

## Supplementary data

### **Divergent Pathogenic Dynamics of Immature Tick-Borne and Mosquito-Borne Flaviviruses: A Paradigm Shift in prM-Containing Particle Infectivity**

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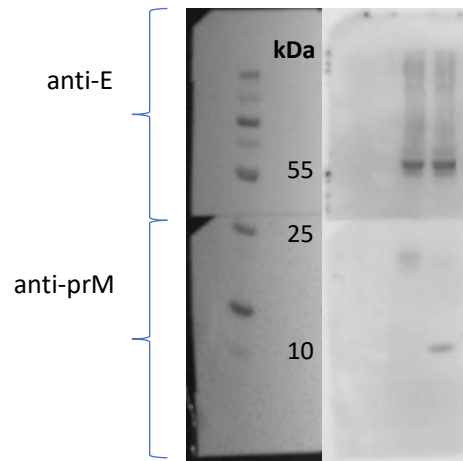
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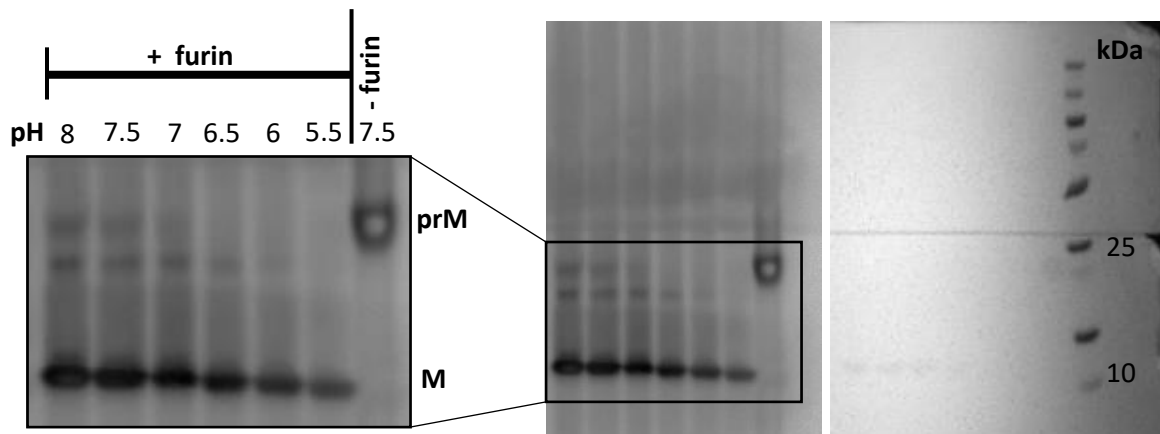
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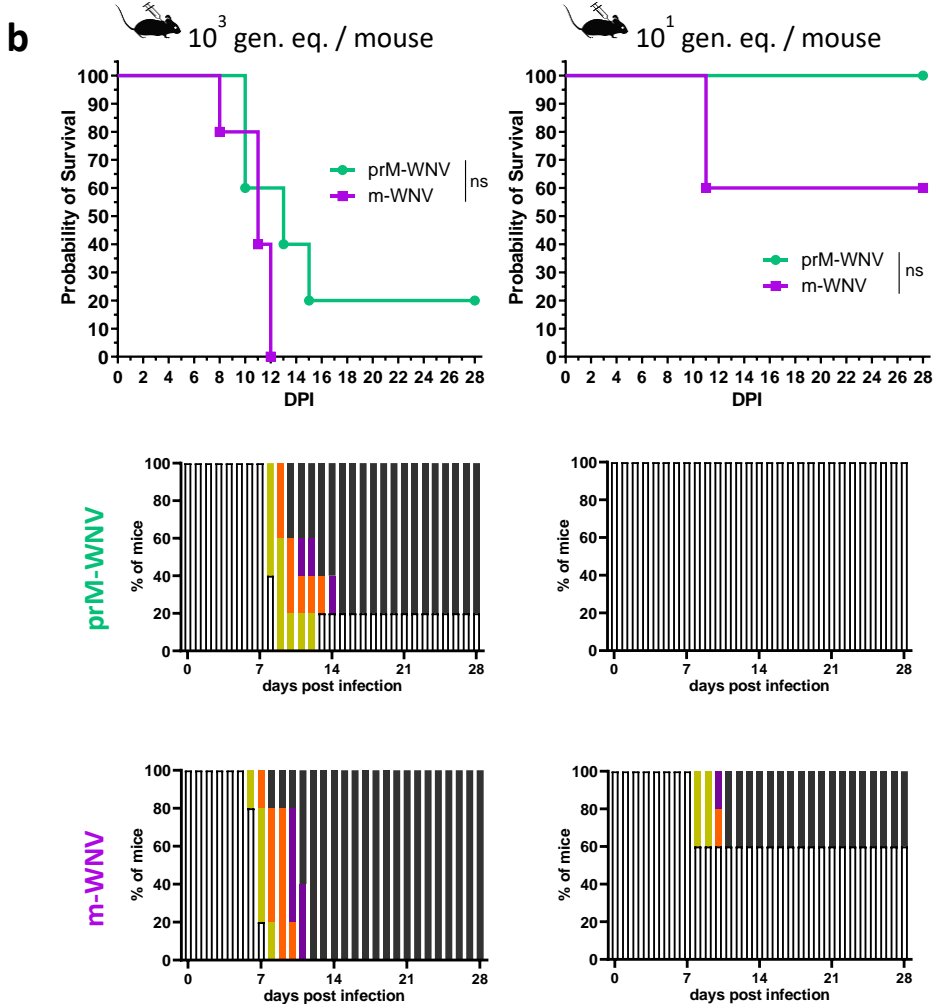
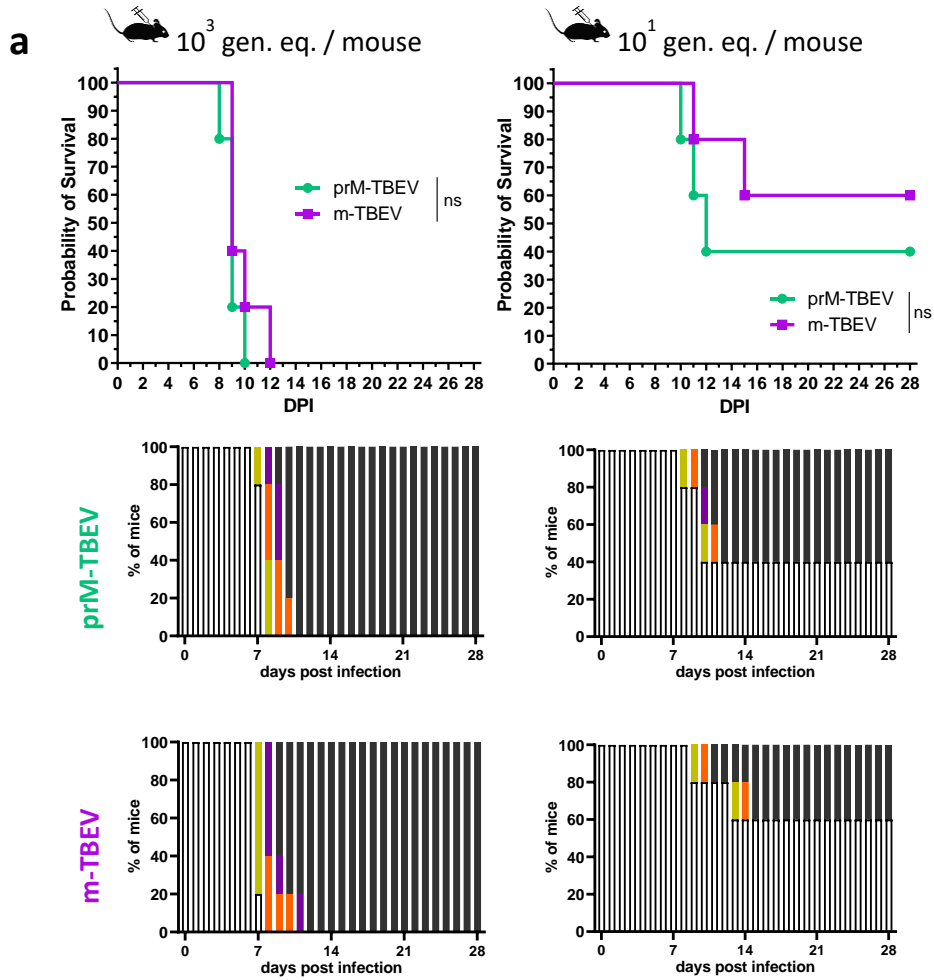
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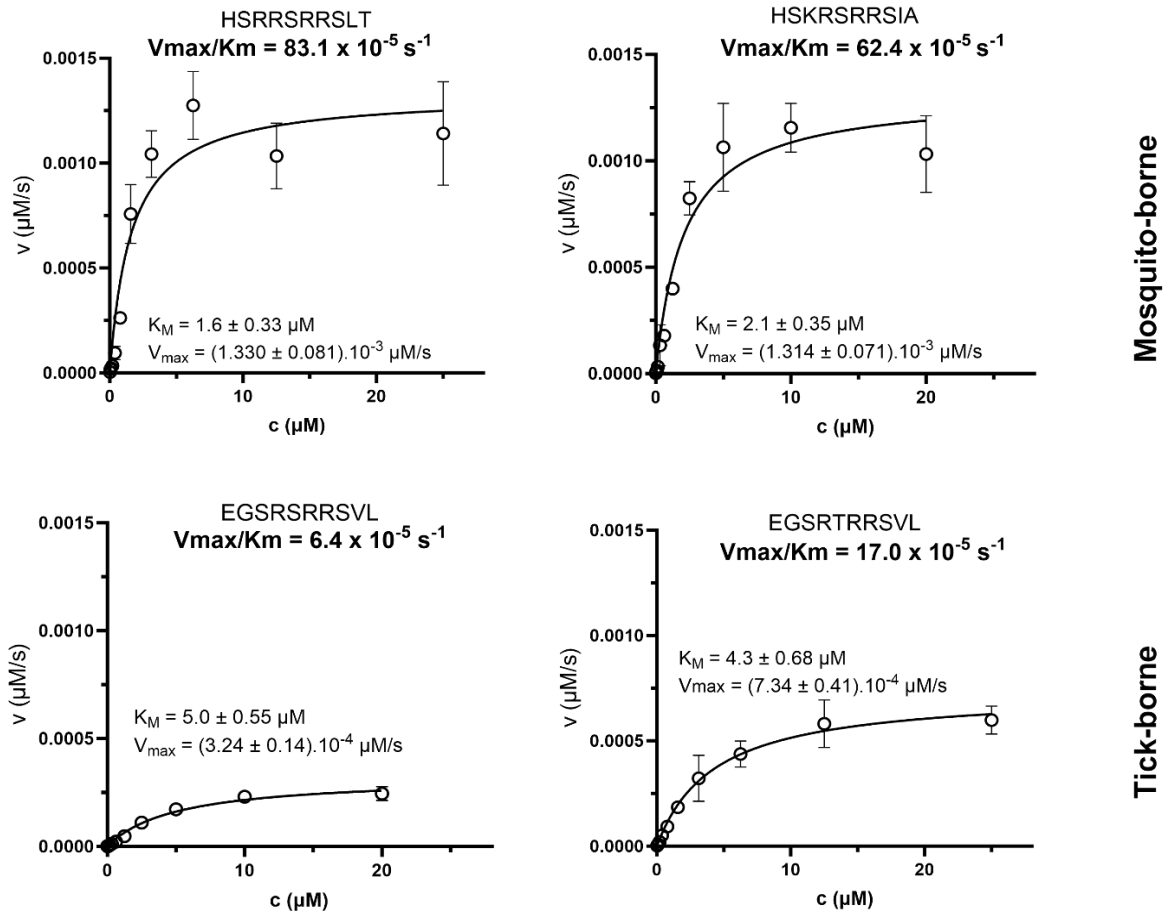
**Supplementary Fig. 1** | Unmodified western blot image corresponding to Fig. 1b. Western blot membrane was divided into two parts to enable separate incubation with different primary and secondary antibodies. Image was acquired with a GE Amersham imager 680.



