
	DENV-4	H241	<u>L</u> <u>R</u> <u>I</u> <u>K</u> <u>G</u> <u>M</u> <u>S</u> <u>Y</u> <u>T</u> <u>M</u> <u>C</u>
TBEV	TBEV	Neudoerfl	<u>L</u> <u>K</u> <u>M</u> <u>K</u> <u>G</u> <u>L</u> <u>T</u> <u>Y</u> <u>T</u> <u>M</u> <u>C</u>
	OHFV	Bogluvovska	<u>L</u> <u>K</u> <u>M</u> <u>K</u> <u>G</u> <u>L</u> <u>T</u> <u>Y</u> <u>T</u> <u>M</u> <u>C</u>

Other residues in the EDI-EDIII linker are representative of serocomplex-specific consensus residues. The WNV E-L297 residue and its equivalent residues exhibit hydrophobic biochemical properties, including methionine within the TBEV serocomplex, leucine within the YFV serocomplex, and leucine or isoleucine within the JEV and DENV serocomplexes. As noted

with DENV-2 E-S298 residue above, the equivalent WNV E-T301 residue contains a polar amino acid in the equivalent position of the E protein, including serine within the YFV and DENV serocomplexes and threonine within the JEV and TBEV serocomplexes. Finally, the equivalent amino acids for the WNV E-V304 residue show the conserved hydrophobic biochemical properties, including methionine within the DENV and TBEV serocomplexes, methionine or valine within the JEV serocomplex, and methionine or isoleucine within the YFV serocomplex. Additionally, the WNV E-V304 residue is equivalent to the wild-type YFV M299 residue. Therefore, the alternative amino acid substitutions of the E-V304 residue can also provide a comparison of the structure-function of the E protein and the attenuating effect between viscerotropic and neurotropic flaviviruses.

Each of the eight flavivirus-conserved residues was substituted with an alanine to eliminate the contribution of the original amino acid side chain as much as possible. The E-G299 residue was also changed to a proline as an alternative strategy in case the alanine substitution was insufficient to produce a phenotypic change (Figure 4-6).

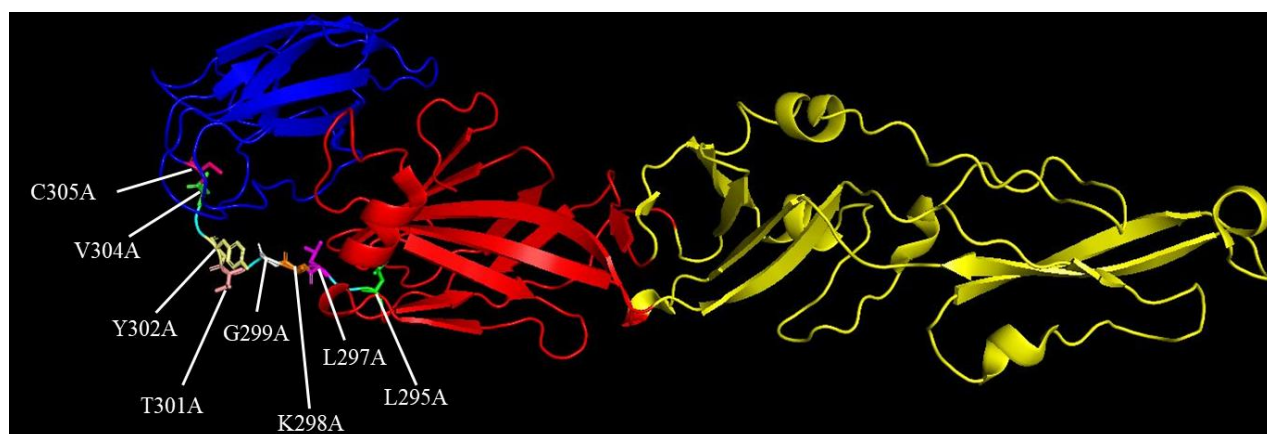


Figure 4-6: Locations of the eight flavivirus conserved hydrophobic residues in the EDI-EDIII linker of the WNV E protein monomer

Each location of the eight flavivirus conserved residues in the EDI-EDIII linker was based on the pre-fusion conformations of the WNV E protein, and each residue is depicted with a different color (PDB ID:2HG0). The E protein monomer is colored red (EDI), yellow (EDII), blue (EDIII), and cyan (EDI-EDII hinge region). The image was generated in PyMOL.

4.2 Results

A. Recovery and infectivity of WNV EDI-EDIII mutants

Each of the eight flavivirus-conserved residues in the EDI-EDIII linker was substituted with alanine to test the importance of the original amino acid of each residue. The E-L295A, E-L297A, E-K298A, E-G299A, E-G299P, E-T301A, E-Y302A, E-V304A, and E-C305A mutants were each rescued via transfection of Vero cells (Table 4-3).

Table 4-3: Phenotype of WNV mutants encoding single alanine substitutions in the EDI-EDIII linker

The WNV NY99ic plasmids were obtained from Dr. Claire Huang's laboratory. Modifications and recovery of WNV EDI-EDIII linker mutants were conducted by Bailey Maloney.

Location	Mutation	Viability	Compensatory Mutations
Motif #1	L295A	Viable	None detected
	L297A	Viable	Partial reversion
	K298A	Lethal	n/a
	G299A	Viable	Reversion
	G299P	Lethal	n/a
	T301A	Lethal	n/a
	Y302A	Lethal	n/a
	V304A	Viable	None detected
	C305A	Lethal	n/a

Alanine substituting the E-K298A, E-G299P, E-T301A, E-Y302A, and E-C305A residues was not tolerated and produced lethal phenotypes. The supernatant of Vero cells transfected with the RNA genome encoding the E-L297A mutation contained a mixed population of mutant and wild-type WNV NY99ic. The E-L297A mutation involved mutations in the first and second codons, i.e., C1855G and U1856C nucleotide substitutions. One of the two independent transfections developed a partial reversion to the wild-type sequence, involving the transition at the second nucleotide from C to U at genome position 1856. The resulting GTG codon encodes valine and was detected by one of the two Sanger sequencing repeats, suggesting a mixed population of the E-L297A and E-L297V mutants. WNV NY99ic did not tolerate the E-G299A mutation. One of the two independent transfection experiments failed to produce viable viruses,

and the other rescue attempt resulted in a reversion to the wild-type sequence and an additional compensatory mutation in the EDI-EDIII linker. The reversion to the wild-type sequence of C to G occurred at genome position 1862, the second nucleotide of the codon, causing the reversion from alanine back to glycine. The compensatory mutation was two individual nucleotide mutations, U1849G and U1850C, resulting in a GCG codon encoding alanine at the E-L295 residue. The E-L295A and E-V304A mutants each showed to retain the engineered amino acid substitution in all of the biological replicates (n=2). The E-L295A mutation in the EDI-EDIII linker showed a 26-fold reduction (average titer: $7.1\log_{10}\text{PFU/ml} \pm 0.5$) in the infectivity compared to the WNV NY99ic ($p=0.0201$). The E-V304A mutation resembled the infectivity of the wild-type NY99ic virus, with its titers exceeding $8\log_{10}\text{PFU/ml}$. We then prioritized the engineering of 19 alternative amino acid substitutions of the E-L295 and E-V304 residues, each tolerating alanine substitution.

B. Optimization of the infectivity of the WNV E-L295 and E-V304 mutants

We hypothesized that 1) WNV NY99ic will tolerate alternative amino acid substitutions of the E-L295 and E-V304 residues, and 2) at least one amino acid substitution of the respective residues will attenuate the mouse neuroinvasive phenotype of WNV NY99ic. Each mutant of the E-L295 and E-V304 residues was rescued from electroporated Vero cells four days after electroporation. Three phenotypes were observed after mutations, including reductions in infectivity, a lethal phenotype, and reversion back to the wild-type NY99ic sequence (Tables 4-4 and 4-5).

Table 4-4: Viability and stability of WNV E-L295 mutants

The WNV NY99ic plasmids were obtained from Dr. Claire Huang's laboratory. Modifications and recovery of WNV E-L295 mutants were conducted by Bailey Maloney and Adrienne Pohl.

Location	Mutation	Viability	Compensatory Mutations
Motif #1	L295C	Lethal	n/a
	L295D	Lethal	n/a
	L295E	Lethal	n/a
	L295F	Viable	None detected
	L295G	Lethal	n/a
	L295H	Lethal	n/a
	L295I	Viable	None detected
	L295K	Lethal	n/a
	L295M	Viable	None detected
	L295N	Viable	Mutated
	L295P	Lethal	n/a
	L295Q	Lethal	n/a
	L295R	Lethal	n/a
	L295S	Viable	None detected
	L295T	Viable	None detected
	L295V	Viable	None detected
	L295W	Lethal	n/a
	L295Y	Lethal	n/a

Table 4-5: Viability and stability of WNV E-V304 mutants

The WNV NY99ic plasmids were obtained from Dr. Claire Huang's laboratory. Modifications and recovery of WNV E-V304 mutants were conducted by Bailey Maloney and Adrienne Pohl.

Location	Mutation	Viability	Compensatory Mutations
Motif #1	V304C	Viable	None detected
	V304D	Lethal	n/a
	V304E	Viable	None detected
	V304F	Viable	None detected
	V304G	Viable	None detected
	V304H	Viable	None detected
	V304I	Viable	None detected
	V304K	Viable	None detected
	V304L	Viable	None detected
	V304M	Viable	None detected
	V304N	Viable	None detected
	V304P	Viable	None detected
	V304Q	Viable	None detected
	V304R	Viable	None detected
	V304S	Viable	None detected
	V304T	Viable	None detected
	V304W	Lethal	n/a
	V304Y	Viable	None detected

Amino acid substitutions that conserve the hydrophobic biochemical property of the E-L295 residue are, in general, tolerated by WNV NY99ic, as observed with the E-L295F, E-L295I, E-L295M, and E-L295V mutants that retained the engineered nucleotide changes in the consensus sequence (Figure 4-7). Amino acid substitutions with large polar side chains are lethal or unstable,

as observed with the lethal phenotype of the E-L295C and E-L295Q mutants and the E-L295N mutant that reverted to the wild-type sequence. The E-L295N mutation required the nucleotide substitutions of all three nucleotides of the codon: U1849A, U1850A, and G1851C. However, transfection of the E-L295N mutant into Vero cells resulted in a reversion of the third nucleotide mutation to the wild-type sequence, resulting in an amino acid change from asparagine to serine. This reversion differs from the E-L295S and E-L295T mutations, which are retained in the consensus sequence and have no demonstrable impact on the infectivity of WNV NY99ic. Most of the amino acid substitutions containing charged, unique, or aromatic side chains of the E-L295 residues are not tolerated by WNV NY99ic, giving rise to the lethal phenotype of the E-L295D, E-L295E, E-L295G, E-L295H, E-L295K, E-L295P, E-L295R, E-L295W, and E-L295Y mutants.

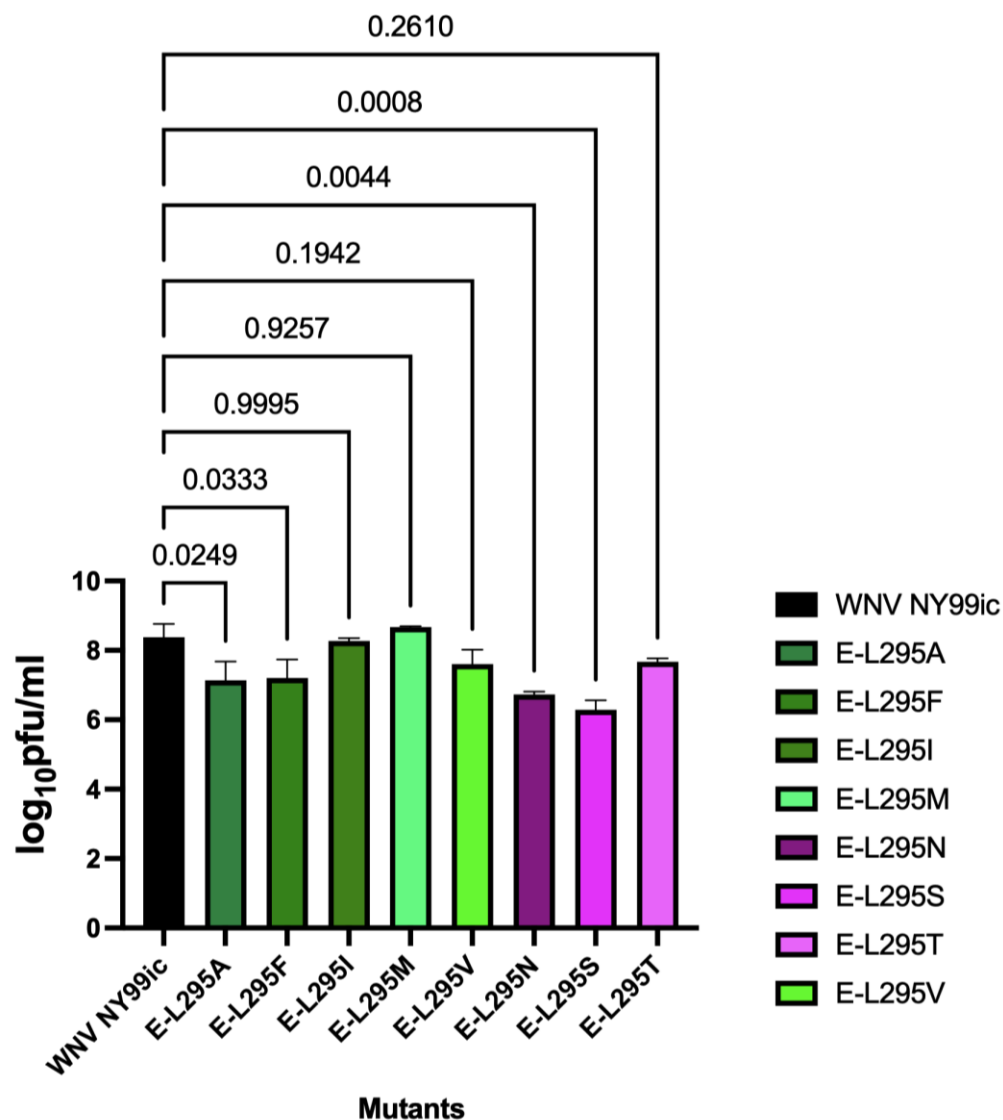


Figure 4-7: Infectivity of WNV E-L295 mutants

Infectivity of WNV NY99ic and two replicates of the viable E-L295 single mutants following transfection of Vero cells. Infectivity between WNV NY99ic and each mutant was compared using one-way ANOVA followed by Dunnett's pairwise comparison. The image was generated in GraphPad. Recovery of mutant viruses and plaque assays were conducted by Bailey Maloney.

The E-L295F and E-L295S mutants showed significantly reduced titers following the transfection of Vero cells. The mutation E-L295F showed a 12-fold reduction in infectivity

(average titer: $7.2 \log_{10}\text{PFU/ml} \pm 0.5$, $p=0.0333$) compared to WNV NY99ic. Further, the E-L295S mutation reduced the infectivity of WNV NY99ic by 125-fold (average titer: $6.29 \log_{10}\text{PFU/ml} \pm 0.27$, $p=0.0008$). Although the E-L295N mutant exhibited reduced infectivity ($p=0.0044$), the mutant was not considered for further evaluation due to the instability of the sequence after Sanger sequencing revealed compensatory mutations that changed the residue from asparagine to serine.

At least 16 alternative amino acid substitutions of the E-V304 residue were well tolerated. The E-V304C, E-V304E, E-V304F, E-V304G, E-V304H, E-V304I, E-V304K, E-V304L, E-V304M, E-V304P, E-V304Q, E-V304R, E-V304S, E-V304T, and E-V304Y mutants not only retained the engineered nucleotide changes but also showed no demonstrable difference in infectivity in comparison with WNV NY99ic. The E-V304N mutation was also stable in the consensus sequence but caused a 4-fold reduction in infectivity (average titer: $7.54 \log_{10}\text{PFU/ml} \pm 0.18$, $p=0.001$) in comparison with WNV NY99ic (Figure 4-8). The single amino acid mutations E-V304D and E-V304W showed to be lethal to WNV NY99ic virions. None of the biological replicates of the E-V304D and E-V304W mutations produced viable viruses.

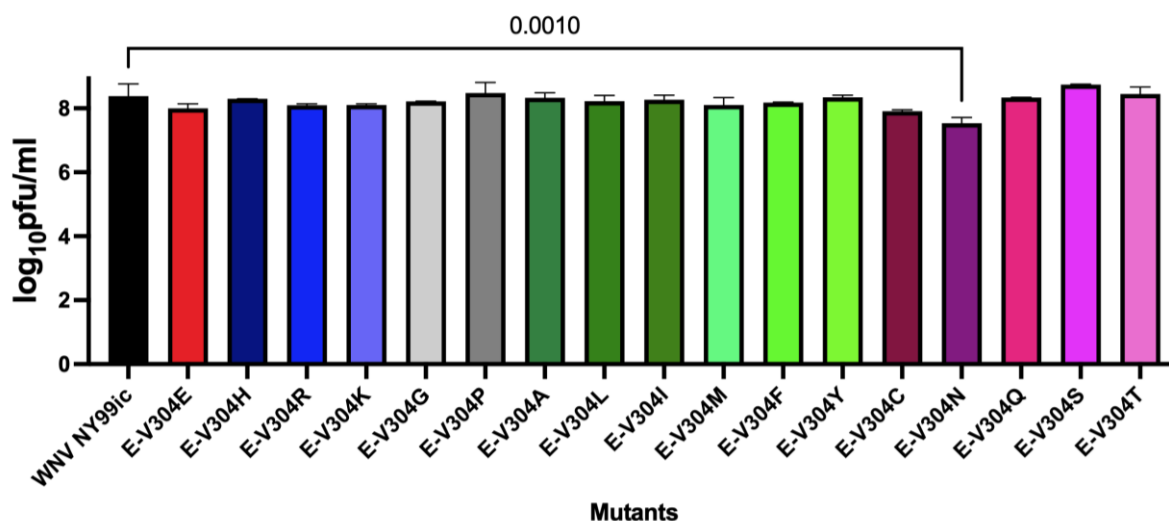


Figure 4-8: Infectivity of WNV E-V304 mutants

Infectivity of WNV NY99ic and two replicates of the viable E-V304 single mutants, following transfection of Vero cells. Infectivity between WNV NY99ic and each mutant was compared using one-way ANOVA followed by Dunnett's pairwise comparison. The image was generated in GraphPad. Recovery of mutant viruses and plaque assays were conducted by Bailey Maloney.

C. Attenuation of the mouse neuroinvasive phenotype of WNV by E-L295 and E-V304 mutant

Groups of five male and five female outbred Swiss mice were each challenged with 500 PFU of the E-L295M, E-L295T, E-L295V, E-L295A, E-L295F, E-L295S, E-V304N mutants, and WNV NY99ic via the intraperitoneal route to determine the neuroinvasive phenotype of the respective mutants (Figure 4-9 and 4-10). We tested seven mutants in two separate studies due to the limited number of cages that can be housed simultaneously. The first experiment examined the neuroinvasive phenotype of the E-L295M, L-295V, E-L295T, E-L295A, and E-L295S mutants, and the second experiment determined the neuroinvasive phenotype of the E-L295F and E-V304N mutants. Significantly, the E-L295S mutant was fully attenuated, i.e., no mortality, at 500 PFU.

The E-L295A mutant showed the second highest level of attenuating effect and caused 20% mortality. The E-L295V and E-L295T mutants caused 70% mortality and are partially attenuated. There is no demonstrable difference in the neuroinvasive phenotype of the E-L295M mutant and WNV NY99ic, resulting in uniform lethality by 12 dpi.

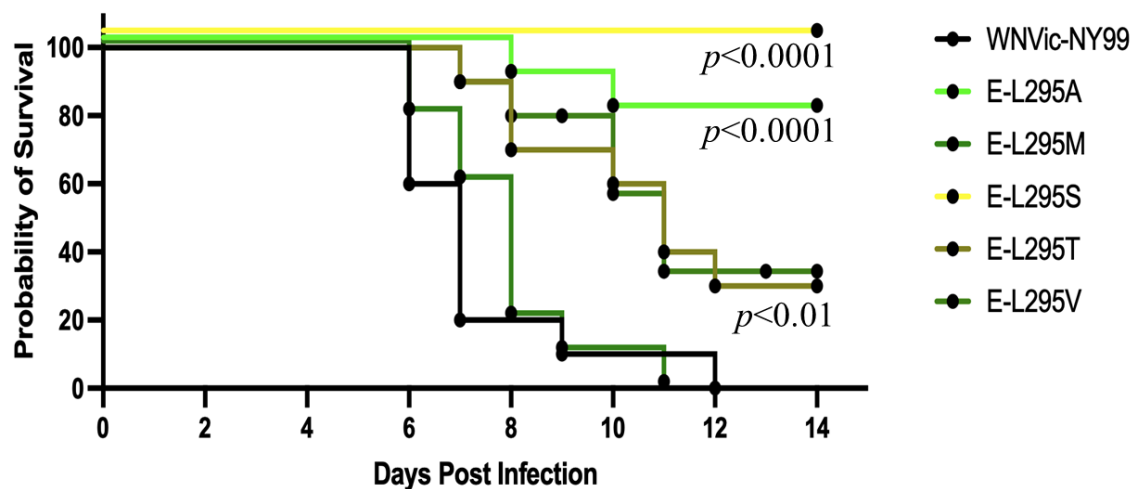


Figure 4-9: Attenuation of the neuroinvasive phenotype of WNV E-L295A, E-L295M, E-L295S, E-L295T, and E-L295V mutants in four-week-old mice

Groups of five male and five female mice received the IP challenge with each WNV mutant or WNV NY99ic at 500 PFU. The Kaplan-Meier survival curve showed differences in survival outcomes and average survival time, and Dunnett's pairwise comparison followed one-way ANOVA. The image was generated in GraphPad. The mouse neuroinvasive disease study was conducted by Bailey Maloney, Natalia Costa Ball, Adrienne Pohl, Alexis Schneider, and Emily Mantlo.

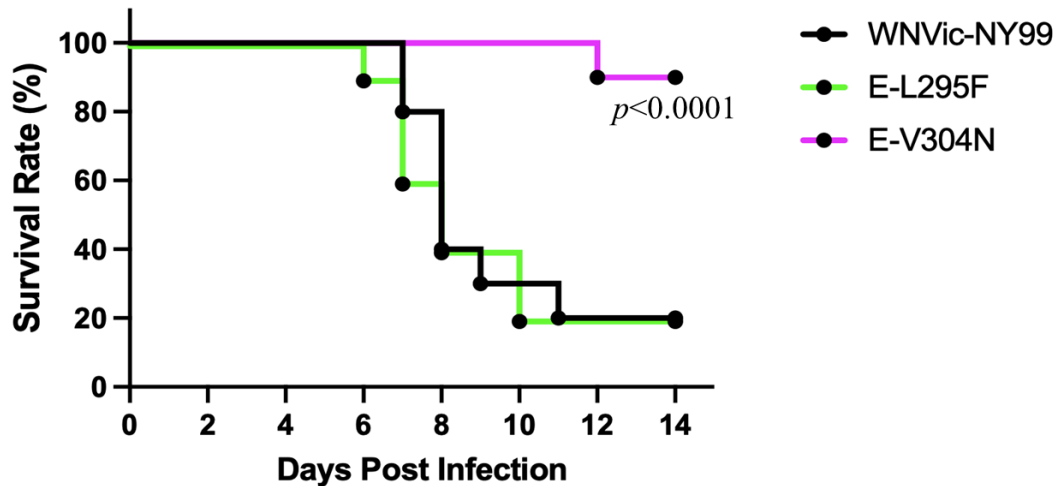


Figure 4-10: Attenuation of the neuroinvasive phenotype of WNV E-L295F and E-V304N mutants in four-week-old mice

Groups of five male and five female mice received the IP challenge with each WNV mutant or WNV NY99ic at 500 PFU. The Kaplan-Meier survival curve showed differences in survival outcomes and average survival time, and Dunnett's pairwise comparison followed one-way ANOVA. The image was generated in GraphPad. The mouse neuroinvasive disease study was conducted by Bailey Maloney, Natalia Costa Ball, Adrienne Pohl, Alexis Schneider, and Emily Mantlo.

Infection with the wild-type WNV NY99ic led to lethality between 6 and 8 dpi. During the first study, uniform lethality was observed, although two female mice showed delayed lethality at 9 and 11 dpi. In the second study, uniform lethality was not observed as one male and one female mouse survived until the study's end. Although the second experiment did not produce uniform lethality in the WNV NY99ic group, we must remember that the mice used are outbred mice and therefore do not always produce the same levels of lethality. While 100% lethality is optimal, the study showed a high mortality rate of 80%.

Significantly, all mice challenged with the E-L295S mutant survived to the endpoint of this study ($p<0.0001$). The average survival time of the E-L295A, E-L295T, and E-L295V mutants was significantly extended (E-L295A: 13.0 days, $p<0.0001$, E-L295T: 10.7 days, $p<0.01$, E-L295V: 10.5 days, $p<0.01$) (Table 4-6). These three E-L295 mutations also showed to be partially attenuated, but only the E-L295A mutant showed to be highly attenuated (E-L295A: 20% mortality, E-L295T: 70% mortality, and E-L295V: 80% mortality). The E- E-L295F and E-L295M mutants showed no significant difference in survival times. The single E-V304 mutant, E-V304N, showed to have a significantly extended survival time of 13.7 days ($p<0.001$) (Table 4-7). Despite this, the E-V304N mutant only exhibited a 10% mortality rate. These data indicate that the E-V304 residue is highly tolerant to change and less likely than other EDI-EDIII linker residues to produce an attenuating mutation in other flaviviruses.

Table 4-6: Average survival times of mice infected with WNV E-L295A, E-L295M, E-L295S, E-L295T, and E-L295V mutants

This table details the average survival time post-infection for each group of inoculated mice and the standard deviation seen within the respective group. The average survival time of each group was calculated using 14 days as the end point of the study. The mouse neuroinvasive disease study was conducted by Bailey Maloney, Natalia Costa Ball, Adrienne Pohl, Alexis Schneider, and Emily Mantlo.

Group	Average Survival Time (days)	Standard Deviation
WNV NY99 (wt)	7.0	1.8
L295A	13.0	2.2
L295M	7.6	1.3
L295S	>14	n/a ³
L295T	10.7	2.7
L295V	10.5	2.4

³ No endpoint data was retrieved in this mouse group so no standard deviation could be calculated.

Table 4-7: Average survival times of mice infected with WNV E-L295F and E-V304N mutants

This table details the average survival time post-infection for each group of inoculated mice and the standard deviation seen within the respective group. The average survival time of each group was calculated using 14 days as the end point of the study. The mouse neuroinvasive disease study was conducted by Bailey Maloney, Natalia Costa Ball, Adrienne Pohl, Alexis Schneider, and Emily Mantlo.

Group	Average Survival Time (days)	Standard Deviation
WNV NY99 (wt)	8.4	3.1
L295F	9.2	2.8
V304N	13.7	0.9

In the first study, weight loss of mice receiving WNV NY99ic began at 5 to 6 dpi (Tables 4-8 and 4-9). In contrast, no signs of weight loss were observed in the mice challenged with the E-L295S mutant. Most mice receiving the E-L295A mutant experienced transient weight loss between 7 and 10 dpi before experiencing weight gain and until the end of the study. The two euthanized male mice exhibited greater than 20% weight loss at 8 and 10 dpi. The mice challenged with E-L295M, E-L295T, and E-L295V showed no clear pattern in their weight changes between different mice of each group. On average, the weight loss progression in mice challenged with the L295M mutant tended to resemble those challenged with WNV NY99ic. In contrast, mice that survived the challenge with the E-L295T and E-L295V mutants tended to experience transient weight loss between 6 and 11 dpi.

In the second study, the weight loss of mice receiving WNV NY99ic began at 4 to 5 dpi. The E-L295F-challenged mice initially showed steady weights before experiencing a weight decrease of 7 dpi. The two female mice that survived showed recovery of weight by 10 dpi. The

mice challenged with E-V304N experienced a mixture of weight patterns, although on average, the mice experienced a fairly steady weight with little increase or decrease.

Table 4-8: Individual weights for mice infected with WNV E-L295A, E-L295M, E-L295S, E-L295T, and E-L295V mutants

The weight of each mouse was measured in grams daily. The starting weight, the peak weight, and the final weight are depicted. “M” represents the male mice, and the notation “F” represents the female mice. Grey coloring represents a mouse that was euthanized or found dead prior to the end of the study, and white represents a mouse that survived to 14 dpi. The mouse neuroinvasive disease study was conducted by Bailey Maloney, Natalia Costa Ball, Adrienne Pohl, Alexis Schneider, and Emily Mantlo.

Group	Mouse (Male)	Starting Weight (g)	Peak Weight (g)	Final Weight (g)	Mouse (Female)	Starting Weight (g)	Peak Weight (g)	Final Weight (g)
WNV NY99 (wt)	1M	31.8	32.6	29.0	1F	25.4	25.7	21.4
	2M	25.0	25.5	21.2	2F	24.0	26.8	22.2
	3M	29.5	30.8	27.7	3F	25.8	26.9	25.4
	4M	27.3	27.6	26.8	4F	26.3	26.5	23.6
	5M	29.4	30.6	30.1	5F	26.5	27.1	26.2
L295A	1M	28.3	32.8	32.4	1F	24.8	25.8	25.6
	2M	24.9	26.8	25.6	2F	26.1	31.1	31.1
	3M	27.9	29.1	28.2	3F	25.6	28.7	28.4
	4M	25.5	29.7	29.7	4F	24.3	27.8	27.8
	5M	27.3	28.4	25.7	5F	26.6	30.4	30.4
L295M	1M	28.3	32.2	31.3	1F	26.1	26.1	24.7
	2M	26.9	28.7	25.0	2F	23.6	25.0	20.4
	3M	28.4	30.5	22.1	3F	23.7	25.2	23.7
	4M	28.6	31.2	27.0	4F	25.0	27.6	26.3

Group	Mouse (Male)	Starting Weight (g)	Peak Weight (g)	Final Weight (g)	Mouse (Female)	Starting Weight (g)	Peak Weight (g)	Final Weight (g)
L295M	5M	28.9	31.4	29.6	5F	23.5	24.8	22.2
L295S	1M	30.5	35.8	35.8	1F	24.8	28.4	28.4
	2M	29.6	31.7	31.7	2F	25.9	28.5	28.5
	3M	28.4	31.2	31.2	3F	25.8	30.6	30.6
	4M	31.5	37.5	37.5	4F	28.5	31.1	31.1
	5M	30.2	33.4	33.4	5F	25.6	35.4	35.4
L295T	1M	27.7	30.5	28.0	1F	24.4	26.1	23.8
	2M	29.5	31.8	25.1	2F	23.7	25.4	25.4
	3M	27.0	30.2	26.7	3F	24.4	25.2	20.2
	4M	31.1	32.7	23.3	4F	26.7	28.0	23.0
	5M	29.5	31.0	25.9	5F	24.3	25.9	25.6
L295V	1M	23.8	27.1	20.6	1F	23.0	24.9	20.5
	2M	25.9	30.9	30.9	2F	26.1	27.4	22.9
	3M	26.2	28.5	19.8	3F	23.3	26.4	22.1
	4M	28.2	31.3	30.3	4F	23.6	26.0	23.3
	5M	29.2	33.3	30.5	5F	23.5	25.2	19.8

Table 4-9: Individual weights for mice infected with E-L295F and E-V304N mutants

The weight of each mouse was measured in grams daily. The starting weight, the peak weight, and the final weight are depicted. “M” represents the male mice, and the notation “F” represents the female mice. Grey coloring represents a mouse that was euthanized or found dead prior to the end of the study, and white represents a mouse that survived to 14 dpi. The mouse neuroinvasive disease study was conducted by Bailey Maloney, Natalia Costa Ball, Adrienne Pohl, Alexis Schneider, and Emily Mantlo.

Group	Mouse (Male)	Starting Weight (g)	Peak Weight (g)	Final Weight (g)	Mouse (Female)	Starting Weight (g)	Peak Weight (g)	Final Weight (g)
WNV NY99 (wt)	1M	29.3	30.2	25.3	1F	31.2	32.1	29.5
	2M	30.1	31.8	24.3	2F	30.7	22.5	17.9
	3M	29.3	32.8	32.5	3F	21.1	22.9	20.1
	4M	30.3	30.3	28.9	4F	25.5	25.6	24.1
	5M	282.7	30.0	26.9	5F	27.1	28.1	24.5
L295F	1M	26.3	27.1	25.9	1F	24.8	26.3	26.3
	2M	29.1	30.7	24.8	2F	25.4	26.6	24.1
	3M	29.4	32.3	25.0	3F	28.5	28.5	26.6
	4M	29.7	30.5	28.5	4F	25.5	26.8	26.8
	5M	32.2	34.0	31.9	5F	25.5	26.0	25.3
V304N	1M	29.2	31.8	31.8	1F	28.3	28.3	26.5
	2M	26.2	28.8	28.8	2F	25.1	27.6	27.6
	3M	25.5	27.2	25.5	3F	26.3	30.8	30.8
	4M	31.0	32.9	28.0	4F	25.3	25.7	19.7
	5M	25.4	26.1	25.1	5F	25.2	28.0	28.0

The fully attenuated E-L295S mutant did not cause any apparent signs of disease during the study. We only observed signs of neurological disease in male mice challenged with the E-

L295A mutant. Symptoms began to resolve in the three mice that survived the challenge at 10 to 11 dpi, while the two that succumbed to the disease experienced rapid progression to neuroinvasive symptoms at 9 to 10 dpi. The onset of neurological disease in mice receiving the E-L295M, E-L295T, and E-L295F mutants all began at 6 to 7 dpi, resembling the kinetics observed in the WNV NY99 control group. However, most of the mice surviving the challenge with their respective mutants either partially or fully recovered from signs of neurological disease by the end of the study. The E-V304N mutation exhibited an onset of symptoms around 7 to 8 dpi. All male mice exhibited mild symptoms until they resolved on 11 dpi. The female mice only exhibited mild symptoms until the morning of 9 dpi. After that, the female mice showed no symptoms except for one female that was euthanized on 12 dpi. The single female showed no symptoms and, on the morning of 12 dpi, showed neurological symptoms and had to be euthanized.

4.3 Discussion

In this study, we investigated the role of eight flavivirus-conserved residues in the EDI-EDIII linker in the structure-function of the E protein. Alanine screening showed the E-L295 and E-V304 residues can tolerate at least one amino acid substitution. We then examined 19 alternative amino acid substitutions of the respective residues and selected E-L295A, E-L295F, E-L295M, E-L295S, E-L295T, L295V, and E-V304N mutants to evaluate the attenuation of the mouse neuroinvasive phenotype of WNV NY99ic. For the first time, the previously assumed structural-functional importance of the EDI-EDIII linker is linked to the mouse neuroinvasive phenotype of at least one flavivirus. Our results outlined the strategy for engineering up to two attenuating mutations in the EDI-EDIII linker.

As a proof-of-concept, we use the WNV E-L295S mutant to prove at least one flavivirus conserved residue in the EDI-EDIII linker can support the engineering of attenuating mutation and

potentially the rational design of candidate LAVs. Not only can the E-L295S mutant fully attenuate the mouse neuroinvasive phenotype of WNV NY99ic up to 500 PFU, but the E-L295 residue also tolerates multiple amino acid substitutions. It stands out from four other strictly or highly conserved amino acids in the EDI-EDIII linker, including the E-L297, E-K298, E-G299, and E-Y302 residues. In contrast, the E-G299 residue exhibits little sequence plasticity because the E-G299A mutation, which only differs by one methyl group from glycine, is lethal to WNV NY99ic. Similarly, the lethal phenotype of the E-Y302A mutant can reflect the little tolerance of amino acid mutation of the flavivirus conserved E-Y302 residue. Although the E-V304N mutation did not fully attenuate the mouse neuroinvasive phenotype of WNV NY99ic, the results are encouraging and warrant further evaluation of other WNV E-V304 mutants that are viable and retain the engineered amino acid substitutions. Importantly, amino acids equivalent to the WNV E-V304 residue tolerate sequencing variation in different flaviviruses, and the YFV E-M299I mutations are among the 20 amino acid substitutions that distinguish the wild-type Asibi strain and the attenuated 17D strain (Hahn et al. 1987, Huang 2014). The lethal phenotype of the E-C305A mutant proves the disulfide bond between the EDI-EDIII linker and the *bc* loop of EDIII is necessary for the EDI-EDIII interdomain movement and is essential for the structure-function of the E protein.

A. Attenuation of the WNV mouse neuroinvasive phenotype by the E-L295 mutants

Of the two residues found to be stable and viable in the WNV EDI-EDIII linker, we anticipate mutations of the E-L295 residue were the most likely to have a more profound effect on the structure and function of the E protein than the E-V304 residue. Unlike the E-V304 residue, the E-L295 residue is strictly conserved across all flaviviruses. The E-L295S mutation can attenuate the mouse neuroinvasive phenotype of WNV NY99ic in full. Furthermore, the

structurally similar E-L295A mutation, which only differs by one hydroxyl group from the E-L295S mutations, also exhibited a high attenuating effect. Therefore, investigation of the immunogenicity of the WNV E-L295S and E-L295A mutants and the attenuating effect of equivalent mutations in other flaviviruses will prove the utility for vaccine development. This shows the phenotypic change caused by mutations of the flavivirus conserved residues in the EDI-EDIII linker are comparable between WNV and DENV-2 as an alanine screening of neighboring residues in the EDI-EDIII linker of DENV-2 showed to be determinants of infectivity (de Wispelaere and Yang 2012). Therefore, the EDI-EDIII linker is crucial for the structure and function of the flavivirus E protein.

The high attenuating effects of the E-L295A and E-L295S mutations indicate that removing a large hydrophobic amino acid and replacing it with a small amino acid will disrupt the structure and function of the E protein. The serine mutation reduces the side chain size from 168 Å³ to 99 Å³, decreasing the hydrophobicity index from 97 to -5. In contrast, the alanine mutation reduces the size of the amino acid to 92 Å³. Presumably, it has a less significant impact on the structure-function of the E protein as its hydrophobicity index is 41. In contrast, substitutions of the E-L295 residue with large hydrophobic amino acids showed no demonstrable impact on the mouse neuroinvasive of WNV NY99ic, as mice challenged with the E-L295F, E-L295M, and E-L295V mutants showed no significant difference from mice challenged with WNV NY99ic in mortality rate and average survival time. Indeed, this may explain 1) the limited attenuating impact of the E-L295T mutation and 2) the reversion of one of the three nucleotide changes encoding the E-L295N mutant to the wild-type sequence, resulting in a serine. While serine and threonine resemble each other in the overall sidechain structure, i.e., a methyl group linked to a hydroxyl or thiol group, threonine has a larger (122 Å³) and more hydrophobic sidechain (hydrophobic index:

13). The large hydrophilic sidechain makes the E-L295N mutation unstable. This hypothesis is further supported by the lethal phenotype of other E-L295 mutants involved in large hydrophobic amino acid substitutions, including the E-L295C, E-L295D, E-L295E, E-L295H, E-L295K, E-L295Q, and E-L295R mutants.

B. Attenuation of the WNV mouse neuroinvasive phenotype by the E-V304 mutants

Alanine screening of the eight flavivirus-conserved residues in the EDI-EDIII linker also identified the E-V304 residue as a putative target for the engineering of attenuating mutations. Amino acids equivalent to the E-V304 residue exhibit the hydrophobic biochemical property across all flaviviruses, although the specific amino acid is not fully conserved across the different serocomplexes.

We demonstrated that 17 different amino acid substitutions of the E-V304 residue support the recovery of mutants retaining the engineered mutations, but only the E-V304N mutant advanced to the mouse neuroinvasive phenotype study, as infectivity of the other 16 E-V304 mutants was indistinguishable from WNV NY99ic. This sequence plasticity can be explained by the proximity of the E-V304 residue near the upper lateral ridge of EDIII, where mutations are often tolerated. Consistent with the highly attenuating effect of the E-V304N mutation, the structurally similar E-V304D mutation is lethal to WNV NY99ic. Likely, the carboxamide sidechain introduced by the E-V304N mutation and the carboxymethyl sidechain introduced by the E-V304D mutation both can have a detrimental impact on the EDI-EDIII interdomain movement.

Although the E-V304 residue has shown to be highly tolerant, the residue is not completely tolerant and resulted in two lethal phenotypes and one highly attenuated phenotype. The one attenuated phenotype, E-V304N, was likely the only attenuating mutation due to its structural

similarities to aspartic acid. The E-V304 mutation was one of the two amino acid substitutions that produced a lethal phenotype, and it has an almost identical structure to asparagine. The only difference is asparagine has a carboxyl amide group at the end of the amino acid side chain instead of the carboxyl group that aspartic acid has. The virus was more tolerant to the E-V304N mutation because it is a more neutral amino acid, whereas the E-V304D mutation is a negatively charged, acidic amino acid. This indicates that the specific structural configuration of the aspartic acid and asparagine amino acid sidechain may interfere with the EDI-EDIII interdomain movement. Overall, the E-V304 residue is a more tolerant but less ideal residue than E-L295 for the engineering of attenuating mutations. Additionally, due to the high tolerance for change, the E-V304 residue is less crucial to the structure and function of the EDI-EDIII linker than the E-L295 residue.

The E-L295S and E-V304N mutations have attenuating effects on the WNV neuroinvasive phenotype. Similar to the EDI-EDII hinge region results in Chapter 3, two attenuating mutations indicate there is a potential of having multiple attenuating mutations in the E protein. Future studies could be used to evaluate the other flavivirus-conserved residues in the EDI-EDIII linker using alternative amino acid substitutions to identify other attenuating mutations.

Chapter 5 - Demonstration of Attenuating Effects by YFV Mutants in the EDI-EDII Hinge Region

The results presented in this chapter involved multiple collaborative projects. Modifications of the YFV infectious clone and analysis of the EDI-EDII hinge region mutants *in vitro* were performed with the assistance of Ashley Bilyeu and Danielle Saunders in Dr. Vanlandingham's laboratory at Kansas State University. The *in vivo* mouse study of the eight YFV mutations tested in Section 5.2.D was performed by the scientists at Bioqual Inc. Specific credits to each individual who contributed to or conducted each experiment are summarized in Table 5.1.

Table 5-1: Acknowledgements for collaborative projects in Chapter 5

Collaborators from Kansas State University: Ashley N Bilyeu – ANB, Bailey E Maloney – BEM, Danielle R D Saunders – DRS

Collaborators from Bioqual Inc.: Hanne Anderson – HA, Brian Backstedt – BB, and Swagata Kar – SK

Key Experiments	Relevant Figures and Tables	Collaborators
Modification and recovery of YFV mutants	Table 5-4	BEM, ANB
Characterization of YFV mutants in cell culture	Figures 5-3, 5-4, 5-5, 5-6	BEM, DRS
Characterization of YFV mutants in mouse neuroinvasive disease model	Figure 5-7 Tables 5-5, 5-6	HA, BB, SK

5.1 Introduction

As a proof-of-concept, we used WNV NY99ic as a model system to investigate the attenuating effect caused by mutations of the eight flavivirus-conserved residues in the EDI-EDII hinge region. The E-A54I and E-Y201P mutations were each shown to fully attenuate the mouse neuroinvasive phenotype of WNV NY99ic, suggesting that at least the first and third motifs of the EDI-EDII hinge region are worthwhile investigating for the engineering of attenuating mutations that can be broadly effective across different flaviviruses. As such, we examined the attenuating mutations of the equivalent flavivirus conserved residues in the EDI-EDII hinge region of YFV Asibi (ic) to investigate the translation of the attenuated phenotype between neurotropic and viscerotropic flaviviruses, based on the conserved structure-function of the E protein involved in the membrane fusion process.

