Figure S1

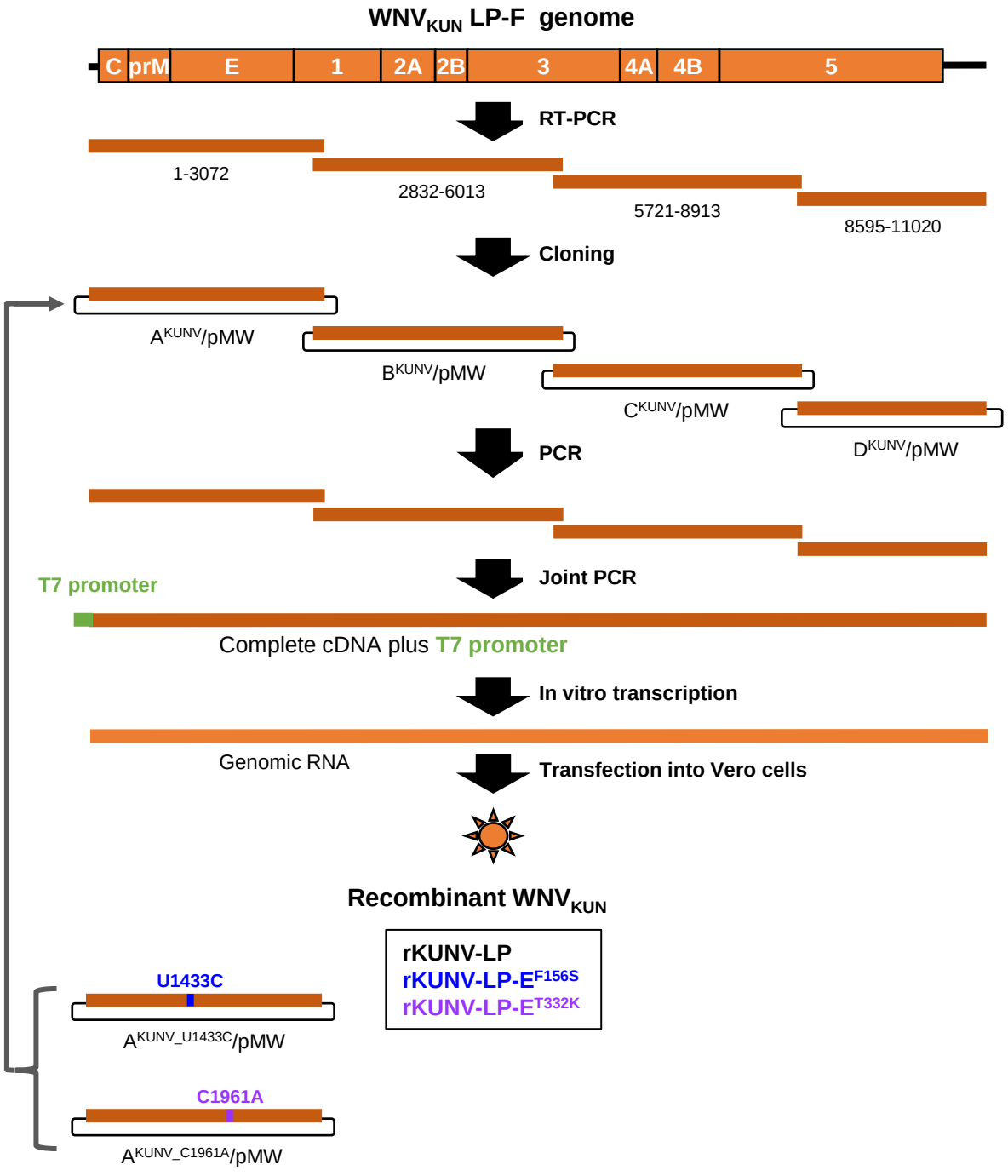


Figure S1. Schematic representation of the recombinant WNV$_{KUN}$ production. The method for the production is described in Materials and Methods section in detail.