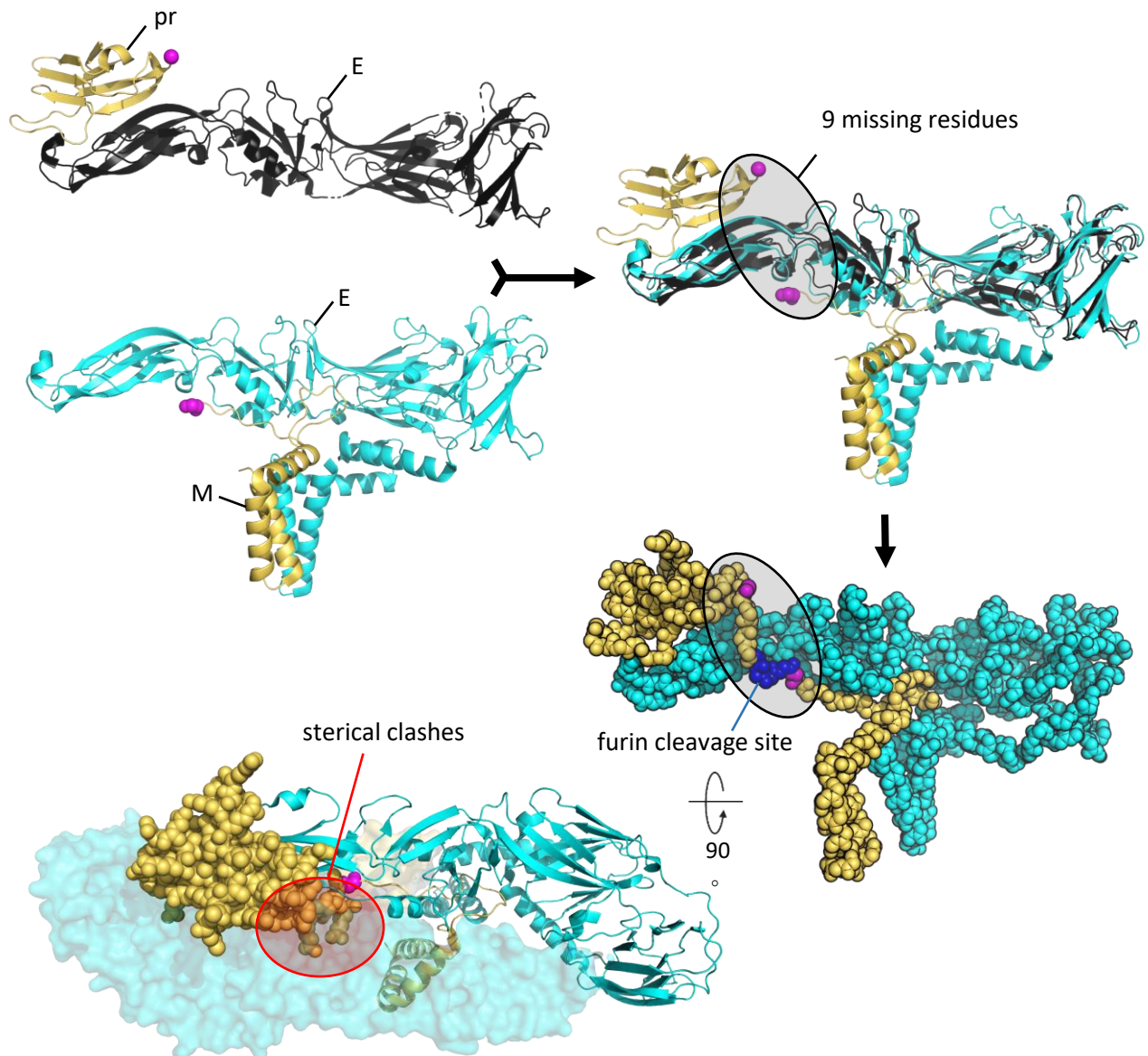
**Supplementary Fig. 2** | pH dependence of furin cleavage of prM-TBEV. Western blot analysis showed efficient furin cleavage over the entire pH range from 5.5 to 8.