A. Yellow fever virus represents the ancestral mosquito-borne flavivirus

In addition to representing viscerotropic flaviviruses, YFV represents an ancestral virus in the evolution of mosquito-borne flaviviruses. The most common hypothesis for the origin of YFV is the virus emerged in Africa approximately 740 years ago (95% highest probability density between 304 and 1320 years ago) (Bryant et al. 2007). Therefore, the emergence of YFV potentially preceded the emergence of the oldest clade 5 (India) of WNV, which was estimated to take place around 95% highest probability density between 304 and 1320 years ago (May et al. 2011). Therefore, examination of the structure-function of the E protein between WNV and YFV provides the comparison of two phylogenetically distant mosquito-borne flaviviruses that emerged in different geographical locations and timeframes in evolution.

B. EDI-EDII hinge region is among the conserved motifs within the E protein

The amino acid sequence of the E protein exhibits varying degrees of sequence homology across different flaviviruses. There are several evolutionarily conserved motifs in the E protein. The structural alignment showed most of the flavivirus conserved sequences are related to the structure-function of the E protein involved in the membrane fusion process, including the fusion loop, the four motifs of the EDI-EDII hinge region, and the EDI-EDIII linker (Gritsun et al. 1995, Medits et al. 2020) Investigating the structural and functional importance of the EDI-EDII hinge region in YFV can provide the basis for the understanding of other flaviviruses, especially tick-borne flaviviruses (Figure 5-1).

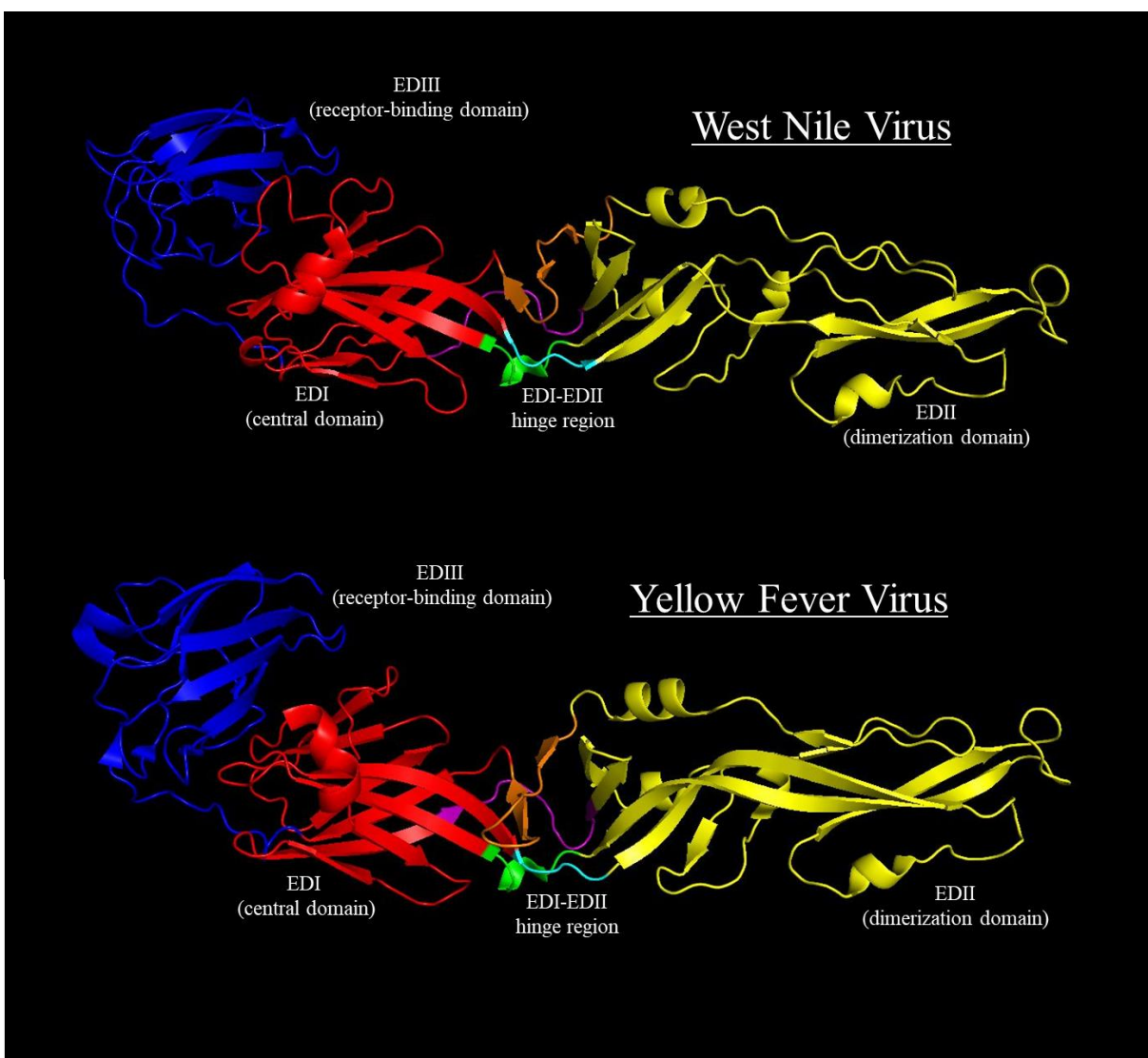


Figure 5-1: Comparison between WNV and YFV E protein monomer

WNV (top image) is derived from PDB structure 2HG0, and YFV (bottom image) is derived from PDB structure 6IW5. The E protein monomer is colored in red (EDI), yellow (EDII), and blue (EDIII). The EDI-EDII hinge region is colored cyan (1st motif), green (2nd motif), purple (3rd motif), and orange (4th motif). The image was generated in PyMOL.

We engineered nine single-site mutations within the conserved hydrophobic residues of the EDI-EDII hinge region of YFV, with eight of the residues being equivalent to the WNV residues tested in Chapter 3 and one additional mutation residue unique to YFV (Table 5-2 and 5-3). We

employed site-directed mutagenesis targeting hydrophobic amino acids across four different motifs of the EDI-EDII hinge region of YFV. The YFV E-A54S mutation was engineered in the first motif of the EDI-EDII hinge region, in comparison with the WNV E-A54S mutation, which produced a partially attenuated phenotype in the outbred Swiss mouse neuroinvasive disease model. This includes the two WNV residues that produced attenuated phenotypes in mice, the WNV E-A54S mutant equivalent to the YFV E-A54S mutant and the WNV E-Y201P mutant being equivalent to the YFV E-Y194P mutant. The other mutants in the 1st to 3rd motifs of EDI-EDII were E-I130N, E-I135N, and E-I196N in WNV. Only the WNV E-I130N residue has an identical amino acid substitution in YFV. The other two residues retain a hydrophobic amino acid but with different amino acids. WNV includes isoleucine at residues 135 and 196, but the equivalent residues in YFV are valines (YFV E-V130 and E-V188 residues). The mutation strategy from Chapter 3 of mutating the original conserved hydrophobic amino acid to a less hydrophobic but similarly structured amino acid was maintained in YFV. The YFV residues E-V130 and E-V188 could not be changed to asparagine. Therefore, the YFV equivalent residues were mutated to threonine, similar to the WNV E-V272T mutants from the 4th motif.

Table 5-2: Comparison of the sequence of the 1st through 3rd motifs of the EDI-EDII hinge region between WNV and YFV

Highly conserved hydrophobic amino acids are labeled with bold text and underlined. The properties of amino acids are shown in green (hydrophobic), pink (polar), red (negatively charged), blue (positively charged), and black (unique). The numbering of amino acids in the motif is based on the YFV Asibi sequence.

Serocomplex	Virus	Strain	Sequence of 1 st Motif (E 51-54)	Sequence of 2 nd Motif (E 130-135)	Sequence of 3 rd Motif (E 183-194)
JEV	WNV	NY99	AN <u>L</u> <u>A</u>	<u>I</u> <u>L</u> <u>K</u> <u>E</u> <u>N</u> <u>I</u>	E <u>P</u> <u>R</u> <u>S</u> <u>G</u> <u>I</u> <u>D</u> <u>T</u> <u>N</u> <u>A</u> - <u>Y</u>
YFV	YFV	Asibi	D <u>G</u> <u>P</u> <u>A</u>	<u>V</u> <u>D</u> <u>Q</u> <u>T</u> <u>K</u> <u>I</u>	<u>Q</u> <u>V</u> <u>Q</u> <u>T</u> <u>A</u> <u>V</u> <u>D</u> <u>F</u> <u>G</u> <u>N</u> <u>S</u> <u>Y</u>

The 4th motif of the YFV EDI-EDII hinge region has high sequence and structure diversity, which is seen particularly clearly in YFV (Table 5-3). The 4th motif of YFV is three amino acids longer than WNV, between YFV E-D270 and YFV E-N272. However, despite the increased length, the distribution of conserved hydrophobic amino acids remains consistent between flaviviruses, with clusters at both the amino and carboxyl termini. In WNV, three conserved residues were tested in the 4th motif. The WNV E-A269S, E-V272T, and E-L281N mutants correspond with the YFV equivalent mutants E-A261S, E-V264T, and E-L276N. Due to the increased length of the 4th motif of YFV, one additional hydrophobic residue was tested. The YFV E-L273 residue is a hydrophobic residue that is only conserved between the different strains of YFV, with the other serocomplexes utilizing a wide variety of amino acids, including polar and basic amino acids. The YFV E-L273N mutant resides in the kl hairpin at the amino terminus of the k β -strand (Figure 5-2). Due to its location, we decided to test the E-L273 residue to determine if the hydrophobic residue has any significance to the hydrophobic pocket of the YFV E protein.

Table 5-3: Comparison of the sequence of the 4th motifs of the EDI-EDII hinge region between WNV and YFV

Highly conserved hydrophobic amino acids are labeled with bold text and underlined across all flaviviruses. An additional residue in the YFV sequences near the C-terminus end of the motif is bold and underlined to represent the YFV-specific hydrophobic amino acid. The properties of amino acids are shown in green (hydrophobic), pink (polar), red (negatively charged), blue (positively charged), and black (unique). The numbering of amino acids in the motif is based on the YFV Asibi sequence.

Serocomplex	Virus	Strain	Sequence of 4 th Motif (E 261-276)
JEV	WNV	NY99	<u>A</u> <u>I</u> P <u>V</u> <u>E</u> FSSN---TV <u>K</u> <u>L</u>
	JEV	SA14	<u>A</u> <u>I</u> <u>V</u> <u>V</u> <u>E</u> YSS----SV <u>K</u> <u>L</u>
	JEV	SA14-14-2	<u>A</u> <u>I</u> <u>V</u> <u>V</u> <u>E</u> YSS----SV <u>M</u> <u>L</u>
	KNV	MRM61C	<u>A</u> <u>I</u> P <u>V</u> <u>E</u> FSSN---TV <u>K</u> <u>L</u>
	SLEV	MS1-7	<u>A</u> <u>I</u> P <u>A</u> <u>T</u> VSSS---TL <u>T</u> <u>L</u>
YFV	YFV	Asibi	<u>A</u> <u>M</u> <u>R</u> <u>V</u> <u>T</u> <u>K</u> <u>D</u> TNDNN <u>L</u> <u>Y</u> <u>K</u> <u>L</u>
	YFV	17D	<u>A</u> <u>M</u> <u>R</u> <u>V</u> <u>T</u> <u>K</u> <u>D</u> TNDNN <u>L</u> <u>Y</u> <u>K</u> <u>L</u>
DENV	DENV-1	Hawaii	<u>A</u> <u>T</u> <u>E</u> <u>I</u> <u>Q</u> TSG----TT <u>T</u> <u>I</u>
	DENV-2	New Guinea C	<u>A</u> <u>T</u> <u>E</u> <u>I</u> <u>Q</u> MSS----GN <u>L</u> <u>L</u>
	DENV-3	H87	<u>A</u> <u>T</u> <u>E</u> <u>I</u> <u>Q</u> TSG----GT <u>S</u> <u>I</u>
	DENV-4	H241	<u>A</u> <u>T</u> <u>E</u> <u>V</u> <u>D</u> SGD----GN <u>H</u> <u>M</u>
TBEV	TBEV	Neudoerfl	<u>V</u> PV <u>A</u> <u>H</u> <u>I</u> EG---TK <u>Y</u> <u>H</u> <u>L</u>
	OHFV	Bogluvska	<u>A</u> PL <u>A</u> <u>H</u> <u>I</u> EG---TK <u>Y</u> <u>H</u> <u>L</u>

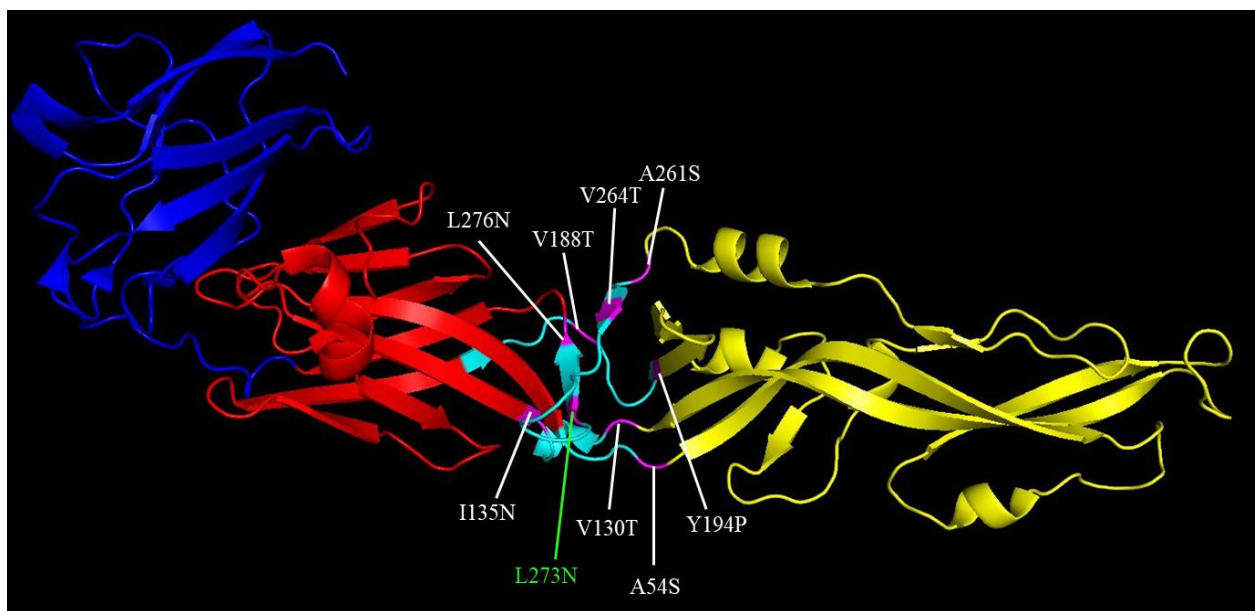


Figure 5-2: Locations of the flavivirus conserved hydrophobic residues in the EDI-EDII hinge region of the YFV E protein monomer

Each location of the conserved residues in the EDI-EDII hinge region is based on the pre-fusion conformations of the YFV E protein shown in magenta (PDB ID: 6IW5). The E protein monomer is colored red (EDI), yellow (EDII), blue (EDIII), and cyan (EDI-EDII hinge region). Mutants labeled in white are equivalent mutations tested in WNV in Chapter 3. The single mutant labeled in green represents an additional YFV-specific hydrophobic residue in the 4th motif. The image was generated in PyMOL.

In Chapter 3, two flavivirus-conserved hydrophobic residues of the EDI-EDII hinge region of WNV were shown to be crucial for the structure-function of the E protein. Such knowledge was applied to engineering two mutations that fully attenuated the neuroinvasive phenotype of WNV NY99ic in outbred Swiss mice. We employed the same site-directed mutagenesis strategy to engineer mutations in the EDI-EDII hinge region of YFV Asibi (ic). Furthermore, similarities and differences in the phenotype of single mutants in the equivalent locations will provide detailed

knowledge of the flavivirus conserved structure-function of the E protein involved in the membrane fusion process.

5.2 Results

A. Recovery and infectivity of YFV mutants

The eight flavivirus conserved hydrophobic residues in the EDI-EDII hinge region of the wild-type YFV Asibi strain was each substituted with an amino acid that has a structurally similar side chain but exhibits higher polarity, including the E-A54S, E-V130T, E-I135N, E-V188T, E-Y194P, E-A261S, E-V264T, and E-L276N mutants. We have also mutated the hydrophobic E-L273 residue in the EDI-EDII hinge region to asparagine to determine whether or not this YFV-specific hydrophobic amino acid is crucial for the structure-function of the E protein. Transfection of Vero cells with RNA transcripts encoding eight mutants produced viable viruses in the culture supernatant at 7 dpi except for the E-I135N mutant (Table 5-4).

Table 5-4: Viability and stability of YFV EDI-EDII mutants

The modification and recovery of the following YFV EDI-EDII mutants was conducted by Bailey Maloney and Ashley Bilyeu.

Location	Mutation	Viability	Compensatory Mutations
Motif #1	A54S	Viable	None detected
Motif #2	V130T	Viable	n/a
	I135N	Lethal	n/a
Motif #3	V188T	Viable	None detected
	Y194P	Viable	n/a
Motif #4	A261S	Viable	None detected
	V264T	Viable	None detected
	L273N	Viable	None detected
	L276N	Viable	Reversion

Although the E-V130T and E-Y194P mutants were viable, they were only detectable by indirect immunofluorescent staining. Neither mutant formed visible plaques detected by the MTT staining and, hence, was excluded from further analysis. Sanger sequencing demonstrated that the E-L276N mutation encoded by AAT nucleotide changes reverted to the wild-type sequence in the second codon, resulting in the ATT codon that gives rise to the E-L276I mutation. This reversion suggests that the presence of a hydrophobic amino acid at the E-L276 residue is very crucial for the structure and function of the E protein, resembling the E-L281N mutation in the equivalent position of WNV E protein, as seen in section 3.2.A. The E-A54S, E-V188T, E-A261S, E-V264T, and E-L273N mutations were tolerated by YFV Asibi (ic), as observed with the retention of the engineered nucleotide substitutions in the consensus sequence and comparable infectivity (Figure 5-3).

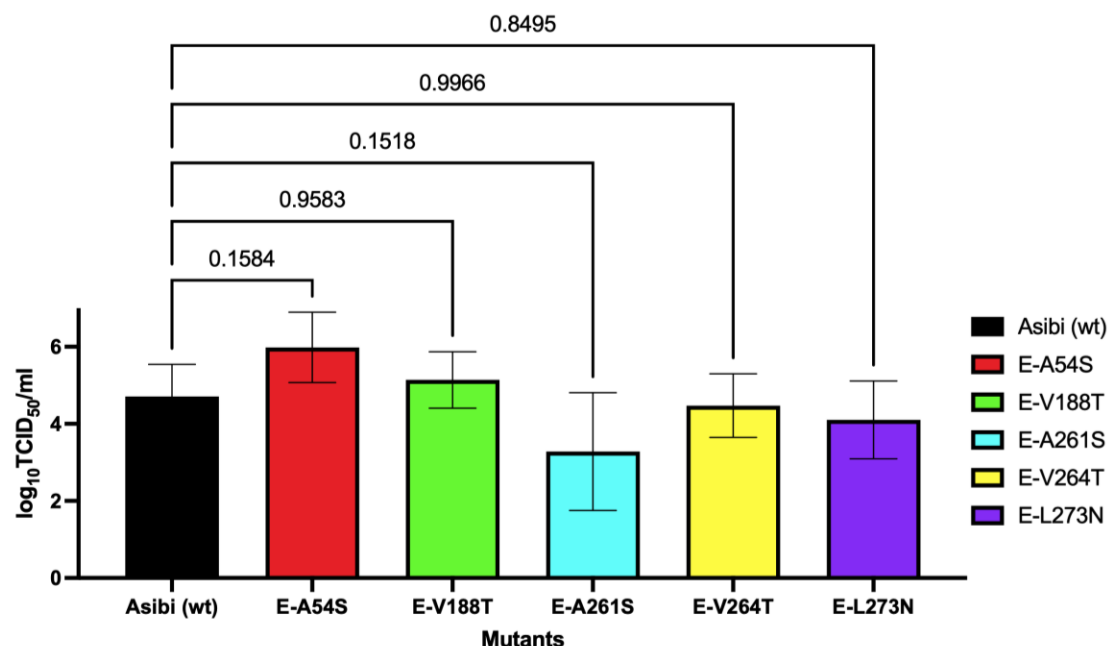


Figure 5-3: Infectivity of YFV EDI-EDII hinge region mutants

Infectivity of YFV Asibi infectious clone and the six single mutants following transfection of Vero cells. Infectivity between YFV Asibi infectious clone and each mutant was compared using one-way ANOVA followed by Dunnett's pairwise comparison. The image was generated in GraphPad. Recovery of mutant viruses and plaque assays was conducted by Bailey Maloney and Danielle Saunders.

All five viable YFV mutations showed no significantly reduced titers compared to the wild-type virus TCID₅₀. The three mutations E-V188T ($5.1\log_{10}\text{TCID}_{50}/\text{mL} \pm 0.7$), E-V264T ($4.5\log_{10}\text{TCID}_{50}/\text{mL} \pm 0.8$), and E-L273N ($4.1\log_{10}\text{TCID}_{50}/\text{mL} \pm 1.0$) showed to have infectivity levels similar to the wild-type which grows to $4.8\log_{10}\text{TCID}_{50}/\text{mL}$. The E-A54S mutation was shown to visibly increase infectivity to $6.0\log_{10}\text{TCID}_{50}/\text{mL} \pm 0.9$ compared to the wild-type, although it was not significant. The only mutation that showed visible reductions in infectivity was the E-A261S mutation, although it was not significant ($p=0.1518$). The E-A261S mutation only grew to over $3.3\log_{10}\text{TCID}_{50}/\text{mL} \pm 1.5$.

B. Reduction in multiplication kinetics of YFV mutants in Vero and BHK-21 cells

We hypothesized that mutations that disrupt the structure-function of the E protein would impair the multiplication kinetics of YFV Asibi (ic). To test this hypothesis, each single mutant was analyzed by growth curve experiments undertaken in Vero cells for five days and BHK-21 cells for seven days (Figures 5-4 and 5-5).

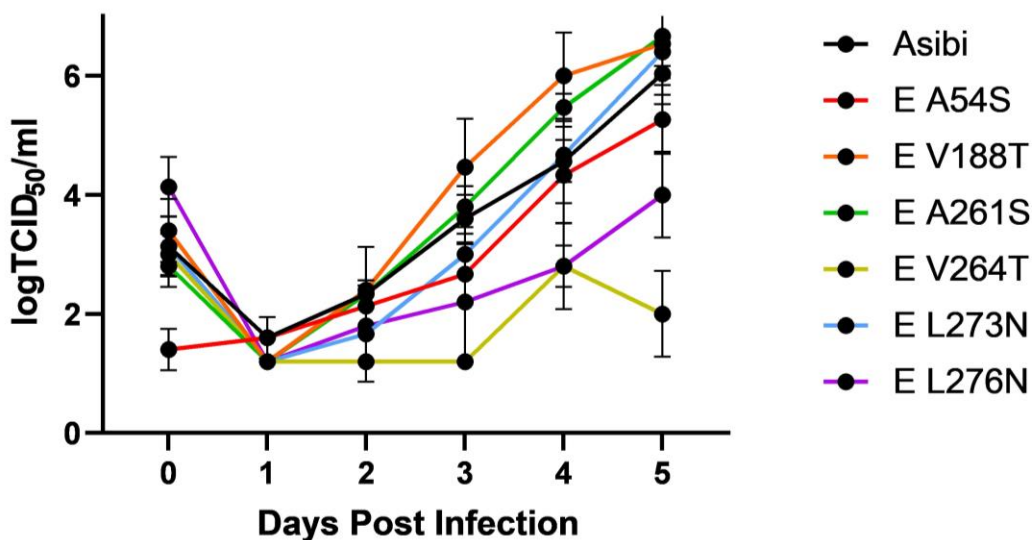


Figure 5-4: Multiplication kinetics of YFV Asibi (ic) and mutants containing single amino acid substitutions of flavivirus conserved hydrophobic residues in the EDI-EDII hinge region in Vero cells

YFV Asibi (ic) and the six single mutants were each inoculated into Vero cells at moi of 0.1 in triplicate. Data points represent the mean viral titer, and error bars represent the standard deviation. The image was generated in GraphPad. Growth curves was conducted by Bailey Maloney and Danielle Saunders.

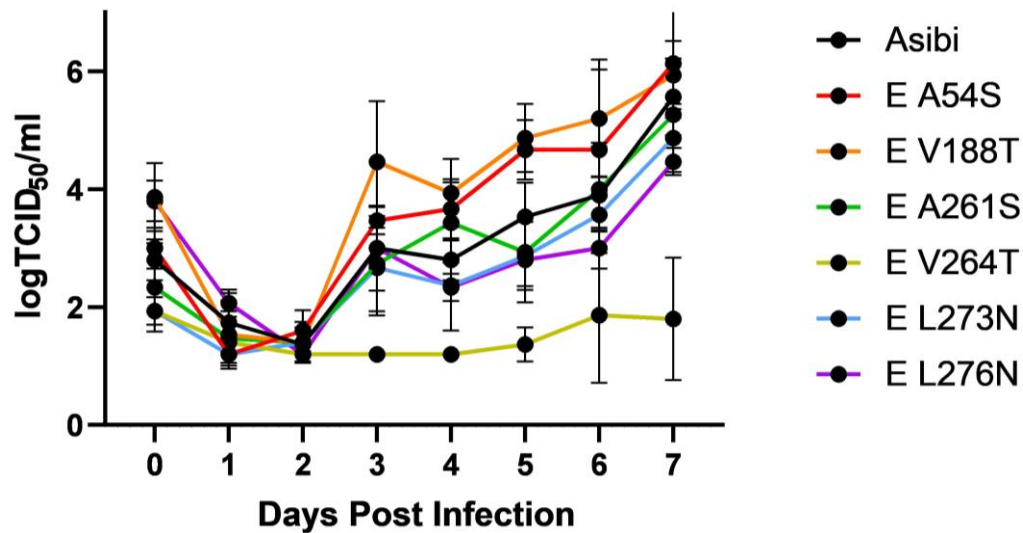


Figure 5-5: Growth curves of YFV mutants over 5 days in BHK-21 cells

YFV Asibi (ic) and the six single mutants were each inoculated into BHK-21 cells at moi of 0.1 in triplicate. Data points represent the mean viral titer, and error bars represent the standard deviation of three biological replicates. The image was generated in GraphPad. Growth curves were performed by Bailey Maloney and Danielle Saunders.

The multiplication kinetics of the E-V264T mutant in Vero and BHK-21 cells showed reduced infectivity compared to YFV Asibi after 3 dpi in Vero cells and 4 dpi in BHK-21 cells. The infectivity of the E-V264T mutant was approximately 4 log₁₀TCID₅₀/ml lower in both cell lines than YFV Asibi (ic) ($p < 0.05$). In contrast, the infectivity of the E-V188T mutant was significantly increased in comparison with YFV Asibi (ic) ($p < 0.05$) in both Vero and BHK-21 cells. The E-L276N mutant encoded a partial reversion to the wild-type sequence, which led to the emergence of the E-L276I mutation in the consensus sequence. Multiplication kinetics of the E-L276N mutant exhibited no demonstrable differences in Vero cells before 3 dpi but failed to reach infectivity at the plateau phase comparable to YFV Asibi (ic). Albeit the lack of significant difference in BHK-21 cells, there was a similar trend in infectivity between YFV Asibi (ic) and

the E-L276N mutant. In both Vero and BHK-21 cells, the E-A54S, E-A261S, and E-L273N mutations had comparable growth curves to YFV Asibi (ic).

C. Mutations of the flavivirus conserved hydrophobic residues in the EDI-EDII hinge region increase the genome-PFU ratio of YFV Asibi (ic) in Vero cell

We compared the genome-PFU ratio between YFV Asibi (ic) and five single mutants (Figure 5-6). Higher genome-PFU ratios showed assembly and release of YFV Asibi (ic) from infected Vero cells (moi: 0.1) is impaired by the E-V264T mutation.

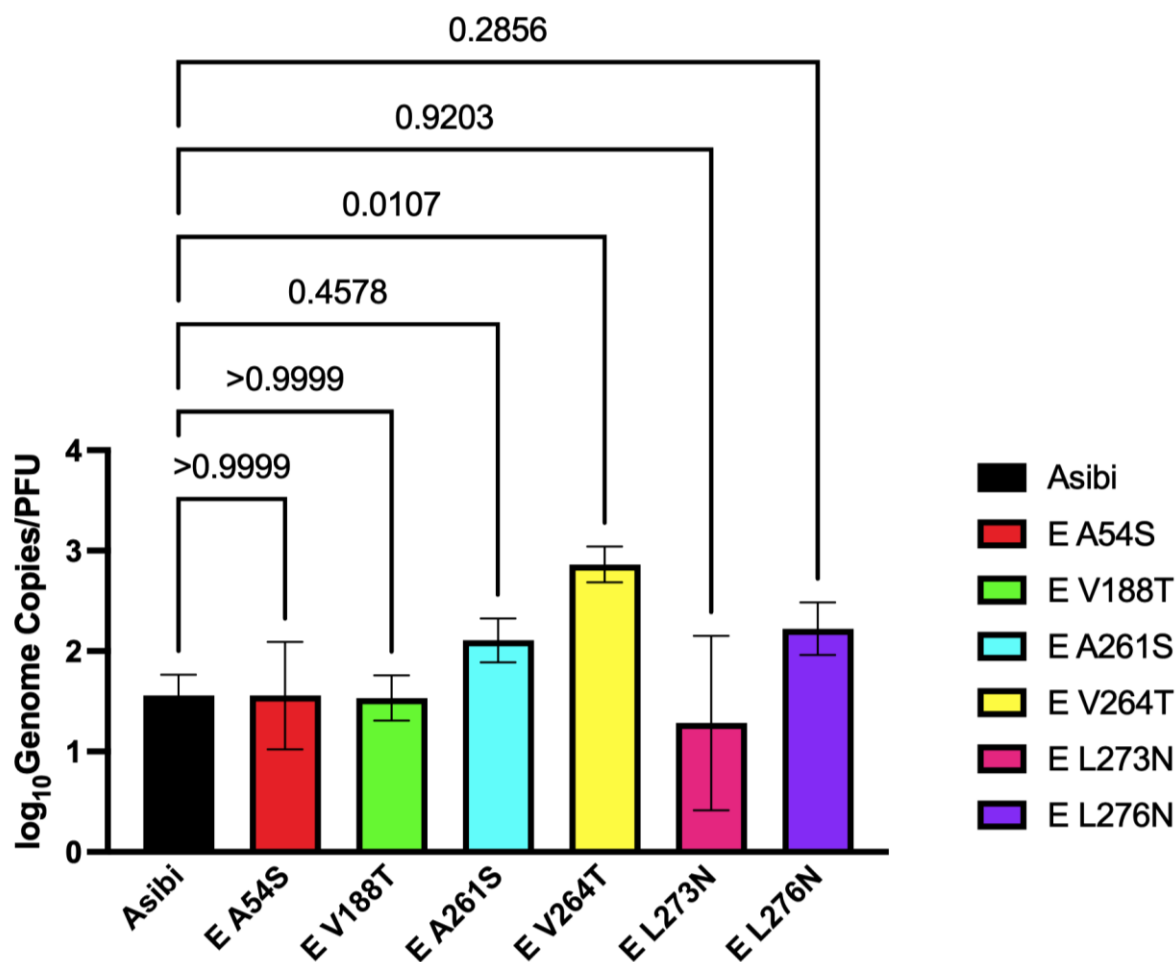


Figure 5-6: PFU-genome ratio of YFV EDI-EDII hinge region mutants

Genome-PFU ratios of YFV Asibi infectious clones and the three replicates of six mutants in the culture supernatants of Vero cells infected at a moi of 0.1. Data points represent the average genome copy-to-PFU ratios, and error bars represent the standard deviation of two biological replicates prepared from Vero cells infected with YFV Asibi or each mutant at moi of 0.1. Comparison of genome copy-to-PFU ratio was performed using one-way ANOVA followed by Dunnett's pairwise comparison. The image was generated in GraphPad. The PFU-genome ratios was conducted by Bailey Maloney and Danielle Saunders.

The genome-PFU ratio of the E-V264Y mutants (3 log₁₀genome-PFU) was higher than YFV Asibi (ic) (1.5 log₁₀genome-PFU) ($p=0.0107$). This result indicates that the E-V264T mutant

can be assembled and secreted into a virion in cell culture that could produce immunogens. However, many of the virions are not infectious. Genome-PFU ratios of the E-A261S and E-L276N mutants had a marginal increase that did not reach statistical significance compared to YFV Asibi (ic). Similarly, neither the E-A54S, E-V188T, or E-L273N mutations led to demonstrable differences in the genome-PFU ratio of YFV Asibi (ic).

D. Attenuation of the mouse viscerotropic phenotype of YFV E-V188T mutant

We investigated the attenuating effect of each YFV mutant in groups of four male and four female six-week-old AG129 immunocompromised mice via IP challenge at 1000 PFU (Figure 5-7). Due to the logistical difficulties and high cost of acquiring immunocompromised AG129 mice, only eight mice per mutant were used instead of ten, like in the WNV mouse studies. Additionally, this study was completed by the scientists at Bioqual Inc.

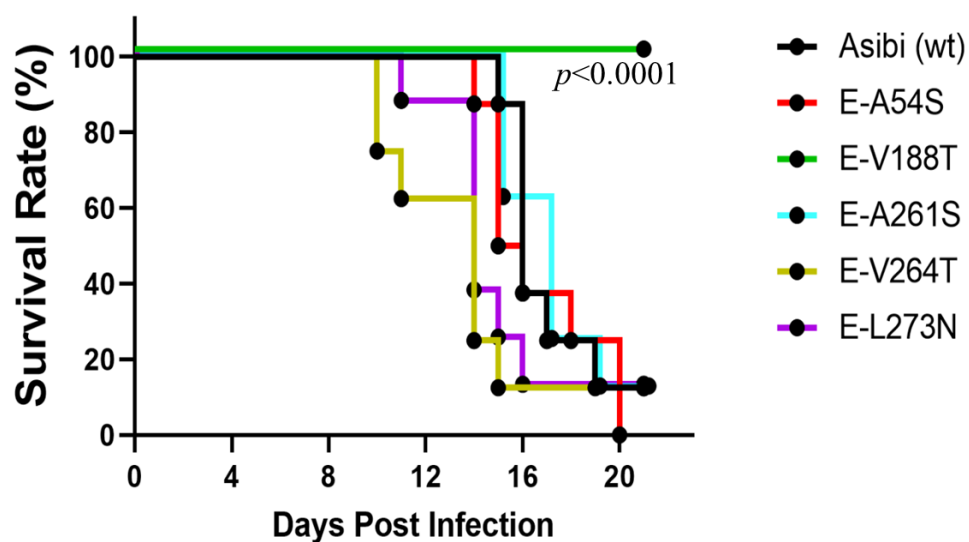


Figure 5-7 Attenuation of the viscerotropic phenotype of YFV in six-week-old mice

Groups of four male mice and four female mice received the IP challenge with each YFV mutant or YFV Asibi (ic) at 1000 PFU. Depiction of the survival rate of mice receiving the E-A54S, E-V188T, E-A261S, E-V264T, E-L273N mutants, or the parental YFV Asibi (ic). The Kaplan-Meier survival curve showed differences in survival outcomes and average survival time, and Dunnett's pairwise comparison followed one-way ANOVA. The image was generated in GraphPad. The YFV mouse viscerotropic disease study was conducted by the scientists at Bioqual Inc.: Hanne Anderson, Brian Backstedt, and Swagata Kar.

The intraperitoneal challenge with YFV Asibi (ic) led to 90% lethality between 15 and 17 dpi. Significantly, no mice developed lethal viscerotropic disease following the IP challenge with the E-V188T mutant ($p < 0.0001$). Therefore, the E-V188T mutation can fully attenuate the virulence phenotype in the viscerotropic disease AG129 mouse model up to 1000 PFU. There was no demonstrable difference in the virulence phenotype between YFV Asibi (ic) and three other single mutants. All eight mice succumbed to the viscerotropic disease induced by IP challenge with the E-A54S mutant, with an average survival time of 16.8 days (Table 5-5). The average survival time was determined by the day post-infection when mortality was observed. The virulence

phenotype of the E-A261S mutant was also indistinguishable from wild-type YFV Asibi, as observed with 90% mortality and an identical average survival time of 17.0 days. Surprisingly, the reduced infectivity of the E-V264T mutant was not translated to the attenuated phenotype in mice. Mice challenged with the E-V264T mutant developed 90% mortality and no significant reduction in average survival time compared to the control group receiving YFV Asibi (ic). Similarly, 90% of mice receiving the challenge with the E-L273N mutant also succumbed to viscerotropic disease and showed no reduction in the average survival time.

Table 5-5: Summary of average survival times of AG129 mice challenged with YFV Asibi (ic) and single-site mutants in the flavivirus conserved residues in the EDI-EDII hinge region

This table details the average survival time post-infection for each group of inoculated mice and the standard deviation seen within the respective group. The average survival time of each group was calculated using 21 days as the end point of the study. Yellow fever virus mouse viscerotropic disease study was conducted by the scientists at Bioqual Inc.: Hanne Anderson, Brian Backstedt, and Swagata Kar.

Group	Average Survival Time (days)	Standard Deviation
YFV Asibi (wt)	17.0	2
A54S	16.8	2.5
V188T	>21	0
A261S	17.0	2.1
V264T	13.5	3.6
L273N	14.9	2.9

The summary of weight changes in each mouse is shown in Table 5-6. Weight loss in mice receiving the IP challenge with YFV Asibi (ic) began between 10 and 12 dpi. In contrast, no significant weight loss, i.e., >20%, was observed in mice challenged with E-V188T mutant

throughout the study. Timing for the onset of significant weight loss in the mice receiving the IP challenge with the E-A54S, E-A261S, and E-L273N mutant resembled the control group challenged with YFV Asibi (ic), i.e., at 10 to 12 dpi. The mice challenged with E-V264T had an earlier onset of severe weight loss compared to YFV Asibi (ic), starting at 7 to 8 dpi. This likely resulted in the slightly earlier death, with two occurring at 10 dpi and one at 11 dpi.

Table 5-6: Summary of weight challenge in groups of four male and four female mice receiving the IP challenge of YFV Asibi (ic) or single mutants containing amino acid substitutions of flavivirus conserved hydrophobic residues in the EDI-EDII hinge region

The weight of each mouse was measured in grams daily. “M” represents the male mice, and the notation “F” represents the female mice. Grey coloring represents a mouse that was euthanized or found dead prior to the end of the study, and white represents a mouse that survived to 21 dpi. Yellow fever virus mouse viscerotropic disease study was conducted by the scientists at Bioqual Inc.: Hanne Anderson, Brian Backstedt, and Swagata Kar.

Group	Mouse (Male)	Starting Weight (g)	Peak Weight (g)	Final Weight (g)	Mouse (Female)	Starting Weight (g)	Peak Weight (g)	Final Weight (g)
YFV Asibi (wt)	1M	20.79	20.79	16.4	1F	24.15	24.15	17.65
	2M	19.69	20.36	14.68	2F	23.94	24.86	20.12
	3M	21.19	21.19	16.03	3F	23.06	23.06	18.12
	4M	20.07	20.07	15.83	4F	22.95	24.28	17.98
A54S	1M	19.05	19.05	12.98	1F	21.94	22.93	17.38
	2M	19.40	19.40	15.33	2F	22.53	22.53	16.69
	3M	20.68	20.68	16.14	3F	21.62	21.74	16.27
	4M	18.31	18.31	13.77	4F	21.89	21.89	17.10
V188T	1M	16.02	17.32	13.21	1F	25.16	25.48	23.58
	2M	18.22	20.00	20.00	2F	25.92	27.79	22.16

Group	Mouse (Male)	Starting Weight (g)	Peak Weight (g)	Final Weight (g)	Mouse (Female)	Starting Weight (g)	Peak Weight (g)	Final Weight (g)
V188T	3M	18.39	20.05	18.98	3F	24.78	26.38	26.29
	4M	18.87	20.34	18.28	4F	22.22	22.38	20.84
A261S	1M	16.87	17.54	12.97	1F	21.78	21.85	17.60
	2M	15.81	17.06	13.28	2F	20.15	20.57	19.41
	3M	16.39	17.64	13.03	3F	20.24	20.80	15.25
	4M	17.35	17.68	13.52	4F	21.02	21.32	16.71
V274T	1M	14.97	15.86	11.63	1F	16.94	18.05	12.96
	2M	15.26	16.46	11.87	2F	16.26	17.10	12.52
	3M	16.65	17.56	13.03	3F	18.29	18.89	14.46
	4M	14.32	15.37	14.55	4F	17.73	17.77	13.45
L273N	1M	23.26	24.77	18.30	1F	14.41	15.34	11.26
	2M	23.87	25.44	18.81	2F	16.84	17.58	13.42
	3M	16.67	18.04	12.94	3F	16.96	17.13	12.76
	4M	19.10	21.46	15.77	4F	16.21	17.20	14.86

The disease onset was initially observed in mice challenged with YFV Asibi (ic) at 14 to 15 dpi. The first signs included hunched spinal posture, ruffled fur, and lethargy or listlessness. Symptoms often progressed quickly, with common symptoms including greater than 20% body weight loss and moribund behavior on top of initial symptoms. Although the E-V188T showed a fully attenuated phenotype with 100% survival, four of the eight mice still developed transient and mild symptoms, including ruffled fur. The mice challenged with the E-A54S mutant started exhibiting symptoms similar to those challenged with YFV Asibi (ic) at 13 to 15 dpi before quickly succumbing to disease. One male mouse challenged with the E-A54S mutant exhibited early symptoms at 7 dpi but succumbed to the disease at a similar time to the other mice in this group.

The three mutants within the fourth motif of the EDI-EDII hinge region all showed similar kinetics in the onset of symptoms. The E-A261S, E-V264T, and E-L273N mutants all, on average, began exhibiting symptoms between 4 to 6 dpi. In general, mice challenged with either of the three mutants began to show mild symptoms for approximately 5 to 7 days, and the numbers or severity of symptoms continued to increase for approximately 3-4 days before succumbing to the disease. The mice in E-A261S, E-V264T, and E-L273N mutant groups that survived the study exhibited transient and mild symptoms, although some exceptions to this pattern were observed. For the E-A261S mutant group, two female mice showed symptoms similar to those challenged with YFV Asibi (ic), with the onset of symptoms at 14 dpi instead of early in the study. Of those two mice, one survived to the end of the study. The E-V264T mutant group had one female mouse shown to have symptoms starting at 11 dpi. While this is still an earlier onset of disease than occurred with the wild-type group, it is a noticeably later first sign of symptoms than the other mice in this group that showed signs at 5 to 6 dpi.

5.3 Discussion

Because of the difficulty and cost of obtaining the immunocompromised AG129 mice, we investigated the attenuation process of wild-type YFV Asibi (ic), following the identification of the E-A54I and E-Y201P mutations that can fully attenuate the mouse neuroinvasive phenotype of WNV NY99ic. We identified the E-V188T mutation that can fully attenuate the virulence phenotype of wild-type YFV Asibi (ic) in the viscerotropic disease AG129 mouse model. Significantly, the E-V188T mutation does not impair the multiplication kinetics of wild-type YFV Asibi (ic) in Vero cells, the acceptable substrate for human vaccine manufacturing. Once proven successful, the E-V188T mutation can be useful for the rational design of second-generate YF LAVs.

Only one substitution was investigated for each flavivirus conserved residue in the EDI-EDII hinge region of YFV, so our conclusions are limited to the specific amino acid substitutions tested. Nevertheless, the comparison of single mutants between YFV and WNV highlights the similarities and differences of the EDI-EDII hinge region between different flaviviruses. The YFV E-V188 residue is the equivalent amino acid to the WNV E-I196 residue in the EDI-EDII hinge region, and the fully attenuated phenotype of the YFV E-V188T mutant is consistent with the lethal phenotype of the WNV E-I196N mutant. The N-terminus of the EDI-EDII hinge region's 3rd motif is crucial for the E protein's structure-function. This suggests that alternative amino acid substitutions of the WNV E-I196 residue may be worthwhile evaluating for the attenuating effect on WNV NY99ic.