Figure S2

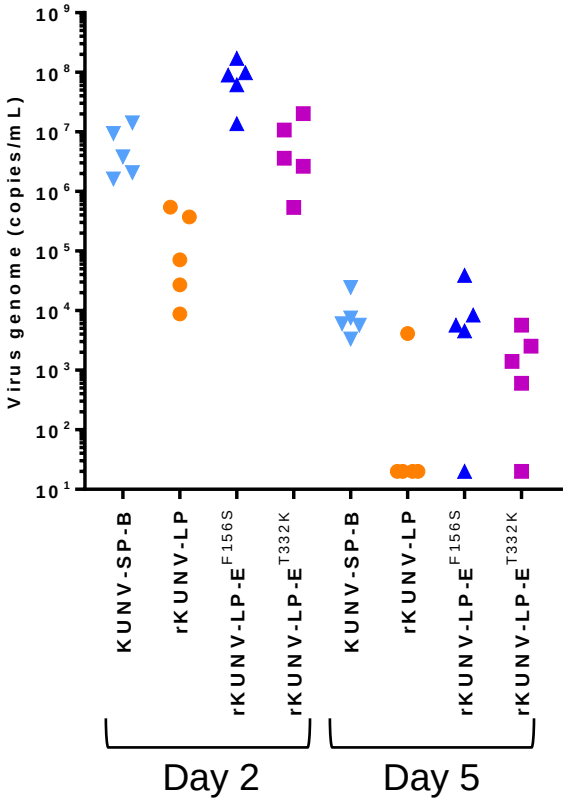


Figure S2. Levels of genomic copy number at 2 and 5 days after inoculation of WNV_{KUN}-infected mice. Mice inoculated i.p. with 1×10^4 PFU of KUNV-SP-B (n = 5), rKUNV-LP (n = 5), rKUNV-LP-$E^{F\ 156S}$ (n = 5), or rKUNV-LP-E^{T332K} (n = 5) were euthanized at two or five days after inoculation, and samples were collected. Sera were used to quantify the genomic copy number (PFU/mL) as described in the Materials and Methods section.

Figure S3



Figure S3. Comparison of the amino acid sequences of the E protein (501 residues) in the WNV_{KUN} and L1 WNV strains NY99. Positions E^{156} and E^{332} are indicated by black frames. The color of residue indicates the property of the side chain. The highlighted residues show minor residues among the strains. We used a software GENETYX version 13 (Nihon Server, Tokyo, Japan) for the analysis.

Figure S4

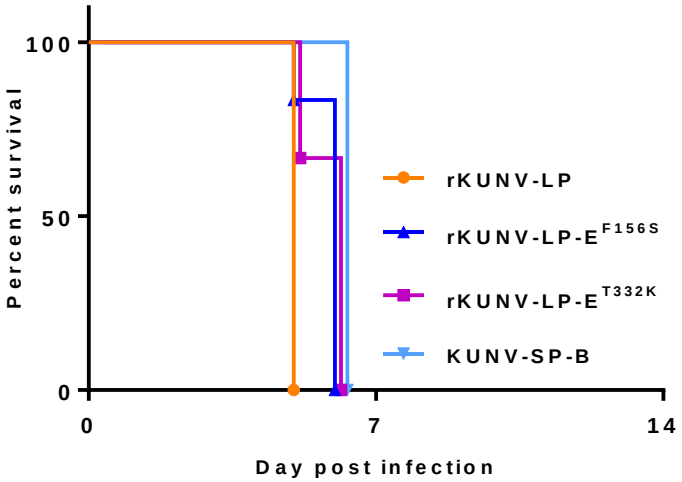


Figure S4. Neurovirulence of the WNV_{KUN} strains. Survival curve of mice inoculated intracerebrally with 1.5×10^4 PFU of the viruses per mouse (n = 6).