**Supplementary Fig. 3 |** Comparison of the pathogenicity of prM-TBEV/m-TBEV and prM-WNV/m-WNV in a mouse model. **a**, Four groups of adult BALB/c mice ( $n = 5$ ) were subcutaneously infected with two different doses ( $10^1$  and  $10^3$  genome equivalents per mouse) of prM-TBEV (two groups) or m-TBEV (two groups), respectively. In the case of prM-TBEV, the infectious dose calculation corresponds to  $\sim 4$  and 400 PFU for  $10^1$  and  $10^3$  gen. eq. per mouse, respectively. Survival rates and clinical scores were monitored for 28 days. Clinical score was evaluated as follows: 1, no signs; 2, piloerection; 3, hunched back; 4, paralysis; and 5, death **b**, Similar to the above experiment, four groups of mice were infected with either prM-WNV or m-WNV, and survival was monitored for 28 days. In the case of prM-WNV, the infectious dose calculation corresponds to  $\sim 0.07$  and 7 PFU for  $10^1$  and  $10^3$  gen. eq. per mouse, respectively. Clinical signs of disease were assessed as described above. Survival rates were statistically evaluated using the log-rank Mantel-Cox test.



**Supplementary Fig. 4 |** Determination of  $K_M/V_{\max}$  values. The substrate-dose-dependent initial velocity of cleavage was measured at pH 7.0 in buffer (25 mM acetic acid, 25 mM MES, 25 mM glycine, 1 mM  $\text{CaCl}_2$ ) for each peptide substrate, in triplicate. Reactions were started by addition of furin (final concentration of  $10 \text{ ng mL}^{-1}$ ).  $K_M$  and  $V_{\max}$  values were fitted to the Michaelis-Menten equation using GraphpadPrism software. The resultant  $V_{\max}/K_M$  indicates that the studied mosquito-borne prM-derived peptides were more efficiently cleaved by furin, compared to substrates derived from tick-borne flaviviruses.



**Supplementary Fig. 5 |** Modelling the prM-E complex of the smooth immature particle. Currently, there is no experimental structure of the immature smooth flavivirus. Therefore, we used the E-pr structure available in PDB 7QRF, which is superimposed with the 7Z51 structure of the E-M complex. Nine residues are missing between the two magenta-coloured residues of these PDB structures. Using AlphaFold, we modelled the heterodimer prM-E and created a complete heterodimeric model together with the two PDB structures. The missing 9 residues do not have much conformational space for modelling because the distance between the purple residues is just enough to connect the 9 residues together. These nine residues are in the middle of the complete prM-E complex. Therefore, they would form clashes with the E protein in a smooth conformation unless they have been previously cleaved.

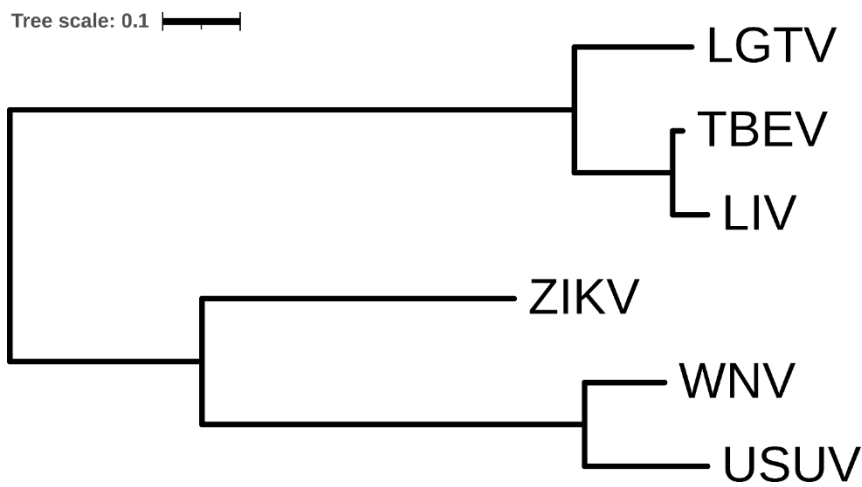
	1	10	20	30	40	50	60	
TBEV_prM	ATVRKERD	ESTVIRAEGRDA	AAT...QVRV	ENGT	CVVILATDM	CSWCDDSL	SYECVTID	QGEFVVDVDFC
LGTV_prM	.TVRRERD	GSMVIRAEGRDA	AAT...QVRV	ENGT	CVVILATDM	CSWCDDSL	SYECVTID	QGEFVVDVDFC
LIV_prM	ATVRKEGD	CTTVIRAEGRDA	AAT...QVRV	ENGT	CVVILATDM	CSWCDDSL	SYECVTID	QGEFVVDVDFC
USUV_prM	.LKLNFQ	GKVMVTINAT	DMADVIVVPTQ	HGKNQ	CWIRAMDV	GYMCDT	TITIECPKLD	AGNDEEDIDCWC
WNV_prM	.VTLSNFQ	GKVMVTINAT	DMADVIVVPTQ	HGKNQ	CWIRAMDV	GYMCDT	TITIECPKLD	AGNDEEDIDCWC
ZIKV_prM	AAEVTRRG	SAYYMLDRND	AGEAISFPTT	LGMNK	CYIQIMDL	GHMCDAT	MSYECPM	LDEGEVIEDVDFCWC