The YFV E-Y194P mutant cannot form visible plaques and was excluded from further analysis in this study. Notably, this is consistent with the small plaque phenotype of the WNV E-Y201P mutant in Vero cells. Detection of viral proteins in Vero cells used for TCID₅₀ assay is encouraging and indicates that the E-Y194 mutant does not prevent the recovery of viable viruses. We have proven WNV NY99ic tolerates 13 alternative amino acid substitutions of the equivalent E-Y201 residue. In addition to the fully attenuated phenotype of the WNV E-Y201P mutant, the WNV E-Y201C and E-Y201D mutants are both highly attenuated. Suppose the equivalent mutations can be proven to attenuate the virulence phenotype of YFV Asibi (ic). In that case, this provides direct evidence that 1) the fg hairpin of EDII has the shared importance for the structure-function of the E protein in at least two different flaviviruses and 2) the amino acid equivalent to the YFV E-Y194 residue and WNV E-Y201 residue may be worthwhile investigating as a target for the engineering of broadly effective attenuating mutations.

The E-L276 residue was crucial for the structure-function of the E protein, as demonstrated by the single nucleotide reversion at the second nucleotide of the codon that results in the YFV E-L276N mutation. The E-L276N residue likely does not tolerate the mutation because of the common role of the kl hairpin structure in stabilizing the EDI-EDII domain interactions across different flaviviruses. Interestingly, the YFV E-L276 residue is equivalent to the WNV E-L281 residue. Both residues exhibited similar reversions to the wild-type sequence, indicating that this residue is not only important to the structure and function of the E protein in WNV and YFV, but to flaviviruses in general. However, we have not investigated all 19 alternative amino acid substitutions of either the YFV E-L276 residue or the WNV E-L281 residue, which may further reveal common attenuating mutations due to the flavivirus conserved kl hairpin structure.

The YFV E-V130 and E-I135 residues are equivalent to the WNV E-I130 and E-I135 residues. The YFV E-V130T and E-I135N mutations preclude the recovery of viable viruses, resembling the lethal phenotype observed with the WNV E-I130N and E-I135N mutants, respectively. Therefore, the specific amino acid substitutions were chosen to disrupt the hydrophobicity of each of the nine residues. We demonstrated that eight of the nine flavivirus conserved hydrophobic amino acids in the EDI-EDII hinge region are essential for the structure-function of WNV E protein: E-V130, E-I135, E-V188, E-Y194, E-A261, E-V264, E-L273, and E-L276 residues.

Comparable to WNV, the YFV mutants E-V130T and E-I135N reside in the 2nd motif of the EDI-EDII hinge region. Both mutants produced lethal phenotypes like their WNV complementary residues. Due to the consistent lethality or instability of mutation to residues of the 2nd motif of the EDI-EDII hinge region, as seen in WNV, DENV-2, and now YFV, the limited plasticity of this region may be conserved among different flaviviruses (Butrapet et al. 2011).

Interestingly the YFV exhibited a viable phenotype after mutations in the E-Y194P mutant in the 3rd motif of the EDI-EDII hinge region, like its WNV equivalent E-Y201P. However, Since the YFV E-Y194P mutant was only detectable by indirect immunofluorescent staining and did not plaque, the mutant was not optimal for further analysis as an attenuating mutation. The lack of growth of this mutant is likely due to the putative interactions between the 2nd and 3rd motifs of the EDI-EDII hinge region (Christian et al. 2013). However, the WNV E-Y201P mutant grew at titers over 4log₁₀PFU/ml lower than wild-type WNV. This proline mutation in both YFV and WNV likely causes significant decreases in the multiplication of the virus. The difference in growth is likely due to the slight differences in the structure and movement of the EDI-EDII hinge region between different flaviviruses, resulting in WNV forming plaques and YFV not showing visible plaques. The E-VI130, E-I135, and E-Y194 residues are located in a structurally and functionally important region that cannot tolerate engineered mutations.

Interestingly, the WNV E-Y201P and YFV E-V188T residues are neighboring residues in 3rd motif of the EDI-EDII hinge region that both produced attenuated phenotypes. This is not surprising as both mutations reside within the “gfeah” β -sheet that controls the orientation of EDII during the EDI-EDII interdomain movement. This makes the conserved hydrophobic residues of the 3rd motif of the EDI-EDII hinge region critical residues to the structure and function of the E protein and ideal targets for the engineering of attenuating mutations. Due to the successful attenuating phenotype produced by the YFV E-V188T mutant, additional attenuating mutations may be possible in the equivalent WNV residue. Although the WNV E-I196N mutant was lethal, it may be worthwhile to investigate a threonine substitution utilized in the equivalent YFV residue.

Somewhat surprising is we did not observe an attenuating effect in AG129 mice receiving the IP challenge with the E-A54S mutant, given the E-A54 residue is strictly conserved across all

flaviviruses. However, this may be explained by the structural variation in the EDI-EDII hinge region between different flaviviruses, as described in the superimposed E protein crystal structures by Luca et al. 2012. A complete picture of the attenuating effect of each alternative amino acid substitution of the YFV E-A54 residue may be valuable not only for the fundamental knowledge of the structure-function of the E protein but also for the rational design of second-generation YF LAVs. While the hydrophobic biochemical property of the E-A54 residue was disrupted with the serine mutation, serine is still a small amino acid (99 Å³), and the WNV data from Chapter 3 indicate the E-A54 residue of WNV can produce an attenuated phenotype with large amino acids.

Overall, mutation of the flavivirus-conserved hydrophobic residues in the EDI-EDII hinge region resulted in fully attenuated virulence phenotypes in both WNV and YFV. Although neither the WNV E-Y201P mutation nor the YFV E-V188T mutation has been proven to produce the attenuated phenotype translated to the other model flavivirus, our findings are still of significance and highlight the third motif of the EDI-EDII hinge region as an attractive region for the engineering of attenuating mutations. Further, the difference reflects the structural differences between the EDI-EDII hinge region of different flaviviruses. Although flaviviruses are closely related, there can be significant differences in the angle of movement of the EDI-EDII hinge region. For example, the JEV E protein and its closely related WNV E protein exhibit a large angular difference of 16.0° (Luca et al. 2012). Therefore, engineering of mutation of the conserved hydrophobic residues of the EDI-EDII hinge region will need to consider structural variations.

Chapter 6 - Identification of Putative Mechanisms of Attenuation in the E Protein Interdomain Regions

Our site-directed mutagenesis of flavivirus conserved residues in the EDI-EDII hinge region, and the EDI-EDIII linker advances our understanding of the common structure-function of the E protein involved in the membrane fusion process. We have demonstrated that mutations of multiple flavivirus conserved residues in the respective E protein interdomain regions can attenuate the mouse virulence phenotype of WNV NY99ic or YFV Asibi (ic) and hence have the potential utility for the rational design of candidate WN and YF LAVs (Figure 6-1). The flavivirus-conserved residues analyzed in this dissertation are located at the interdomain junctions, each connecting the β -sheets of two respective domains. Rearrangement of the β -sheets in each domain drives the interdomain movement and, consequently, the conformational change of the E protein from dimer in the virion to trimer. Therefore, we can use the location and phenotypic changes caused by the mutations examined in this project to provide a detailed view into 1) flavivirus conserved residues involved in the conformational change of the E protein from dimer in the virion to trimer inserted into the host cell and 2) potential targets for the establishment of a flavivirus common attenuation strategy.

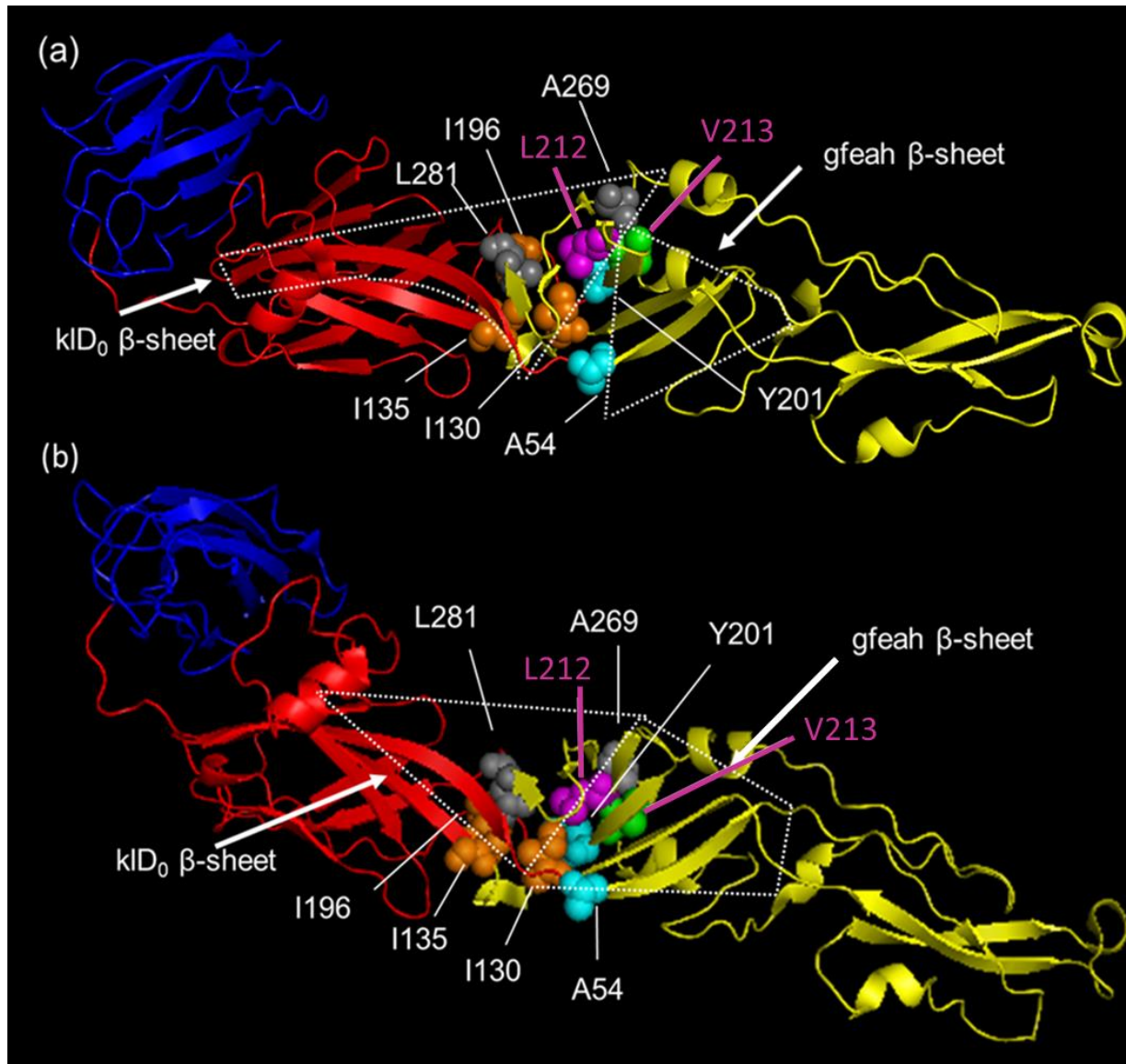


Figure 6-1: Seven flavivirus conserved hydrophobic residues crucial for the structure-function of the E protein

Each of the seven flavivirus-conserved residues in the EDI-EDII hinge region of the pre-fusion (a) and post-fusion (b) conformations of WNV E protein is shown in spheres (PDB ID:2HG0). The post-fusion conformation of WNV E protein is predicted using SLEV E protein (PDB ID: 4FG0). The E protein is colored in red (EDI), yellow (EDII), and blue (EDIII). The gfeah and klD0 β-sheets are marked with dashed white boxes. The seven conserved hydrophobic residues are

labeled in white, with neighboring hydrophobic residues labeled in magenta. The E-A54 and E-Y201 residues are highlighted with cyan. The fg hairpin residues E-L212 and E-V213 are highlighted in green and magenta, respectively. The E-I130, E-135, and E-I196 residues are labeled orange. The E-A269S and the E-L281N mutation are shown in gray and are part of the kID0 β -sheet. The image was generated in PyMOL.

A. Structural-functional importance of the flavivirus EDI-EDII hinge region conserved residue and putative mechanism of attenuation

As described in section 3.1, we selected the eight flavivirus-conserved hydrophobic amino acids in the EDI-EDII hinge region for site-directed mutagenesis. The eight residues can be categorized based on their locations about the β -barrel of EDI and the “gfeah” β -sheet at the base of EDII. The attenuating effect caused by the E-A54 and E-Y201 mutations potentially complements each other as both the E-A54 and E-Y201 residues are located at the base of the “gfeah” β -sheet. The E-A54 residue is the first residue from the N-terminus of the β -strand, and the E-Y201 residue immediately precedes the f β -strand (Figure 6-2).

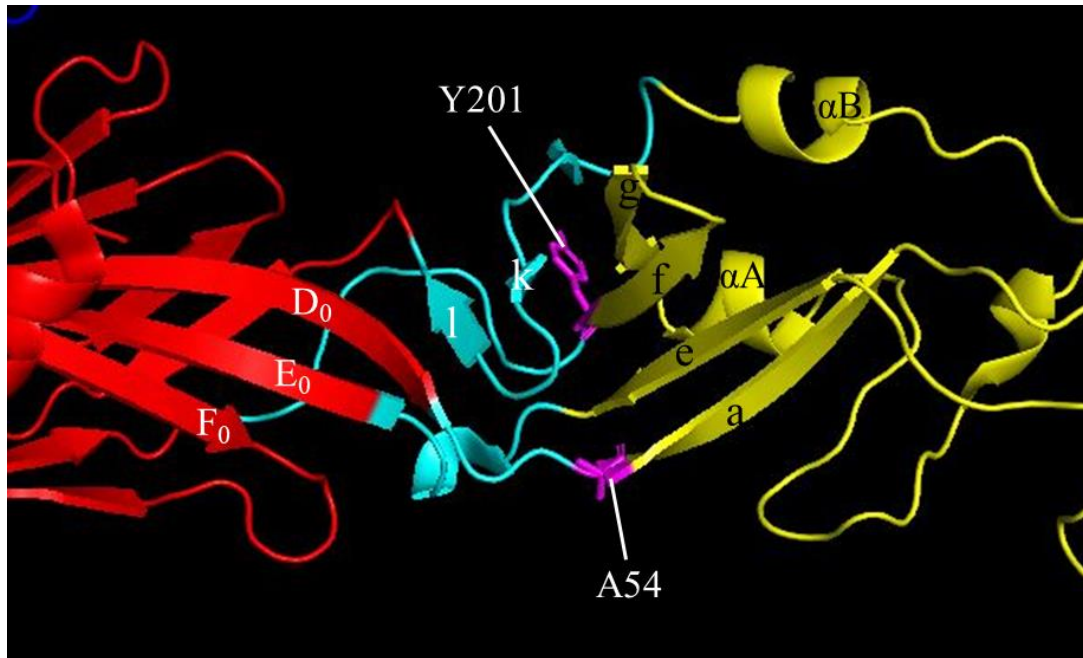


Figure 6-2: Location of the E-A54 and E-Y201 residues in the EDI-EDII hinge region (PDB ID: 2HG0)

The E protein monomer is colored red (EDI), yellow (EDII), and cyan (EDI-EDII hinge region). WNV E-A54 and E-Y201 residues are highlighted in magenta. The image was generated in PyMOL.

The E-A54 residue is sandwiched between the e and h β -strands. The stability of β -sheets typically depends on multiple hydrogen bonds between neighboring β -strands. This explains that WNV NY99ic can tolerate up to 13 alternative amino acid substitutions of the E-A54 residue because the proper folding of the “gfeah” β -sheet may not be entirely abolished by mutations of the E-A54 residue (Modis et al. 2003).

Rotation of the “gfeah” β -sheet is responsible for the EDI-EDII interdomain movement. Cryo-EM structures suggest that the radius of flavivirus virion expands by 10% due to the conformational change of the E protein from dimer in the virion to trimer inserted into the host cell (Kuhn et al. 2002). The increased space avoids the collision between the rotating “gfeah” β -

sheets into the neighboring E protein homodimer arranged in the herringbone pattern. Notably, the E-A54 residue is on the periphery of the E protein homodimer and proximal to the disordered structure between the bc loop of EDII in a neighboring E protein homodimer arranged in the herringbone pattern (Figure 6-3).

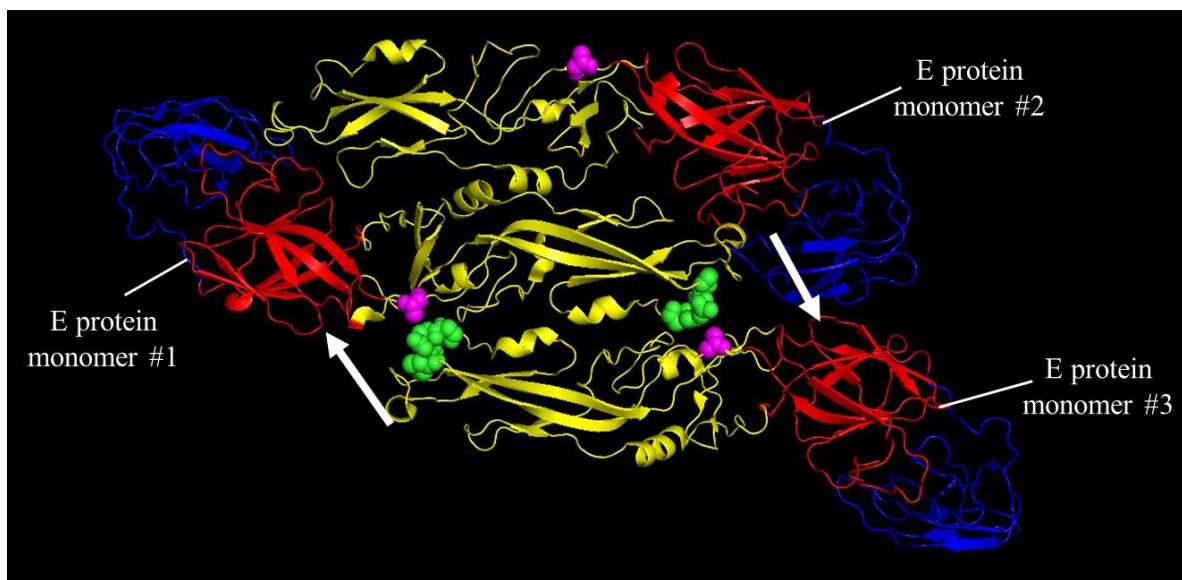


Figure 6-3: Location of WNV E-A54 residue on the periphery of the E protein dimer, modeled using JEV E protein (PDB ID: 3J27).

The E protein monomer is colored red (EDI), yellow (EDII), and cyan (EDI-EDII hinge region). There are three copies of the E protein monomer. Two copies of the E protein monomer arranged in the antiparallel direction to form the E protein homodimer are labeled “E protein monomer #1 and #2.” For ease of viewing, only one copy of the E protein monomer in the neighboring E protein homodimer is labeled “E protein monomer #3.” The WNV E-A54 residue is highlighted in magenta spheres, and the disordered motif of the bc loop in the EDII of the neighboring E protein homodimer is shown in green spheres. White arrows indicate the direction of EDII rotation. The image was generated in PyMOL.

It is likely that amino acid substitutions with large side chains of the E-A54 residue, i.e., the E-A54I mutation and the E-A54E mutation, can effectively interfere with rotation of the “gfeah” β -sheet due to the steric hindrance and consequently achieve a high level of attenuation effect (Figure 6-4). The impaired rotation of the “gfeah” β -sheet may change the orientation of EDII and, consequently, the exposure of the fusion loop from the protected position. A comparison of the pre-fusion and post-fusion conformations of DENV-2 and TBEV E proteins showed a fundamental difference in the EDI-EDII interdomain movement. For example, the TBEV E protein trimer formation requires the rotation of EDII about EDI by 19° (Bressanelli et al. 2004). In contrast, the EDI-EDII interdomain movement of DENV-2 E protein involves a 30° rotation (Modis et al. 2004). This difference in EDI-EDII hinge region movements helps explain that the same amino acid substitution of the E-A54 residue may exhibit different levels of attenuating effect in two different flaviviruses, e.g., the difference in the attenuating effect of the E-A54S mutation between WNV and YFV.

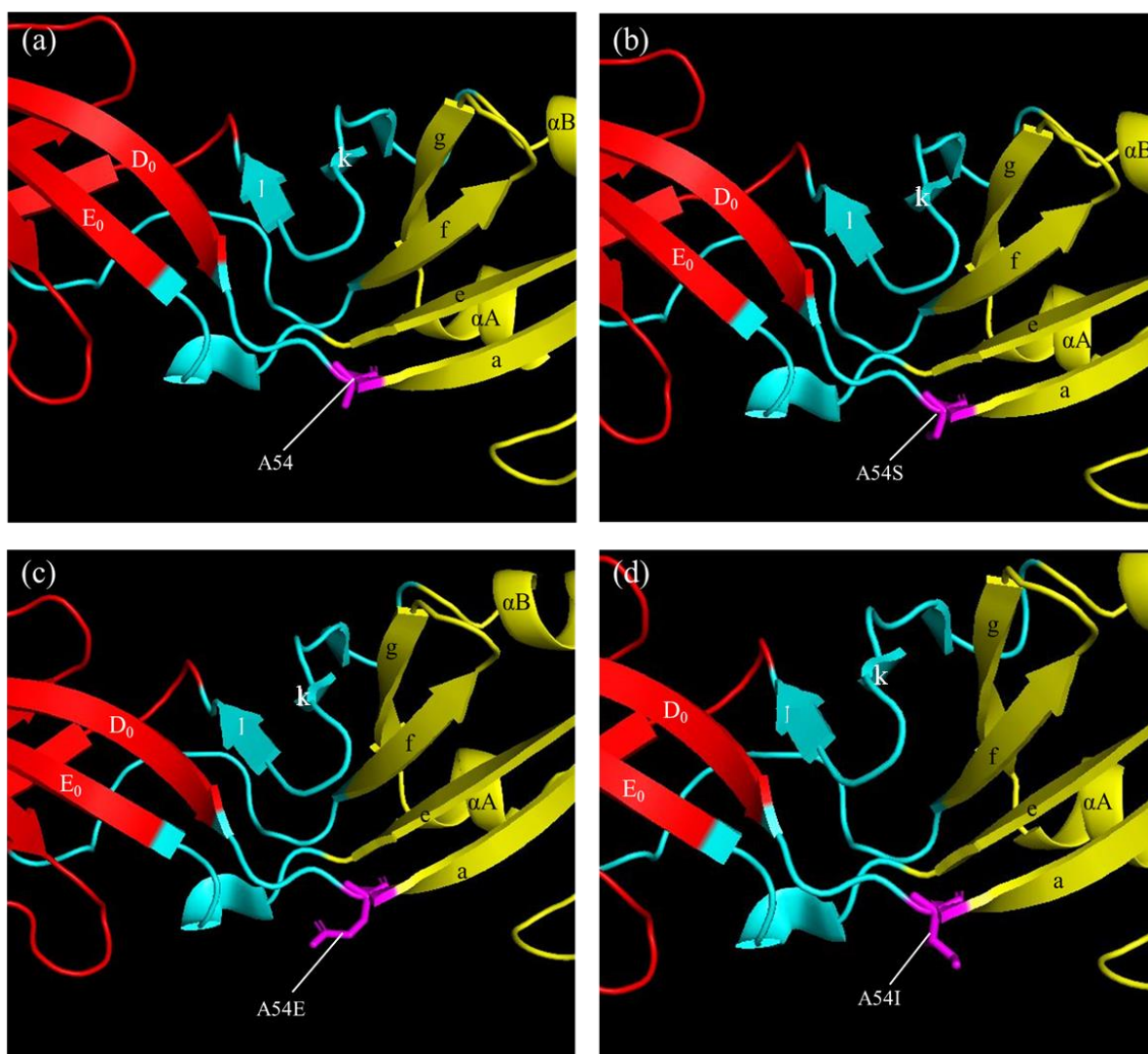


Figure 6-4: Mutations of the WNV E-A54 residue in the peripheral region of the “gfeah” β - sheet of the E protein (PDB ID: 2HG0)

The E protein monomer is colored red (EDI), yellow (EDII), and cyan (EDI-EDII hinge region).

(a) Wild-type alanine residue. (b) Serine mutation from initial screening of mutations in the EDI-EDII hinge region. (c) Glutamic acid mutation from optimization of E-A54 amino acid substitution. (d) Isoleucine mutation from optimization of E-A54 amino acid substitution. The image was generated in PyMOL.

The attenuated phenotype of WNV E-Y201 mutants is also likely related to interference with the rotation of the “gfeah” β -sheet of EDII. Specifically, the WNV E-Y201 residue

immediately precedes the hairpin structure consisting of the f and g β -strands, namely, the fg hairpin. Superimposed cryo-EM structures of immature, mature, and post-fusion flavivirus virions show all interdomain movements of the E protein use the fg hairpin as an axle for rotation of the “gfeah” β -sheet (Zhang et al. 2012). Here we propose the fg hairpin is potentially stabilized by the interaction of three flavivirus-conserved residues, including the WNV E-Y201, E-L212, and E-V213 residues (Table 6-1 and Figure 6-5). The WNV E-Y201 residue is within the third motif of the EDI-EDII hinge region, and the E-L212 and E-V213 residues are within the neighboring g β -strand. Moreover, the amino acids equivalent to the WNV E-Y201, E-L212, and E-V213 residues show the conserved hydrophobic biochemical properties based on structural alignment.

Table 6-1: Amino acid sequence alignment of the hydrophobic triad in EDII

Three residues of the hydrophobic triad are highlighted with bold text and underlined. The properties of amino acids are shown in green (hydrophobic), pink (polar), red (negatively charged), blue (positively charged), and black (unique). The numbering of amino acids in the motif is based on the WNV NY99 sequence.

Serocomplex	Virus	Strain	Sequence Hydrophobic Triad (E 201-213)
JEV	WNV	NY99	<u>Y</u> YVM <u>T</u> VG <u>T</u> ----- <u>K</u> <u>T</u> <u>F</u> <u>L</u> <u>V</u>
	JEV	SA14	<u>F</u> YVM <u>T</u> VG <u>S</u> ----- <u>K</u> <u>S</u> <u>F</u> <u>L</u> <u>V</u>
	JEV	SA14-14-2	<u>F</u> YVM <u>T</u> VG <u>S</u> ----- <u>K</u> <u>S</u> <u>F</u> <u>L</u> <u>V</u>
	KNV	MRM61C	<u>Y</u> YVM <u>T</u> VG <u>T</u> ----- <u>K</u> <u>T</u> <u>F</u> <u>L</u> <u>V</u>
	SLEV	MS1-7	<u>Y</u> YV <u>F</u> <u>T</u> V <u>K</u> <u>E</u> ----- <u>K</u> <u>S</u> <u>W</u> <u>L</u> <u>V</u>
YFV	YFV	Asibi	<u>Y</u> YIA <u>E</u> MG <u>K</u> ----- <u>E</u> <u>S</u> <u>W</u> <u>I</u> <u>V</u>
	YFV	17D	<u>Y</u> YIA <u>E</u> MG <u>K</u> ----- <u>E</u> <u>S</u> <u>W</u> <u>I</u> <u>V</u>
DENV	DENV-1	Hawaii	<u>M</u> VLL <u>T</u> M <u>K</u> <u>E</u> ----- <u>K</u> <u>S</u> <u>W</u> <u>L</u> <u>V</u>
	DENV-2	New Guinea C	<u>M</u> VLL <u>Q</u> M <u>E</u> <u>N</u> ----- <u>K</u> <u>A</u> <u>W</u> <u>L</u> <u>V</u>
	DENV-3	H87	<u>M</u> ILL <u>T</u> M <u>K</u> <u>N</u> ----- <u>K</u> <u>A</u> <u>W</u> <u>M</u> <u>V</u>
	DENV-4	H241	<u>M</u> ILM <u>K</u> M <u>K</u> <u>T</u> ----- <u>K</u> <u>T</u> <u>W</u> <u>L</u> <u>V</u>
TBEV	TBEV	Neudoerfl	<u>V</u> ILELD <u>K</u> T <u>V</u> E <u>H</u> L <u>P</u> T <u>A</u> <u>W</u> <u>Q</u> <u>V</u>
	OHFV	Bogluvovska	<u>V</u> VLELD <u>K</u> T <u>A</u> E <u>H</u> L <u>P</u> T <u>A</u> <u>W</u> <u>Q</u> <u>V</u>

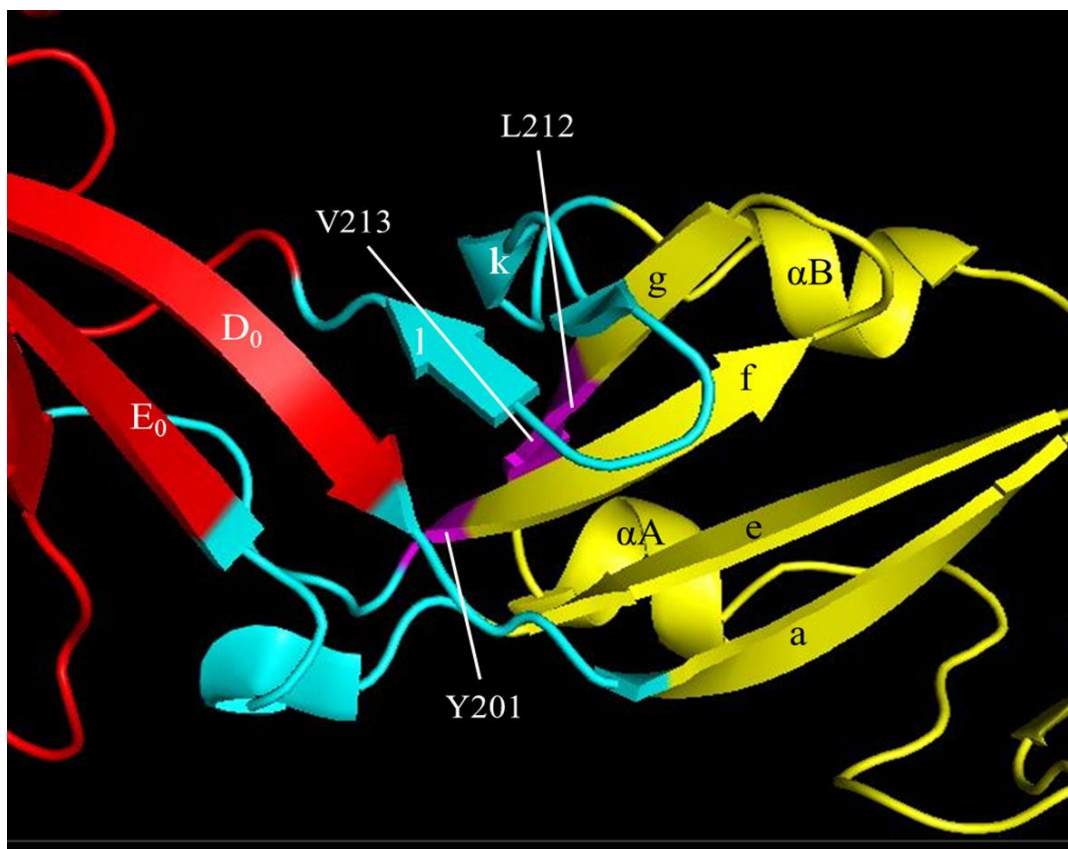


Figure 6-5: Hydrophobic triad of the fg hairpin of the E protein (PDB ID: 2HG0)

The E protein monomer is colored red (EDI), yellow (EDII), and cyan (EDI-EDII hinge region). WNVE-Y201, E-L212, and E-V213 residues are highlighted in magenta. The image was generated in PyMOL.

The amino acid sequence of the three hydrophobic residues in the fg hairpin is conserved within each serocomplex, consistent with the variations in the EDI-EDII hinge region across different flaviviruses. The E-Y201P, E-Y201C, and E-Y201D mutations can likely reduce the stability of the fg hairpin to interfere with the membrane fusion mechanism of WNV NY99ic, as observed with the change of the optimal pH for the HA activity (Figure 6-6). Indeed, there is considerable agreement between our finding on WNV and the previously published studies using small molecules targeting the flavivirus-conserved hydrophobic residues in the EDI-EDII hinge region of DENV-2 (de Wispelaere et al. 2018). Serial passage of WNV E-Y201P mutant in Vero

cells resulted in the partial reversion to the wild-type sequence encoding the E-Y201L mutation, suggesting a strong preference for a hydrophobic amino acid occupying the E-Y201 residue. Serial passage of DENV-2 in the presence of 2,4-diamino and 4,6-disubstituted pyrimidines that preferentially bind to the EDI-EDII hinge region also cause mutations of the equivalent E-M196 residue. Furthermore, there is no coincidence only hydrophobic amino acid substitutions of the DENV-2 E-M196 residue were reported. This means the amino acid equivalents to the WNV E-Y201 or the DENV-2 E-M196 residue can potentially support the engineering of flavivirus-common attenuation.

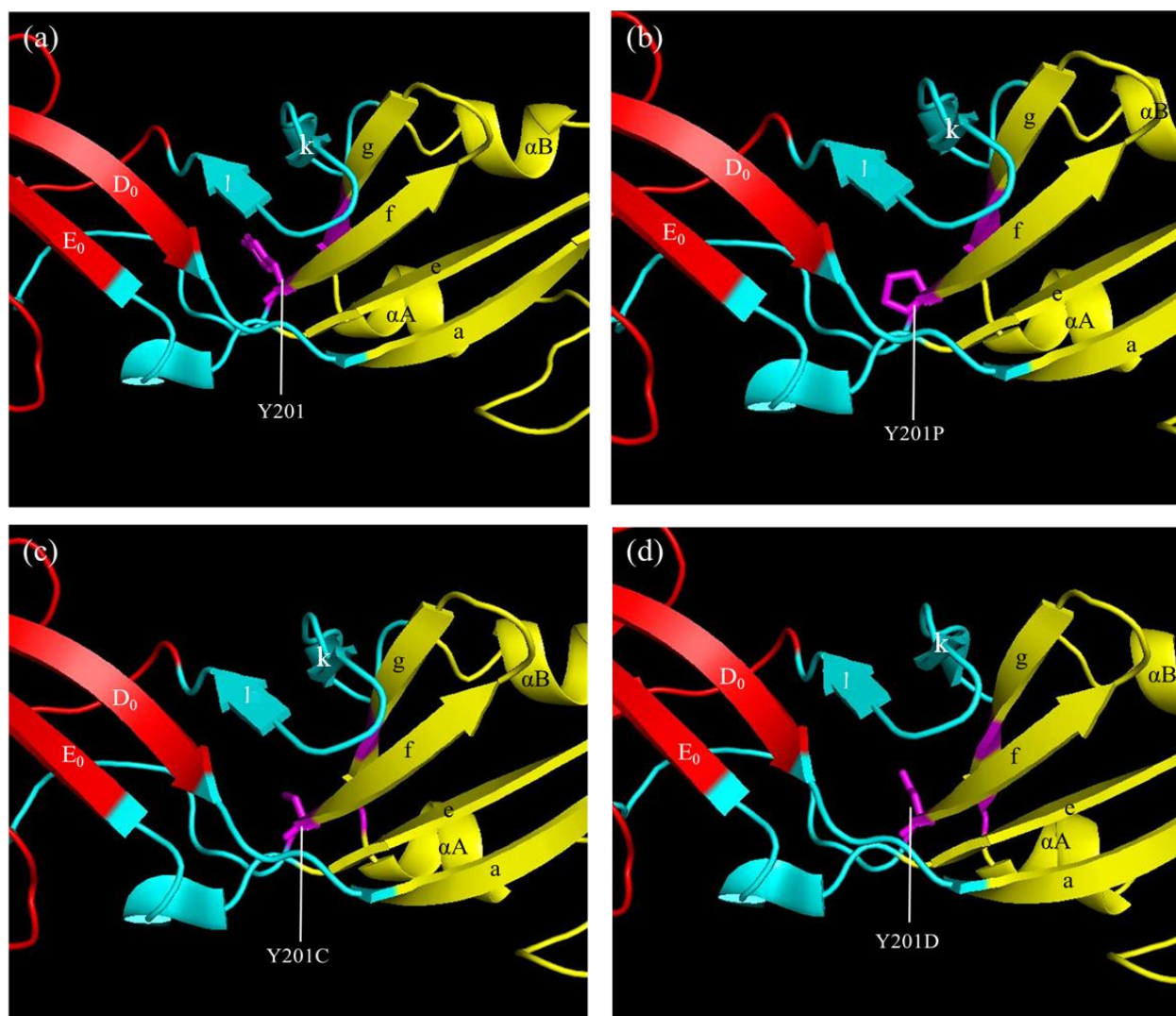


Figure 6-6: Mutation of the WNV E-Y201 residue at the edge of the fg hairpin of the E protein (PDB ID: 2HG0)

The E protein monomer is colored red (EDI), yellow (EDII), cyan (EDI-EDII hinge region), and magenta is the hydrophobic triad. (a) Wild-type tyrosine residue. (b) Proline mutation from initial screening of mutations in the EDI-EDII hinge region. (c) Cysteine mutation from optimization of E-Y201 amino acid substitution. (d) Aspartic acid mutation from optimization of E-Y201 amino acid substitution. The image was generated in PyMOL.

The attenuated phenotype of the YFV E-V188T mutant may be due to the interactions between the second and third motifs of the EDI-EDII hinge region proposed in the previous model based on the DENV-3 E protein (Christian et al. 2013). Substitution of the flavivirus-conserved YFV E-V188 residue with threonine may reduce the hydrophobic interaction with the YFV E-I130 and YFV E-V135 residues without ultimately impairing the proper function of the EDI-EDII hinge region (Figure 6-7) (Christian et al. 2013, Onofrio et al. 2014). Substitution of the equivalent WNV E-I196 residue with an asparagine can potentially induce structural and functional changes that cannot be tolerated. The YFV E-V188T mutation introduced a polar electrostatically neutral residue. In contrast, the WNV E-I195N mutation introduced the positively charged basic residue expected to cause a more profound impact on the structure-function of the E protein.

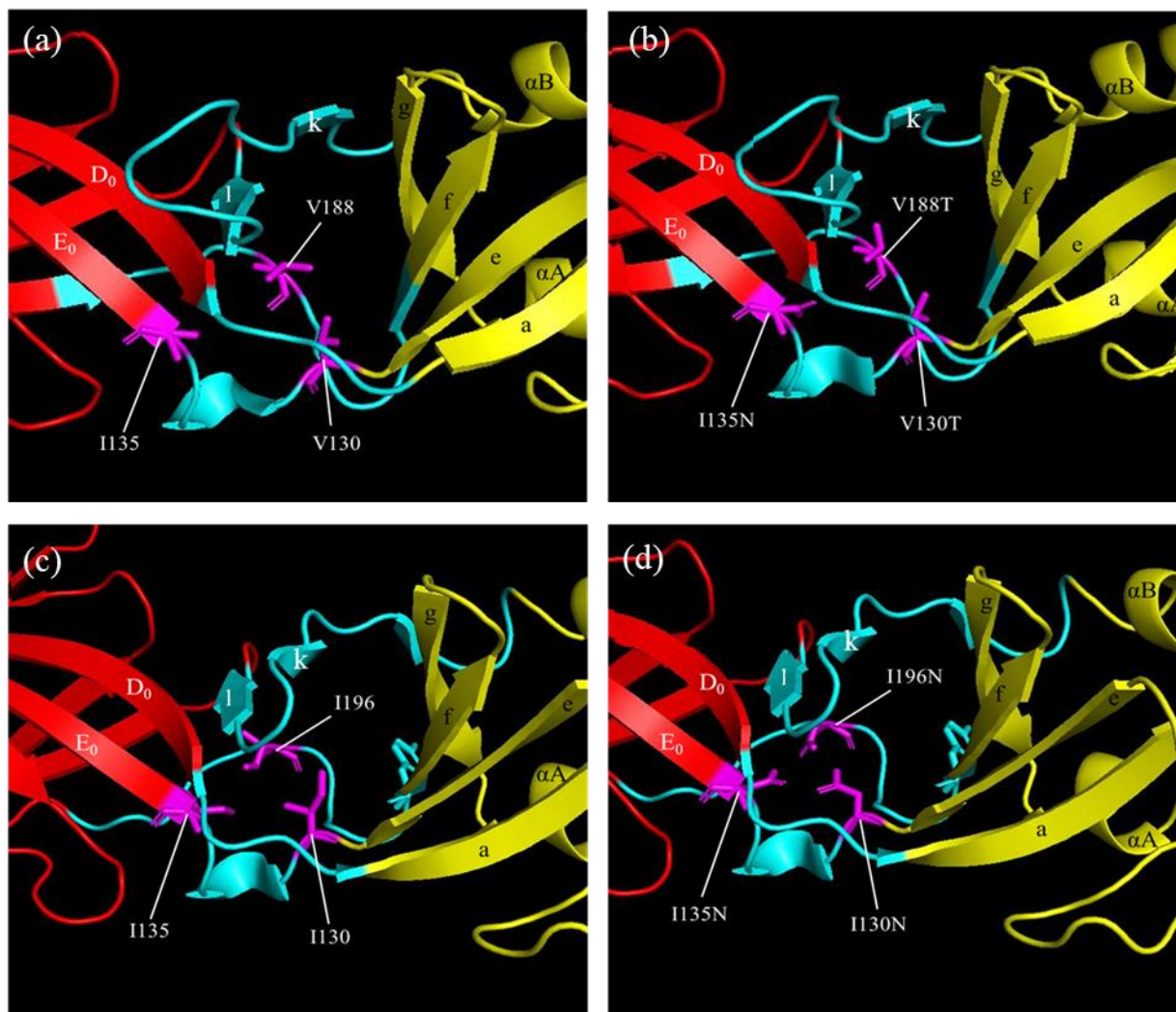


Figure 6-7: Mutation of the YFV and WNV residues located within the 2nd and 3rd motifs that are important to the gfeah rotation around the fg hairpin of the E protein

YFV (top row) is derived from PDB structure 6IW5, and WNV (bottom row) is derived from PDB structure 2HG0. The E protein monomer is colored red (EDI), yellow (EDII), and cyan (EDI-EDII hinge region). (a) Original E-V130, E-I135, and E-V188 residues in YFV. (b) Threonine mutations in the YFV E-V130 and E-V188 residues and asparagine mutation in the YFV E-I135 residue. (c) Wild-type E-I130, E-I135, and E-I196 residues in WNV. (d) Asparagine mutations in the E-I130, E-I135, and E-I196 residues in WNV. The image was generated in PyMOL.

The kl hairpin controls the interactions between the D₀ β -strand of EDI and the kl β -strands of EDII during the conformational change of the E protein from dimer to trimer (Modis et al. 2004). The kl hairpin is pulled apart in the E protein trimer, and the l β -strand of EDII forms new hydrogen bonds with the D₀ β -strand of EDI, presumably to stabilize the post-fusion conformation of the E protein (Figure 6-8).