Figure S5

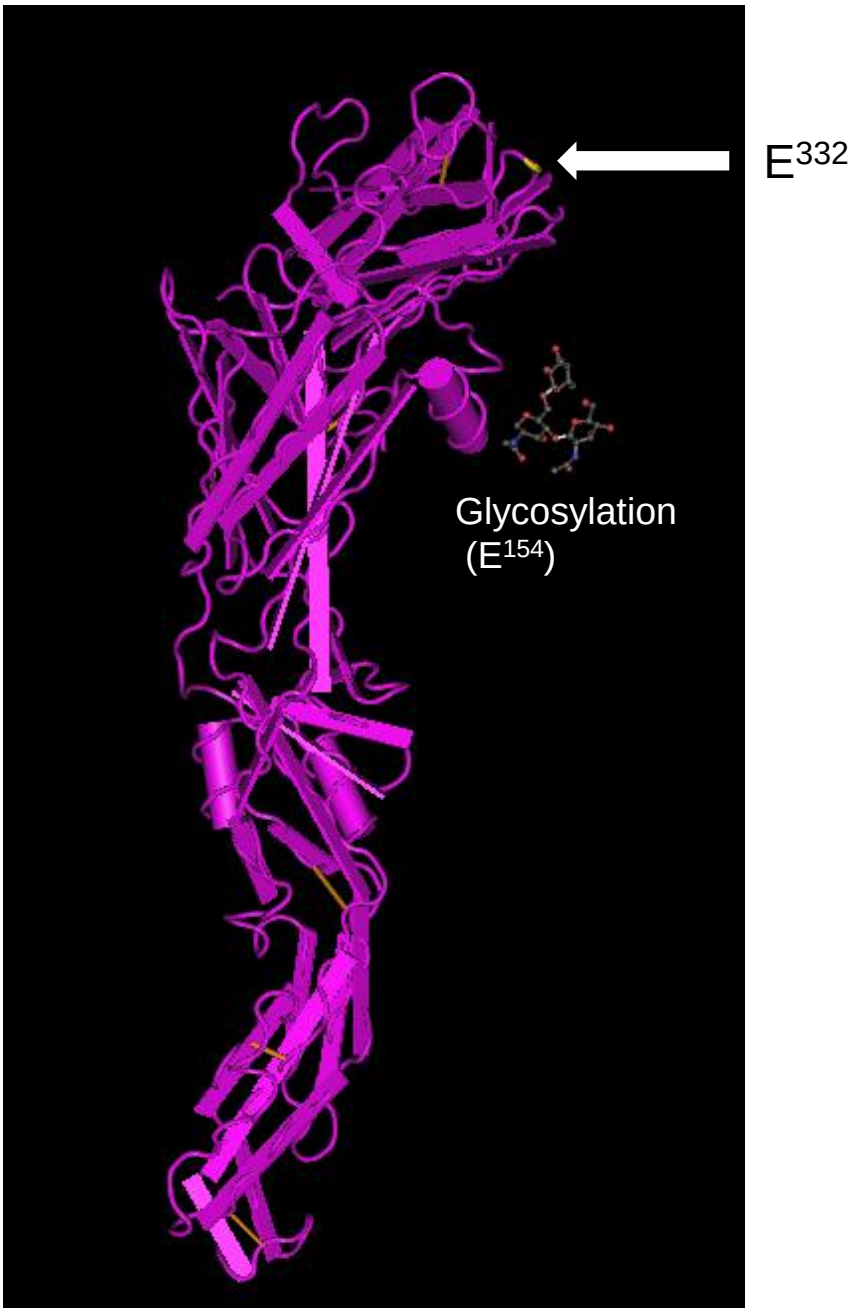


Figure S5. Structure of West Nile virus E protein (PDB ID: 2I69). E^{T332} is indicated by white arrow. The molecular graphic was constructed using Cn3D 4.3 (NCBI).