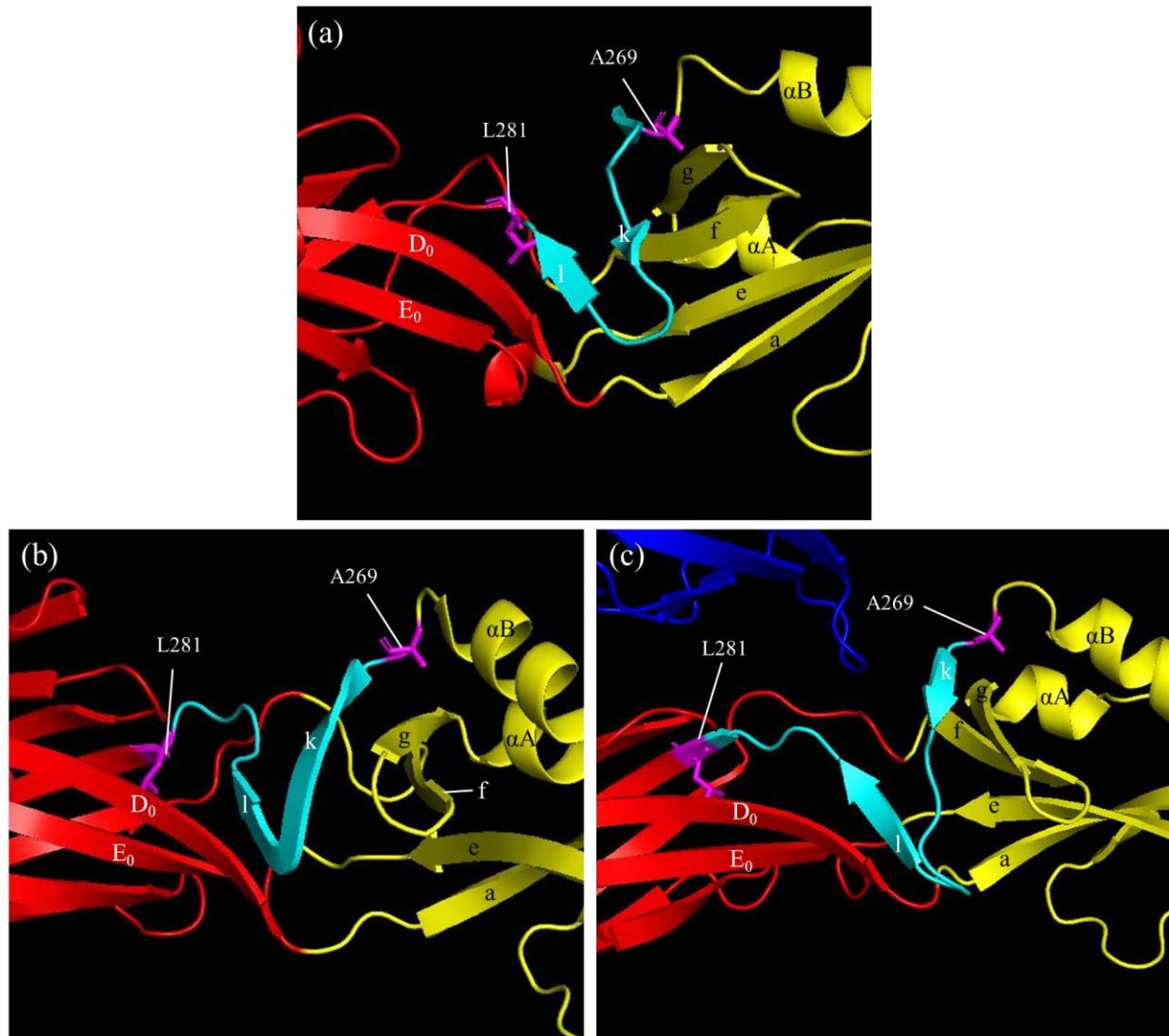


Figure 6-8: Mutations in the kl hairpin of WNV E protein

The E protein monomer is colored in red (EDI), yellow (EDII), and cyan (kl hairpin/4th motif of the EDI-EDII hinge region). (a) depiction of the locations of the E-A269 and E-L281 residues in WNV (PDB ID: 2HG0). Using DENV-2 as a model, depiction of the conformational change with WNV E-A269 and E-L281 residues (b) before fusion (PDB ID: 1OAN) and (c) after fusion (PDB ID: 1OK8). The image was generated in PyMOL.

Reversion of the WNV E-L281N mutation in the l β -strand is consistent with the structural-functional importance of the kl hairpin despite the engineering of nucleotide changes in all three

codon positions. All flaviviruses except for DENV-4 contain a leucine or isoleucine residue at the position equivalent to the WNV E-L281 residue, which likely indicates this leucine or isoleucine is important for in controlling the shift of the l β -strand towards the D₀ β -strand of EDI. We have made a similar observation with YFV Asibi (ic) based on the development of the compensatory E-L276I mutation identified in the consensus sequence of the E-L276 mutant. Similarly, the WNV E-A269S mutation in the k β -strand resulted in the emergence of the E-I270L compensatory mutation, presumably to compensate for the loss of hydrophobic interactions. Although we can only draw conclusions based on the engineered amino acid substitutions, i.e., the E-A269S and E-L281N mutations of WNV and the E-L276N mutation of YFV, the results have important implications as evaluation of alternative amino acid substitutions of the two residues in the kl hairpin of EDII may help establish a different attenuation mechanism (Figure 6-9).

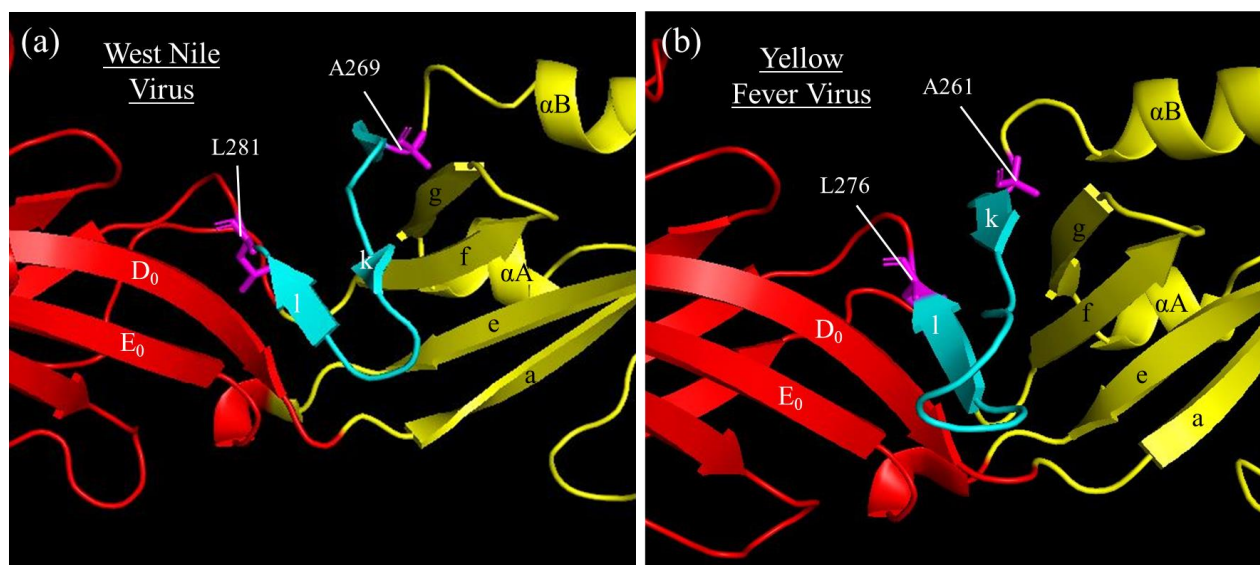


Figure 6-9: WNV E-L281 and YFV E-L276 residues kl hairpin of the E protein

The E protein monomer is colored in red (EDI), yellow (EDII), and cyan (kl hairpin/4th motif of the EDI-EDII hinge region). (a) WNV structure is derived from PDB structure 2HG0 with highlighted E-A296 and E-L281 residues (b) YFV structure is derived from PDB structure 6IW5 with highlighted E-A261 and E-L276 residues. The image was generated in PyMOL.

B. Putative mechanism of attenuation by mutations in the EDI-EDIII linker

The EDI-EDIII interdomain movement leads to the formation of the metastable post-fusion E protein trimer. It is controlled by the single motif at the domain interface, i.e., the EDI-EDIII linker. Comparison between the prefusion and post-fusion conformations of the E protein shows the EDI-EDIII linker changes from the disordered single linear chain of amino acid to an extended motif with putative interactions with both EDI and EDIII (Figure 6-10). The N-terminus of the EDI-EDIII linker must move towards the A₀ and C₀ β-strands of EDI in the E protein trimer, leading to the reorganization of both the A₀C₀D₀E₀F₀ and B₀I₀H₀G₀ β-sheets. The original A₀ β-strand is separated into two smaller β-strands. The N-terminus of the A₀ β-strand remains as part

of the A₀C₀D₀E₀F₀ β-sheet. Notably, the C-terminus of the original A₀ β-strand becomes exposed and potentially stabilizes the interface of the E protein trimer.

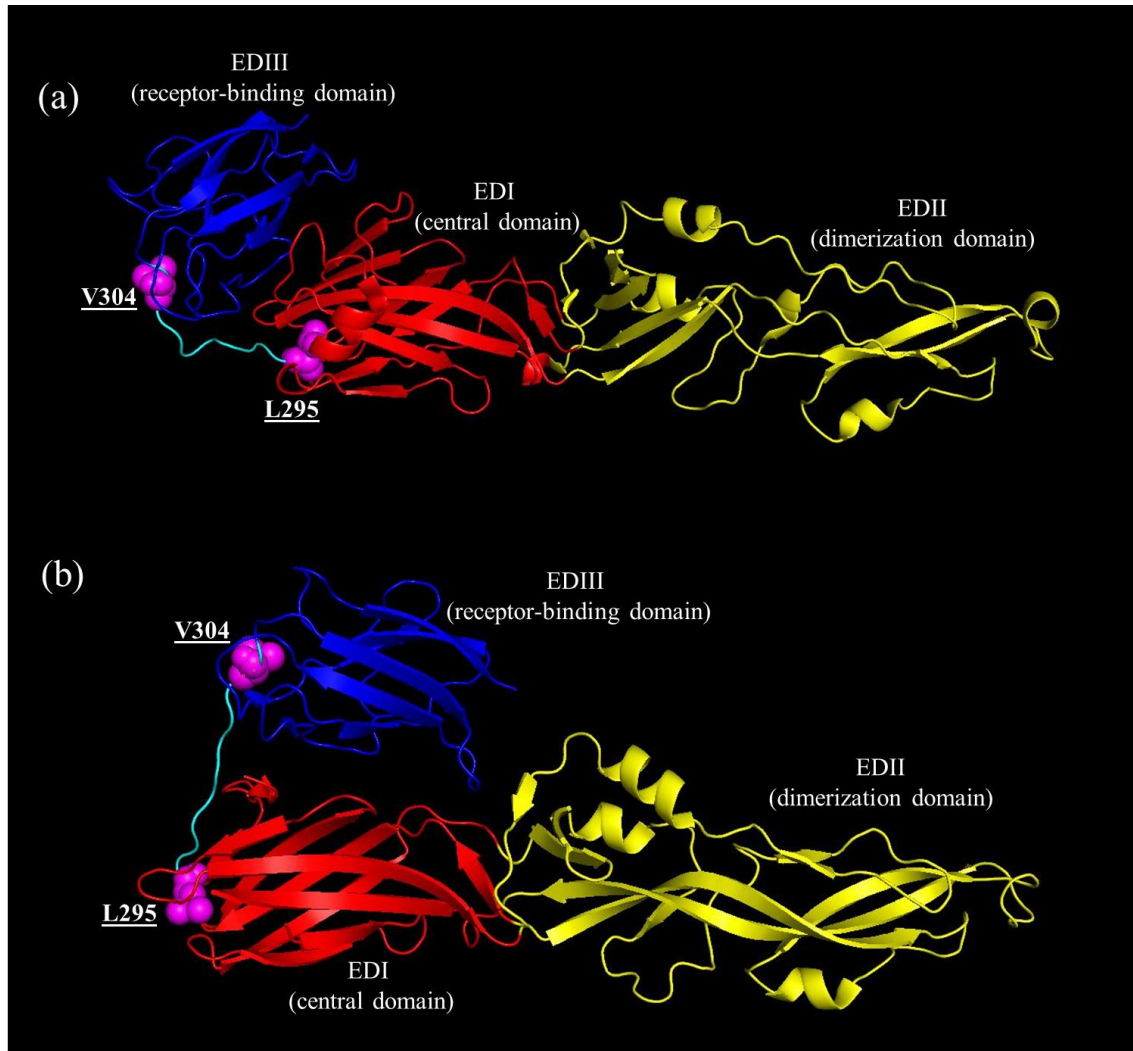


Figure 6-10: Location of WNV E-L295 and E-V304 residues in the EDI-EDIII linker before and after membrane fusion

The E protein monomer is colored -red (EDI), yellow (EDII), and cyan (EDI-EDII hinge region). The WNV E-L295 and E-V304 residues are highlighted in magenta. (a) represents the prefusion conformation of WNV (PDB ID: 2HG0). (b) the location of the WNV residues in the post-fusion conformation is shown in DENV-2 E protein as a model due to the lack of crystal structure of WNV E protein in post-fusion conformation (PDB ID: 1OK8). The image was generated in PyMOL.

The separation of the A₀ β-strand during the conformational change of the E protein explains why the C-terminus of the A₀ β-strand consists of an amino acid sequence that is almost strictly conserved across all flaviviruses. So, the N-terminus of the EDI-EDIII linker may act as a flavivirus-conserved switch mechanism for the assembly of the post-fusion E protein trimer, explaining the high level of amino acid sequence homology across flaviviruses. It is noteworthy that two of the three flavivirus conserved residues within the C-terminus of the A₀ β-strand are hydrophobic amino acids, and so is the flavivirus conserved E-L295 residue in the EDI-EDIII linker. The E-L295S mutation fully attenuated the mouse neuroinvasive phenotype, and the attenuated phenotype of the E-L295T mutant is partial. Substitution of the E-L295 residue with the small hydrophobic alanine also has a high level of attenuating effect in contrast to the virulence phenotype of the WNV E-L295V mutant resembling WNV NY99ic. Substitution of alternative hydrophobic amino acids at the E-L295 residue, such as the E-L295F and E-L295M mutations, had no changes to the infectivity or virulence phenotype of WNV NY99ic (Figure 6-11 and 6-12). So, it is likely the proper packing of the E protein trimer will require a large hydrophobic amino acid present in the equivalent position of the E-L295 residue. Potential hydrophobic interactions exist between the E-L295 residue and the hydrophobic amino acids in the C₀ β-strand, including the flavivirus-conserved E-V31, E-I33, and E-M34 residues.

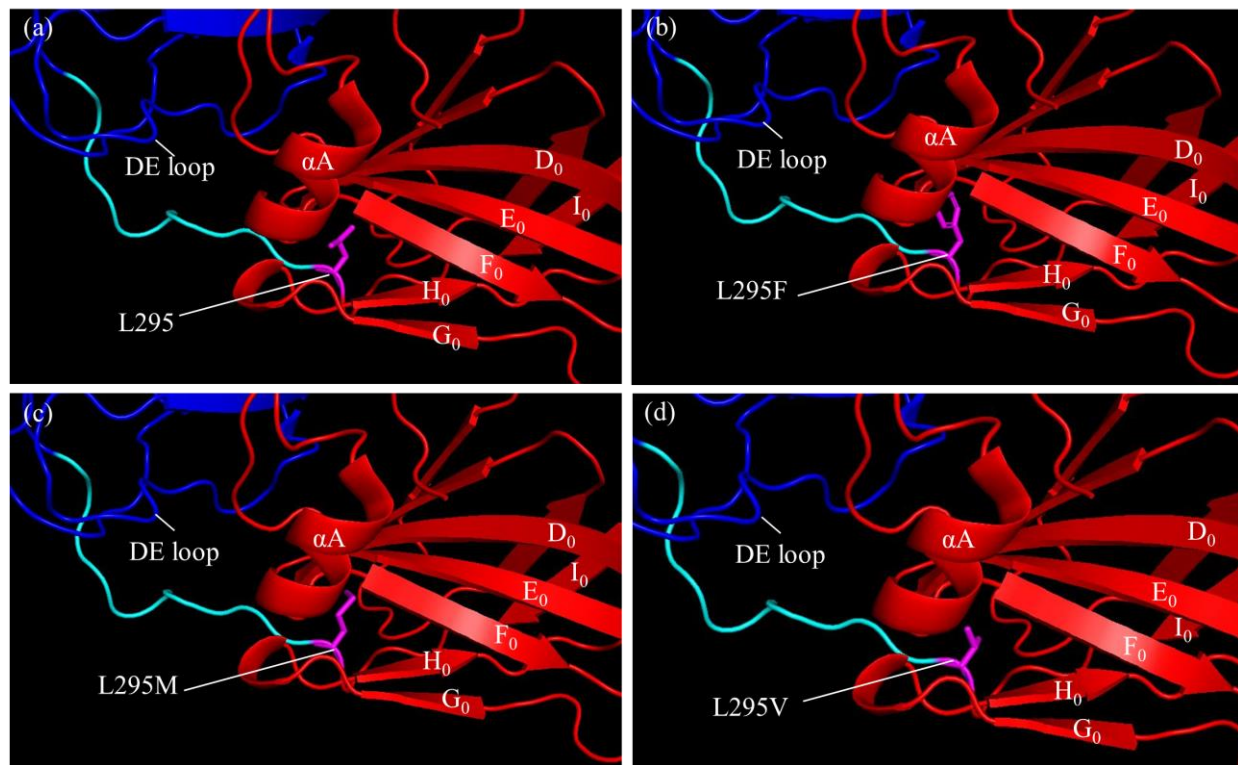


Figure 6-11: Mutation with large hydrophobic amino acids of the WNV E-L295 residue at the N-terminus of the EDI-EDIII linker (PDB ID: 2HG0)

The E protein monomer is colored in red (EDI), blue (EDIII), and cyan (EDI-EDIII linker). (a) Wild-type leucine residue. (b) Phenylalanine mutation with a hydrophobic index of 100. (c) Methionine mutation with a hydrophobic index of 74. (d) Valine mutation with a hydrophobic index of 76. The image was generated in PyMOL.

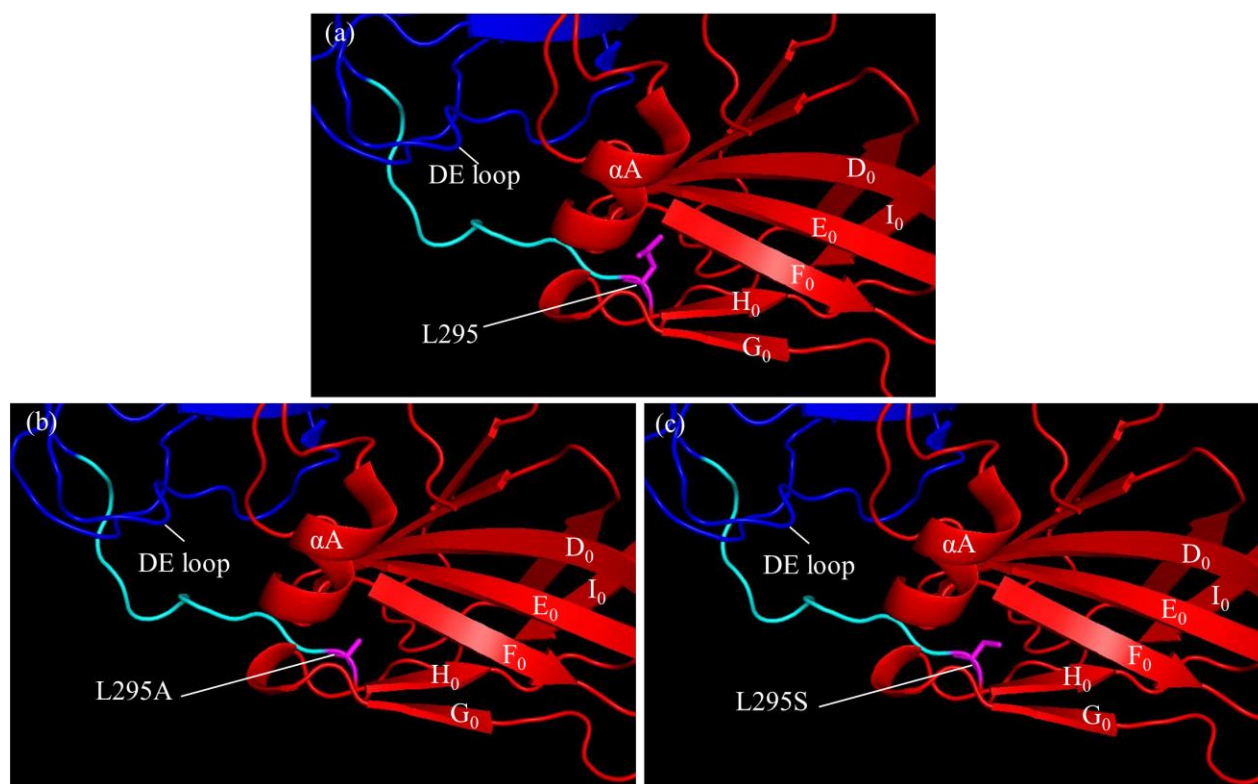


Figure 6-12: Mutations of the WNV E-L295 residue at the N-terminus of the EDI-EDIII linker that produced an attenuated neuroinvasive phenotype (PDB ID: 2HG0)

The E protein monomer is colored in red (EDI), blue (EDIII), and cyan (EDI-EDIII linker). (a) Wild-type leucine residue. (b) Alanine mutation from original alanine screening (c) Serine mutation from optimization of E-L295 amino acid substitution. The image was generated in PyMOL.

Mutation of the E-V304 residue resulted in very different results than the E-L295 residue. The E-V304 residue is at the C-terminus of the EDI-EDIII linker and is connected to the A β -strand of EDIII. This region of the ED-EDIII linker is more intrinsically disordered than the N-terminus of the EDI-EDIII linker, presumably becoming more tolerant to amino acid substitutions. Viable E-V304 mutants include 17 of 19 alternative amino acid substitutions, most of which had no demonstrable difference in infectivity compared to the parental WNV NY99ic. This high

sequence plasticity can be explained by the relative position of the E-V304 residue to the BC and DE loops in the upper lateral surface of EDIII, which has a high level of sequence variation between different flaviviruses. The structural plasticity of the upper lateral surface of EDIII can likely accommodate the respective E-V304 mutations without significantly impairing the structure-function of the E protein.

The E-V304N mutant is an exception and exhibited reduced infectivity in transfected Vero cells and a partially attenuated phenotype in mice (Figure 6-13). This is consistent with the lethal phenotype exhibited by the E-V304D mutation because asparagine and aspartic acid are structurally similar and only differ in the amide or hydroxyl group at the end of the side chain.

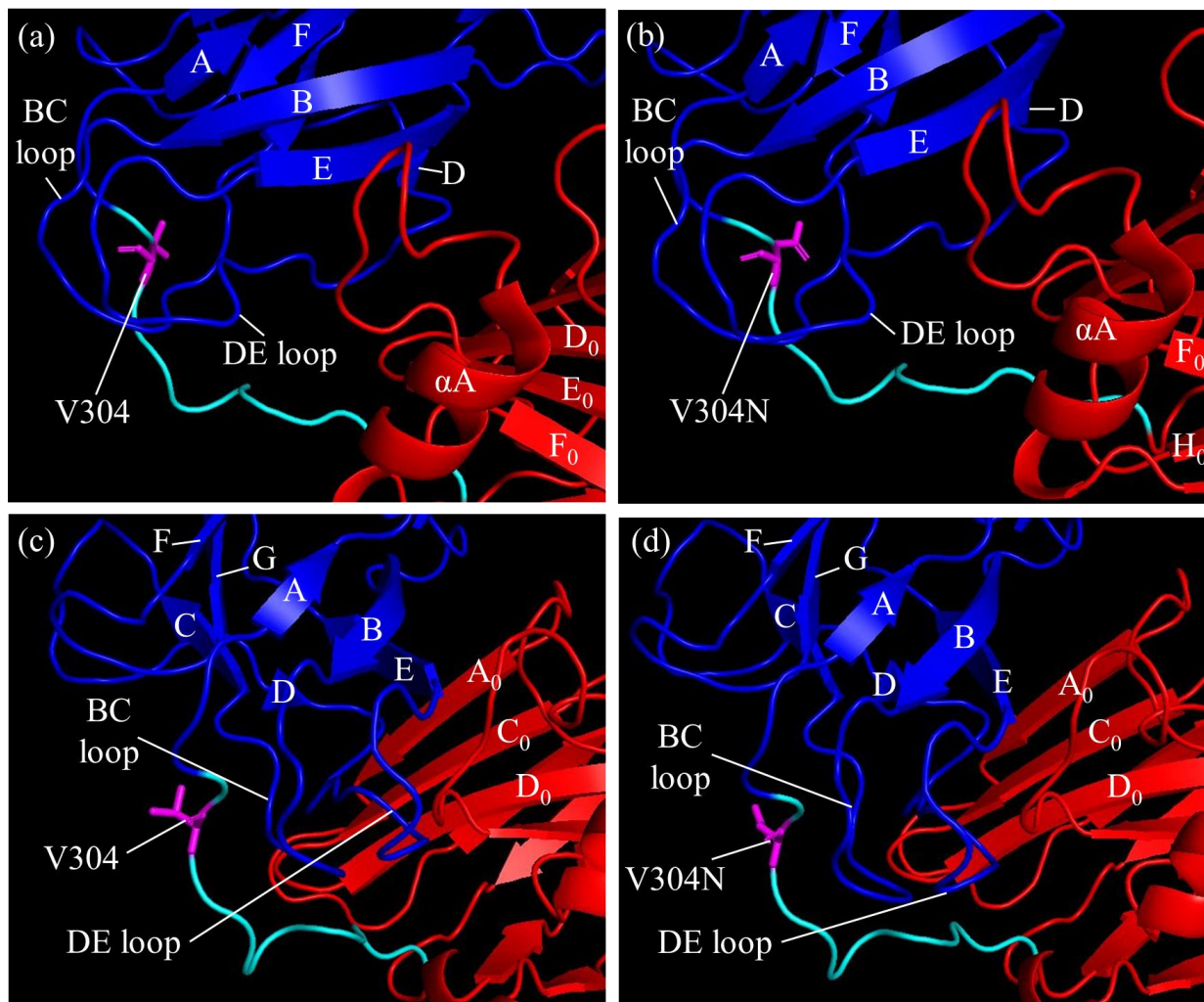


Figure 6-13: Mutations of the WNV E-V304 residue of the EDI-EDIII linker (PDB ID: 2HG0)

The E protein monomer is colored in red (EDI), blue (EDIII), and cyan (EDI-EDIII linker). The (a) and (b) images represent the view of the E-V304 residue from the top of the ABDE and CFG β -sheets, with (a) being the wild-type valine residue and (b) being the asparagine mutation. The (c) and (d) images represent the view of the E-V304 residue from the side of the ABDE and CFG β -sheets, with (c) being the wild-type valine residue and (d) being the asparagine mutation. The image was generated in PyMOL.

Chapter 7 - Discussion and Future Directions

The research described in this dissertation has provided the basis for a flavivirus-common attenuation strategy, targeting the flavivirus-conserved residues in the EDI-EDII hinge region and the EDI-EDIII linker. Further, mutations engineered in the EDI-EDII hinge region do not compromise the immunogenicity of WNV, suggesting the presentation of at least some neutralizing epitopes in the E protein is maintained. As a proof-of-concept, we successfully translated the fully attenuated phenotype from WNV to YFV, using mutations in the third motif of the EDI-EDII hinge region. Therefore, the EDI-EDII hinge region is a putative region for the engineering of attenuating mutations for a broad range of flaviviruses.

We have also shown mutation of at least one flavivirus-conserved residue in the EDI-EDIII linker can fully attenuate the mouse neuroinvasive phenotype of WNV. The EDI-EDIII linker controls the arbovirus-conserved interdomain movement leading to the metastable E protein trimer. Therefore, WNV provides a proof-of-concept for a potential common attenuation strategy for arboviruses based on the conserved structure-function of class II fusion proteins involved in the membrane fusion process. Comparison of the WNV and YFV mutants generated in this project highlights several motifs crucial for the structure-function of the E protein involved in the membrane fusion process. It warrants detailed analysis to provide a complete picture of the flavivirus cell entry mechanism.

A. Structural variations of the EDI-EDII hinge region between different flaviviruses need to be considered for the rational design of candidate live-attenuated vaccines

In contrast to the sequence homology, the orientation of the EDI-EDII hinge region differs significantly between different flaviviruses. West Nile virus stands out from other flaviviruses in

the EDI-EDII hinge region. Surprisingly, the most considerable variation known to date is approximately a 16° difference in the orientation of the EDI-EDII hinge region between WNV and JEV, both of which are in the JEV serocomplex (Luca et al. 2012). This comparison was made before the crystal structure of the YFV E protein became available (Lu et al. 2019). Based on the superimposed E protein structure between WNV, YFV, DENV-2, TBEV, JEV, and ZIKV, the orientation of the EDI-EDII hinge region may have a greater difference between WNV and YFV. Because superimposition of the E protein placed the EDI-EDII hinge region at a nearly identical angle, the difference in the orientation of the EDI-EDII hinge region leads to different positions of the fusion loop within EDII (Figure 7-1). This not only explains the different attenuating effect of the E-A54S mutation between WNV and YFV but also provides a hypothesis for the failure of translating the attenuated phenotype from JEV to WNV, using the equivalent mutations in or near the EDI-EDII hinge region. Specifically, the E-E138K mutation near the second motif contributes to the attenuated phenotype of the JEV SA14-14-2 vaccine strain but is unstable in WNV NY99ic (Wang et al. 2017, Kaiser et al. 2019b).

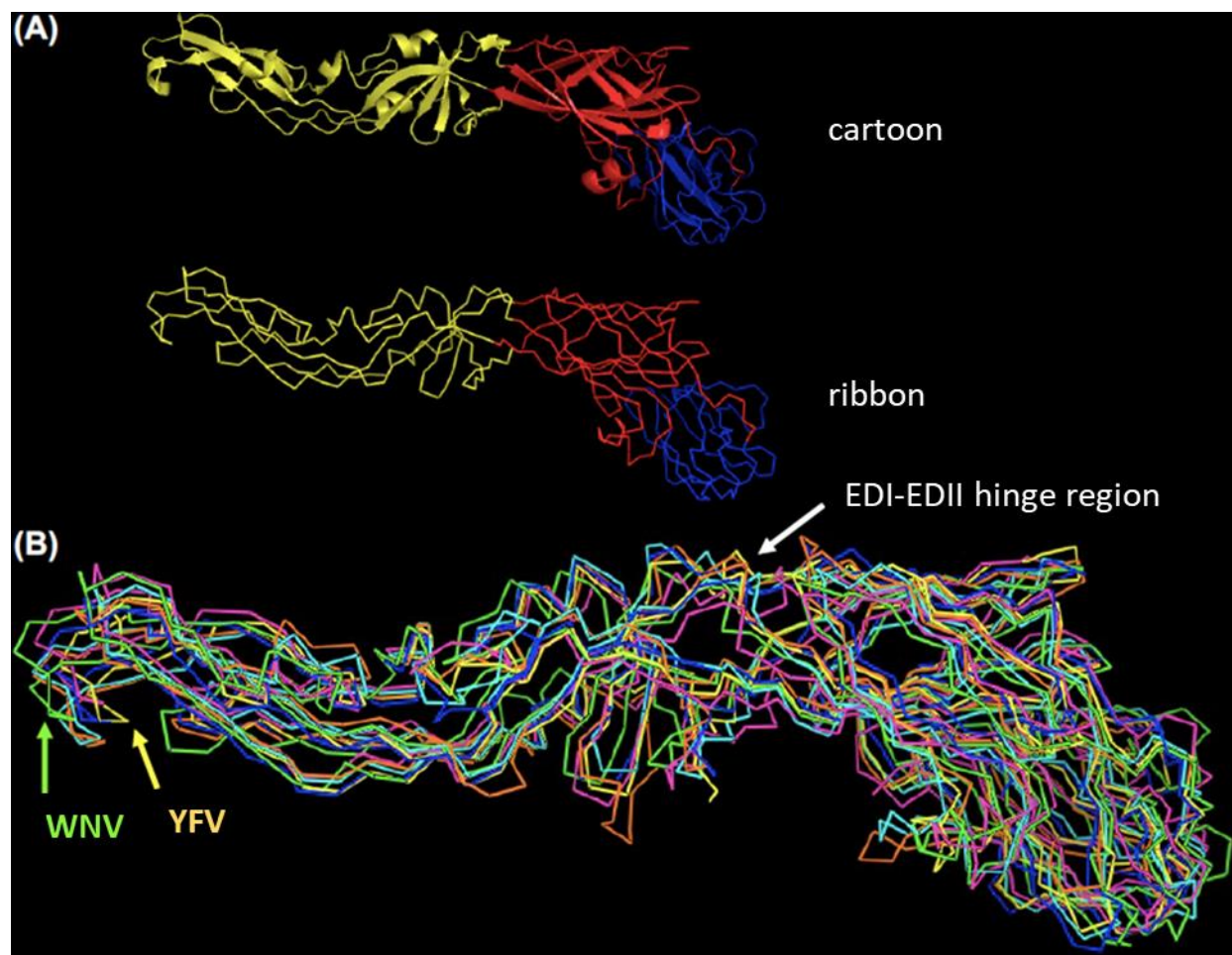


Figure 7-1: Comparison of different flavivirus E protein crystal structures

(a) Cartoon and ribbon diagram of WNV E protein crystal structure (PDB ID: 2HG0). The EDI-EDII hinge region is between EDI, shown in red, and EDII, shown in yellow. (b) Superimposed E protein crystal structures of WNV (green), YFV (yellow, PDB ID: 6IW5), DENV-2 (cyan, PDB ID: 1OAN), TBEV (orange, PDB ID: 1SVB), JEV (magenta, PDB ID: 5MV1), and ZIKV (blue, PDB ID: 5JHM). The location of the EDI-EDII hinge region is highlighted with a white arrow. Yellow and green arrows highlight the tip of EDII in the YFV and WNV E proteins, respectively. The image was generated in PyMOL.

The lack of stability of the JEV E-E138K mutation in WNV also raises an important point that the application of attenuating mutations in the E protein of the JEV SA14-14-2 vaccine strain

to the rational design of candidate WN LAVs may need to be carefully revisited. There is no coincidence at least three mouse virulence phenotype determinants in the E protein of the JEV SA14-14-2 strain have putative importance for the membrane fusion mechanism, including the E-L107F mutation in the fusion loop and the E-E138K and E-Q264H mutations near the EDI-EDII hinge region (Figure 7-2) (Gromowski et al. 2015a). The introduction of the E-L107F and E-E138K mutations individually to the equivalent residues of WNV E protein did not achieve a significant attenuation level. It can be associated with reversing the wild-type sequence (Zhang et al. 2006, Kaiser et al. 2019b).

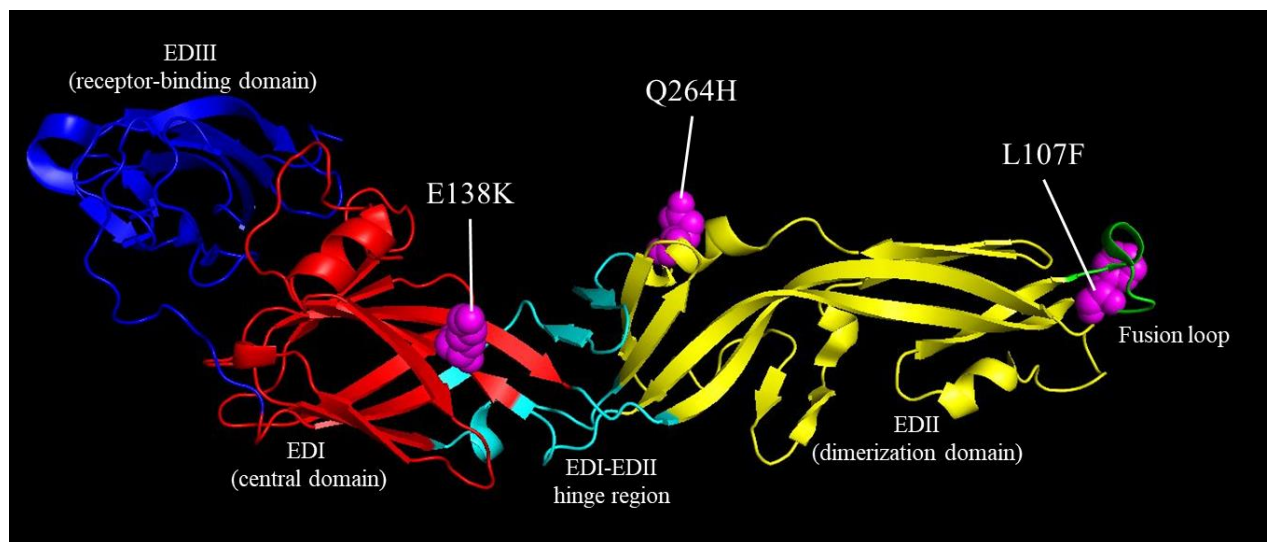


Figure 7-2: Locations of the E-L107F, E-E138K, and E-Q264H virulence determinants in the JEV SA14-14-2 E protein (PDB ID: 3P54)

The E protein monomer is colored red (EDI), yellow (EDII), blue (EDIII), cyan (EDI-EDII hinge region), and green (fusion loop). The JEV SA14-14-2 E-L107F, E-E138K, and E-Q264H virulence determinants are highlighted in magenta. The image was generated in PyMOL.

A putative hypothesis is a more complicated interaction may involve the attenuated phenotype and stability associated with the JEV SA14-14-2 strain E protein mutations. The previous success of applying the attenuating mutations in the E protein of JEV SA14-14-2 strain

to further attenuate the mouse neuroinvasive phenotype of ChimeriVax-WN01 may also be due to the attenuated YFV 17D backbone that limits the multiplication of the resulting ChimeriVax-WN02 in mice (Arroyo et al. 2004). Furthermore, the E protein attenuating mutations, including the E-L107F mutation, further reduced the multiplication level. Broadly, this also makes the application of the YFV E-G52R and E-A54V mutations in the first motif of the EDI-EDII hinge region of the 17D vaccine strain and FNV strain, respectively, to the rational design of candidate flavivirus LAVs (Wang et al. 1995, Huang 2014). It is important to note that YFV stands out from the antigenicity of other mosquito-borne flaviviruses, supported by its position in the phylogenetic tree produced using the E protein sequence (Gould and Solomon 2008, Beck et al. 2014, Beck et al. 2018).

B. Attenuating mutations in the EDI-EDIII linker of WNV and formation of the E protein trimer

We have evaluated the 19 alternative amino acid substitutions of the E-L295 and E-V304 residues in the EDI-EDIII linker and believe fundamental knowledge is generated beyond the attenuated phenotype of WNV. As discussed in section 6.2, one potential explanation combining the attenuating effect of mutations in the EDI-EDIII linker and the available structures of E protein pre-fusion and post-fusion conformations, we hypothesize the attenuated phenotype of mutants containing amino acid substitutions in the EDI-EDIII linker is due to interference with the formation of the E protein trimer. Specifically, amino acids equivalent to the WNV E-L295 residue may play an important role in the interaction(s) with the A₀ and C₀ β -strands of EDI. We observed a putative preference for a hydrophobic amino acid in the position of WNV E-L295 residue to maintain the structure-function of the E protein. Furthermore, this putative preference can explain why the YFV 17D vaccine strain encodes the E-M299I mutation in the EDI-EDIII linker. Due to

the conserved hydrophobic biochemical property and side chain structure, the methionine residue was replaced with isoleucine. Importantly, methionine often is a preferred hydrophobic residue at the protein interface (Mbaye et al. 2019). The most notable example is the ability of methionine to stabilize protein structures via interactions with amino acids containing aromatic-ring side chains, consistent with the strictly conserved E-F11 residue in the A₀ β -strand, which can potentially interact with the EDI-EDIII linker (Modis et al. 2004).

C. Selection of attenuating mutations in the E protein interdomain regions with utility for the rational design of candidate LAVs

In addition to the identification of mutations that can attenuate the virulence phenotype of multiple flaviviruses, a continuation of this project will also need 1) to demonstrate that the mutations in the EDI-EDII hinge region and the EDI-EDIII linker are stable and compatible with mutations in other viral proteins and can achieve a safe level of attenuation by multigenic mutations and 2) to investigate the molecular basis of immunogenicity, using fully attenuated mutants that contain amino acid substitutions of flavivirus conserved residues in the EDI-EDII hinge region and EDI-EDIII linker. Mutations leading to the balance of attenuation and immunogenicity will directly apply to the rational design of candidate flavivirus LAVs.

However, these mutations were designed based on the structure and function of the E protein involved in virus membrane fusion, which is the viral entry process. The cryo-EM structure has also demonstrated that the EDI-EDII hinge region and EDI-EDIII linker are involved in virion maturation and assembly. However, a previously published study demonstrated that mutations in the EDI-EDIII linker could impair the virion assembly of DENV-2 (de Wispelaere and Yang 2012). Therefore, we cannot rule out the possibility that the mutations designed for this study may lead to attenuation through disruption of virion assembly in addition to disruption of viral

membrane fusion. Many of these mutations remain to be investigated using serially passaged viruses, with next-generation sequencing being used to discover compensatory mutations that emerge as SNVs and become consensus sequences. Individually examining these compensatory mutations does not increase the virulence phenotype. As such, it is important to incorporate the seed lot manufacturing system to ensure the attenuated phenotype is also stable.

As observed with the manufacturing of the YFV 17D, Dengvaxia, and DENVax LAVs, it is expected that rationally designed flavivirus LAVs must utilize a seed lot system following rescue via transfection of Vero cells. The sequence of LAVs can be determined to ensure no reversion to the wild-type sequence encoding the virulence phenotype. We have demonstrated that the E-A54I, E-Y201P, and E-L295S mutations can fully attenuate the virulence phenotype of WNV NY99ic and be retained in the consensus sequence. Similarly, the YFV E-V188T mutation is also retained in the consensus sequence. However, serial passage of the fully attenuated WNV E-Y201P mutant resulted in the loss of the attenuating mutation. Furthermore, the E-Y201P mutant engineered with the most preferred proline codon by WNV was only partially attenuated. Because the CCC codon encodes the fully attenuated E-Y201P mutant, it will be helpful to investigate whether or not the remaining CCU and CCG proline codon can fulfill the requirement of full attenuation and genetic stability.

This dissertation research focused on site-directed mutagenesis of flavivirus-conserved residues in the E protein interdomain regions. Importantly, we have demonstrated that both the fully attenuated E-A54I and E-Y201P WNV mutants are immunogenic and can elicit neutralizing antibody responses in mice. Neither of the fully attenuated mutants contains mutations in NS proteins. Therefore, we also anticipate the attenuation strategy established in this project will not compromise T-cell responses. Indeed, it will be worthwhile to examine each fully attenuated

mutant using monoclonal antibodies targeting the EDI-EDII hinge region and the EDI-EDIII linker. This will provide direct evidence that neutralizing epitopes can still be appropriately presented to elicit immune responses.

D. Translation of the attenuated phenotype from mosquito-borne flaviviruses to tick-borne flaviviruses

Fundamentally there are differences in the structure and function of the E protein between mosquito-borne and tick-borne flaviviruses (Figure 7-3). While the generalized functions of the E proteins are conserved, the details of how the E protein conformational change takes place between mosquito and tick-borne flaviviruses show differences. For example, one striking difference is the conformational switching of the peripheral and transmembrane helices in the M proteins of the virion structure to compensate for a lower number of hydrogen bonding contacts in the E proteins of TBEV compared to other mosquito-borne flaviviruses. The changes in the M protein play an essential role in stabilizing the E protein dimer for TBEV (Pulkkinen et al. 2022).

Although there are slight differences in the function of the E proteins, tick-borne flaviviruses and mosquito-borne flaviviruses share sequences in their interdomain regions, as seen in sections 3.1.C and 4.1.C. The tick-borne viruses share identical conserved residues in the EDI-EDII hinge region and EDI-EDIII linker as the mosquito-borne flaviviruses. Since the attenuation strategy of mutating the conserved residues in the interdomain regions is translatable between WNV and YFV, the attenuation strategy can also be translated to tick-borne flaviviruses. We have seen differences between WNV and YFV, presumably because of structural variation. We would likely see different mutations or residues producing an attenuated phenotype, especially with the functional differences between tick-borne and mosquito-borne flaviviruses (Pulkkinen et al. 2018).

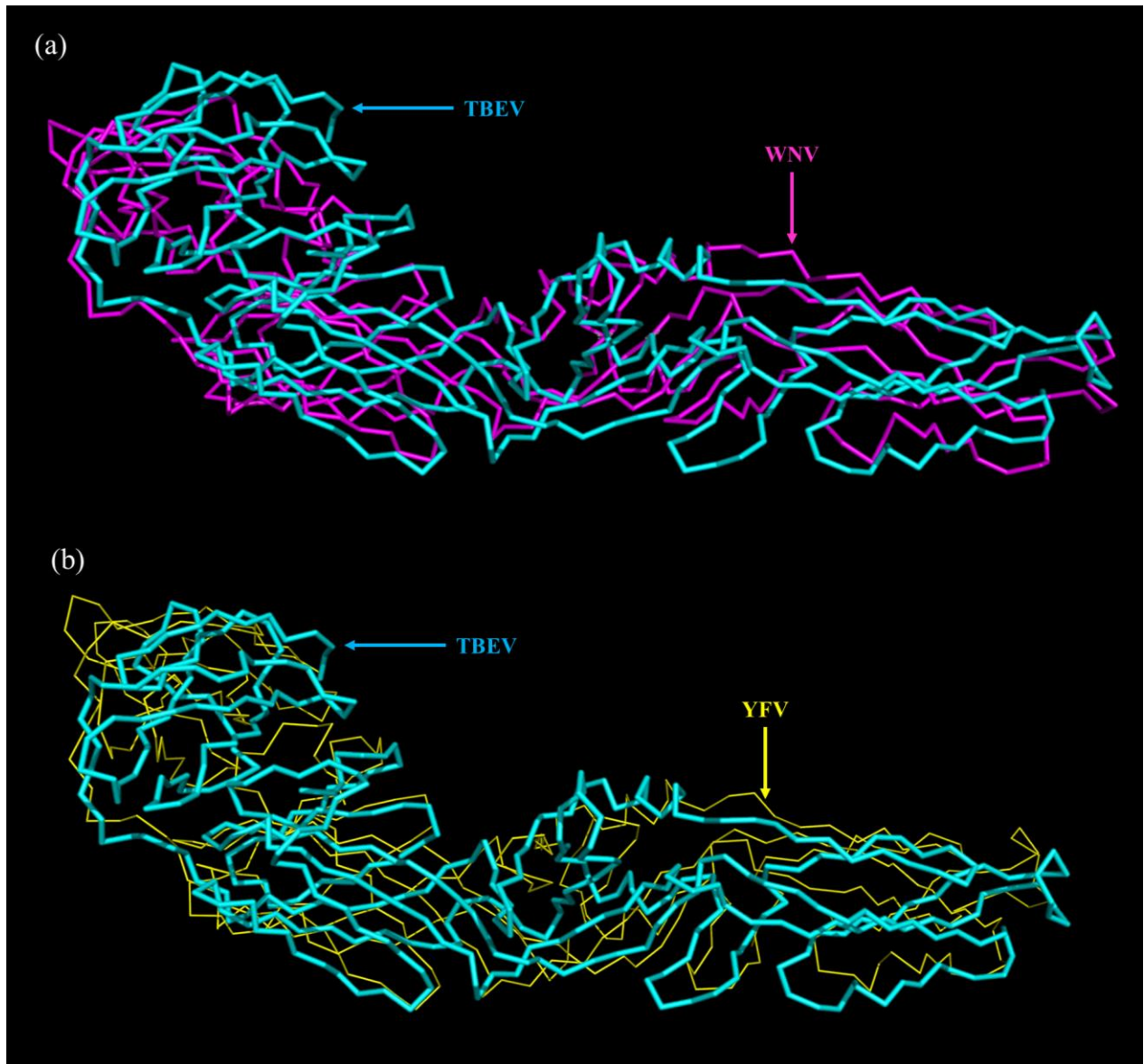


Figure 7-3: Comparison of the E protein crystal structure of mosquito-borne flaviviruses and tick-borne flaviviruses

(a) Superimposed images of the E protein of TBEV (PDB ID: 7Z51) and WNV (PDB ID: 2HG0) in its prefusion conformation. (b) Superimposed images of the E protein of TBEV (PDB ID: 7Z51) and YFV (PDB ID: 6IW5) in its prefusion conformation. The image was generated in PyMOL.

E. Future directions for the development of a broadly effective attenuating strategy based on the common structure-function of class II fusion proteins

The results of this dissertation have provided the molecular basis for the attenuation caused by mutations in the interdomain regions of the flavivirus E protein. However, those results are limited to only the JEV and YFV serocomplexes. The EDI-EDII hinge region and the EDI-EDIII linker contain conserved residues that are conserved across all flaviviruses, i.e., not limited to two of the serocomplexes tested in this dissertation (Table 7-1) (Bressanelli et al. 2004, Modis et al. 2004). The attenuation process could be adapted to work with other mosquito-borne flaviviruses, such as DENV and tick-borne flaviviruses. A handful of residues, especially within the EDI-EDIII linker, are strictly conserved across all flaviviruses, including the WNV E-A54 residue. A fully attenuated phenotype was discovered by optimizing the amino acid substitution. Additional experiments into the YFV E-A54 residue and its equivalent residues in any other flavivirus may all yield a fully attenuated phenotype in mice.

Table 7-1: Conserved sequences of the EDI-EDII hinge region and EDI-EDIII linker across the different serocomplexes of flaviviruses

The properties of amino acids are shown in green (hydrophobic), pink (polar), red (negatively charged), blue (positively charged), and black (unique). Underlines and bolded residues are highly conserved residues. The numbering corresponds to amino acids in the WNV E protein.

Sero-complex	Virus	EDI EDII Hinge Region				EDI-EDIII Linker (295-305)
		Motif #1 (51-54)	Motif #2 (130-135)	Motif #3 (191-201)	Motif #4 (269-281)	
JEV	WNV	<u>ANLA</u>	<u>ILKENI</u>	<u>EPRSGIDTNA-Y</u>	<u>AIPVEFS</u> --- <u>SNTVKL</u>	<u>LQLKGT</u> <u>TYGVC</u>
	JEV	<u>SQLA</u>	<u>IQPENI</u>	<u>EPRSGINTEA-F</u>	<u>AIVVEYS</u> --- <u>S-SVKL</u>	<u>LALKGT</u> <u>TYGMC</u>
	KNV	<u>ANLA</u>	<u>ILKENI</u>	<u>EPRSGIDTNA-Y</u>	<u>AIPVEFS</u> --- <u>SNTVKL</u>	<u>LQLKGT</u> <u>TYGVC</u>
	SLEV	<u>TELA</u>	<u>ILRENI</u>	<u>EARSGINTEA-Y</u>	<u>AIPATVS</u> --- <u>SSTLT</u>	<u>VKIKGT</u> <u>TYGMC</u>
YFV	YFV	<u>DGPA</u>	<u>VDQTKI</u>	<u>QVQTAVDFGNSY</u>	<u>AMRVTKDTNDNNLYKL</u>	<u>LT</u> <u>TLKG</u> <u>TSYKMC</u>
DENV	DENV-1	<u>TNPA</u>	<u>VQYENL</u>	<u>SPRTGLDFNE-M</u>	<u>ATEIQTS</u> --- <u>GT-TTI</u>	<u>LT</u> <u>TLKG</u> <u>MSYVMC</u>
	DENV-2	<u>KQPA</u>	<u>VQPENL</u>	<u>SPRTGLDFNE-M</u>	<u>ATEIQMS</u> --- <u>SG-NLL</u>	<u>LQLKGM</u> <u>SYSMC</u>
	DENV-3	<u>TQLA</u>	<u>VQHENL</u>	<u>SPRTGLDFNE-M</u>	<u>ATEIQTS</u> --- <u>GG-TSI</u>	<u>LE</u> <u>TLKG</u> <u>MSYAMC</u>
	DENV-4	<u>KEVA</u>	<u>VQIENL</u>	<u>EPRSGIDFNE-M</u>	<u>ATEVDSG</u> --- <u>DG-NHM</u>	<u>LR</u> <u>IKGM</u> <u>SYTMC</u>
TBEV	TBEV	<u>ENPA</u>	<u>YDANKI</u>	<u>RVASGVDLAQTV</u>	<u>VPVAHIE</u> --- <u>GTKYHL</u>	<u>LKMKGL</u> <u>TYTMC</u>
	POWV	<u>ESPA</u>	<u>YDSTKI</u>	<u>KVASGIDVAQTV</u>	<u>VPLASVE</u> --- <u>GQKYHL</u>	<u>LK</u> <u>IKGT</u> <u>TYSMC</u>
	OHFV	<u>ENPA</u>	<u>YDANKI</u>	<u>RVASGVDLAQTV</u>	<u>VPVAHIE</u> --- <u>GTKYHL</u>	<u>LKMKGL</u> <u>TYTMC</u>

Available crystal structures of alphavirus E1 protein and bunyavirus Gc protein showed the EDI-EDII hinge region and the EDI-EDIII linker play a crucial role in the structure-function of class II fusion proteins. Stabilizing the extended trimeric intermediate of all three viral proteins involves the relocation of EDIII towards the gravity center (Dessau and Modis 2013). Thus, there is a putative common mechanism underlying the formation of a stable post-fusion trimer shared by flavivirus E protein, alphavirus E1 protein, and bunyavirus Gc protein, as observed with the

presence of the homologous EDI-EDII hinge region and EDI-EDIII linker (Figures 7-4, 7-5, and 7-6).

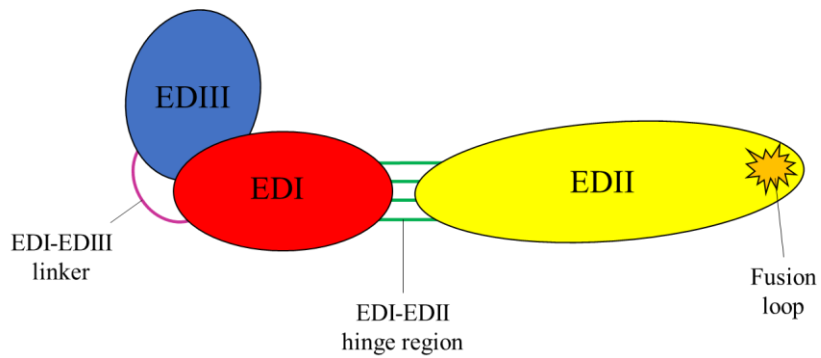


Figure 7-4: Cartoon depiction of the class II fusion protein in the pre-fusion conformation

The image was generated in PowerPoint.

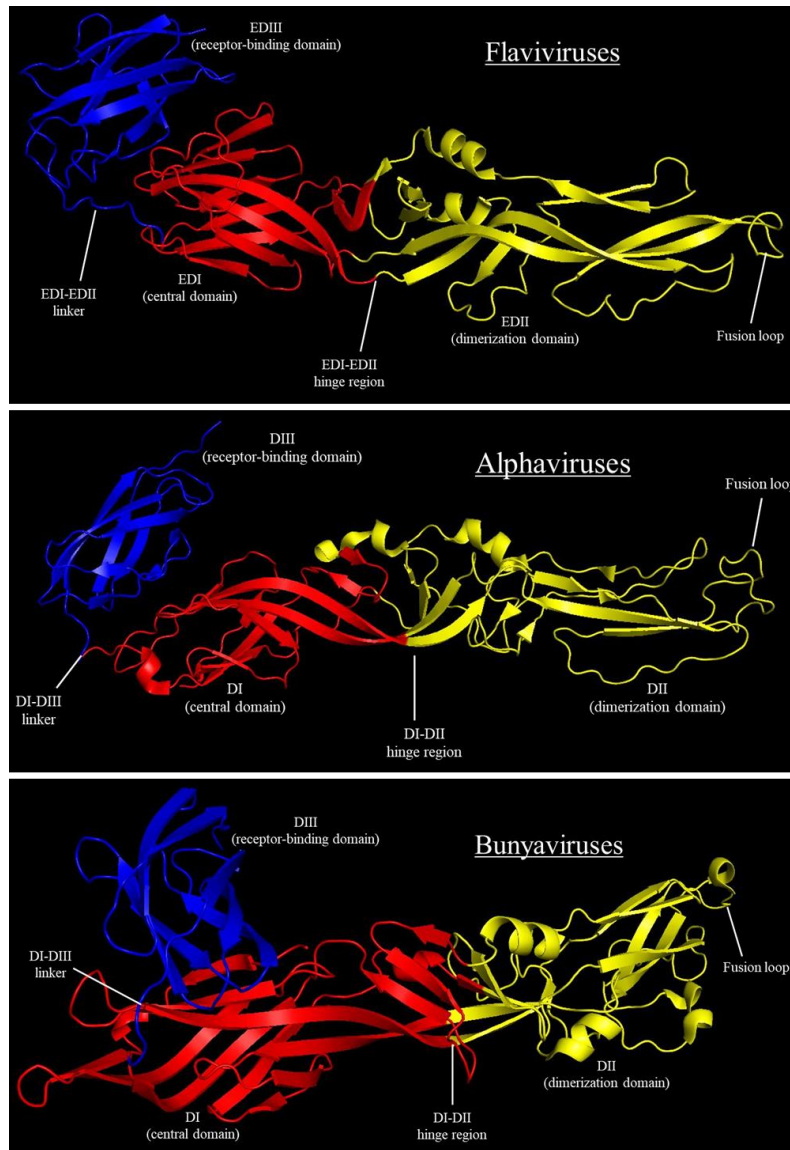


Figure 7-5: Comparison of the flavivirus E protein, alphavirus E1 protein, and bunyavirus Gc protein in its pre-fusion conformation

The three domains are labeled in red for DI, yellow for DII, and blue for DIII. The flavivirus glycoprotein is based on the DENV-2 E protein (PDB ID: 1OAN), the alphavirus glycoprotein is based on the Semliki Forest virus (SFV) E1 protein (PDB ID: 2ALA), and the bunyavirus glycoprotein is based on the Rift Valley fever virus (RVFV) Gc protein (PDB ID: 4HJ1). The image was generated in PyMOL.

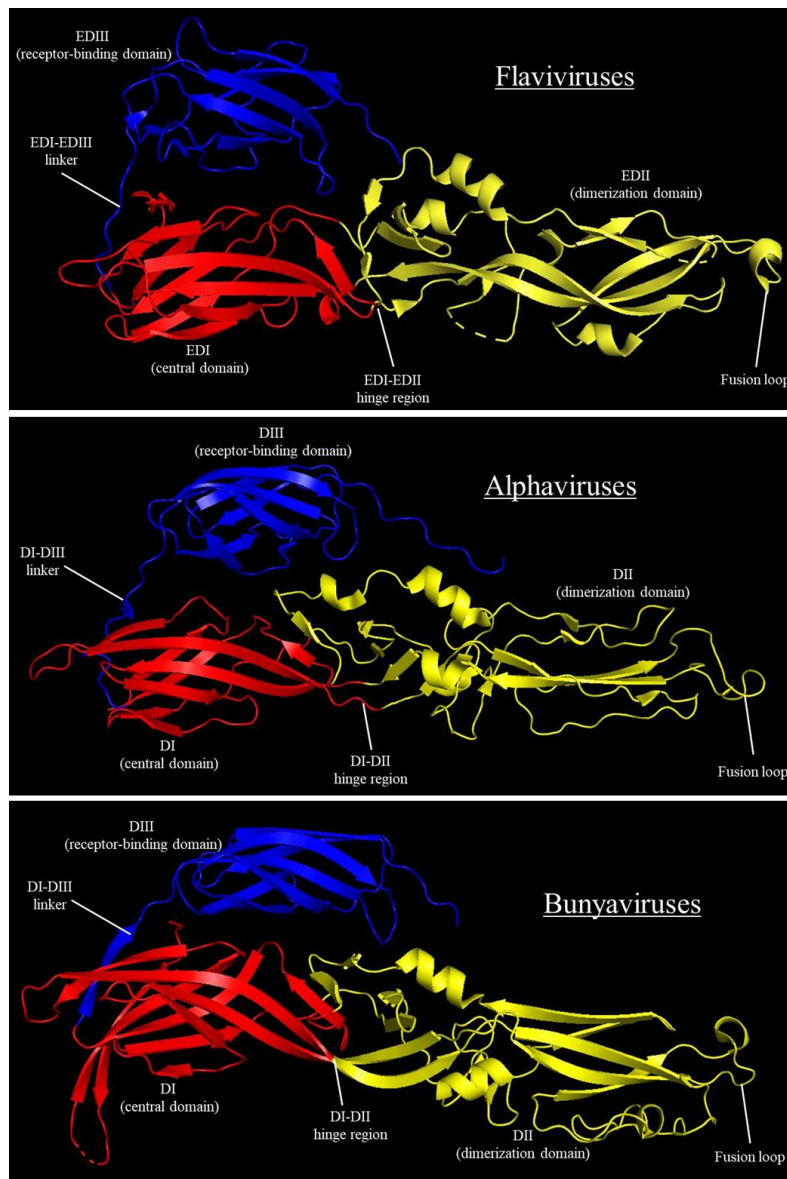


Figure 7-6: Comparison of the flavivirus E protein, alphavirus E1 protein, and bunyavirus Gc protein in its post-fusion conformation

The three domains are labeled in red for DI, yellow for DII, and blue for DIII. The flavivirus glycoprotein is based on the DENV-2 E protein (PDB ID: 3G7T), the alphavirus glycoprotein is based on the Semliki Forest virus (SFV) E1 protein (PDB ID: 8D87), and the bunyavirus glycoprotein is based on the Rift Valley fever virus (RVFV) Gc protein (PDB ID: 6EGU). The image was generated in PyMOL.

It is important to note that the role of the gfeah and kID₀ β -sheets in governing the EDI-EDII interdomain movement and the stabilization movement of the EDI-EDIII linker is conserved between class II fusion proteins. The structural alignment shows the corresponding residues in alphavirus E1 and bunyavirus Gc proteins (Figure 7-7). Therefore, these residues can be the targets for the engineering of mutations that interfere with the membrane fusion process to develop a common attenuation process for arboviruses in different genera (Gibbons et al. 2004, Zheng et al. 2011, Dessau and Modis 2013, Guardado-Calvo et al. 2016, Serris et al. 2020).

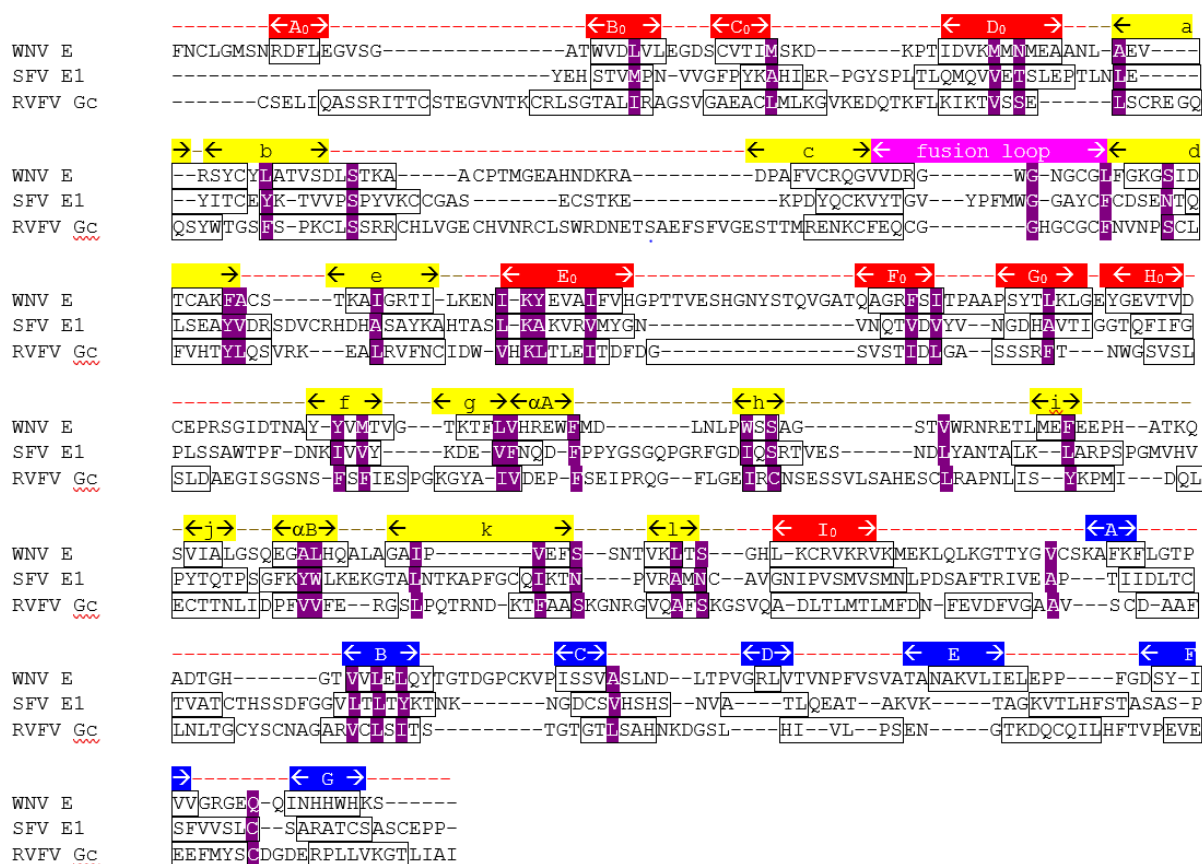


Figure 7-7: Structural homology between the glycoproteins of different arboviruses

A protein alignment between the flavivirus E protein (WNV), the alphavirus E1 protein (SFV), and the bunyavirus Gc protein (RVFV). Colored notations above the sequence represent the homologous secondary protein structure of the glycoproteins in the prefusion structure, with red for domain I, yellow for domain II, and blue for domain III. Dark purple represents residues that have conserved biochemical properties or conserved amino acids across the different arbovirus. The image was generated in Word.

In addition to the structural alignment between the glycoproteins of different arbovirus families, alphavirus E1 proteins and bunyavirus Gc proteins also have highly conserved amino acid residues in their interdomain regions. For example, the EDI-EDIII linker of alphavirus E1 has three strictly conserved residues across all alphaviruses, represented by the SFV E1-F287, E1-

R289, and E1-P294 residues (Table 7-2). The SFV E1-E292 residue is another highly conserved residue across all alphaviruses. The E1-E292 residue represents negatively charged acidic biochemical properties with glutamic acid represented for SFV and New World alphaviruses and aspartic acid for all other equivalent residues of the alphavirus E1 protein. Four amino acids between the SFV E1-P283 and E1-A286 residues exhibit a highly conserved consensus sequence between alphaviruses. All alphaviruses share the SFV E1-P283 residue except for the Mayaro virus (MAYV), which possesses the E1-A284 residue. The SFV E1-D284 residue represents the equivalent amino acids' negatively charged acidic biochemical properties in the alphavirus E1 protein except for the Sindbis virus (SINV) E1-N284 residue. The SFV E1-S285 residue is representative of the shared Barmah Forest virus (BFV) E1-S283 residue. In contrast, chikungunya virus (CHIKV), O'nyong'nyong virus (ONNY), and all New World encephalitic alphaviruses encode an alanine in the equivalent position of the E1 protein. The SFV E1-A286 residue is conserved across all alphaviruses except for the Venezuelan equine encephalitis virus (VEEV) E1-L286 residue, which exhibits the shared hydrophobic biochemical property.

Table 7-2: Sequences of the DI-DIII linker in the alphavirus E1 proteins

Highly conserved amino acids are labeled with bold text and underlined. Highly conserved amino acids are labeled with bold text and underlined. The properties of amino acids are shown in green (hydrophobic), pink (polar), red (negatively charged), blue (positively charged), and black (unique). The numbering of amino acids in the motif is based on the SFV sequence. GenBank protein ID: SFV-AAK66741.1, Ross River virus (RRV)-UQW95928.1, CHIKV-WFP31717.1, O'nyong'nyong virus (ONNY)-AAA46784.1, MAYV-AZM66146.1, BFV-QVM797755.1, SINV-AWT57845.1, Western equine encephalitis virus (WEEV-QEX51909.1, Eastern equine encephalitis virus (EEEV)-AAU95735.1, and VEEV-QWY73223.1.

Serocomplex	Clade	Virus	Sequence of DI-DIII linker (E1 283-294)
SFV	Old World	SFV	PDS <u>AFT</u> <u>RI</u> <u>VE</u> <u>AP</u>
		RRV	PDS <u>AFT</u> <u>RV</u> <u>VD</u> <u>AP</u>
		CHIKV	PEA <u>AFT</u> <u>RV</u> <u>VD</u> <u>AP</u>
		ONNV	PDA <u>AFT</u> <u>RV</u> <u>TD</u> <u>AP</u>
	New World	MAYV	ADS <u>AFT</u> <u>RL</u> <u>TD</u> <u>AP</u>
BFV	Old World	BFV	PDS <u>AFT</u> <u>RV</u> <u>VD</u> <u>AP</u>
WEEV	Old World	SINV	PNA <u>AFT</u> <u>RI</u> <u>TS</u> <u>DA</u> <u>AP</u>
	New World	WEEV	PDA <u>AFV</u> <u>RS</u> <u>ES</u> <u>AP</u>
EEEV	New World	EEEV	PDA <u>AFT</u> <u>RI</u> <u>SE</u> <u>TP</u>
VEEV	New World	VEEV	PDA <u>LEF</u> <u>TV</u> <u>SE</u> <u>TP</u>

The bunyaviruses are a diverse group of arboviruses or zoonotic viruses across 63 genera, including hantaviruses, phleboviruses, and orthobunyaviruses. Their EDI-EDIII linker also shows to have genera-specific consensus sequences. Hantaviruses contain four strictly conserved residues, represented by Hantaan virus (HTNV) Gc-D959, Gc-F962, Gc-L965, and Gc-P969

(Table 7-3). An additional four other residues exhibit conservation of the biochemical properties of the residue. The HTNV Gc-K958 is a positively charged lysine, whereas the other equivalent hantavirus residues encode for an arginine. The HTNV Gc-I960 conserves the hydrophobic biochemical properties but utilizes different amino acids, including valine for Sin Nombre virus (SNV) and Lechingunas virus (ECV), and leucine for Puumala virus (PUUV). Residue 967 in HTNV E1 protein is represented by a negatively charged acidic amino acid, including aspartic acid for SNV and LECV and glutamic acid for HTNV and PUUV. All hantaviruses share the final HTNV Gc-N968 residue except for PUUV, which possesses the Gc-T977 residue.

Table 7-3: Amino acid sequences alignment of the DI-DIII linker of hantaviruses

Residues with highly conserved amino acids or biochemical properties within each genus are labeled with bold text and underlined. Highly conserved amino acids are labeled with bold text and underlined. The properties of amino acids are shown in green (hydrophobic), pink (polar), red (negatively charged), blue (positively charged), and black (unique). The numbering of amino acids in the motif is based on the HTNV sequence. GenBank protein ID: HTNV-AAK66741.1, SNV-AAC42202.1, PUUV-AAC37847.1, and LECV-AAB87908.1.

Genus	Virus	Strain	Sequence of DI-DIII linker (SFV Gc 958-969)
Hantavirus	HTNV	76-118	<u>K</u> <u>D</u> <u>I</u> <u>D</u> <u>F</u> <u>D</u> <u>N</u> <u>L</u> <u>G</u> <u>E</u> <u>N</u> <u>P</u>
	SNV	NM R11	<u>R</u> <u>D</u> <u>V</u> <u>S</u> <u>F</u> <u>Q</u> <u>D</u> <u>L</u> <u>S</u> <u>D</u> <u>N</u> <u>P</u>
	PUUV	K27	<u>R</u> <u>D</u> <u>L</u> <u>S</u> <u>F</u> <u>Q</u> <u>D</u> <u>L</u> <u>S</u> <u>E</u> <u>T</u> <u>P</u>
	LECV	Of22819	<u>R</u> <u>D</u> <u>V</u> <u>S</u> <u>F</u> <u>Q</u> <u>D</u> <u>L</u> <u>S</u> <u>D</u> <u>N</u> <u>P</u>

Phleboviruses have five conserved residues, four conserving the hydrophobic biochemical property (Table 7-4). Three strictly conserved residues across the phleboviruses are represented by the RVFV Gc-V1020, Gc-C1029, and Gc-A1031 residues. The RVFV Gc-F1022 residue

represents the hydrophobic biochemical property of the equivalent amino acid in the bunyavirus Gc protein except for the Toscana virus (TOCV) virus Gc-Y989 residue. The RVFV Gc-V1027 residue also represents the hydrophobic biochemical property of the equivalent amino acid in the bunyavirus Gc protein, except for the TOCV virus Gc-F993 residue.

Table 7-4: Amino acid sequences alignment of the DI-DIII linker of phleboviruses

Residues with highly conserved amino acids or biochemical properties within each genus are labeled with bold text and underlined. Highly conserved amino acids are labeled with bold text and underlined. The properties of amino acids are shown in green (hydrophobic), pink (polar), red (negatively charged), blue (positively charged), and black (unique). The numbering of amino acids in the motif is based on the RVFV sequence. GenBank protein ID: RVFV-AYA44411.1, sandfly fever Sicilian virus (SFSV)-AIS25027.1, and TOSV-QIQ30940.1.

Genus	Virus	Strain	Sequence of DI-DIII linker (RVFV Gc 1020-1031)
Phlebovirus	RVFV	RVFBJ01/2016	<u>V</u> <u>D</u> <u>F</u> <u>V</u> GAA <u>V</u> <u>S</u> <u>C</u> <u>D</u> <u>A</u>
	SFSV	Ethiopia-2011	<u>V</u> <u>E</u> <u>F</u> <u>E</u> <u>E</u> <u>E</u> <u>R</u> <u>V</u> <u>S</u> <u>C</u> <u>L</u> <u>A</u>
	TOSV	SI-283660	<u>V</u> <u>V</u> <u>Y</u> <u>Q</u> <u>N</u> <u>S</u> <u>Q</u> <u>F</u> <u>D</u> <u>C</u> <u>S</u> <u>A</u>

Orthobunyaviruses contain four strictly conserved residues, represented by La Crosse virus (LACV) Gc-D1251, Gc-Y1254, Gc-K1255, Gc-F1257, and Gc-D1262 (Table 7-5). Two additional residues exhibit conserved biochemical properties: the LACV Gc-V1252 and Gc-S1260. The LACV Gc-V1252 residue conserved the hydrophobic biochemical properties of the residue utilizing a variety of hydrophobic amino acids, including valine, leucine, and isoleucine. The LACV Gc-S1260 and its equivalent residues are represented by polar biochemical properties with a serine or an asparagine.

Table 7-5: Amino acid sequences alignment of the DI-DIII linker of orthobunyaviruses

Residues with highly conserved amino acids or biochemical properties within each genus are labeled with bold text and underlined. Highly conserved amino acids are labeled with bold text and underlined. The properties of amino acids are shown in green (hydrophobic), pink (polar), red (negatively charged), blue (positively charged), and black (unique). The numbering of amino acids in the motif is based on the LACV sequence. GenBank protein ID: LACV-AAC54270.1, Ngari virus (NRIV)-AGM34030.1, and Oropouche virus (OROV)-UYI36405.1.

Genus	Virus	Strain	Sequence of DI-DIII linker (LACV Gc 1251-1262)
Orthobunyavirus	LACV	LAC22988-89	<u>D</u> <u>V</u> <u>K</u> <u>Y</u> <u>K</u> <u>T</u> <u>F</u> <u>A</u> <u>D</u> <u>S</u> <u>V</u> <u>D</u>
	NRIV	Dakar D28542/4e	<u>D</u> <u>L</u> <u>R</u> <u>Y</u> <u>K</u> <u>S</u> <u>F</u> <u>A</u> <u>E</u> <u>N</u> <u>P</u> <u>D</u>
	OROV	LET-882	<u>D</u> <u>I</u> <u>N</u> <u>Y</u> <u>K</u> <u>I</u> <u>F</u> <u>T</u> <u>K</u> <u>S</u> <u>V</u> <u>D</u>

Our hypothesis can be tested with SFV and RVFV as model systems for alphaviruses and bunyaviruses, respectively. Both SFV and RVFV have excellent animal models to test for attenuation of the virulent phenotype. Specifically, SFV causes neurotropic diseases in BALB/c mice, which exhibit a lethal infection following the IP challenge with the wild-type L10 strain. Therefore, any attenuation to the virulence phenotype will be exhibited by the survival of the infection (Atkins et al. 1985, Atkins and Sheahan 2016). The attenuation process of RVFV can be investigated in rats, which can develop lethal encephalitis after aerosol exposure, allowing detailed studies of the pathogenic events during RVFV encephalitis (Ikegami and Makino 2010, Connors and Hartman 2022). Another reason for utilizing RVFV as a model system for bunyaviruses is RVFV has a conditionally licensed vaccine (MP12) that could be used as a benchmark for any future candidate LAVs to be compared to (Ikegami et al. 2015, Kitandwe et al. 2022).

Specifically, we can target amino acids equivalent to the flavivirus conserved amino acids in the WNV E protein interdomain regions within the SFV E1 and RVFV Gc protein. The alphavirus E1 and bunyavirus Gc proteins resemble the structure-function of flavivirus E protein, including the EDI-EDII hinge region (Figure 7-8). The fg hairpin is present in all three glycoproteins. It contains a set of three hydrophobic residues that act as a hydrophobic triad that mediates the hydrophobic interactions of the EDI-EDII hinge movement. This is observed with the alphavirus-conserved SFV E1-I177, E1-V184, and E1-F185 residues and the bunyavirus-conserved RVFV Gc-F905, Gc-I915, and Gc-V916 residues. Within the body of the fg hairpin also resides a triad of hydrophobic residues that make up the core of the hydrophobic interactions. The A124, L129, and F173 residues of SFV E1 protein and the W855, L859, and F903 residues of RVFV Gc protein resemble the relative location of the I130, I135, and I196 residues central to the WNV EDI-EDII hinge region. There is also proximity between flavivirus-conserved WNV E-A54 and I130 residues, followed by the analogous SFV E1-L44 and E-A124 residues and RVFV Gc-L754 and Gc-W855 residues, connecting the core and peripheral hydrophobic interactions. The E-L281 residue in the 1 β -strand of WNV E protein is also conserved in alphavirus E1 protein and bunyavirus Gc protein, corresponding to the SFV E1-A268 residue and RVFV Gc-A1001 residues (Li et al. 2010, Dessau and Modis 2013).

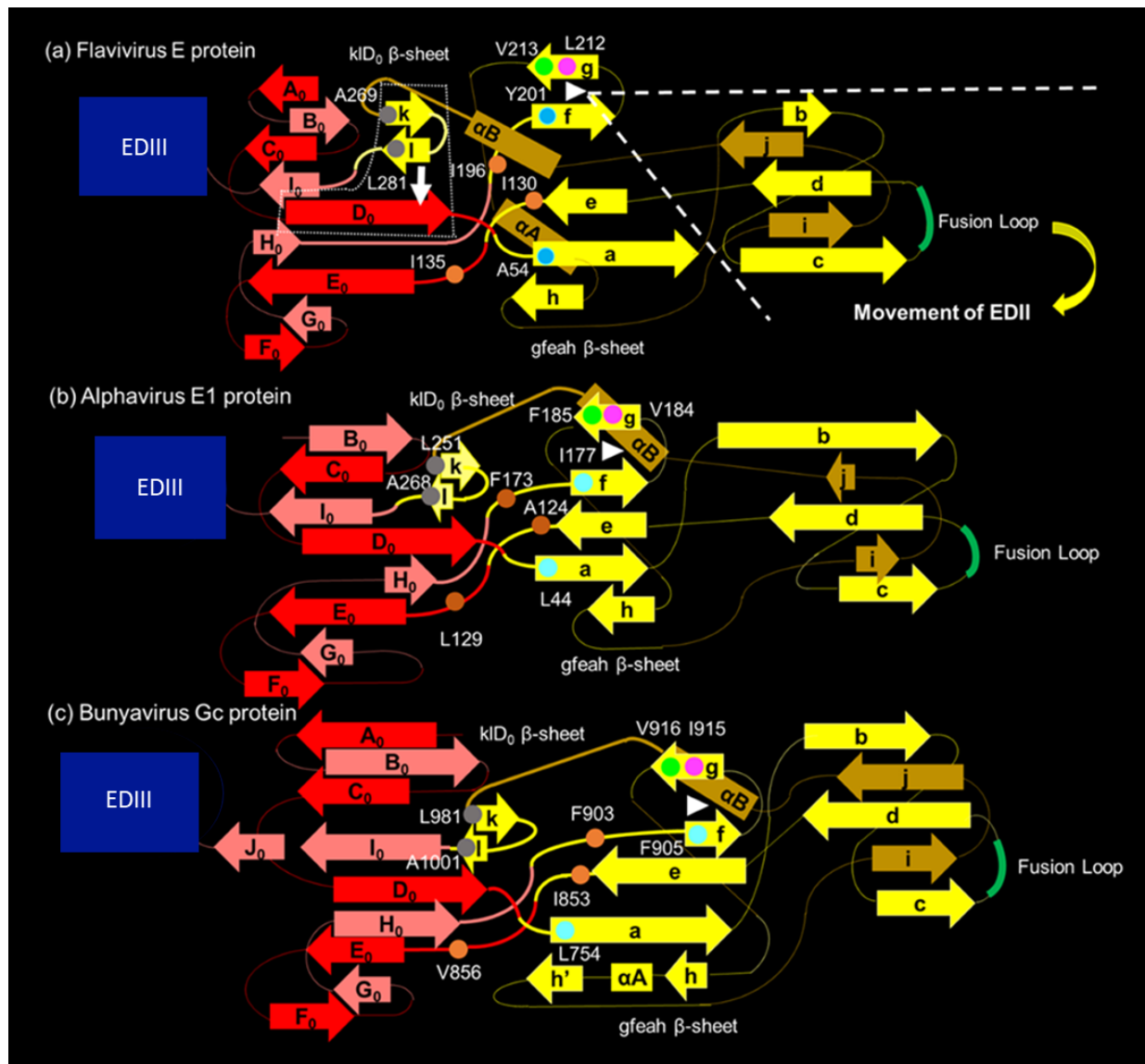


Figure 7-8: Proposed model for the hinge effect to control the EDI-EDII interdomain movement

(a) Represents the proposed role of each residue in exerting the hinge effect at the EDI-EDII interface. The four motifs of the EDI-EDII hinge region are highlighted in thick lines. The WNV E-A54 and E-Y201 residues are at the base of the gfeah β -sheet in the EDII. The E-Y201 residue and the flavivirus conserved E-L212 (magenta) and E-V213 (green) residues form the fg hairpin (white triangle). The gfeah β -sheet rotates along the fg hairpin to change the orientation of EDII (highlighted by the white dash line). The movement of EDII triggers the rotation of the A₀C₀D₀E₀F₀

β -sheet of the EDI. The new conformation of the D0 β -strand is stabilized by the hydrogen bonds with the I β -strand separated from the kl hairpin structure of EDII (indicated by the white arrow). Two other class II fusion proteins, the alphavirus E1 proteins (b) and bunyavirus Gc proteins (c), contain residues corresponding to the seven conserved hydrophobic amino acids in the flavivirus EDI-EDII hinge region. The numbering is based on the SFV E1 protein and RVFV Gc protein. The image was generated in PowerPoint.

The EDI-EDIII linker of flaviviruses E, alphavirus E1, and bunyavirus Gc proteins all resemble a single structurally disorganized motif residing at the base of the protein, farthest away from the fusion loop. Similar movements occur with the EDIII domain moving towards the protein's gravity center using the EDI-EDIII linker's N-terminus as an anchorage point. In the SFV E1 protein, this movement moves the EDIII domain 37 Å towards the center of the protein, whereas the RVFV induces a 132.6° rotation (Gibbons et al. 2004, Guardado-Calvo et al. 2016). At the N-terminus of the EDI-EDIII linker sits conserved hydrophobic residues that initiate hydrophobic bonds with the C₀ β -strand of EDI. These hydrophobic residues are observed in the conserved SFV E1-A286 and E1-F287 residues and the RVFV Gc-V1020 and Gc-F1022 residues. Mutation of these residues could lead to similar attenuated phenotypes exhibited by the WNV E-L295 residue.

In conclusion, the study described in this dissertation has demonstrated that mutations of flavivirus-conserved residues in the E protein interdomain region can provide the basis for attenuation. Understanding the attenuation mechanisms can contribute to the future development of candidate LAVs for flaviviruses and potentially arboviruses of different genera.