Table S1. List of oligonucleotides used in this study

Name	Sequence (5’ – 3’)	Applications
KUNV_001f	AGT AGT TCG CCT GTG TGA GCT	PCR amplification
KUNV_2832f	CAT TCT GTT CGC ACC AGA ACT	PCR amplification
KUNV_5721f	TGA GAT AGC CCT CTG TCT GCA	PCR amplification
KUNV_8595f	GAA CTA CCA CGG TAG CTA CGA	PCR amplification
KUNV_3072r	CCA GTA GGA TAG ATC GCT ATG	PCR amplification
KUNV_6013r	GTG ATG GAT TCC TAC CAA TGC	PCR amplification
KUNV_8913r	CAG AGC TGC ATT ACT ATT GAC	PCR amplification
KUNV_11020r	AGA TCC TGT GTT CTC GCA CCA	PCR amplification
IF_KV_OR393_001f	CGG TAC CCG GGG ATC AGT AGT TCG CCT GTG TGA GCT	PCR amplification, Cloning
IF_KV_OR393_2832f	CGG TAC CCG GGG ATC CAT TCT GTT CGC ACC AGA ACT	PCR amplification, Cloning
IF_KV_OR393_5721f	CGG TAC CCG GGG ATC TGA GAT AGC CCT CTG TCT GCA	PCR amplification, Cloning
IF_KV_OR393_8595f	CGG TAC CCG GGG ATC GAA CTA CCA CGG TAG CTA CGA	PCR amplification, Cloning
IF_KV_OR393_3072r	ATG CCT GCA GGT CGA CCA ATA GGA TAG ATC GCT ATG	PCR amplification, Cloning
IF_KV_OR393_6013r	ATG CCT GCA GGT CGA GTG ATG GAC TCC TAC CAA TGC	PCR amplification, Cloning
IF_KV_OR393_8913r	ATG CCT GCA GGT CGA CAG AGC TGC ATT ACT ATT GAC	PCR amplification, Cloning
IF_KV_OR393_11020r	ATG CCT GCA GGT CGA AGA TCC TGT GTT CTC GCA CCA	PCR amplification, Cloning
T7-KUNV_001f	TAA TAC GAC TCA CTA TAG AGT AGT TCG CCT GTG TGA GCT	PCR amplification
KUNV_1429(1433C)f	TAC TCC ACG CAA ACC GGA GCT	Site-directed mutagenesis
KUNV_1428r	GTT CCC ATG CGA TTC CAC GGT	Site-directed mutagenesis
KUNV_1957(1961A)f	GGC AAG GAT GGA CCC TGT AAG	Site-directed mutagenesis
KUNV_1956r	TGT GTA CTG CAG TTC CAA TAC	Site-directed mutagenesis
KUNV_6013r.v2	GTG ATG GAC TCC TAC CAA TGC	Site-directed mutagenesis
KUNV_1279(1285T)f	TGC GGA TTT TTT GGT AAA GGA	Site-directed mutagenesis
KUNV_1293(1285A)r	ACC AAA AAA TCC GCA CCC ATT	Site-directed mutagenesis
KUNV_1906(1913T)f	ACC CCC GTA GAC ACG GGC CAT	Site-directed mutagenesis
KUNV_1920(1913A)r	CGT GTC TAC GGG GGT CCC AAG	Site-directed mutagenesis
KUNV_2278(2285G)f	GTG GGA AGG GCT GTC CAT CAA	Site-directed mutagenesis
KUNV_2292(2285C)r	GAC AGC CCT TCC CAC GGA GGT	Site-directed mutagenesis
WNVcom.3451f	GGH TGT TGG TAT GGH ATG GA	Real time RT-PCR
WNVcom.3590r	TC CTG GGT GGC CAA GAA CAC	Real time RT-PCR
WNV_3538p	FAM-ATG ATT GAY CCT TTT CAG YTG GGC CTT CTG-TAMRA	Real time RT-PCR (probe)

Table S2. P values in Figure 4

Cell (panel)	Virus strain compared	P value			
		Day 1	Day 2	Day 3	Day 4
Vero (C)	rKUN-LP vs. rKUNV-LP-E^{F156S}	0.0019	<0.0001	0.0310	Not tested
	rKUNV-LP vs. rKUNV-LP-E^{T332K}	0.0187	<0.0001	0.0028	Not tested
C6/36 (D)	rKUN-LP vs. rKUNV-LP-E^{F156S}	0.0067	> 0.05	<0.0001	0.0001
	rKUNV-LP vs. rKUNV-LP-E^{T332K}	0.0054	0.0033	<0.0001	<0.0001
IMR-32 (E)	rKUN-LP vs. rKUNV-LP-E^{F156S}	> 0.05	> 0.05	0.0010	<0.0001
	rKUNV-LP vs. rKUNV-LP-E^{T332K}	<0.0001	0.0010	0.0001	<0.0001
Neuro-2a (F)	rKUN-LP vs. rKUNV-LP-E^{F156S}	0.0118	> 0.05	0.0023	0.0091
	rKUNV-LP vs. rKUNV-LP-E^{T332K}	0.0208	0.0113	0.0050	0.0038

Table S3. Amino acid residues at positions E^{156} and E^{332} in viruses recovered from brain samples in Figure 6.

Day 5 mouse brain infected with:	Amino acid at position in E	
	156	332
SP-B.2	Ser	Lys
SP-B.3	Ser	Lys
SP-B.5	Ser	Lys
rKUNV-LP.2	Phe	Thr
rKUNV-LP.3	Phe and Ser	Thr
rKUNV-LP-E^{F156S}.1	Ser	Thr
rKUNV-LP-E^{F156S}.2	Ser	Thr
rKUNV-LP-E^{F156S}.3	Ser	Thr
rKUNV-LP-E^{F156S}.4	Ser	Thr
rKUNV-LP-E^{F156S}.5	Ser	Thr
rKUNV-LP-E^{T332K}.1	Ser	Lys
rKUNV-LP-E^{T332K}.2	Ser	Lys
rKUNV-LP-E^{T332K}.3	Ser	Lys