References

- AAEP (2022). "Core Vaccination Guidelines." from <https://aaep.org/guidelines/vaccination-guidelines/core-vaccination-guidelines>.
- Acosta-Ampudia, Y., Monsalve, D. M., Rodríguez, Y., Pacheco, Y., Anaya, J.-M. and Ramírez-Santana, C. (2018). Mayaro: an emerging viral threat? *Emerg Microbes Infect* 7(1): 1-11. 10.1038/s41426-018-0163-5
- Akondy, R. S., Monson, N. D., Miller, J. D., Edupuganti, S., Teuwen, D., Wu, H., Quyyumi, F., Garg, S., Altman, J. D., Rio, C. D., Keyserling, H. L., Ploss, A., Rice, C. M., Orenstein, W. A., Mulligan, M. J. and Ahmed, R. (2009). The yellow fever virus vaccine induces a broad and polyfunctional human memory CD8⁺ T cell response. *J Immunol* 183(12): 7919-7930. 10.4049/jimmunol.0803903
- Aktepe, T. E. and Mackenzie, J. M. (2018). Shaping the flavivirus replication complex: It is curvaceous! *Cell Microbiol* 20(8): e12884. 10.1111/cmi.12884
- Aliota, M. T., Jones, S. A., II, A. P. D., Ciota, A. T., Hubalek, Z. and Kramer, L. D. (2012). Characterization of Rabensburg Virus, a Flavivirus Closely Related to West Nile Virus of the Japanese Encephalitis Antigenic Group. *PLoS One* 7(6): e39387. 10.1371/journal.pone.0039387
- Allison, S. L., Schlich, J., Stiasny, K., Mandl, C. W. and Heinz, F. Z. (2001). Mutational Evidence for an Internal Fusion Peptide in Flavivirus Envelope Protein E. *J Virol* 75(9): 4268-4275. 10.1128/JVI.75.9.4268-4275.2001
- Allison, S. L., Schlich, J., Stiasny, K., Mandl, C. W., Kunz, C. and Heinz, F. X. (1995). Oligomeric rearrangement of tick-borne encephalitis virus envelope proteins induced by an acidic pH. *J Virol* 69(2): 695-700. 10.1128/jvi.69.2.695-700.1995
- Alzahrani, A. G., Shaiban, H. M. A., Mazroa, M. A. A., Al-Hayani, O., MacNeil, A., Rollin, P. E. and Memish, Z. A. (2010). Alkhurma Hemorrhagic Fever in Humans, Najran, Saudi Arabia. *Emerg Infect Dis* 16(12): 1882-1888. 10.3201/eid1612.100417
- Amanna, I. J. and Slifka, M. K. (2014). Current Trends in West Nile Virus Vaccine Development. *Expert Rev Vaccines* 13(5): 589-608. 10.1586/14760584.2014.906309
- Amicizia, D., Domnich, A., Panatto, D., Lai, P. L., Cristina, M. L., Avio, U. and Gasparini, R. (2013). Epidemiology of tick-borne encephalitis (TBE) in Europe and its prevention by available vaccines. *Hum Vaccin Immunother* 9(5): 1163-1171. 10.4161/hv.23802
- Amicizia, D., Zangrillo, F., Lai, P. L., Iovine, M. and Panatto, D. (2019). Overview of Japanese encephalitis disease and its prevention. Focus on IC51 vaccine (IXIARO®). *J Prev Med Hyg* 59(1): E99-E107. 10.15167/2421-4248/jpmh2018.59.1.962

- Anwar, M. N., Akhtar, R., Abid, M., Khan, S. A., Rehman, Z. U., Tayyub, M., Malik, M. I., Shahzad, M. K., Mubeen, H., Qadir, M. S., Hameed, M., Wahaab, A., Li, Z., Liu, K., Li, B., Qiu, Y., Ma, Z. and Wei, J. (2022). The interactions of flaviviruses with cellular receptors: Implications for virus entry. *Virology* 568: 77-85. 10.1016/j.virol.2022.02.001
- Appaiahgari, M. B. and Vрати, S. (2010). IMOJEV®: a Yellow fever virus-based novel Japanese encephalitis vaccine. *Expert Rev Vaccines* 9(12): 1371-1384. 10.1586/ERV.10.139
- Araujo, S. C., Pereira, L. R., Alves, R. P. S., Andreato-Santos, R., Kanno, A. I., Ferreira, L. C. S. and Gonçalves, V. M. (2020). Anti-Flavivirus vaccines: review of the present situation and perspectives of subunit vaccines produced in *Escherichia coli*. *Vaccines* 8(3): 492. 10.3390/vaccines8030492
- Arroyo, J., Guirakhoo, F., Fenner, S., Zhang, Z.-X., Monath, T. P. and Chambers, T. J. (2001). Molecular Basis for Attenuation of Neurovirulence of a Yellow Fever Virus/Japanese Encephalitis Virus Chimera Vaccine (ChimeriVax-JE). *J Virol* 75(2): 934-942. 10.1128/JVI.75.2.934-942.2001
- Arroyo, J., Miller, C., Catalan, J., Myers, G. A., Ratterree, M. S., Trent, D. W. and Monath, T. P. (2004). ChimeriVax-West Nile Virus Live-Attenuated Vaccine: Preclinical Evaluation of Safety, Immunogenicity, and Efficacy. *J Virol* 78(22): 124997-112507. 10.1128/JVI.78.22.12497-12507.2004
- Atkins, G. J. and Sheahan, B. J. (2016). Molecular determinants of alphavirus neuropathogenesis in mice. *J Gen Virol* 97(6): 1283-1696. 10.1099/jgv.0.000467
- Atkins, G. J., Sheahan, B. J. and Dimmock, N. J. (1985). Semliki Forest Virus Infection of Mice: A Model for Genetic and Molecular Analysis of Viral Pathogenicity *J Gen Virol* 66(Pt 3): 395-408. 10.1099/0022-1317-66-3-395
- Avirutnan, P., Fuchs, A., Hauhart, R. E., Somnuk, P., Youn, S., Diamond, M. S. and Atkinson, J. P. (2010). Antagonism of the complement component C4 by flavivirus nonstructural protein NS1. *J Exp Med* 207(4): 793-806. 10.1084/jem.20092545
- Bai, F., Thompson, E. A., Vig, P. J. S. and Leis, A. A. (2019). Current Understanding of West Nile Virus Clinical Manifestations, Immune Responses, Neuroinvasion, and Immunotherapeutic Implications. *Pathogens* 8(4): 193. 10.3390/pathogens8040193
- Banet-Noach, C., Malkinson, M., Brill, A., Samina, I., Yadin, H., Weisman, Y., Pokamunski, S., King, R., Deubel, V. and Stram, Y. (2003). Phylogenetic Relationships of West Nile Viruses Isolated from Birds and Horses in Israel from 1997 to 2001. *Virus Genes* 26: 135-141. 10.1023/A:1023431328933
- Barker, J., daSilva, L. L. P. and Crump, C. M. (2023). Mechanisms of bunyavirus morphogenesis and egress Open Access. *J Gen Virol* 104(4). 10.1099/jgv.0.001845

- Barrett, A. D. T. (1997). Yellow Fever Vaccines. *Biologicals* 25: 17-25. 10.1006/biol.1997.0056
- Barrett, A. D. T. (2014). Economic Burden of West Nile Virus in the United States. *Am J Trop Med Hyg* 90(3): 389-390. 10.4269/ajtmh.14-0009
- Barrett, A. D. T. and Higgs, S. (2007). Yellow Fever: A Disease that Has Yet to be Conquered. *Annu Rev Entomol* 52: 209-229. 10.1146/annurev.ento.52.110405.091454
- Barrett, A. D. T. and Teuwen, D. E. (2009). Yellow fever vaccine — how does it work and why do rare cases of serious adverse events take place? *Curr Opin Immunol* 21(3): 308-313. 10.1016/j.coi.2009.05.018
- Barrett, P. N., Schober-Bendixen, S. and Ehrlich, H. J. (2003). History of TBE vaccines. *Vaccine* 21: S41-S49. 10.1016/S0264-410X(02)00814-9
- Basset, J., Burlaud-Gaillard, J., Feher, M., Roingeard, P., Rey, F. A. and Pardigon, N. (2020). A Molecular Determinant of West Nile Virus Secretion and Morphology as a Target for Viral Attenuation. *J Virol* 94(12): e00086-00020. 10.1128/JVI.00086-20
- Beasley, D. W. C., Davis, C. T., Guzman, H., Vanlandingham, D. L., Rosa, A. P. A. T. d., Parsons, R. E., Higgs, S., Tesh, R. B. and Barrett, A. D. T. (2003). Limited evolution of West Nile virus has occurred during its southwesterly spread in the United States. *Virology* 309(2): 190-195. 10.1016/s0042-6822(03)00150-8
- Beasley, D. W. C., Li, L., Suderman, M. T. and Barrett, A. D. T. (2002). Mouse neuroinvasive phenotype of West Nile virus strains varies depending upon virus genotype. *Virology* 296(1): 17-23. 10.1006/viro.2002.1372
- Beasley, D. W. C., Whiteman, M. C., Zhang, S., Huang, C. Y.-H., Schneider, B. S., Smith, D. R., Gromowski, G. D., Higgs, S., Kinney, R. M. and Barrett, A. D. T. (2005). Envelope protein glycosylation status influences mouse neuroinvasion phenotype of genetic lineage 1 West Nile virus strains. *J Virol* 79(13): 8339-8347. 10.1128/JVI.79.12.8339-8347.2005
- Beatty, P. R., Puerta-Guardo, H., Killingbeck, S. S., Glasner, D. R., Hopkins, K. and Harris, E. (2015). Dengue virus NS1 triggers endothelial permeability and vascular leak that is prevented by NS1 vaccination. *Sci Transl Med* 7(304): 304ra141. 10.1126/scitranslmed.aaa3787
- Beck, A., Guzman, H., Li, L., Ellis, B., Tesh, R. B. and Barrett, A. D. T. (2013). Phylogeographic Reconstruction of African Yellow Fever Virus Isolates Indicates Recent Simultaneous Dispersal into East and West Africa. *PLoS Negl Trop Dis* 7(3): e1910. 10.1371/journal.pntd.0001910
- Beck, A., Tesh, R. B., Wood, T. G., Widen, S. G., Ryman, K. D. and Barrett, A. D. T. (2014). Comparison of the Live Attenuated Yellow Fever Vaccine 17D-204 Strain to Its Virulent Parental Strain Asibi by Deep Sequencing. *J Infect Dis* 209(3): 334-344. 10.1093/infdis/jit546

Beck, A. S. and Barrett, A. D. T. (2015). Current Status and Future Prospects of Yellow Fever Vaccines. *Expert Rev Vaccines* 14(11): 1479-1492. 10.1586/14760584.2015.1083430

Beck, A. S., Wood, T. G., Widen, S. G., Thompson, J. K. and Barrett, A. D. T. (2018). Analysis By Deep Sequencing of Discontinued Neurotropic Yellow Fever Vaccine Strains. *Sci Rep* 8: 13408. 10.1038/s41598-018-31085-2

Bhamarapravati, N., Yoksan, S., Chayaniyayothin, T., Angsubphakorn, S. and Bunyaratvej, A. (1987). Immunization with a live attenuated dengue-2-virus candidate vaccine (16681-PDK 53): clinical, immunological and biological responses in adult volunteers. *Bull World Health Organ* 65(2): 189-195

Bhatt, T. R., Crabtree, M. B., Guirakhoo, F., Monath, T. P. and Miller, B. R. (2000). Growth characteristics of the chimeric Japanese encephalitis virus vaccine candidate, ChimeriVax-JE (YF/JE SA14--14--2), in *Culex tritaeniorhynchus*, *Aedes albopictus*, and *Aedes aegypti* mosquitoes. *Am J Trop Med Hyg* 62(4): 480-484. 10.4269/ajtmh.2000.62.480

Bialosuknia, S. M., II, A. P. D., Zink, S. D., Koetzner, C. A., Maffei, J. G., Owen, J. C., Landwerlen, H., Kramer, L. D. and Ciota, A. T. (2022). Adaptive evolution of West Nile virus facilitated increased transmissibility and prevalence in New York State. *Emerg Microbes Infect* 11(1): 988-999. 10.1080/22221751.2022.2056521

Bilyeu, A. N. (2023). Comparative evaluation of single-site mutations in the West Nile virus envelope protein using a mouse model [Master's Thesis, Kansas State University]. K-State Research Exchange.

Blackmore, C. G., Blackmore, M. S., and Grimstad, P. R. (1998). Role of *Anopheles quadrimaculatus* and *Coquillettidia perturbans* (Diptera: Culicidae) in the transmission cycle of Cache Valley virus (Bunyaviridae: Bunyavirus) in the midwest, USA. *J Med Entomol* 35(5): 660-664. 10.1093/jmedent/35.5.660

Blitvich, B. J. and Firth, A. E. (2015). Insect-Specific Flaviviruses: A Systematic Review of Their Discovery, Host Range, Mode of Transmission, Superinfection Exclusion Potential and Genomic Organization. *Viruses* 7(4): 1927-1959. 10.3390/v7041927

Bondre, V. P., Jadi, R. S., Mishra, A. C., Yergolkar, P. N. and Arankalle, V. A. (2007). West Nile virus isolates from India: evidence for a distinct genetic lineage. *J Gen Virol* 88(Pt. 3): 875-884. 10.1099/vir.0.82403-0

Boonstra, S., Blijleven, J. S., Roos, W. H., Onck, P. R., Giessen, E. v. d. and Oijen, A. M. v. (2018). Hemagglutinin-Mediated Membrane Fusion: A Biophysical Perspective. *Annu Rev Biophys* 47: 153-173. 10.1146/annurev-biophys-070317-033018

Borucki, M. K., Kempf, B. J., Blitvich, B. J., Blair, C. D. and Beaty, B. J. (2002). La Crosse virus: replication in vertebrate and invertebrate hosts. *Microbes Infect* 4(3): 341-350. 10.1016/S1286-4579(02)01547-2

Botha, E. M., Markotter, W., Wolfaardt, M., Paweska, J. T., Swanepoel, R., Palacios, G., Nel, L. H. and Venter, M. (2008). Genetic Determinants of Virulence in Pathogenic Lineage 2 West Nile Virus Strains. *Emerg Infect Dis* 14(2): 222-230. 10.3201/eid1402.070457

Bouloy, M. and Weber, F. (2010). Molecular Biology of Rift Valley Fever Virus. *Open Virol J* 4: 8-14. 10.2174/1874357901004010008

Brackney, D. E., and Armstrong, P. M. (2016). Transmission and evolution of tick-borne viruses. *Curr Opin Virol* 21: 67-74. 10.1016/j.coviro.2016.08.005

Brecher, M., Zhang, J. and Li, H. (2013). The Flavivirus Protease As a Target for Drug Discovery. *Virol Sin* 28(6): 326-336. 10.1007/s12250-013-3390-x

Bredenbeek, P. J., Kooi, E. A., Lindenbach, B., Huijckman, N., Rice, C. M. and Spaan, W. J. M. (2003). A stable full-length yellow fever virus cDNA clone and the role of conserved RNA elements in flavivirus replication. *J Gen Virol* 84(5): 1261-1268. 10.1099/vir.0.18860-0

Bressanelli, S., Stiasny, K., Allison, S. L., Stura, E. A., Duquerroy, S., Lescar, J., Heinz, F. X. and Rey, F. A. (2004). Structure of a flavivirus envelope glycoprotein in its low-pH-induced membrane fusion conformation. *EMBO J* 23(4): 728-738. 10.1038/sj.emboj.7600064

Bryant, J. E., Holmes, E. C. and Barrett, A. D. T. (2007). Out of Africa: A Molecular Perspective on the Introduction of Yellow Fever Virus into the Americas. *PLoS Pathog* 3(5): e75. 10.1371/journal.ppat.0030075

Buescher, E. L., Scherer, W. F., McClure, H. E., Moyer, J. T., Rosenberg, M. Z., Yoshii, M. and Okada, Y. (1959). Ecologic Studies of Japanese Encephalitis Virus in Japan. *Am J Trop Med Hyg* 8(6): 678-688. 10.4269/ajtmh.1959.8.678

Burt, F. J., Grobbelaar, A. A., Leman, P. A., Anthony, F. S., Gibson, G. V. F. and Swanepoel, R. (2002). Phylogenetic Relationships of Southern African West Nile Virus Isolates. *Emerg Infect Dis* 8(8): 820-826. 10.3201/eid0808.020027

Busch, M. P., Kleinman, S. H., Tobler, L. H., Kamel, H. T., Norris, P. J., Walsh, I., Matud, J. L., Prince, H. E., Lanciotti, R. S., Wright, D. J., Linnen, J. M. and Caglioti, S. (2008). Virus and Antibody Dynamics in Acute West Nile Virus Infection *J Infect Dis* 198(7): 984-993. 10.1086/591467

Butantan-Institute (2022). Butantan's dengue vaccine has 79.6% efficacy, partial results from 2-year follow-up show.

Butrapet, S., Childers, T., Moss, K. J., Erb, S. M., Luy, B. E., Calvert, A. E., Blair, C. D., Roehrig, J. T. and Huang, C. Y.-H. (2011). Amino acid changes within the E protein hinge region that affect dengue virus type 2 infectivity and fusion. *Virology* 413(1): 118-127. 10.1016/j.virol.2011.01.030

Caldwell, H. S., Pata, J. D. and Ciota, A. T. (2022). The Role of the Flavivirus Replicase in Viral Diversity and Adaptation. *Viruses* 14(5): 1076. 10.3390/v14051076

Campbell, G. L., Ceianu, C. S. and Savage, H. M. (2001). Epidemic West Nile encephalitis in Romania: waiting for history to repeat itself. *Ann N Y Acad Sci* 951: 94-101. 10.1111/j.1749-6632.2001.tb02688.x

Capeding, M. R., Tran, N. H., Hadinegoro, S. R. S., Ismail, H. I. H. J. M., Chotpitayasunondh, T., Chua, M. N., Luong, C. Q., Rusmil, K., Wirawan, D. N., Nallusamy, R., Pitisuttithum, P., Thisyakorn, U., Yoon, I.-K., Vliet, D. v. d., Langevin, E., Laot, T., Hutagalung, Y., Frago, C., Boaz, M., Wartel, T. A., Tornieporth, N. G., Saville, M., Bouckennooghe, A. and Group, C. S. (2014). Clinical efficacy and safety of a novel tetravalent dengue vaccine in healthy children in Asia: a phase 3, randomised, observer-masked, placebo-controlled trial. *Lancet* 384(9951): 1358-1365. 10.1016/S0140-6736(14)61060-6

Carpio, K. L. and Barrett, A. D. T. (2021). Flavivirus NS1 and Its Potential in Vaccine Development. *Vaccines (Basel)* 9: 622. 10.3390/vaccines9060622

Carpio, K. L., Thompson, J. K., Widen, S. G., Smith, J. K., Juelich, T. L., Clements, D. E., Freiberg, A. N. and Barrett, A. D. T. (2023). Differences in Genetic Diversity of Mammalian Tick-Borne Flaviviruses. *Viruses* 15(2): 281. 10.3390/v15020281

CDC (2002). Provisional Surveillance Summary of the West Nile Virus Epidemic --- United States, January--November 2002. *MMRW* 51(50): 1129-1133

CDC (2017) International catalogue of arboviruses; including certain other viruses of vertebrates. Centers for Disease Control and Prevention (U.S.)

CDC (2018). "Global Health - Yellow Fever." from <https://www.cdc.gov/globalhealth/newsroom/topics/yellowfever/index.html>.

CDC (2021a). "Arboviral Diseases, Neuroinvasive and Non-neuroinvasive." from <https://ndc.services.cdc.gov/case-definitions/arboviral-diseases-neuroinvasive-and-non-neuroinvasive-2015/>.

CDC (2021b). "Bunyavirales." from <https://www.cdc.gov/vhf/virus-families/bunyaviridae.html>.

CDC (2021c). "St. Louis Encephalitis Virus - Transmission." from <https://www.cdc.gov/sle/transmission/index.html>.

CDC (2022a). "Powassan Virus." from <https://www.cdc.gov/powassan/index.html>.

CDC (2022b). "Tick-borne encephalitis (TBE)." from <https://www.cdc.gov/tick-borne-encephalitis/index.html>.

CDC (2023a). "Final Cumulative Maps & Data for 1999–2021." West Nile Virus. from <https://www.cdc.gov/westnile/statsmaps/cummapsdata.html>.

CDC (2023b). St. Louis Encephalitis Virus - Historical Data (2003-2022).

CDC (2023c). "West Nile Virus: Final Annual Maps & Data for 1999-2021." from <https://www.cdc.gov/westnile/statsmaps/finalmapsdata/index.html>.

Chaintoutis, S. C., Diakakis, N., Papanastassopoulou, M., Banos, G. and Dovas, C. I. (2015). Evaluation of Cross-Protection of a Lineage 1 West Nile Virus Inactivated Vaccine against Natural Infections from a Virulent Lineage 2 Strain in Horses, under Field Conditions. *Clin Vaccine Immunol* 22(9): 1040-1049. 10.1128/CVI.00302-15

Chambers, T. J., Hahn, C. S., Galler, R. and Rice, C. M. (1990). Flavivirus genome organization, expression, and replication. *Annu Rev Microbiol* 44: 649-688. 10.1146/annurev.mi.44.100190.003245

Chambers, T. J., Nestorowicz, A., Mason, P. W. and Rice, C. M. (1999). Yellow Fever/Japanese Encephalitis Chimeric Viruses: Construction and Biological Properties. *J Virol* 73(4): 3095-3101. 10.1128/JVI.73.4.3095-3101.1999

Chancey, C., Grinev, A., Volkova, E. and Rios, M. (2015). The Global Ecology and Epidemiology of West Nile Virus. *Biomed Res Int* 2015: 376230. 10.1155/2015/376230

Chao, D.-Y., Davis, B. S. and Chang, G.-J. J. (2007). Development of multiplex real-time reverse transcriptase PCR assays for detecting eight medically important flaviviruses in mosquitoes. *J Clin Microbiol* 45(2): 584-589. 10.1128/JCM.00842-06

Chen, L. H. and Wilson, M. E. (2005). Non-Vector Transmission of Dengue and Other Mosquito-Borne Flaviviruses. *Dengue Bulletin* 29: 18-31

Chen, Y., Maguire, T., Hileman, R. E., Fromm, J. R., Esko, J. D., Linhardt, R. J. and Marks, R. M. (1997). Dengue virus infectivity depends on envelope protein binding to target cell heparan sulfate. *Nat Med* 3(8): 866-871. 10.1038/nm0897-866

Chippaux, J.-P. and Chippaux, A. (2018). Yellow fever in Africa and the Americas: a historical and epidemiological perspective. *J Venom Anim Toxins Incl Trop Dis* 24: 20. 10.1186/s40409-018-0162-y

Chokephaibulkit, K., Houillon, G., Feroldi, E. and Bouckenooghe, A. (2015). Safety and immunogenicity of live attenuated Japanese encephalitis chimeric virus vaccine (Imojev®) in children. *Expert Rev Vaccines* 15(2): 153-166. 10.1586/14760584.2016.1123097

Choithia, C. (1975). Structural invariants in protein folding. *Nature* 254(5498): 304-308. 10.1038/254304a0

- Chowdhury, P., Khan, S. A., Dutta, P., Topno, R. and Mahanta, J. (2014). Characterization of West Nile virus (WNV) isolates from Assam, India: Insights into the circulating WNV in northeastern India. *Comp Immunol Microbiol Infect Dis* 37(1): 39-47. 10.1016/j.cimid.2013.10.006
- Christian, E. A., Kahle, K. M., Mattia, K., Puffer, B. A., Pfaff, J. M., Miller, A., Paes, C., Davidson, E. and Doranz, B. J. (2013). Atomic-level functional model of dengue virus Envelope protein infectivity. *PNAS* 110(46): 18662-18667. 10.1073/pnas.1310962110
- Ciota, A. T. and Kramer, L. D. (2013). Vector-Virus Interactions and Transmission Dynamics of West Nile Virus. *Viruses* 5(12): 3021-3047. 10.3390/v5123021
- Clarke, D. H. and Casals, J. (1958). Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. *Am J Trop Med Hyg* 7(5): 561-573. 10.4269/ajtmh.1958.7.561
- Connors, K. A. and Hartman, A. L. (2022). Advances in Understanding Neuropathogenesis of Rift Valley Fever Virus. *Annu Rev Virol* 9(1): 437-450. 10.1146/annurev-virology-091919-065806
- Crabtree, M. B., Kinney, R. M. and Miller, B. R. (2005). Deglycosylation of the NS1 protein of dengue 2 virus, strain 16681: construction and characterization of mutant viruses. *Arch Virol* 150(4): 771-786. 10.1007/s00705-004-0430-8
- Daffis, S., Szretter, K. J., Schriewer, J., Li, J., Youn, S., Errett, J., Lin, T.-Y., Schneller, S., Züst, R., Dong, H., Thiel, V., Sen, G. C., Fensterl, V., Klimstra, W. B., Pierson, T. C., Buller, R. M., Jr, M. G., Shi, P.-Y. and Diamond, M. S. (2010). 2'-O methylation of the viral mRNA cap evades host restriction by IFIT family members. *Nature* 468: 452-456. 10.1038/nature09489
- Dandawate, C. N., Desai, G. B., Achar, T. R. and Banerjee, K. (1994). Field evaluation of formalin inactivated Kyasanur forest disease virus tissue culture vaccine in three districts of Karnataka state. *Indian J Med Res* 99: 152-158
- Dandawate, C. N., Upadhyaya, S. and Banerjee, K. (1980). Serological response to formolized Kyasanur forest disease virus vaccine in humans at Sagar and Sorab Talukas of Shimoga district. *J Biol Stand* 8(1): 1-6. 10.1016/S0092-1157(80)80041-2
- Danko, J. R., Kochel, T., Teneza-Mora, N., Luke, T. C., Raviprakash, K., Sun, P., Simmons, M., Moon, J. E., Barrera, R. D. L., Martinez, L. J., Thomas, S. J., Kenney, R. T., Smith, L. and Porter, K. R. (2018). Safety and Immunogenicity of a Tetravalent Dengue DNA Vaccine Administered with a Cationic Lipid-Based Adjuvant in a Phase 1 Clinical Trial. *Am J Trop Med Hyg* 98(3): 849-856. 10.4269/ajtmh.17-0416

- Davis, C. T., Galbraith, S. E., Zhang, S., Whiteman, M. C., Li, L., Kinney, R. M. and Barrett, A. D. T. (2007). A Combination of Naturally Occurring Mutations in North American West Nile Virus Nonstructural Protein Genes and in the 3' Untranslated Region Alters Virus Phenotype ▽ . *J Virol* 81(11): 6111-6116. 10.1128/JVI.02387-06
- Davis, E. H., Beck, A. S., Li, L., White, M. M., Greenberg, M. B., Thompson, J. K., Widen, S. G., Barrett, A. D. T. and Bourne, N. (2021). Japanese encephalitis virus live attenuated vaccine strains display altered immunogenicity, virulence and genetic diversity. *npj Vaccines* 6: 112. 10.1038/s41541-021-00371-y
- Dayan, G. H., Bevilacqua, J., Coleman, D., Buldo, A. and Risi, G. (2012). Phase II, dose ranging study of the safety and immunogenicity of single dose West Nile vaccine in healthy adults ≥ 50 years of age. *Vaccine* 30(47): 6656-6664. 10.1016/j.vaccine.2012.08.063
- Dayan, G. H., Pugachev, K., Bevilacqua, J., Lang, J. and Monath, T. P. (2013). Preclinical and Clinical Development of a YFV 17 D-Based Chimeric Vaccine against West Nile Virus. *Viruses* 5(12): 3048-3070. 10.3390/v5123048
- de Oliveira Figueiredo, P., Stoffella-Dutra, A. G., Costa, G. B., de Oliveira, J. S., Amaral, C. D., Santos, J. D., Rocha, K. L. S., Araújo Júnior, J. P., Nogueira, M. L., Borges, M. A. Z., Paglia, A. P., LaBeaud, A. D., Abrahão, J. S., Kroon, E. G., de Oliveira, D. B., Drumond, B. P. and de Souza Trindade, G. (2020). Re-Emergence of Yellow Fever in Brazil during 2016–2019: Challenges, Lessons Learned, and Perspectives. *Viruses* 12(11): 1233. 10.3390/v12111233
- de Silva, A. and White, L. (2023). Immunogenicity of a Live Dengue Vaccine (TAK-003). *J Infect Dis* 227: 163-164. 10.1093/infdis/jiac424
- de Wispelaere, M., Khou, C., Frenkiel, M.-P., Desprès, P. and Pardigona, N. (2016). A Single Amino Acid Substitution in the M Protein Attenuates Japanese Encephalitis Virus in Mammalian Hosts. *J Virol* 90(5): 2676-2689. 10.1128/JVI.01176-15
- de Wispelaere, M., Lian, W., Potisophon, S., Li, P.-C., Jang, J., Ficarro, S., Clark, M. J., Zhu, X., Kaplan, J. B., Pitts, J. D., Wales, T. E., Wang, J., Engen, J. R., Marto, J., Gray, N. S. and Yang, P. L. (2018). Inhibition of Flaviviruses by Targeting a Conserved Pocket on the Viral Envelope Protein. *Cell Chem Biol* 25(8): 1006-1016.e1008. 10.1016/j.chembiol.2018.05.011
- de Wispelaere, M. and Yang, P. L. (2012). Mutagenesis of the DI/DIII Linker in Dengue Virus Envelope Protein Impairs Viral Particle Assembly. *J Virol* 86(13): 7072-7083. 10.1128/JVI.00224-12
- Defraites, R. F., Gambel, J. M., Jr, C. H. H., Sanchez, J. L., Withers, B. G., Karabatsos, N., Shope, R. E., Tirrell, S., Yoshida, I., Takagi, M., Meschievitz, C. K. and Tsai, T. F. (1999). Japanese encephalitis vaccine (inactivated, BIKEN) in U.S. soldiers: immunogenicity and safety of vaccine administered in two dosing regimens. *Am J Trop Med Hyg* 61(2): 288-293. 10.4269/ajtmh.1999.61.288

- Demicheli, V., Debalini, M. G., Rivetti, A. and Group, C. I. D. (2009). Vaccines for preventing tick-borne encephalitis. *Cochrane Database of Syst Rev* 2009(1): CD000977. 10.1002/14651858.CD000977.pub2
- Dessau, M. and Modis, Y. (2013). Crystal structure of glycoprotein C from Rift Valley fever virus. *PNAS* 110(5): 1696-1701. 10.1073/pnas.1217780110
- Diamond, M. S., Sitati, E. M., Friend, L. D., Higgs, S., Shrestha, B. and Engle, M. (2003). A Critical Role for Induced IgM in the Protection against West Nile Virus Infection. *J Exp Med* 198(12): 1853-1862. 10.1084/jem.20031223
- Diaz, A., Coffey, L. L., Burkett-Cadena, N. and Day, J. F. (2018). Reemergence of St. Louis Encephalitis Virus in the Americas. *Emerg Infect Dis* 24(12): 2150-2157. 10.3201/eid2412.180372
- Douam, F. and Ploss, A. (2018). Yellow fever virus: Knowledge gaps impeding the fight against an old foe. *Trends Microbiol* 26(11): 913-928. 10.1016/j.tim.2018.05.012
- Duan, Y., Zeng, M., Jiang, B., Zhang, W., Wang, M., Jia, R., Zhu, D., Liu, M., Zhao, X., Yang, Q., Wu, Y., Zhang, S., Liu, Y., Zhang, L., Yu, Y., Pan, L., Chen, S. and Cheng, A. (2019). Flavivirus RNA-Dependent RNA Polymerase Interacts with Genome UTRs and Viral Proteins to Facilitate Flavivirus RNA Replication. *Viruses* 11(10): 929. 10.3390/v11100929
- Durbin, A. P., Kirkpatrick, B. D., Pierce, K. K., Schmidt, A. C. and Whitehead, S. S. (2011). Development and clinical evaluation of multiple investigational monovalent DENV vaccines to identify components for inclusion in a live attenuated tetravalent DENV vaccine. 29(7242-7250). 10.1016/j.vaccine.2011.07.023
- Durbin, A. P., Wright, P. F., Cox, A., Kagucia, W., Elwood, D., Henderson, S., Wanionek, K., Speicher, J., Whitehead, S. S. and Pletnev, A. G. (2013). The live attenuated chimeric vaccine rWN/DEN4Δ30 is well-tolerated and immunogenic in healthy flavivirus-naïve adult volunteers. *Vaccine* 31(48): 5772-5777. 10.1016/j.vaccine.2013.07.064
- Dusek, R. J., McLean, R. G., Kramer, L. D., Ubico, S. R., II, A. P. D., Ebel, G. D. and Guptill, S. C. (2009). Prevalence of West Nile Virus in Migratory Birds during Spring and Fall Migration. *Am J Trop Med Hyg* 81(6): 1151-1158. 10.4269/ajtmh.2009.09-0106
- Ebel, G. D., Foppa, I., Spielman, A. and 2nd, S. R. T. (1999). A focus of deer tick virus transmission in the northcentral United States. *Emerg Infect Dis* 5(4): 570-574. 10.3201/eid0504.990423
- Eckels, K. H., Scott, R. M., Bancroft, W. H., Brown, J., Dubois, D. R., Summers, P. L., Russell, P. K. and Halstead, S. B. (1984). Selection of attenuated dengue 4 viruses by serial passage in primary kidney cells. V. Human response to immunization with a candidate vaccine prepared in fetal rhesus lung cells. *Am J Trop Med Hyg* 33(4): 684-689. 10.4269/ajtmh.1984.33.684

Eckels, K. H., Yong-Xin, Y., Dubois, D. R., Marchette, N. J., Trent, D. W. and Johnson, A. J. (1988). Japanese encephalitis virus live-attenuated vaccine, Chinese strain SA14-14-2; adaptation to primary canine kidney cell cultures and preparation of a vaccine for human use. *Vaccine* 6(6): 513-518. 10.1016/0264-410X(88)90103-X

Egloff, M.-P., Benarroch, D., Selisko, B., Romette, J.-L. and Canard, B. (2002). An RNA cap (nucleoside-2'-O-)-methyltransferase in the flavivirus RNA polymerase NS5: crystal structure and functional characterization. *EMBO J* 21(11): 2757-2768. 10.1093/emboj/21.11.2757

Ehrlich, H. J., Pavlova, B. G., Fritsch, S., Poellabauer, E.-M., Loew-Baselli, A., Obermann-Slupetzky, O., Maritsch, F., Cil, I., Dorner, F. and Barrett, P. N. (2003). Randomized, phase II dose-finding studies of a modified tick-borne encephalitis vaccine: evaluation of safety and immunogenicity. *Vaccine* 22(2): 217-223. 10.1016/s0264-410x(03)00563-2

Eldridge, B. F. and Edman, J. D. (2003). Medical entomology: a textbook on public health and veterinary problems caused by arthropods. Boston, Kluwer Academic Publishers.

Ellis, B. R. and Barrett, A. D. T. (2008). The enigma of yellow fever in East Africa. *Rev Med Virol* 18(5): 331-346. 10.1002/rmv.584

Erra, E. O., Askling, H. H., Yoksan, S., Rombo, L., Riutta, J., Vene, S., Lindquist, L., Vapalahti, O. and Kantele, A. (2013). Cross-Protective Capacity of Japanese Encephalitis (JE) Vaccines Against Circulating Heterologous JE Virus Genotypes *Clin Infect Dis* 56(2): 267-270. 10.1093/cid/cis883

European Centre for Disease Prevention and Control (2017). Epidemiological update: West Nile virus transmission season in Europe, 2017.

European Medicines Agency (2023). "Ixiaro." from <https://www.ema.europa.eu/en/medicines/human/EPAR/ixiaro>

EVIP (2013). Austria: Recommended vaccinations.

Fang, Y. and Reisen, W. K. (2006). Previous infection with West Nile or St. Louis encephalitis viruses provides cross protection during reinfection in house finches. *Am J Trop Med Hyg* 75(3): 480-485

FDA (2019). "IXIARO." from <https://www.fda.gov/vaccines-blood-biologics/vaccines/ixiaro>

FDA (2023). "Dengvaxia." from <https://www.fda.gov/vaccines-blood-biologics/dengvaxia>.

Fenner, F., Pereira, H. G., Portfield, J. S., Joklik, W. K., and Downie, A. W. (1974). Family and generic names for viruses approved by the International Committee on Taxonomy of Viruses, June 1974. *Intervirology*. 3(3): 193-198. 10.1159/000149755

Fernandez-Garcia, M. D., Meertens, L., Chazal, M., Hafirassou, M. L., Dejarnac, O., Zamborlini, A., Despres, P., Sauvonnnet, N., Arenzana-Seisdedos, F., Jouvenet, N. and Amara, A. (2016). Vaccine and Wild-Type Strains of Yellow Fever Virus Engage Distinct Entry Mechanisms and Differentially Stimulate Antiviral Immune Responses. *mBio* 7(1): e01956-01915. 10.1128/mBio.01956-15

Ferreira, C. d. C., Campi-Azevedo, A. C., Peruhype-Magalhães, V., Costa-Pereira, C., Albuquerque, C. P. d., Muniz, L. F., Souza, T. Y. d., Oliveira, A. C. V., Martins-Filho, O. A. and Mota, L. M. H. d. (2018). The 17D-204 and 17DD yellow fever vaccines: an overview of major similarities and subtle differences. *Expert Rev Vaccines* 17(1): 79-90. 10.1080/14760584.2018.1406800

Fiacre, L., Pagès, N., Albina, E., Richardson, J., Lecollinet, S. and Gonzalez, G. (2020). Molecular Determinants of West Nile Virus Virulence and Pathogenesis in Vertebrate and Invertebrate Hosts. *Int J Mol Sci* 21(23): 9117. 10.3390/ijms21239117

Files, M. A., Hansen, C. A., Herrera, V.C., Schindewolf, C., Barrett, A. D. T., Beasley, D. W. C., Bourne, N., and Milligan, G. N. (2022). Baseline mapping of Oropouche virology, epidemiology, therapeutics, and vaccine research and development. *npj Vaccines* 7: 38. 10.1038/s41541-022-00456-2

Fox, J. P. (1956). Yellow Fever Vaccination. World Health Organization Monograph Series, No. 30. World Health Organization, Geneva, 1956 (order from Columbia University Press, New York 27). *Science* 124(3217): 370-371. 10.1126/science.124.3217.370.c

Friberg, H., Martinez, L. J., Lin, L., Blaylock, J. M., Barrera, R. A. D. L., Rothman, A. L., Putnak, J. R., Eckels, K. H., Thomas, S. J., Jarman, R. G. and Currier, J. R. (2020). Cell-Mediated Immunity Generated in Response to a Purified Inactivated Vaccine for Dengue Virus Type 1. *mSphere* 5(1): e00671-00619. 10.1128/mSphere.00671-19

Frierson, J. G. (2010). The Yellow Fever Vaccine: A History. *Yale J Biol Med* 83(2): 77-85

Fulton, C. D. M., Beasley, D. W. C., Bente, D. A. and Dineley, K. T. (2020). Long-term, West Nile virus-induced neurological changes: A comparison of patients and rodent models. *Brain Behav Immun Health* 100105. 10.1016/j.bbih.2020.100105

Gabiane, G., Yen, P. S. and Failloux, A. B. (2022). Aedes mosquitoes in the emerging threat of urban yellow fever transmission. *Rev Med Virol* 32(4): e2333. 10.1002/rmv.2333

Gallichotte, E. N., Baric, T. J., Widman, D. G., Whitehead, S., Baric, R. S. and Silva, A. M. d. (2018). Human dengue virus serotype 2 neutralizing antibodies target two distinct quaternary epitopes. *PLoS Pathog* 14(2): e1006934. 10.1371/journal.ppat.1006934

Garch, H. E., Minke, J. M., Rehder, J., Richard, S., Toulemonde, C. E., Dinic, S., Andreoni, C., Audonnet, J. C., Nordgren, R. and Juillard, V. (2008). A West Nile virus (WNV) recombinant canarypox virus vaccine elicits WNV-specific neutralizing antibodies and cell-mediated immune

responses in the horse. *Vet Immunol Immunopathol* 123(3-4): 230-239.
10.1016/j.vetimm.2008.02.002

Gardner, C. L. and Ryman, K. D. (2010). Yellow Fever: A Reemerging Threat. *Clin Lab Med* 30(1): 237-260. 10.1016/j.cll.2010.01.001

Gaunt, M. W., Sall, A. A., Lamballerie, X. d., Falconar, A. K. I., Dzhivanian, T. I. and Gould, E. A. (2001). Phylogenetic relationships of flaviviruses correlate with their epidemiology, disease association and biogeography. *J Gen Virol* 82(Pt. 8): 1867-1876. 10.1099/0022-1317-82-8-1867

Gaythorpe, K. A., Hamlet, A., Jean, K., Ramos, D. G., Cibrelus, L., Garske, T. and Ferguson, N. (2021). The global burden of yellow fever. *eLife* 10: e64670. 10.7554/eLife.64670

Gianhecchi, E., Cianchi, V., Torelli, A. and Montomoli, E. (2022). Yellow Fever: Origin, Epidemiology, Preventive Strategies and Future Prospects. *Vaccines (Basel)* 10(3): 372. 10.3390/vaccines10030372

Gibbons, D. L., Vaney, M.-C., Roussel, A., Vigouroux, A., Reilly, B., Lepault, J., Kielian, M. and Rey, F. A. (2004). Conformational change and protein–protein interactions of the fusion protein of Semliki Forest virus. *Nature* 427(6972): 320-325. 10.1038/nature02239

Giladi, M., Metzkor-Cotter, E., Martin, D. A., Siegman-Igra, Y., Korczyn, A. D., Rosso, R., Berger, S. A., Campbell, G. L. and Lanciotti, R. S. (2001). West Nile encephalitis in Israel, 1999: the New York connection. *Emerg Infect Dis* 7(4): 659-661. 10.3201/eid0704.010410

Go, Y. Y., Balasuriya, U. B. R. and Lee, C.-k. (2014). Zoonotic encephalitides caused by arboviruses: transmission and epidemiology of alphaviruses and flaviviruses. *Clin Exp Vaccine Res* 3(1): 58-77. 10.7774/cevr.2014.3.1.58

Goo, L., VanBlargan, L. A., Dowd, K. A., Diamond, M. S. and Pierson, T. C. (2017). A single mutation in the envelope protein modulates flavivirus antigenicity, stability, and pathogenesis. *PLoS Pathog* 13(2): e1006178. 10.1371/journal.ppat.1006178

Gotuzzo, E., Yactayo, S. and Córdova, E. (2013). Efficacy and Duration of Immunity after Yellow Fever Vaccination: Systematic Review on the Need for a Booster Every 10 Years. *Am J Trop Med Hyg* 89(3): 434-444. 10.4269/ajtmh.13-0264

Gould, C. V., Staples, J. E., Huang, C. Y.-H., Brault, A. C. and Nett, R. J. (2023). Combating West Nile Virus Disease — Time to Revisit Vaccination. *N Engl J Med* 388(18): 1633-1636. 10.1056/NEJMp2301816

Gould, E. A. and Solomon, T. (2008). Pathogenic flaviviruses. *Lancet* 371(9611): 500-509. 10.1016/s0140-6736(08)60238-x

- Gritsun, T. S., Holmes, E. C. and Gould, E. A. (1995). Analysis of flavivirus envelope proteins reveals variable domains that reflect their antigenicity and may determine their pathogenesis. *Virus Res* 35(3): 307-321. 10.1016/0168-1702(94)00090-y
- Gromowski, G. D., Firestone, C.-Y., Bustos-Arriaga, J. and Whitehead, S. S. (2015a). Genetic and Phenotypic Properties of Vero Cell-Adapted Japanese Encephalitis Virus SA14-14-2 Vaccine Strain Variants and a Recombinant Clone, which Demonstrates Attenuation and Immunogenicity in Mice. *Am J Trop Med Hyg* 92(1): 98-107. 10.4269/ajtmh.14-0427
- Gromowski, G. D., Firestone, C.-Y. and Whitehead, S. S. (2015b). Genetic Determinants of Japanese Encephalitis Virus Vaccine Strain SA14-14-2 That Govern Attenuation of Virulence in Mice. *J Virol* 89(12): 6328-6337. 10.1128/JVI.00219-15
- Grosenbaugh, D. A., C S Backus, K. K., Minke, J. M. and Nordgren, R. M. (2004). The anamnestic serologic response to vaccination with a canarypox virus-vectored recombinant West Nile virus (WNV) vaccine in horses previously vaccinated with an inactivated WNV vaccine. *Vet Ther* 5(4): 251-257
- Guardado-Calvo, P., Bignon, E. A., Stettner, E., Jeffers, S. A., Pérez-Vargas, J., Pehau-Arnaudet, G., Tortorici, M. A., Jestin, J.-L., England, P., Tischler, N. D. and Rey, F. A. (2016). Mechanistic Insight into Bunyavirus-Induced Membrane Fusion from Structure-Function Analyses of the Hantavirus Envelope Glycoprotein Gc. *PLoS Pathog* 12(10): e1005813. 10.1371/journal.ppat.1005813
- Guarner, J. and Hale, G. L. (2019). Four human diseases with significant public health impact caused by mosquito-borne flaviviruses: West Nile, Zika, dengue and yellow fever. *Semin Diagn Pathol* 36(3): 170-176. 10.1053/j.semdp.2019.04.009
- Gubler, D. J. (2002). The global emergence/resurgence of arboviral diseases as public health problems. *Arch Med Res* 33(4): 330-342. 10.1016/s0188-4409(02)00378-8
- Guirakhoo, F., Pugachev, K., Arroyo, J., Miller, C., Zhang, Z.-X., Weltzin, R., Georgakopoulos, K., Catalan, J., Ocran, S., Draper, K. and Monath, T. P. (2002). Viremia and immunogenicity in nonhuman primates of a tetravalent yellow fever-dengue chimeric vaccine: genetic reconstructions, dose adjustment, and antibody responses against wild-type dengue virus isolates. *Virology* 298(1): 146-195. 10.1006/viro.2002.1462
- Guu, T. S. Y., Zheng, W. and Tao, Y. J. (2011). Bunyavirus: Structure and Replication. Viral Molecular Machines. Advances in Experimental Medicine and Biology, 726. Boston, MA, Springer.
- Guy, B., Guirakhoo, F., Barban, V., Higgs, S., Monath, T. P. and Lang, J. (2010a). Preclinical and clinical development of YFV 17D-based chimeric vaccines against dengue, West Nile and Japanese encephalitis viruses. *Vaccine* 28(3): 632-649. 10.1016/j.vaccine.2009.09.098

Guy, B., Saville, M. and Lang, J. (2010b). Development of Sanofi Pasteur tetravalent dengue vaccine. *Vaccine* 6(9): 696-705. 10.4161/hv.6.9.12739

Guzman, M. G., Halstead, S. B., Artsob, H., Buchy, P., Farrar, J., Gubler, D. J., Hunsperger, E., Kroeger, A., Margolis, H. S., Martínez, E., Nathan, M. B., Pelegriño, J. L., Simmons, C., Yoksan, S. and Peeling, R. W. (2015). Dengue: a continuing global threat. *Nat Rev Microbiol* 8(12 Suppl): S7-16. 10.1038/nrmicro2460

Guzman, M. G. and Vazquez, S. (2010). The Complexity of Antibody-Dependent Enhancement of Dengue Virus Infection. *Viruses* 2(12): 2649-2662. 10.3390/v2122649

Hackett, B. A. and Cherry, S. (2018). Flavivirus internalization is regulated by a size-dependent endocytic pathway. *PNAS* 115(16): 4246-4251. 10.1073/pnas.1720032115

Haddow, A. D., Perez-Sautu, U., Wiley, M. R., Miller, L. J., Kimmel, A. E., Principe, L. M., Wollen-Roberts, S. E., Shamblin, J. D., Valdez, S. M., Cazares, L. H., Pratt, W. D., Rossi, F. D., Lugo-Roman, L., Bavari, S., Palacios, G. F., Nalca, A., Nasar, F. and M. Louise M. Pitt (2020). Modeling mosquito-borne and sexual transmission of Zika virus in an enzootic host, the African green monkey. *PLoS Negl Trop Dis* 14(6): e0008107. 10.1371/journal.pntd.0008107

Hahn, C. S., Dalrymple, J. M., Strauss, J. H. and Rice, C. M. (1987). Comparison of the virulent Asibi strain of yellow fever virus with the 17D vaccine strain derived from it. *PNAS* 84: 2019-2023. 10.1073/pnas.84.7.2019

Hall, R. A., Scherret, J. H. and Machenzie, J. S. (2006). Kunjin Virus: An Australian Variant of West Nile? *Ann N Y Acad Sci* 951(1): 153-160. 10.1111/j.1749-6632.2001.tb02693.x

Halstead, S. B. (2018). Safety issues from a Phase 3 clinical trial of a live-attenuated chimeric yellow fever tetravalent dengue vaccine. *Hum Vaccin Immunother* 14(9): 2158-2162. 10.1080/21645515.2018.1445448

Halstead, S. B. and Thomas, S. J. (2010). New Vaccines for Japanese Encephalitis. *Curr Infect Dis Rep* 12: 174-180. 10.1007/s11908-010-0098-z

Hamlet, A., Gaythorpe, K. A. M., Garske, T. and Ferguson, N. M. (2021). Seasonal and inter-annual drivers of yellow fever transmission in South America. *PLoS Negl Trop Dis* 15(1): e0008974. 10.1371/journal.pntd.0008974

Hammon, W. M. (1947). The Arthropod-Borne Virus Encephalitides. *Am J Trop Med Hyg* s1-28(4): 515-525. 10.4269/ajtmh.1948.s1-28.515

Harrison, S. C. (2015). Viral membrane fusion. *Virology* 479-480: 498-507. 10.1016/j.virol.2015.03.043

Hartlage, A. S., Cullen, J. M. and Kapoor, A. (2017). The Strange, Expanding World of Animal Hepaciviruses. *Annu Rev Virol* 3(1): 53-75. 10.1146/annurev-virology-100114-055104

Hasan, S. S., Dey, D., Singh, S. and Martin, M. (2021). The Structural Biology of Eastern Equine Encephalitis Virus, an Emerging Viral Threat. *Pathogens* 10(8): 973. 10.3390/pathogens10080973

Hayes, C. G. and Monath, T. (1989). West Nile fever. Arboviruses: epidemiology and ecology, Vol. V.

Hayes, E. (2010). Is it time for a new yellow fever vaccine? *Vaccine* 28(51): 8073-8076. 10.1016/j.vaccine.2010.10.015

Hayes, E. B., Komar, N., Nasci, R. S., Montgomery, S. P., O'Leary, D. R. and Campbell, G. L. (2005). Epidemiology and Transmission Dynamics of West Nile Virus Disease. *Emerg Infect Dis* 11(8): 1167-1173. 10.3201/eid1108.050289a

Hegde, N. R. and Gore, M. M. (2017). Japanese encephalitis vaccines: Immunogenicity, protective efficacy, effectiveness, and impact on the burden of disease. *Hum Vaccin Immunother* 13(6): 1320-1337. 10.1080/21645515.2017.1285472

Henein, S., Adams, C., Bonaparte, M., Moser, J. M., Munteanu, A., Baric, R. and Silva, A. M. d. (2021). Dengue vaccine breakthrough infections reveal properties of neutralizing antibodies linked to protection. *J Clin Invest* 131(13): e147066. 10.1172/JCI147066

Henriques, H. R., Rampazo, E. V., Gonçalves, A. J. S., Vicentin, E. C. M., Amorim, J. H., Panatieri, R. H., Amorim, K. N. S., Yamamoto, M. M., Ferreira, L. C. S., Alves, A. M. B. and Boscardin, S. B. (2013). Targeting the Non-structural Protein 1 from Dengue Virus to a Dendritic Cell Population Confers Protective Immunity to Lethal Virus Challenge. *PLoS Negl Trop Dis* 7(7): e2330. 10.1371/journal.pntd.0002330

Higgs, S., Schneider, B. S., Vanlandingham, D. L., Klingler, K. A. and Gould, E. A. (2005). Nonviremic transmission of West Nile virus. *PNAS* 102(25): 8871-8874. 10.1073/pnas.0503835102

Higgs, S., Vanlandingham, D. L., Klingler, K. A., McElroy, K. L., McGee, C. E., Harrington, L., Lang, J., Monath, T. P. and Guirakhoo, F. (2006). Growth characteristics of ChimeriVax-Den vaccine viruses in *Aedes aegypti* and *Aedes albopictus* from Thailand. *Am J Trop Med Hyg* 75(5): 986-993

Holbrook, M. R. (2012). Kyasanur Forest Disease. *Antiviral Res* 96(3): 353-362. 10.1016/j.antiviral.2012.10.005

Holmes, E. C. and Twiddy, S. S. (2003). The origin, emergence and evolutionary genetics of dengue virus. *Infect Genet Evol* 3: 19-28. 10.1016/S1567-1348(03)00004-2

Huang, C. Y.-H., Butrapet, S., Moss, K. J., Childers, T., Erb, S. M., Calvert, A. E., Silengo, S. J., Kinney, R. M., Blair, C. D. and Roehrig, J. T. (2010). The dengue virus type 2 envelope protein

fusion peptide is essential for membrane fusion. *Virology* 396(2): 305-315.
10.1016/j.virol.2009.10.027

Huang, C. Y.-H., Butrapet, S., Pierro, D. J., Chang, G.-J. J., Hunt, A. R., Bhamarapravati, N., Gubler, D. J. and Kinney, R. M. (2000). Chimeric Dengue Type 2 (Vaccine Strain PDK-53)/Dengue Type 1 Virus as a Potential Candidate Dengue Type 1 Virus Vaccine. *J Virol* 74(7): 3020-3028. 10.1128/jvi.74.7.3020-3028.2000

Huang, Y.-J. (2014). Identification and characterization of the genetic determinants for yellow fever virus infection and dissemination in *Aedes aegypti* [Doctoral Dissertation, Kansas State University]. K-State Research Exchange.

Huang, Y.-J. S., Nuckols, J. T., Horne, K. M., Vanlandingham, D., Lobigs, M. and Higg, S. (2014). Mutagenesis analysis of T380R mutation in the envelope protein of yellow fever virus. *Virol J* 11(60). 10.1186/1743-422X-11-60

Hulswit, R. J. G., Paesen, G. C., Bowden, T. A. and Shi, X. (2021). Recent Advances in Bunyavirus Glycoprotein Research: Precursor Processing, Receptor Binding and Structure. *Viruses* 13: 353. 10.3390/v13020353

Hung, J., Hsieh, M., Young, M., Kao, C., King, C., and Chang, W. (2004). An External Loop Region of Domain III of Dengue Virus Type 2 Envelope Protein Is Involved in Serotype-Specific Binding to Mosquito but Not Mammalian Cells. *J Virol* 78(1): 378-388.
10.1128/JVI.78.1.378-388.2004

Hurlbut, H. S., Rizk, F., Taylor, R. M. and Work, T. H. (1956). A Study of the Ecology of West Nile Virus in Egypt. *Am J Trop Med Hyg* 5(4): 579-620. 10.4269/ajtmh.1956.5.579

Hurrelbrink, R. J. and McMinn, P. C. (2001). Attenuation of Murray Valley Encephalitis Virus by Site-Directed Mutagenesis of the Hinge and Putative Receptor-Binding Regions of the Envelope Protein. *J Virol* 75(16): 7692-7702. 10.1128/JVI.75.16.7692-7702.2001

Hurrelbrink, R. J. and McMinn, P. C. (2003). Molecular determinants of virulence: the structural and functional basis for flavivirus attenuation. *Adv Virus Res* 60: 1-42. 10.1016/s0065-3527(03)60001-1

Hyde, J. L., Chen, R., Trobaugh, D. W., Diamond, M. S., Weaver, S. C., Klimstra, W. B. and Wilusz, J. (2015). The 5' and 3' ends of alphavirus RNAs – Non-coding is not non-functional. *Virus Res* 206: 99-107. 10.1016/j.virusres.2015.01.016

ICTV (2022). Current ICTV Taxonomy Release - Taxonomy Browser.

Ikegami, T., Hill, T. E., Smith, J. K., Zhang, L., Juelich, T. L., Gong, B., Slack, O. A. L., Ly, H. J., Lokugamage, N. and Freiberg, A. N. (2015). Rift Valley Fever Virus MP-12 Vaccine Is Fully Attenuated by a Combination of Partial Attenuations in the S, M, and L Segments. *J Virol* 89(14): 7262-7276. 10.1128/JVI.00135-15

- Ikegami, T. and Makino, S. (2010). Rift Valley fever vaccines. *Vaccine* 27: D69-D72. 10.1016/j.vaccine.2009.07.046
- Isakov, N. (1997). ITIMs and ITAMs. The Yin and Yang of antigen and Fc receptor-linked signaling machinery. *Immunol Res* 16(1): 85-100. 10.1007/BF02786325
- Jarvi, S. I., Lieberman, M. M., Hofmeister, E., Nerurkar, V. R., Wong, T. and Levy, C. W. (2008). Protective efficacy of a recombinant subunit West Nile virus vaccine in domestic geese (Anser anser). *Vaccine* 26(42): 5338-5344. 10.1016/j.vaccine.2008.08.011
- Jordan, I., Briese, T. and Lipkin, W. I. (2009). Discovery and Molecular Characterization of West Nile Virus NY 1999. *Viral Immunol* 13(4): 435-446. 10.1089/vim.2000.13.435
- Jose, J., Snyder, J. E. and Kuhn, R. J. (2009). A structural and functional perspective of alphavirus replication and assembly. *Future Microbiol* 4: 837-856. 10.2217/fmb.09.59
- Kaiser, J. A. and Barrett, A. D. T. (2019). Twenty Years of Progress Toward West Nile Virus Vaccine Development. *Viruses* 11(9): 823. 10.3390/v11090823
- Kaiser, J. A., Luo, H., Widen, S. G., Wood, T. G., Huang, C. Y.-H., Wang, T. and Barrett, A. D. T. (2019a). Genotypic and phenotypic characterization of West Nile virus NS5 methyltransferase mutants. *Vaccine* 37(48): 7155-7164. 10.1016/j.vaccine.2019.09.045
- Kaiser, J. A., Luo, H., Widen, S. G., Wood, T. G., Huang, C. Y.-H., Wang, T. and Barrett, A. D. T. (2019b). Japanese encephalitis vaccine-specific envelope protein E138K mutation does not attenuate virulence of West Nile virus. *npj Vaccines* 4(50). 10.1038/s41541-019-0146-0
- Kaiser, J. A., Wang, T. and Barrett, A. D. (2017). Virulence determinants of West Nile virus: how can these be used for vaccine design? *Future Virol* 12(5): 283-295. 10.2217/fvl-2016-0141
- Kallas, E. G., Precioso, A. R., Palacios, R., Thomé, B., Braga, P. E., Vanni, T., Campos, L. M. A., Ferrari, L., Mondini, G., Salomão, M. d. G., Silva, A. d., Espinola, H. M., Santos, J. d. P., Santos, C. L. S., Timenetsky, M. d. C. S. T., Miraglia, J. L., Gallina, N. M. F., Weiskopf, D., Sette, A., Goulart, R., Salles, R. T., Maestri, A., Sallum, A. M. E., Farhat, S. C. L., Sakita, N. K., Ferreira, J. C. O. A., Silveira, C. G. T., Costa, P. R., Raw, I., Whitehead, S. S., Durbin, A. P. and Kalil, J. (2020). Safety and immunogenicity of the tetravalent, live-attenuated dengue vaccine Butantan-DV in adults in Brazil: a two-step, double-blind, randomised placebo-controlled phase 2 trial. *Lancet Infect Dis* 20(7): 839-850. 10.1016/S1473-3099(20)30023-2
- Kanai, R., Kar, K., Anthony, K., Gould, L. H., Ledizet, M., Fikrig, E., Marasco, W. A., Koski, R. A. and Modis, Y. (2006). Crystal Structure of West Nile Virus Envelope Glycoprotein Reveals Viral Surface Epitopes. *J Virol* 80(22): 11000-11008. 10.1128/jvi.01735-06
- Kasabi, G. S., Murhekar, M. V., Sandhya, V. K., Raghunandan, R., Kiran, S. K., Channabasappa, G. H. and Mehendale, S. M. (2013). Coverage and Effectiveness of Kyasanur Forest Disease

(KFD) Vaccine in Karnataka, South India, 2005–10. *PLoS Negl Trop Dis* 7(1): e2025. 10.1371/journal.pntd.0002025

Kaufmann, B., Vogt, M. T., Goudsmit, J., Holdaway, H. A., Aksyuk, A. A., Chipman, P. R., Kuhn, R. J., Diamond, M. S., and Rossmann, M. G. (2010). Neutralization of West Nile virus by cross-linking of its surface proteins with Fab fragments of the human monoclonal antibody CR4354. *PNAS* 107(44): 18950-18955. 10.1073/pnas.1011036107

Kemenesi, G. and Bányai, K. (2019). Tick-Borne Flaviviruses, with a Focus on Powassan Virus. *Clin Microbiol Rev* 32(1): e00106-00117. 10.1128/CMR.00106-17

Kim, A. S. and Diamond, M. S. (2022). A molecular understanding of alphavirus entry and antibody protection. *Nat Rev Microbiol*. 10.1038/s41579-022-00825-7

Kim, I. S., Jenni, S., Stanifer, M. L., Roth, E., Whelan, S. P. J., Oijen, A. M. v. and Harrison, S. C. (2017). Mechanism of membrane fusion induced by vesicular stomatitis virus G protein. *PNAS* 114(1): E28-E36. 10.1073/pnas.1618883114

Kinney, R. M., Huang, C. Y.-H., Whiteman, M. C., Bowen, R. A., Langevin, S. A., Miller, B. R. and Brault, A. C. (2006). Avian virulence and thermostable replication of the North American strain of West Nile virus. *J Gen Virol* 87(12): 3611-3622. 10.1099/vir.0.82299-0

Kitandwe, P. K., McKay, P. F., Kaleebu, P. and Shattock, R. J. (2022). An Overview of Rift Valley Fever Vaccine Development Strategies. *Vaccines* 10(11): 1794. 10.3390/vaccines10111794

Klaitong, P. and Smith, D. R. (2021). Roles of Non-Structural Protein 4A in Flavivirus Infection. *Viruses* 13(10): 2077. 10.3390/v13102077

Klein, D. E., Choi, J. L. and Harrison, S. C. (2012). Structure of a dengue envelope protein late-stage fusion intermediate. *J Virol* 87(4): 2287-2293. 10.1128/JVI.02957-12

Kleinschmidt-DeMasters, B. K. and Beckham, J. D. (2015). West Nile Virus Encephalitis 16 Years Later. *Brain Pathol* 25(5): 625-633. 10.1111/bpa.12280

Kling, K., Harder, T., Younger, Z., Burchard, G., Schmidt-Chanasit, J. and Wichmann, O. (2020). Vaccination against Japanese encephalitis with IC51: systematic review on immunogenicity, duration of protection and safety *J Travel Med* 27(2). 10.1093/jtm/taaa016

Klug, B., Robertson, J. S., Condit, R. C., Seligman, S. J., Laderoute, M. P., Sheets, R., Williamson, A.-L., Chapman, L., Carbery, B., Mac, L. M. and Chen, R. T. (2016). Adventitious Agents and Live Viral Vected Vaccines: Considerations for Archiving Samples of Biological Materials for Retrospective Analysis. *Vaccine* 34(51): 6617-6625. 10.1016/j.vaccine.2016.02.015

Knipe, D. M. and Howley, P. M. (2013). *Fields Virology*. Philadelphia, PA, Wolters Kluwer/Lippincott Williams & Wilkins Health.

Konishi, E., Yamaoka, M., Khin-Sane-Win, Kurane, I. and Mason, P. W. (1998). Induction of Protective Immunity against Japanese Encephalitis in Mice by Immunization with a Plasmid Encoding Japanese Encephalitis Virus Premembrane and Envelope Genes. *J Virol* 72(6): 4925-4930. 10.1128/JVI.72.6.4925-4930.1998

Kovacs, J. M., Mant, C. T. and Hodges, R. S. (2006). Determination of intrinsic hydrophilicity/hydrophobicity of amino acid side chains in peptides in the absence of nearest-neighbor or conformational effects. *Biopolymers* 84(3): 283-297. 10.1002/bip.20417

Ku, C. C., King, C. C., Lin, C. Y., Hsu, H. C., Chen, L. Y., Yueh, Y. Y. and Chang, G. J. (1994). Homologous and heterologous neutralization antibody responses after immunization with Japanese encephalitis vaccine among Taiwan children. *J Med Virol* 44(2): 122-131. 10.1002/jmv.1890440204

Kuhn, R. J., Zhang, W., Rossmann, M. G., Pletnev, S. V., Corver, J., Lenches, E., Jones, C. T., Mukhopadhyay, S., Chipman, P. R., Strauss, E. G., Baker, T. S. and Strauss, J. H. (2002). Structure of Dengue Virus: Implications for Flavivirus Organization, Maturation, and Fusion. *Cell* 108(5): 717-725. 10.1016/S0092-8674(02)00660-8

Kumar, R., Tripathi, P. and Rizvi, A. (2009). Effectiveness of One Dose of SA 14-14-2 Vaccine against Japanese Encephalitis. *N Engl J Med* 360: 1465-1466. 10.1056/NEJMc0808664

Kuno, G., Chang, G.-J. J., Tsuchiya, K. R., Karabatsos, N. and Cropp, C. B. (1998). Phylogeny of the Genus Flavivirus. *J Virol* 72(1): 73-83. 10.1128/jvi.72.1.73-83.1998

Kunz, C. (2003). TBE vaccination and the Austrian experience. *Vaccine* 21: S50-S55. 10.1016/S0264-410X(02)00813-7

Lambert, A. J. and Hughes, H. R. (2021). Clinically Important Phleboviruses and Their Detection in Human Samples. *Viruses* 13(8): 1500. 10.3390/v13081500

Lasecka, L. and Baron, M. D. (2014). The molecular biology of nairoviruses, an emerging group of tick-borne arboviruses. *Arch Virol* 159(6): 1249-1265. 10.1007/s00705-013-1940-z

Lecomte, E., Laureys, G., Verbeke, F., Carrasco, C. D., Esbroeck, M. V. and Huits, R. (2020). A clinician's perspective on yellow fever vaccine-associated neurotropic disease. *J Travel Med* 27(7): taaa172. 10.1093/jtm/taaa172

Lee, E. and Lobigs, M. (2008). E Protein Domain III Determinants of Yellow Fever Virus 17D Vaccine Strain Enhance Binding to Glycosaminoglycans, Impede Virus Spread, and Attenuate Virulence. *J Virol* 82(12): 6024-6033. 10.1128/JVI.02509-07

- Lefevre, A., Marianneau, P. and Deubel, V. (2004). Current Assessment of Yellow Fever and Yellow Fever Vaccine. *Curr Infect Dis Rep* 6(2): 96-104. 10.1007/s11908-996-0005-9
- Lei, Y., Takeda, K. and Yu, L. (2018). Impaired heterologous protein-protein interaction is an essential cause for non-viability of WNV/DENV recombinants. *Virology* 524: 140-150. 10.1016/j.virol.2018.08.013
- Lequime, S. and Lambrechts, L. (2014). Vertical transmission of arboviruses in mosquitoes: A historical perspective. *Infect Genet Evol* 28: 681-690. 10.1016/j.meegid.2014.07.025
- Leung, J. Y.-S., Ng, M. M.-L. and Chu, J. J. H. (2011). Replication of Alphaviruses: A Review on the Entry Process of Alphaviruses into Cells. *Adv Virol*(249640). 10.1155/2011/249640
- Leung, J. Y., Pijlman, G. P., Kondratieva, N., Hyde, J., Mackenzie, J. M. and Khromykh, A. A. (2008). Role of Nonstructural Protein NS2A in Flavivirus Assembly. *J Virol* 82(10): 4731-4741. 10.1128/JVI.00002-08
- Li, G., Adam, A., Luo, H., Shan, C., Cao, Z., Fontes-Garfias, C. R., Sarathy, V. V., Teleki, C., Winkelmann, E. R., Liang, Y., Sun, J., Bourne, N., Barrett, A. D. T., Shi, P.-Y. and Wang, T. (2019). An attenuated Zika virus NS4B protein mutant is a potent inducer of antiviral immune responses. *npj Vaccines* 4(48). 10.1038/s41541-019-0143-3
- Li, H.-C., Yang, C.-H. and Lo, S.-Y. (2021). Hepatitis C Viral Replication Complex. *Viruses* 13(3): 520. 10.3390/v13030520
- Li, L., Jose, J., Xiang, Y., Kuhn, R. J. and Rossmann, M. G. (2010). Structural changes of envelope proteins during alphavirus fusion. *Nature* 468(7324): 705-708. 10.1038/nature09546
- Li, X., Ma, S.-J., Liu, X., Jiang, L.-N., Zhou, J.-H., Xiong, Y.-Q., Ding, H. and Chen, Q. (2014). Immunogenicity and safety of currently available Japanese encephalitis vaccines: A systematic review. *Hum Vaccin Immunother* 10(12): 3579-3593. 10.4161/21645515.2014.980197
- Lieberman, M. M., Clements, D. E., Ogata, S., Wang, G., Corpuz, G., Wong, T., Martyak, T., Gilson, L., Collier, B.-A., Leung, J., Watts, D. M., Tesh, R. B., Siirin, M., Rosa, A. T. d., Humphreys, T. and Weeks-Levy, C. (2007). Preparation and immunogenic properties of a recombinant West Nile subunit vaccine. *Vaccine* 25(3): 414-423. 10.1016/j.vaccine.2006.08.018
- Lieberman, M. M., Nerurkar, V. R., Luo, H., Cropp, B., Ricardo Carrion, J., Garza, M. d. l., Collier, B.-A., Clements, D., Ogata, S., Wong, T., Martyak, T. and Weeks-Levy, C. (2009). Immunogenicity and Protective Efficacy of a Recombinant Subunit West Nile Virus Vaccine in Rhesus Monkeys. *Clin Vaccine Immunol* 16(9): 1332-1337. 10.1128/CI.00119-09
- Lindenbach, B. D., Thiel, H. J. and Rice, C. M. (2007). Flaviviridae :The Viruses and Their Replication. PA, USA, Lippincott-Raven Publishers.

- Lindquist, L. and Vapalahti, O. (2008). Tick-borne encephalitis. *Lancet* 371(9627): 1861-1871. 10.1016/S0140-6736(08)60800-4
- Liu, Z.-L., Hennessy, S., Strom, B. L., Tsai, T. F., Wan, C.-M., Tang, S.-C., Xiang, C.-F., Bilker, W. B., Pan, X.-P., Yao, Y.-J., Xu, Z.-W. and Halstead, S. B. (1997). Short-Term Safety of Live Attenuated Japanese Encephalitis Vaccine (SA14-14-2): Results of a Randomized Trial with 26,239 Subjects *J Infect Dis* 176(5): 1366-1369. 10.1086/517323
- Lorenz, I. C., Allison, S. L., Heinz, F. X. and Helenius, A. (2002). Folding and Dimerization of Tick-Borne Encephalitis Virus Envelope Proteins prM and E in the Endoplasmic Reticulum. *J Virol* 76(11): 5480-5491. 10.1128/JVI.76.11.5480-5491.2002
- Lousa, D. and Soares, C. M. (2021). Molecular mechanisms of the influenza fusion peptide: insights from experimental and simulation studies. *FEBS Open Bio* 11(12): 3253-3261. 10.1002/2211-5463.13323
- Low, J. G. H., Ooi, E. E. and Vasudevan, S. G. (2017). Current Status of Dengue Therapeutics Research and Development *J Infect Dis* 215(suppl_2): S96-S102. 10.1093/infdis/jiw423
- Lu, X., Xiao, H., Li, S., Pang, X., Song, J., Liu, S., Cheng, H., Li, Y., Wang, X., Huang, C., Guo, T., Meulen, J. t., Daffis, S., Yan, J., Dai, L., Rao, Z., Klenk, H.-D., Qi, J., Shi, Y. and Gao, G. F. (2019). Double Lock of a Human Neutralizing and Protective Monoclonal Antibody Targeting the Yellow Fever Virus Envelope. *Cell Rep* 26(2): 438-446. 10.1016/j.celrep.2018.12.065
- Luca, V. C., AbiMansour, J., Nelson, C. A. and Fremont, D. H. (2012). Crystal Structure of the Japanese Encephalitis Virus Envelope Protein. *J Virol* 86(4): 2337-2346. 10.1128/JVI.06072-11
- Luethy, D. (2023). Eastern, Western, and Venezuelan Equine Encephalitis and West Nile Viruses: Clinical and Public Health Considerations. *Vet Clin N Am Equine Pract* 39(1): 99-113. 10.1016/j.cveq.2022.11.007
- Lumley, S., Horton, D. L., Hernandez-Triana, L. L. M., Johnson, N., Fooks, A. R. and Hewson, R. (2017). Rift Valley fever virus: strategies for maintenance, survival and vertical transmission in mosquitoes. *J Gen Virol* 98(5): 875-887. 10.1099/jgv.0.000765
- Lustig, Y., Mannasse, B., Koren, R., Katz-Likvornik, S., Hindiyeh, M., Mandelboim, M., Dovrat, S., Sofer, D. and Mendelson, E. (2016). Superiority of West Nile Virus RNA Detection in Whole Blood for Diagnosis of Acute Infection. *J Clin Microbiol* 54(9): 2294-2297. 10.1128/JCM.01283-16
- Lyons, A. C., Huang, Y.-J. S., Park, S. L., Ayers, V. B., Hettenbach, S. M., Higgs, S., McVey, D. S., Noronha, L., Hsu, W.-W. and Vanlandingham, D. L. (2018). Shedding of Japanese Encephalitis Virus in Oral Fluid of Infected Swine. *Vector Borne Zoonotic Dis* 18(9): 469-474. 10.1089/vbz.2018.2283

- Mackenzie, J. S., William, D. T., Hurk, A. F. v. d., Smith, D. W. and Currie, B. J. (2022). Japanese Encephalitis Virus: The Emergence of Genotype IV in Australia and Its Potential Endemicity. *Viruses* 14(11): 2480. 10.3390/v14112480
- MacNamara, F. N. (1953). Reactions following neurotropic yellow fever vaccine given by scarification in Nigeria. *Trans R Soc Trop Med Hyg* 47(3): 199-208. 10.1016/0035-9203(53)90003-0
- Mandary, M. B., Masomian, M. and Poh, C. L. (2019). Impact of RNA Virus Evolution on Quasispecies Formation and Virulence. *Int J Mol Sci* 20(18): 4657. 10.3390/ijms20184657
- Mann, B. R., McMullen, A. R., Swetnam, D. M. and Barrett, A. D. T. (2013). Molecular Epidemiology and Evolution of West Nile Virus in North America. *Int J Environ Res Public Health* 10(10): 5111-5129. 10.3390/ijerph10105111
- Mansfield, K. L., Folly, A. J., Hernández-Triana, L. M., Sewgobind, S. and Johnson, N. (2022). Batai Orthobunyavirus: An Emerging Mosquito-Borne Virus in Europe. *Viruses* 14: 1868. 10.3390/v14091868
- Mansfield, K. L., Johnson, N., Phipps, L. P., Stephenson, J. R., Fooks, A. R. and Solomon, T. (2009). Tick-borne encephalitis virus - a review of an emerging zoonosis. *J Gen Virol* 90(Pt 8): 1781-1794. 10.1099/vir.0.011437-0
- Manson-Bahr, P. H. and Alcock, A. (1927). The life and work of Sir Patrick Manson. New York, William Wood and Company.
- Marquardt, W. C. (2005). Biology of Disease Vectors. Burlington, MA, Elsevier Academic Press.
- Martin, J. E., Pierson, T. C., Hubka, S., Rucker, S., Gordon, I. J., Enama, M. E., Andrews, C. A., Xu, Q., Davis, B. S., Nason, M. C., Fay, M. P., Koup, R. A., Roederer, M., Bailer, R. T., Gomez, P. L., Mascola, J. R., Chang, G.-J. J., Nabel, G. J., Graham, B. S. and Teama, V. R. C. S. (2009). A West Nile Virus DNA Vaccine Induces Neutralizing Antibody in Healthy Adults during a Phase 1 Clinical Trial. *J Infect Dis* 196(12): 1732-1740. 10.1086/523650
- Martinez, L. J., Lin, L., Blaylock, J. M., Lyons, A. G., Bauer, K. M., Barrera, R. D. L., Simmons, M., Jarman, R. G., Currier, J. R., Friberg, H., Danko, J. R., Teneza-Mora, N. C., Putnak, J. R., Eckels, K. H. and Thomas, S. J. (2015). Safety and Immunogenicity of a Dengue Virus Serotype-1 Purified-Inactivated Vaccine: Results of a Phase 1 Clinical Trial. *Am J Trop Med Hyg* 93(3): 454-460. 10.4269/ajtmh.14-0819
- Mason, R. A., Tauraso, N. M., Ginn, R. K., O'Brien, T. C. and Trimmer, R. W. (1972). Yellow fever vaccine. V. Antibody response in maonkeys inoculated with graded doses of the 17D vaccine. *Appl Microbiol* 23(5): 908-913. 10.1128/am.23.5.908-913.1972

Mason, R. A., Tauraso, N. M., Spertzel, R. O. and Ginn, R. K. (1973). Yellow Fever Vaccine: Direct Challenge of Monkeys Given Graded Doses of 17D Vaccine. *Appl Microbiol* 25(4): 539-544. 10.1128/am.25.4.539-544.1973

Mattingly, P. T. (1957). Genetical aspects of the *Aedes aegypti* problem. I. Taxonom: and bionomics. *Ann Trop Med Parasitol* 51(4): 392-408

May, F. J., Davis, C. T., Tesh, R. B. and Barrett, A. D. T. (2011). Phylogeography of West Nile Virus: from the Cradle of Evolution in Africa to Eurasia, Australia, and the Americas. *J Virol* 85(6): 2964-2974. 10.1128/JVI.01963-10

Mbaye, M. N., Hou, Q., Basu, S., Teheux, F., Pucci, F. and Rooman, M. (2019). A comprehensive computational study of amino acid interactions in membrane proteins. *Sci Rep* 9(1): 12043. 10.1038/s41598-019-48541-2

McArthur, J. H., Durbin, A. P., Marron, J. A., Wanionek, K. A., Thumar, B., Pierro, D. J., Schmidt, A. C., Jr, J. E. B., Murphy, B. R. and Whitehead, S. S. (2008). Phase I clinical evaluation of rDEN4Delta30-200,201: a live attenuated dengue 4 vaccine candidate designed for decreased hepatotoxicity. *Am J Trop Med Hyg* 79(5): 678-684

McAuley, A. J., Sawatsky, B., Ksiazek, T., Torres, M., Korva, M., Lotrič-Furlan, S., Avšič-Županc, T., Messling, V. v., Holbrook, M. R., Freiberg, A. N., Beasley, D. W. C. and Bente, D. A. (2017). Cross-neutralisation of viruses of the tick-borne encephalitis complex following tick-borne encephalitis vaccination and/or infection. *npj Vaccines* 2(5). 10.1038/s41541-017-0009-5

McElroy, K. L., Girard, Y. A., McGee, C. E., Tsetsarkin, K. A., Vanlandingham, D. L. and Higgs, S. (2008). Characterization of the antigen distribution and tissue tropisms of three phenotypically distinct yellow fever virus variants in orally infected *Aedes aegypti* mosquitoes. *Vector Borne and Zoonotic Dis* 8(5): 675-687. 10.1089/vbz.2007.0269

McElroy, K. L., Tsetsarkin, K. A., Vanlandingham, D. L. and Higgs, S. (2005). Characterization of an infectious clone of the wild-type yellow fever virus Asibi strain that is able to infect and disseminate in mosquitoes *J Gen Virol* 86(6): 1747-1751. 10.1099/vir.0.80746-0

McElroy, K. L., Tsetsarkin, K. A., Vanlandingham, D. L. and Higgs, S. (2006a). Manipulation of the yellow fever virus non-structural genes 2A and 4B and the 3'non-coding region to evaluate genetic determinants of viral dissemination from the *Aedes aegypti* midgut. *Am J Trop Med Hyg* 75(6): 1158-1164

McElroy, K. L., Tsetsarkin, K. A., Vanlandingham, D. L. and Higgs, S. (2006b). Role of the yellow fever virus structural protein genes in viral dissemination from the *Aedes aegypti* mosquito midgut. *J Gen Virol* 87(Pt 10): 2993-3001. 10.1099/vir.0.82023-0

McKee Jr, K. T., Bancroft, W. H., Eckels, K. H., Redfield, R. R., Summers, P. L. and Russell, P. K. (1987). Lack of attenuation of a candidate dengue 1 vaccine (45AZ5) in human volunteers. *Am J Trop Med Hyg* 36(2): 435-442. 10.4269/ajtmh.1987.36.435

Médits, I., Vaney, M.-C., Rouvinski, A., Rey, M., Chamot-Rooke, J., Rey, F. A., Heinz, F. X. and Stiasny, K. (2020). Extensive flavivirus E trimer breathing accompanies stem zippering of the post-fusion hairpin. *EMBO Rep* 21(8): e50096. 10.15252/embr.202050069

Meertens, L., Carnec, X., Lecoïn, M. P., Ramdasi, R., Guivel-Benhassine, F., Lew, E., Lemke, G. and Olivier Schwartz, A. A. (2012). The TIM and TAM families of phosphatidylserine receptors mediate dengue virus entry. *Cell Host Microbe* 12(4): 544-557. 10.1016/j.chom.2012.08.009

Meier, K. C., Gardner, C. L., Khoretonenko, M. V., Klimstra, W. B. and Ryman, K. D. (2009). A mouse model for studying viscerotropic disease caused by yellow fever virus infection. *PLoS Pathog* 5(10): e1000614. 10.1371/journal.ppat.1000614

Mignotte, A., Garros, C., Dellicour, S., Jacquot, M., Gilbert, M., Gardés, L., Balenghien, T., Duhayon, M., Rakotoarivony, I., de Wavrechin, M., and Huber, K. (2021). High dispersal capacity of *Culicoides obsoletus* (Diptera: Ceratopogonidae), vector of bluetongue and Schmallenberg viruses, revealed by landscape genetic analyses. *Parasit Vectors* 14(1): 93. 10.1186/s13071-020-04522-3

Modis, Y., Ogata, S., Clements, D. and Harrison, S. C. (2003). A ligand-binding pocket in the dengue virus envelope glycoprotein. *PNAS* 100(12): 6986-6991. 10.1073/pnas.0832193100

Modis, Y., Ogata, S., Clements, D. and Harrison, S. C. (2004). Structure of the dengue virus envelope protein after membrane fusion. *Nature* 427: 313-319. 10.1038/nature02165

Mokaya, J., Kimathi, D., Lambe, T. and Warimwe, G. M. (2021). What Constitutes Protective Immunity Following Yellow Fever Vaccination? *Vaccines (Basel)* 9(6): 671. 10.3390/vaccines9060671

Monath, T. P. (2001). Yellow fever: an update. *Lancet Infect Dis* 1(1): 11-20. 10.1016/S1473-3099(01)00016-0

Monath, T. P. (2008). Treatment of yellow fever. *Antiviral Res* 78(1): 116-124. 10.1016/j.antiviral.2007.10.009

Monath, T. P. (2010). Short Report: Suspected Yellow Fever Vaccine-Associated Viscerotropic Adverse Events (1973 and 1978), United States. *Am J Trop Med Hyg* 82(5): 919-921. 10.4269/ajtmh.2010.10-0001

Monath, T. P., Soike, K., Levenbook, I., Zhang, Z.-X., Arroyo, J., Delagrave, S., Myers, G., Barrett, A. D. T., Shope, R. E., Ratterree, M., Chambers, T. J. and Guirakhoo, F. (1999). Recombinant, chimeric live, attenuated vaccine (ChimeriVax™) incorporating the envelope genes of Japanese encephalitis (SA14-14-2) virus and the capsid and nonstructural genes of yellow fever (17D) virus is safe, immunogenic and protective in non-human primates. *Vaccine* 17(15-16): 1869-1882. 10.1016/S0264-410X(98)00487-3

- Monera, O. D., Sereda, T. J., Zhou, N. E., Kay, C. M. and Hodges, R. S. (1995). Relationship of sidechain hydrophobicity and alpha-helical propensity on the stability of the single-stranded amphipathic alpha-helix. *J Pept Sci* 1(5): 319-329. 10.1002/psc.310010507
- Morrison, D., Legg, T. J., Billings, C. W., Forrat, R., Yoksan, S. and Lang, J. (2010). A Novel Tetravalent Dengue Vaccine Is Well Tolerated and Immunogenic against All 4 Serotypes in Flavivirus-Naive Adults. *J Infect Dis* 201(3): 370-377. 10.1086/649916
- Mukhopadhyay, S., Kim, B.-S., Chipman, P. R., Rossmann, M. G. and Kuhn, R. J. (2003). Structure of West Nile Virus. *Science* 302(5643): 248. 10.1126/science.1089316
- Mukhopadhyay, S., Kuhn, R. J. and Rossmann, M. G. (2005). A structural perspective of the flavivirus life cycle. *Nat Rev Microbiol* 3: 13-22. 10.1038/nrmicro1067
- Munivenkatappa, A., Sahay, R. R., Yadav, P. D., Viswanathan, R. and Mourya, D. T. (2018). Clinical & epidemiological significance of Kyasanur forest disease. *Indian J Med Res* 148(2): 145-150. 10.4103/ijmr.IJMR_688_17
- Mutebi, J.-P. and Barrett, A. D. T. (2002). The epidemiology of yellow fever in Africa. *Microbes Infect* 4(14): 1459-1468. 10.1016/s1286-4579(02)00028-x
- Mutebi, J.-P., Wang, H., Li, L., Bryant, J. E. and Barrett, A. D. T. (2001). Phylogenetic and Evolutionary Relationships among Yellow Fever Virus Isolates in Africa. *J Virol* 75(15): 6999-7008. 10.1128/JVI.75.15.6999-7008.2001
- Myles, K. M., Pierro, D. J. and Olson, K. E. (2003). Deletions in the putative cell receptor-binding domain of Sindbis virus strain MRE16 E2 glycoprotein reduce midgut infectivity in *Aedes aegypti*. *J Virol* 77(16): 8872-8881. 10.1128/jvi.77.16.8872-8881.2003
- NIAID (2015). "West Nile Virus Vaccines." from <https://www.niaid.nih.gov/diseases-conditions/wnv-vaccines>.
- NIAID (2018). "NIAID Emerging Infectious Diseases/ Pathogens." from <https://www.niaid.nih.gov/research/emerging-infectious-diseases-pathogens>.
- Nickells, M. and Chambers, T. J. (2003). Neuroadapted Yellow Fever Virus 17D: Determinants in the Envelope Protein Govern Neuroinvasiveness for SCID Mice. *J Virol* 77(22): 12232-12242. 10.1128/JVI.77.22.12232-12242.2003
- NIH (2009). "Safety Study of HBV-002 West Nile Vaccine in Healthy Adults." from <https://clinicaltrials.gov/ct2/show/NCT00707642>.
- NIH (2016). "Safety and Immunogenicity of ChimeriVax-WN02 West Nile Vaccine in Healthy Adults." from <https://classic.clinicaltrials.gov/ct2/show/NCT00442169>.

NIH (2019). Phase 1 Trial of Inactivated West Nile Virus Vaccine.

NIH (2022). "Treatment of West Nile Virus With MGAWN1 (PARADIGM)." from <https://classic.clinicaltrials.gov/ct2/show/NCT00927953?cond=west+nile&draw=3&rank=18>.

Nybakken, G. E., Nelson, C. A., Chen, B. R., Diamond, M. S. and Fremont, D. H. (2006). Crystal Structure of the West Nile Virus Envelope Glycoprotein. *J Virol* 80(23): 11467-11474. 10.1128/JVI.01125-06

Okada, K., Iwasa, T., Group, J. V. C. S., Namazue, J., Akechi, M. and Ueda, S. (2012). Safety and immunogenicity of a freeze-dried, cell culture-derived Japanese encephalitis vaccine (Inactivated) (JEBIK®V) in children. *Vaccine* 30(41): 5967-5972. 10.1016/j.vaccine.2012.07.034

Oliveira, A., Selvara, K., Tripathy, J. P., Betodkar, U., Cacodcar, J. and Wadkar, A. (2019). Kyasanur Forest Disease vaccination coverage and its perceived barriers in Goa, India—A mixed methods operational research. *PLoS One* 14(12): e0226141. 10.1371/journal.pone.0226141

Onofrio, A., Parisi, G., Punzi, G., Todisco, S., Noia, M. A. D., Bossis, F., Turi, A., Grassi, A. D. and Pierri, C. L. (2014). Distance-dependent hydrophobic–hydrophobic contacts in protein folding simulations. *Phys Chem Chem Phys* 16(35): 18907-18917. 10.1039/c4cp01131g

Orlinger, K. K., Hofmeister, Y., Fritz, R., Holzer, G. W., Falkner, F. G., Unger, B., Loew-Baselli, A., Poellabauer, E.-M., Ehrlich, H. J., Barrett, P. N. and Kreil, T. R. (2011). A Tick-borne Encephalitis Virus Vaccine Based on the European Prototype Strain Induces Broadly Reactive Cross-neutralizing Antibodies in Humans *J Infect Dis* 203(11): 1556-1564. 10.1093/infdis/jir122

Ortiz-Martínez, Y., Vega-Useche, L., Villamil-Gómez, W. E. and Rodriguez-Morales, A. J. (2017). Saint Louis Encephalitis Virus, another re-emerging arbovirus: a literature review of worldwide research. *Infez Med* 25(1): 77-79

Osorio, J. E., Brewoo, J. N., Silengo, S. J., Arguello, J., Moldovan, I. R., Tary-Lehmann, M., Powell, T. D., Livengood, J. A., Kinney, R. M., Huang, C. Y.-H. and Stinchcomb, D. T. (2011). Efficacy of a Tetravalent Chimeric Dengue Vaccine (DENVax) in Cynomolgus Macaques. *Am J Trop Med Hyg* 84(6): 978-987. 10.4269/ajtmh.2011.10-0592

Pardi, N., Hogan, M. J., Pelc, R. S., Muramatsu, H., Andersen, H., DeMaso, C. R., Dowd, K. A., Sutherland, L. L., Scarce, R. M., Parks, R., Wagner, W., Granados, A., Greenhouse, J., Walker, M., Willis, E., Yu, J.-S., McGee, C. E., Sempowski, G. D., Mui, B. L., Tam, Y. K., Huang, Y.-J., Vanlandingham, D., Holmes, V. M., Balachandran, H., Sahu, S., Lifton, M., Higgs, S., Hensley, S. E., Madden, T. D., Hope, M. J., Karikó, K., Santra, S., Graham, B. S., Lewis, M. G., Pierson, T. C., Haynes, B. F. and Weissman, D. (2017). Zika virus protection by a single low dose nucleoside modified mRNA vaccination. *Nature* 543(7644): 248-251. 10.1038/nature21428

- Patel, H., Sander, B. and Nelder, M. P. (2015). Long-term sequelae of West Nile virus-related illness: a systematic review. *Lancet Infect Dis* 15(8): 951-959. 10.1016/S1473-3099(15)00134-6
- Patterson, K. D. (1992). Yellow fever epidemics and mortality in the United States, 1693-1905. *Soc Sci Med* 34(8): 855-865. 10.1016/0277-9536(92)90255-o
- Peltier, M. (1947). Yellow fever vaccination, simple or associated with vaccination against smallpox, of the populations of French West Africa by the method of the Pasteur Institute of Dakar. *Am J Public Health Nations Health* 37(8): 1026-1032
- Pfizer (2023). "TicoVac - Important Safety Information and Indication." from <https://www.ticovac.com/>.
- Philip, C. B. and Smadel, J. E. (1943). Transmission of West Nile Virus by Infected *Aedes albopictus*. *Proc Soc Exp Biol Med* 53: 49-50
- Pierce, K. K., Whitehead, S. S., Kirkpatrick, B. D., Grier, P. L., Jarvis, A., Kenney, H., Carmolli, M. P., Reynolds, C., Tibery, C. M., Lovchik, J., Janiak, A., Luke, C. J., Durbin, A. P. and Pletnev, A. G. (2017). A Live Attenuated Chimeric West Nile Virus Vaccine, rWN/DEN4Δ30, Is Well Tolerated and Immunogenic in Flavivirus-Naive Older Adult Volunteers. *J Infect Dis* 5(1): 52-55. 10.1093/infdis/jiw501
- Pierson, T. C. and Diamond, M. S. (2020). The continued threat of emerging flaviviruses. *Nat Microbiol* 5(6): 796-812. 10.1038/s41564-020-0714-0
- Pletnev, A. G., Putnak, R., Speicher, J., Wagar, E. J. and Vaughn, D. W. (2002). West Nile virus/dengue type 4 virus chimeras that are reduced in neurovirulence and peripheral virulence without loss of immunogenicity or protective efficacy. *PNAS* 99(5): 3036-3041. 10.1073/pnas.022652799
- Poland, J. D., Calisher, C. H., Monath, T. P., Downs, W. G. and Murphy, K. (1981). Persistence of neutralizing antibody 30-35 years after immunization with 17D yellow fever vaccine. *Bull World Health Organ* 59(6): 895-900
- Porterfield, J. S. (1975). The basis of arbovirus classification. *Med Biol* 53(5): 400-405
- Powers, A. M., Brault, A. C., Shirako, Y., Strauss, E. G., Kang, W., Strauss, J. H. and Weaver, S. C. (2001). Evolutionary relationships and systematics of the alphaviruses. *J Virol* 75(21): 10118-10131. 10.1128/JVI.75.21.10118-10131.2001
- Prommalikit, O. and Thisyakorn, U. (2015). Dengue virus virulence and disease severity. *Southeast Asian J Trop Med Public Health* 46: 35-42
- Puerta-Guardo, H., Glasner, D. R., Espinosa, D. A., Biering, S. B., Patana, M., Ratnasiri, K., Wang, C., Beatty, P. R. and Harris, E. (2019). Flavivirus NS1 Triggers Tissue-Specific Vascular

- Endothelial Dysfunction Reflecting Disease Tropism. *Cell Rep* 26(6): 1598-1613.e1598. 10.1016/j.celrep.2019.01.036
- Puerta-Guardo, H., Glasner, D. R. and Harris*, E. (2016). Dengue Virus NS1 Disrupts the Endothelial Glycocalyx, Leading to Hyperpermeability. *PLoS Pathog* 12(7): e1005738. 10.1371/journal.ppat.1005738
- Pugachev, K. V., Guirakhoo, F., Ocran, S. W., Mitchell, F., Parsons, M., Penal, C., Girakhoo, S., Pougatcheva, S. O., Arroyo, J., Trent, D. W. and Monath, T. P. (2004). High Fidelity of Yellow Fever Virus RNA Polymerase. *J Virol* 78(2): 1032-1038. 10.1128/JVI.78.2.1032-1038.2004
- Pulkkinen, L. I. A., Barrass, S. V., Domanska, A., Överby, A. K., Anastasina, M. and Butcher, S. J. (2022). Molecular Organisation of Tick-Borne Encephalitis Virus. *Viruses* 14(4): 792. 10.3390/v14040792
- Pulkkinen, L. I. A., Butcher, S. J. and Anastasina, M. (2018). Tick-Borne Encephalitis Virus: A Structural View. *Viruses* 10(7): 350. 10.3390/v10070350
- Pybus, O. G. and Thézé, J. (2016). Hepacivirus cross-species transmission and the origins of the hepatitis C virus. *Curr Opin Virol* 16: 1-7. 10.1016/j.coviro.2015.10.002
- Querec, T., Bennouna, S., Alkan, S., Laouar, Y., Gorden, K., Flavell, R., Akira, S., Ahmed, R. and Pulendran, B. (2006). Yellow fever vaccine YF-17D activates multiple dendritic cell subsets via TLR2, 7, 8, and 9 to stimulate polyvalent immunity. *J Exp Med* 203(2): 413-424. 10.1084/jem.20051720
- Randolph, S. E. (2011). Transmission of tick-borne pathogens between co-feeding ticks: Milan Labuda's enduring paradigm. *Ticks Tick Borne Dis* 2(4): 179-182. 10.1016/j.ttbdis.2011.07.004
- Randolph, S. E., Gern, L. and Nuttall, P. A. (1996). Co-feeding ticks: Epidemiological significance for tick-borne pathogen transmission. *Parasitol Today* 12(12): 472-479. 10.1016/S0169-4758(96)10072-7
- Razinkov, V. I., Melikyan, G. B. and Cohen, F. S. (1999). Hemifusion between cells expressing hemagglutinin of influenza virus and planar membranes can precede the formation of fusion pores that subsequently fully enlarge. *Biophys J* 77(6): 3144-3151. 10.1016/S0006-3495(99)77144-4
- Reiner Jr., R. C., Achee, N., Barrera, R., Burkot, T. R., Chadee, D. D., Devine, G. J., Endy, T., Gubler, D., Hombach, J., Kleinschmidt, I., Lenhart, A., Lindsay, S. W., Longini, I., Mondy, M., Morrison, A. C., Perkins, T. A., Vazquez-Prokopec, G., Reiter, P., Ritchie, S. A., Smith, D. L., Strickman, D. and Scott, T. W. (2016). Quantifying the Epidemiological Impact of Vector Control on Dengue. *PLoS Negl Trop Dis* 10(5): e0004588. 10.1371/journal.pntd.0004588

- Rey, F. A., Heinz, F. X., Mandlt, C., Kunz, C. and Harrison, S. C. (1995). The envelope glycoprotein from tick-borne encephalitis virus at 2 Å resolution. *Nature* 375(6529): 291-298. 10.1038/375291a0
- Riedel, C., Aitkenhead, H., Omari, K. E. and Rümenapf, T. (2021). Atypical Porcine Pestiviruses: Relationships and Conserved Structural Features. *Viruses* 13(5): 760. 10.3390/v13050760
- Rivera, L., Biswal, S., Sáez-Llorens, X., Reynales, H., López-Medina, E., Borja-Tabora, C., Bravo, L., Sirivichayakul, C., Kosalaraksa, P., Vargas, L. M., Yu, D., Watanaveeradej, V., Espinoza, F., Dietze, R., Fernando, L., Wickramasinghe, P., Jr, E. D. M., Fernando, A. D., Gunasekera, D., Luz, K., Cunha, R. V., Rauscher, M., Zent, O., Liu, M., Hoffman, E., LeFevre, I., Tricou, V., Wallace, D., Alera, M. and Borkowski, A. (2022). Three-year Efficacy and Safety of Takeda's Dengue Vaccine Candidate (TAK-003). *Clin Infect Dis* 75(1): 107-117. 10.1093/cid/ciab864
- Rizzo, S., Imperato, P., Mora-Cárdenas, E., Konstantinidou, S., Marcello, A. and Sblattero, D. (2020). Selection and characterization of highly specific recombinant antibodies against West Nile Virus E protein. *J Biotechnol* 311: 35-43. 10.1016/j.jbiotec.2020.02.004
- Robich, R. M., Cosenza, D. S., Elias, S. P., Henderson, E. F., Lubelczyk, C. B., Margret Welch and Smith, R. P. (2019). Prevalence and Genetic Characterization of Deer Tick Virus (Powassan Virus, Lineage II) in Ixodes scapularis Ticks Collected in Maine. *Am J Trop Med Hyg* 101(2): 467-471. 10.4269/ajtmh.19-0281
- Roby, J. A., Setoh, Y. X., Hall, R. A. and Khromykh, A. A. (2015). Post-translational regulation and modifications of flavivirus structural proteins *J Gen Virol* 96(7): 1551-1569. 10.1099/vir.0.000097
- Roehrig, J. T. (2013). West Nile Virus in the United States — A Historical Perspective. *Viruses* 5(12): 3088-3108. 10.3390/v5123088
- Roehrig, J. T., Hombach, J. and Barrett, A. D. T. (2008). Guidelines for plaque-reduction neutralization testing of human antibodies to dengue viruses. *Viral Immunol* 21(2): 123-132. 10.1089/vim.2008.0007
- Ronca, S. E., Ruff, J. C. and Murray, K. O. (2021). A 20-year historical review of West Nile virus since its initial emergence in North America: Has West Nile virus become a neglected tropical disease? *PLoS Negl Trop Dis* 15(5): e0009190. 10.1371/journal.pntd.0009190
- Rosenzweig, E. C., Babione, R. W. and Jr, C. L. W. (1963). Immunological studies with group B arthropod-borne viruses. IV. Persistence of yellow fever antibodies following vaccination with 17D strain yellow fever vaccine. *Am J Trop Med Hyg* 12: 230-235
- Roukens, A. H. and Visser, L. G. (2008). Yellow fever vaccine: past, present and future *Expert Opin Biol Ther* 8(11): 1787-1795. 10.1517/14712598.8.11.1787

Roussel, A., Lescar, J., Vaney, M.-C., Wengler, G., Wengler, G. and Rey, F. A. (2006). Structure and Interactions at the Viral Surface of the Envelope Protein E1 of Semliki Forest Virus. *Stu* 14(1): 75-86. 10.1016/j.str.2005.09.014

Russell, C. J., Jardetzky, T. S. and Lamb, R. A. (2001). Membrane fusion machines of paramyxoviruses: capture of intermediates of fusion. *EMBO J* 20(15): 4024-4034. 10.1093/emboj/20.15.4024

Růžek, D. D., Yakimenko, V. V., Karan, L. S. and Tkachev, S. E. (2010). Omsk haemorrhagic fever. *The Lancet* 376(9758): 2104-2113. 10.1016/S0140-6736(10)61120-8

Ryman, K. D., Ledger, T. N., Campbell, G. A., Watowich, S. J. and Barrett, A. D. (1998). Mutation in a 17D-204 Vaccine Substrain-Specific Envelope Protein Epitope Alters the Pathogenesis of Yellow Fever Virus in Mice. *Virology* 244(1): 59-65. 10.1006/viro.1998.9057

Ryman, K. D., Xie, H., Ledger, T. N., Campbell, G. A. and Barrett, A. D. (1997). Antigenic Variants of Yellow Fever Virus with an Altered Neurovirulence Phenotype in Mice. *Virology* 230(2): 376-380. 10.1006/viro.1997.8496

Sabchareon, A., Wallace, D., Sirivichayakul, C., Limkittikul, K., Chanthavanich, P., Suvannadabha, S., Jiwariyavej, V., Dulyachai, W., Pengsaa, K., Wartel, T. A., Moureau, A., Saville, M., Bouckennooghe, A., Viviani, S., Tornieporth, N. G. and Lang, J. (2012). Protective efficacy of the recombinant, live-attenuated, CYD tetravalent dengue vaccine in Thai schoolchildren: a randomised, controlled phase 2b trial. *Lancet* 380(9853): 1559-1567. 10.1016/S0140-6736(12)61428-7

Sáez-Llorens, X., Biswal, S., Borja-Tabora, C., Fernando, L., Liu, M., Wallace, D., Folschweiller, N., Reynales, H. and LeFevre, I. (2023). Effect of the Tetravalent Dengue Vaccine TAK-003 on Sequential Episodes of Symptomatic Dengue. *Am J Trop Med Hyg* 108(4): 722-726. 10.4269/ajtmh.22-0673

Sanchayan, K., Fernandopulle, R., Amarasinghe, A., Thiyahiny, S. N. and Ranganathan, S. S. (2016). Safety of live attenuated Japanese encephalitis vaccine given at the age of 9 months in National Immunisation Programme of Sri Lanka. *Ceylon Med J* 61: 99-105. 10.4038/cmj.v61i3.8344

Santos, C. N. D. d., Post, P. R., Carvalho, R., Ferreira, I. I., Rice, C. M. and Galler, R. (1994). Complete nucleotide sequence of yellow fever virus vaccine strains 17DD and 17D-213 *Virus Res* 35(1): 35-41. 10.1016/0168-1702(94)00076-O

Satchidanandam, V. (2020). Japanese Encephalitis Vaccines. *Curr Treat Options Infect Dis* 14(4): 375-386. 10.1007/s40506-020-00242-5

Scherer, W. F. and Buescher, E. L. (1959). Ecologic Studies of Japanese Encephalitis Virus in Japan. *Am J Trop Med Hyg* 8(6): 644-650. 10.4269/ajtmh.1959.8.644

Schiøler, K. L., Samuel, M. and Wai, K. L. (2007). Vaccines for preventing Japanese encephalitis. *Cochrane Database Syst Rev* 2007(3). 10.1002/14651858.CD004263.pub2

Schlesinger, J. J., Chapman, S., Nestorowicz, A., Rice, C. M., Ginocchio, T. E. and Chambers, T. J. (1996). Replication of yellow fever virus in the mouse central nervous system: comparison of neuroadapted and non-neuroadapted virus and partial sequence analysis of the neuroadapted strain. *J Gen Virol* 77(Pt 6): 1277-1285. 10.1099/0022-1317-77-6-1277

Schulz, K., Staubach, C. and Blome, S. (2017). African and classical swine fever: similarities, differences and epidemiological consequences. *Vet Res* 48: 84. 10.1186/s13567-017-0490-x

Sejvar, J. J. (2003). West Nile Virus: An Historical Overview. *Ochsner J* 5(3): 6-10

Serris, A., Stass, R., Bignon, E. A., Muenz, N. A., Manuguerra, J.-C., Jangra, R. K., Li, S., Chandran, K., Tischler, N. D., Huiskonen, J. T., Rey, F. A. and Guardado-Calvo, P. (2020). The Hantavirus Surface Glycoprotein Lattice and Its Fusion Control Mechanism. *Cell* 183(2): 442-456.e416. 10.1016/j.cell.2020.08.023

Shiryaev, S. A., Chernov, A. V., Aleshin, A. E., Shiryaeva, T. N. and Strongin, A. Y. (2009). NS4A regulates the ATPase activity of the NS3 helicase: a novel cofactor role of the non-structural protein NS4A from West Nile virus. *J Gen Virol* 90(9): 2081-2085. 10.1099/vir.0.012864-0

Sitati, E. M. and Diamond, M. S. (2006). CD4+ T-Cell Responses Are Required for Clearance of West Nile Virus from the Central Nervous System. *J Virol* 80(24): 12060-12069. 10.1128/JVI.01650-06

Skidmore, A. M. and Bradfute, S. B. (2023). The life cycle of the alphaviruses: from an antiviral perspective. *Antiviral Res* 209: 105476. doi: 10.1016/j.antiviral.2022.105476

Smith, H. H. and Theiler, M. (1937). The adaptation of unmodified strain of yellow fever virus to cultivation in vitro. *J Exp Med* 65(6): 801-808. 10.1084/jem.65.6.801

Smithburn, K. C., Hughes, T. P., Burke, A. W. and Paul, J. H. (1940). A neurotropic virus isolated from the blood of a native of Uganda. *Am J Trop Med Hyg* 20: 471-492. 10.4269/ajtmh.1940.s1-20.471

Smura, T., Kuivanen, S. and Vapalahti, O. (2021). Tick-Borne Encephalitis Virus (Flaviviridae). Encyclopedia of Virology, Academic Press.

Solomon, T., Ni, H., Beasley, D. W. C., Ekkelenkamp, M., Cardoso, M. J. and Barrett, A. D. T. (2003). Origin and Evolution of Japanese Encephalitis Virus in Southeast Asia. *J Virol* 77(5): 3091-3098. 10.1128/jvi.77.5.3091-3098.2003

Soverow, J. E., Wellenius, G. A., Fisman, D. N., and Mittleman, M. A. (2009). Infectious Disease in a Warming World: How Weather Influenced West Nile Virus in the United States (2001-2005). *Environ Health Perspect* 117(7): 1049-1052. 10.1289/ehp.0800487

Sridhar, S., Luedtke, A., Langevin, E., Zhu, M., Bonaparte, Machabert, T., Savarino, S., Zambrano, B., Moureau, A., Khromava, A., Moodie, Z., Westling, T., Mascareñas, C., Frago, C., Cortés, M., Chansinghakul, D., Noriega, F., Bouckennooghe, A., Chen, J., Ng, S.-P., Gilbert, P. B., Gurunathan, S. and DiazGranados, C. A. (2018). Effect of Dengue Serostatus on Dengue Vaccine Safety and Efficacy. *N Engl J Med* 379(4): 327-340. 10.1056/NEJMoa1800820

Stapleton, J. T., Fong, S., Muerhoff, A. S., Bukh, J. and Simmonds, P. (2011). The GB viruses: a review and proposed classification of GBV-A, GBV-C (HGV), and GBV-D in genus Pegivirus within the family Flaviviridae. *J Gen Virol* 92(Pt 2): 233-246. 10.1099/vir.0.027490-0

Steel, J. J., Franz, A. W. E., Sanchez-Vargas, I., Olson, K. E. and Geiss, B. J. (2013). Subgenomic Reporter RNA System for Detection of Alphavirus Infection in Mosquitoes. *PLoS One* 8(12): e84930. 10.1371/journal.pone.0084930

Stiasny, K., Allison, S. L., Schalich, J. and Heinz, F. X. (2002). Membrane Interactions of the Tick-Borne Encephalitis Virus Fusion Protein E at Low pH. *J Virol* 76(8): 3784-3790. 10.1128/JVI.76.8.3784-3790.2002

Stiasny, K., Medits, I., Roßbacher, L. and Heinz, F. X. (2022). Impact of structural dynamics on biological functions of flaviviruses. *FEBS J.* 10.1111/febs.16419

Stokes, A., Bauer, J. H. and Hudson, N. P. (2001). The transmission of yellow fever to *Macacus rhesus*. 1928. *Rev Med Virol* 11(3): 141-148. 10.1002/rmv.311

Stromberg, Z. R., Fischer, W., Bradfute, S. B., Kubicek-Sutherland, J. Z. and Hraber, P. (2020). Vaccine Advances against Venezuelan, Eastern, and Western Equine Encephalitis Viruses. *Vaccines (Basel)* 8(2): 273. 10.3390/vaccines8020273

Takeda (2022). "Takeda's QDENGAR[®] ▼ (Dengue Tetravalent Vaccine [Live, Attenuated]) Approved for Use in European Union." from <https://www.takeda.com/newsroom/newsreleases/2022/takedas-qdenga-dengue-tetravalent-vaccine-live-attenuated-approved-for-use-in-european-union>.

Tandan, J. B., Ohrr, H., Sohn, Y. M., Yoksan, S., Ji, M., Nam, C. M. and Halstead, S. B. (2007). Single dose of SA 14-14-2 vaccine provides long-term protection against Japanese encephalitis: a case-control study in Nepalese children 5 years after immunization. *Vaccine* 25(27): 5041-5045. 10.1016/j.vaccine.2007.04.052

Tassaneetrithep, B., Burgess, T. H., Granelli-Piperno, A., Trumpfheller, C., Finke, J., Sun, W., Eller, M. A., Pattanapanyasat, K., Sarasombath, S., Bix, D. L., Steinman, R. M., Schlesinger, S.

and Marovich, M. A. (2003). DC-SIGN (CD209) mediates dengue virus infection of human dendritic cells. *J Exp Med* 197(7): 823-829. 10.1084/jem.20021840

Taylor, R. M., Hurlbut, H. S., Work, T. H., Kingston, J. R. and Frothingham, T. E. (1954). Sindbis Virus: A Newly Recognized Arthropod-Transmitted Virus. *Am J Trop Med Hyg* 4(5): 844-862. 10.4269/ajtmh.1955.4.844

Telford, S. R. and Piantadosi, A. L. (2023). Powassan virus persistence after acute infection. *mBio*: e0071223. 10.1128/mbio.00712-23

Teoh, E. P., Kukkaro, P., Teo, E. W., Lim, A. P. C., Tan, T. T., Yip, A., Schul, W., Aung, M., Kostyuchenko, V. A., Leo, Y. S., Chan, S. H., Smith, K. G. C., Chan, A. H. Y., Zou, G., Ooi, E. E., Kemeny, D. M., Tan, G. K., Ng, J. K. W., Ng, M. L., Alonso, S., Fisher, D., Shi, P.-Y., Hanson, B. J., Lok, S.-M. and MacAry, P. A. (2012). The structural basis for serotype-specific neutralization of dengue virus by a human antibody. *Sci Transl Med* 4(139): 139ra183. 10.1126/scitranslmed.3003888

Tesh, R. B., Arroyo, J., Rosa, A. P. A. T. d., Guzman, H., Xiao, S.-Y. and Monath, T. P. (2002). Efficacy of Killed Virus Vaccine, Live Attenuated Chimeric Virus Vaccine, and Passive Immunization for Prevention of West Nile virus Encephalitis in Hamster Model. *Emerg Infect Dis* 8(12): 1392-1397. 10.3201/eid0812.020229

Theiler, M. and Smith, H. H. (1937a). The effect of prolonged cultivation in vitro upon the pathogenicity of yellow fever virus. *J Exp Med* 65(6): 767-786. 10.1084/jem.65.6.767

Theiler, M. and Smith, H. H. (1937b). The Use of Yellow Fever Virus Modified by in Vitro Cultivation for Human Immunization. *J Exp Med* 65(6): 787-800. 10.1084/jem.65.6.787

Tomori, O. (2004). Yellow Fever: The Recurring Plague. *Crit Rev Clin Lab Sci* 41(4): 391-427. 10.1080/10408360490497474

Torresi, J., Ebert, G. and Pellegrini, M. (2017). Vaccines licensed and in clinical trials for the prevention of dengue. *Hum Vaccin Immunother* 13(5): 1059-1072. 10.1080/21645515.2016.1261770

Tricou, V., Low, J. G., Oh, H. M., Leo, Y.-S., Kalimuddin, S., Wijaya, L., Pang, J., Ling, L. M., Lee, T. H., Brose, M., Hutagalung, Y., Rauscher, M., Borkowski, A. and Wallace, D. (2019). Safety and immunogenicity of a single dose of a tetravalent dengue vaccine with two different serotype-2 potencies in adults in Singapore: A phase 2, double-blind, randomised, controlled trial. *Vaccine* 38(6). 10.1016/j.vaccine.2019.11.061

Tsetsarkin, K. A., Maximova, O. A., Liu, G., Kenney, H., Teterina, N. L. and Pletnev, A. G. (2019). Stable and Highly Immunogenic MicroRNA-Targeted Single-Dose Live Attenuated Vaccine Candidate against Tick-Borne Encephalitis Constructed Using Genetic Backbone of Langat Virus. *mBio* 10(2): e02904-02918. 10.1128/mBio.02904-18

- Tuells, J., Henao-Martínez, A. F. and Franco-Paredes, C. (2022). Yellow Fever: A Perennial Threat. *Arch Med Res* 53(7): 649-657. 10.1016/j.arcmed.2022.10.005
- Urbanowski, M. D. and Hobman, T. C. (2013). The West Nile Virus Capsid Protein Blocks Apoptosis through a Phosphatidylinositol 3-Kinase-Dependent Mechanism. *J Virol* 82(2): 872-881. 10.1128/JVI.02030-12
- Valentine, M. J., Murdock, C. C. and Kelly, P. J. (2019). Sylvatic cycles of arboviruses in non-human primates. *Parasit Vectors* 12(1): 463. 10.1186/s13071-019-3732-0
- van den Brom, R., de Jong, A., van Engelen, E., Heuvelink, A., and Vellema, P. (2020). Zoonotic risks of pathogens from sheep and their milk borne transmission. *Small Rumin Res* 189: 106123. 10.1016/j.smallrumres.2020.106123
- van den Elsen, K., Chew, B. L. A., Ho, J. S. and Luo, D. (2023). Flavivirus nonstructural proteins and replication complexes as antiviral drug targets. *Curr Opin Virol* 59: 101305. 10.1016/j.coviro.2023.101305
- van den Elsen, K., Quek, J. P. and Luo, D. (2021). Molecular Insights into the Flavivirus Replication Complex. *Viruses* 13(6): 956. 10.3390/v13060956
- van den Hurk, A. F., Ritchie, S. A. and Mackenzie, J. S. (2009). Ecology and geographical expansion of Japanese encephalitis virus. *Annu Rev Entomol* 54: 17-35. 10.1146/annurev.ento.54.110807.090510
- van den Hurk, A. F., Skinner, E., Ritchie, S. A. and Mackenzie, J. S. (2022). The Emergence of Japanese Encephalitis Virus in Australia in 2022: Existing Knowledge of Mosquito Vectors. *Viruses* 14(6): 1208. 10.3390/v14061208
- Vaney, M. C., Dellarole, M., Duquerroy, S., Medits, I., Tsouchnikas, G., Rouvinski, A., England, P., Stiasny, K., Heinz, F. X., and Rey, F. A. (2022). *Nat Commun* 13(1): 3718. 10.1038/s41467-022-31111-y
- Vannice, K. S., Hills, S. L., Schwartz, L. M., Barrett, A. D., Heffelfinger, J., Hombach, J., Letson, G. W., Solomon, T. and Marfin, A. A. (2021). The future of Japanese encephalitis vaccination: expert recommendations for achieving and maintaining optimal JE control. *npj Vaccines* 6: 82. 10.1038/s41541-021-00338-z
- Vogels, C. B. F., Brackney, D. E., Dupuis, A. P., Robich, R. M., Fauver, J. R., Brito, A. F., Williams, S. C., Anderson, J. F., Lubelczyk, C. B., Lange, R. E., Prusinski, M. A., Kramer, L. D., Gangloff-Kaufmann, J. L., Goodman, L. B., Baele, G., Smith, R. P., Armstrong, P. M., Ciota, A. T., Dellicour, S. and Grubaugh, N. D. (2023). Phylogeographic reconstruction of the emergence and spread of Powassan virus in the northeastern United States. *PNAS* 120(16): e2218012120. 10.1073/pnas.2218012120

Volk, D. E., May, F. J., Gandham, S. H. A., Anderson, A., Lindern, J. J. V., Beasley, D. W. C., Barrett, A. D. T. and Gorenstein, D. G. (2009). Structure of yellow fever virus envelope protein domain III. *Virology* 394(1): 12-18. 10.1016/j.virol.2009.09.001

von Lindern, J. J., Aroner, S., Barrett, N. D., Wicker, J. A., Davis, C. T. and Barrett, A. D. T. (2006). Genome analysis and phylogenetic relationships between east, central and west African isolates of Yellow fever virus. *J Gen Virol* 87(Pt 4): 895-907. 10.1099/vir.0.81236-0

Waggoner, J. J., Rojas, A. and Pinsky, B. A. (2018). Yellow Fever Virus: Diagnostics for a Persistent Arboviral Threat. *J Clin Microbiol* 56(10): e00827-00818. 10.1128/JCM.00827-18

Wahaab, A., Mustafa, B. E., Hameed, M., Stevenson, N. J., Anwar, M. N., Liu, K., Wei, J., Qiu, Y. and Ma, Z. (2022). Potential Role of Flavivirus NS2B-NS3 Proteases in Viral Pathogenesis and Anti-flavivirus Drug Discovery Employing Animal Cells and Models: A Review. *Viruses* 14(1): 44. 10.3390/v14010044

Walter, C. T. and Barr, J. N. (2011). Recent advances in the molecular and cellular biology of bunyaviruses. *J Gen Virol* 92(11): 2467-2484. 10.1099/vir.0.035105-0

Wang, C., Puerta-Guardo, H., Biering, S. B., Glasner, D. R., Tran, E. B., Patana, M., Gomberg, T. A., Malvar, C., Lo, N. T. N., Espinosa, D. A. and Harris, E. (2019). Endocytosis of flavivirus NS1 is required for NS1-mediated endothelial hyperpermeability and is abolished by a single N-glycosylation site mutation. *PLoS Pathog* 15(7): e1007938. 10.1371/journal.ppat.1007938

Wang, E., Ryman, K. D., Jennings, A. D., Wood, D. J., Taffs, F., Minor, P. D., Sanders, P. G. and Barrett, A. D. T. (1995). Comparison of the genomes of the wild-type French viscerotropic strain of yellow fever virus with its vaccine derivative French neurotropic vaccine. *J Gen Virol* 76(11): 2749-2755. 10.1099/0022-1317-76-11-2749

Wang, X., Li, S.-H., Zhu, L., Nian, Q.-G., Yuan, S., Gao, Q., Hu, Z., Ye, Q., Li, X.-F., Xie, D.-Y., Shaw, N., Wang, J., Walter, T. S., Huiskonen, J. T., Fry, E. E., Qin, C.-F., Stuart, D. I. and Rao, Z. (2017). Near-atomic structure of Japanese encephalitis virus reveals critical determinants of virulence and stability. *Nat Commun* 8(14). 10.1038/s41467-017-00024-6

Wang, Y., Xie, X. and Shi, P.-Y. (2022). Flavivirus NS4B protein: Structure, function, and antiviral discovery. *Antiviral Res* 207: 105423. 10.1016/j.antiviral.2022.105423

Weaver, S. C. and Reisen, W. K. (2010). Present and future arboviral threats. *Antiviral Res* 85: 328-345. 10.1016/j.antiviral.2009.10.008

Wei, H., Tan, J., Zhou, B., Guan, X., Zhong, Q. and Wang, J. (2022). Charged Residue Implantation Improves the Affinity of a Cross-Reactive Dengue Virus Antibody. *Int J Mol Sci* 23(8): 4197. 10.3390/ijms23084197

Whiteman, M. C., Li, L., Wicker, J. A., Kinney, R. M., Huang, C., Beasley, D. W. C., Chung, K. M., Diamond, M. S., Solomon, T. and Barrett, A. D. T. (2010). Development and

characterization of non-glycosylated E and NS1 mutant viruses as a potential candidate vaccine for West Nile virus. *Vaccine* 28(4): 1075-1083. 10.1016/j.vaccine.2009.10.112

Whiteman, M. C., Wicker, J. A., Kinney, R. M., Huang, C. Y.-H., Solomon, T. and Barrett, A. D. T. (2011). Multiple amino acid changes at the first glycosylation motif in NS1 protein of West Nile virus are necessary for complete attenuation for mouse neuroinvasiveness. *Vaccine* 29(52): 9702-9710. 10.1016/j.vaccine.2011.09.036

Whitman, L. (1939). Failure of *Aedes Aegypti* to Transmit Yellow Fever Cultured Virus (17D). *Am J Trop Med Hyg* s1-19(1): 19-26. 10.4269/ajtmh.1939.s1-19.19

WHO (1967). Arboviruses and human disease. Report of a WHO Scientific Group. World Health Organ Tech Rep Ser. 369: 1-84

WHO (1985). Arthropod-borne and rodent-borne viral diseases. Report of a WHO Scientific Group. World Health Organ Tech Rep Ser. 719: 1-116

WHO (2017). "West Nile virus." from <https://www.who.int/news-room/fact-sheets/detail/west-nile-virus>.

WHO (2018). "Vaccines and immunization: Dengue." from <https://www.who.int/news-room/questions-and-answers/item/dengue-vaccines>.

WHO (2019). "Japanese encephalitis." from <https://www.who.int/news-room/fact-sheets/detail/japanese-encephalitis>.

WHO (2022a). "Dengue and severe dengue." from <https://www.who.int/news-room/fact-sheets/detail/dengue-and-severe-dengue>

WHO (2022b). "Eliminate yellow fever epidemics (EYE) strategy 2017-2026." from <https://www.who.int/initiatives/eye-strategy>.

WHO (2022c). "Yellow fever - number of reported cases." from <https://www.who.int/data/gho/data/indicators/indicator-details/GHO/yellow-fever---number-of-reported-cases>.

WHO (2023a). "Japanese Encephalitis (JE) vaccination coverage." from https://immunizationdata.who.int/pages/schedule-by-disease/japenc.html?ISO_3_CODE=&TARGETPOP_GENERAL=.

WHO (2023b). "Tick-borne encephalitis." from https://www.who.int/health-topics/tick-borne-encephalitis/#tab=tab_2.

WHO (2023c). "Vaccine schedule for Japan." from https://immunizationdata.who.int/pages/schedule-by-country/jpn.html?DISEASECODE=&TARGETPOP_GENERAL=

WHO (2023d). "Vaccination schedule for Republic of Korea." from https://immunizationdata.who.int/pages/schedule-by-country/kor.html?DISEASECODE=&TARGETPOP_GENERAL=

Wicker, J. A., Whiteman, M. C., Beasley, D. W. C., Davis, T., McGee, C. E., Lee, C., Higgs, S., Kinney, R. M., Huang, C. Y.-H. and Barrett, A. D. T. (2012). Mutational analysis of the West Nile virus NS4B protein. *Virology* 426(1): 22-33. 10.1016/j.virol.2011.11.022

Wicker, J. A., Whiteman, M. C., Beasley, D. W. C., Davis, T., Zhang, S., Schneider, B. S., Higgs, S., Kinney, R. M. and Barrett, A. D. T. (2006). A single amino acid substitution in the central portion of the West Nile virus NS4B protein confers a highly attenuated phenotype in mice. *Virology* 349(2): 245-253. 10.1016/j.virol.2006.03.007

Wilder-Smith, A., Hombach, J., Ferguson, N., Selgelid, M., O'Brien, K., Vannice, K., Barrett, A., Ferdinand, E., Flasche, S., Guzman, M., Novaes, H. M., Ng, L.-C., Smith, P. G., Tharmaphornpilas, P., Yoon, I.-K., Cravioto, A., Farrar, J. and Nolan, T. M. (2019). Deliberations of the Strategic Advisory Group of Experts on Immunization on the use of CYD-TDV dengue vaccine. *Lancet Infect Dis* 19(1): e31-e38. 10.1016/S1473-3099(18)30494-8

Wimberly, M. C., Lamsal, A., Giacomo, P. and Chuang, T.-W. (2014). Regional Variation of Climatic Influences on West Nile Virus Outbreaks in the United States. *Am J Trop Med Hyg* 91(4): 677-684. 10.4269/ajtmh.14-0239

Woo, J. H., Jeong, Y. E., Jo, J. E., Shim, S.-M., Ryou, J., Kim, K.-C., Lee, W. J. and Lee, J.-Y. (2020). Genetic Characterization of Japanese Encephalitis Virus Genotype 5 Isolated from Patient, South Korea, 2015. *Emerg Infect Dis* 26(5): 1002-1006. 10.3201/eid2605.190977

WRBU (2021a). "Aedes aegypti (Linnaeus, 1762)." Retrieved 6-22-2023, from <https://wrbu.si.edu/index.php/vectorspecies/mosquitoes/aegypti>.

WRBU (2021b). "Aedes albopictus (Skuse, 1895)." from <https://wrbu.si.edu/index.php/vectorspecies/mosquitoes/albopictus>.

Wright, D., Kortekaas, J., Bowden, T. A. and Warimwe, G. M. (2019). Rift Valley fever: biology and epidemiology. *J Gen Virol* 100(8): 1187-1199. 10.1099/jgv.0.001296

Wu, S.-J., Ewing, D., Sundaram, A. K., Chen, H.-W., Liang, Z., Cheng, Y., Jani, V., Sun, P., Gromowski, G. D., Barrera, R. A. D. L., Schilling, M. A., Petrovsky, N., Porter, K. R. and Williams, M. (2022). Enhanced Immunogenicity of Inactivated Dengue Vaccines by Novel Polysaccharide-Based Adjuvants in Mice. *Microorganisms* 10(5): 1034. 10.3390/microorganisms10051034

Xie, G., Luo, H., Tian, B., Mann, B., Bao, X., McBride, J., Tesh, R., Barrett, A. D. and Wang, T. (2016). A West Nile virus NS4B-P38G mutant strain induces cell intrinsic innate cytokine

responses in human monocytic and macrophage cells. *Vaccine* 33(7): 869-878.
10.1016/j.vaccine.2014.12.056

Xie, H., Cass, A. R. and Barrett, A. D. T. (1998). Yellow fever 17D vaccine virus isolated from healthy vaccinees accumulates very few mutations. *Virus Res* 55(1): 93-99. 10.1016/S0168-1702(98)00036-7

Yang, D., Li, X.-F., Ye, Q., Wang, H.-J., Deng, Y.-Q., Zhu, S.-Y., Zhang, Y., Li, S.-H. and Qin, C.-F. (2014). Characterization of live-attenuated Japanese encephalitis vaccine virus SA14-14-2. *Vaccine* 32(23): 2675-2681. 10.1016/j.vaccine.2014.03.074

Yang, J., Yang, H., Li, Z., Wang, W., Lin, H., Liu, L., Ni, Q., Liu, X., Zeng, X., Wu, Y. and Li, Y. (2017). Envelope Protein Mutations L107F and E138K Are Important for Neurovirulence Attenuation for Japanese Encephalitis Virus SA14-14-2 Strain. *Viruses* 9(1): 20.
10.3390/v9010020

Young, E., Carnahan, R. H., Andrade, D. V., Kose, N., Nargi, R. S., Fritch, E. J., Munt, J. E., Doyle, M. P., White, L., Baric, T. J., Stoops, M., DeSilva, A., Tse, L. V., Martinez, D. R., Zhu, D., Metz, S., Wong, M. P., Espinosa, D. A., Montoya, M., Biering, S. B., Sukulpolvi-Petty, S., Kuan, G., Balmaseda, A., Diamond, M. S., Harris, E., Jr, J. E. C. and Baric, R. S. (2020). Identification of Dengue Virus Serotype 3 Specific Antigenic Sites Targeted by Neutralizing Human Antibodies. *Cell Host & Microbe* 27(5): 710-724e717. 10.1016/j.chom.2020.04.007

Yu, I.-M., Holdaway, H. A., Chipman, P. R., Kuhn, R. J., Rossmann, M. G. and Chen, J. (2009). Association of the pr Peptides with Dengue Virus at Acidic pH Blocks Membrane Fusion. *J Virol* 83(23): 12101-12107. 10.1128/JVI.01637-09

Yu, I.-M., Zhang, W., Holdaway, H. A., Li, L., Kostyuchenko, V. A., Chipman, P. R., Kuhn, R. J., Rossmann, M. G. and Chen, J. (2008). Structure of the immature dengue virus at low pH primes proteolytic maturation. *Science* 319(5871): 1834-1837. 10.1126/science.1153264

Yu, X. and Cheng, G. (2022). Adaptive Evolution as a Driving Force of the Emergence and Re-Emergence of Mosquito-Borne Viral Diseases. *Viruses* 14(2): 435. 10.3390/v14020435

Yu, Y. (2010). Phenotypic and genotypic characteristics of Japanese encephalitis attenuated live vaccine virus SA14-14-2 and their stabilities. *Vaccine* 28(21): 3635-3641.
10.1016/j.vaccine.2010.02.105

Yu, Y. (2013). Development of Japanese Encephalitis Attenuated Live Vaccine Virus SA14-14-2 and its Characteristics. Encephalitis, Intechopen.

Yu, Y., Wan, Z., Wang, J.-H., Yang, X. and Zhang, C. (2022). Review of human pegivirus: Prevalence, transmission, pathogenesis, and clinical implication. *Virulence* 13(1): 324-341.
10.1080/21505594.2022.2029328

Yun, K. W., Lee, H. J., Kang, J. H., Eun, B. W., Kim, Y.-J., Kim, K.-H., Kim, N. H., Hong, Y. J., Kim, D. H., Kim, H. M. and Cha, S.-H. (2015). Safety and immunogenicity of a freeze-dried, Vero cell culture-derived, inactivated Japanese encephalitis vaccine (KD-287, ENCEVAC®) versus a mouse brain-derived inactivated Japanese encephalitis vaccine in children: a phase III, multicenter, double-blinded, randomized trial. *BMC Infect Dis* 15(7). 10.1186/s12879-014-0744-4

Yun, S.-I., Song, B.-H., Polejaeva, I. A., Davies, C. J., White, K. L. and Lee, Y.-M. (2016). Comparison of the live-attenuated Japanese encephalitis vaccine SA14 -14-2 strain with its pre-attenuated virulent parent SA14 strain: similarities and differences in vitro and in vivo *J Gen Virol* 97(10): 2575-2591. 10.1099/jgv.0.000574

Zacks, M. A. and Paessler, S. (2010). Encephalitic alphaviruses. *Vet Microbiol* 140(3-4): 281. 10.1016/j.vetmic.2009.08.023

Zanotto, P. M. D. A., Gould, E. A., Gao, G. F., Harvey, P. H. and Holmes, E. C. (1996). Population Dynamics of Flaviviruses Revealed by Molecular Phylogenies. *PNAS* 93(2): 548-553. 10.1073/pnas.93.2.548

Zehender, G., Ebranati, E., Bernini, F., Presti, A. L., Rezza, G., Delogu, M., Galli, M. and Ciccozzi, M. (2011). Phylogeography and epidemiological history of West Nile virus genotype 1a in Europe and the Mediterranean basin. *Infect Genet Evol* 11(3): 646-653. 10.1016/j.meegid.2011.02.003

Zhang, S., Li, L., Woodson, S. E., Huang, C. Y.-H., Kinney, R. M., Barrett, A. D. T. and Beasley, D. W. C. (2006). A mutation in the envelope protein fusion loop attenuates mouse neuroinvasiveness of the NY99 strain of West Nile virus. *Virology* 353(1): 35-40. 10.1016/j.virol.2006.05.025

Zhang, W., Wu, J., Li, Y., Li, F. and Njoo, H. (2009). Rapid and Accurate In Vitro Assays for Detection of West Nile Virus in Blood and Tissues. *Transfus Med Rev* 23(2): 146-154. 10.1016/j.tmr.2008.12.008

Zhang, X., Ge, P., Yu, X., Brannan, J. M., Bi, G., Zhang, Q., Schein, S. and Zhou, Z. H. (2012). Cryo-EM structure of the mature dengue virus at 3.5-Å resolution. *Nat Struct Mol Biol* 20(1): 105-110. 10.1038/nsmb.2463

Zhang, X., Zhang, Y., Jia, R., Wang, M., Yin, Z. and Cheng, A. (2021). Structure and function of capsid protein in flavivirus infection and its applications in the development of vaccines and therapeutics. *Vet Res* 52: 98. 10.1186/s13567-021-00966-2

Zhang, Y., Zhang, W., Ogata, S., Clements, D., Strauss, J. H., Baker, T. S., Kuhn, R. J. and Rossmann, M. G. (2004). Conformational Changes of the Flavivirus E Glycoprotein. *Structure* 12(9): 1607-1618. 10.1016/j.str.2004.06.019

- Zhao, Z., Date, T., Li, Y., Kato, T., Miyamoto, M., Yasui, K. and Wakita, T. (2005). Characterization of the E-138 (Glu/Lys) mutation in Japanese encephalitis virus by using a stable, full-length, infectious cDNA clone Free. *J Gen Virol* 86(8): 2209-2220. 10.1099/vir.0.80638-0
- Zheng, Y., Martín, C. S.-S., Qin, Z.-l. and Kielian, M. (2011). The Domain I-Domain III Linker Plays an Important Role in the Fusogenic Conformational Change of the Alphavirus Membrane Fusion Protein. *J Virol* 85(13): 6334-6342. 10.1128/JVI.00596-11
- Zhou, Y., Ray, D., Zhao, Y., Dong, H., Ren, S., Li, Z., Guo, Y., Bernard, K. A., Shi, P.-Y. and Li, H. (2007). Structure and Function of Flavivirus NS5 Methyltransferase. *J Virol* 81(8): 3891-3903. 10.1128/JVI.02704-06
- Züst, R., Dong, H., Li, X.-F., Chang, D. C., Zhang, B., Balakrishnan, T., Toh, Y.-X., Jiang, T., Li, S.-H., Deng, Y.-Q., Ellis, B. R., Ellis, E. M., Poidinger, M., Zolezzi, F., Qin, C.-F., Shi, P.-Y. and Fink, K. (2013). Rational Design of a Live Attenuated Dengue Vaccine: 2'-O-Methyltransferase Mutants Are Highly Attenuated and Immunogenic in Mice and Macaques. *PLoS Pathog* 9(8): e1003521. 10.1371/journal.ppat.1003521