	70	80	90	100	110	120	130												
TBEV_prM	RNVDG	VYLE	YGRCK	QOE	.GSRTRRS	SVLIP	SHAQGE	LTRGRGHK	WLEGD	SLRTH	LTRVE	GWVW	KNRLL	ALA					
LGTV_prM	RGVEK	VYLE	YGRCK	QOE	.GSRTRRS	SVLIP	SHAQGE	LTRGRGHK	WLEGD	SLRTH	LTRVE	GWVW	KNRLL	ALA					
LIV_prM	RNVDG	VYLE	YGRCK	QOE	.GSRTRRS	SVLIP	SHAQGE	LTRGRGHK	WLEGD	SLRTH	LTRVE	GWVW	KNRLL	ALA					
USUV_prM	DK.QE	MYVY	YGRCK	QOE	.HSKRS	RRSIAV	QTHG	ESMLANK	KKDA	WLD	STKAS	RYL	MKTE	NWIT	IRNPGY	ALV			
WNV_prM	TK.SS	VYVY	YGRCK	QOE	.HSRRS	RRSIAV	QTHG	ESMLANK	KKDA	WLD	STKAS	RYL	MKTE	NWIT	IRNPGY	ALV			
ZIKV_prM	NT.TS	TWVY	YGTC	HKK	GEARR	SRR	AVT	PSH	STRK	LQTR	SQT	WLE	SRE	YTKH	LIR	VEN	WIF	IRNPGY	ALV

	140	150	160				
TBEV_prM	MVTV	WLTLE	SVVTRVA	LVVLL	CLAP	VYA	
LGTV_prM	LVMVA	WLMVD	GLLPRIL	VVVA	LALAP	VYA	
LIV_prM	MVAV	WLALE	SVVTRVA	LVVLL	CLAP	VYA	
USUV_prM	AVLL	GWML	GSNNG	QRVVF	VVLL	LVVAP	VYS
WNV_prM	AAVI	GWML	GSNTM	QRVVF	VVLL	LVVAP	VYS
ZIKV_prM	AAAIA	WLLGS	SSTS	QKVI	VVMI	LIVAP	VYS



**Supplementary Fig. 6 |** Multiple sequence alignment of flavivirus prM proteins, with the highlighted furin cleavage site (blue) and amino acid residues K81 and E83 (orange), which were mutated in rTBEV. Percentage of equivalent residues was calculated considering the physico-chemical properties. Fully conserved residues are shown on a black background, and residues in black frames indicate that over 70% of residues share similar properties. Alignment was performed using ClustalW, with subsequent graphical rendering using ESPrnt 3.0. The numbering reflects the position within the alignment. The highlighted residues were numbered and used in the study based on the position of the respective residue in the amino acid sequence for a particular virus. The phylogenetic tree was based on amino acid multiple sequence alignment (prM protein) using MAFFT, and then constructed using the neighbor-joining method with JTT substitution model. Visualization and graphic processing were performed with the iTOL online tool<sup>68</sup>.

	1	10	20	30	40	50	60	70
TBEV	SRC	THLE	NRDF	VIT	GVO	CT	TR	TL
LIV	SRC	THLE	NRDF	VIT	GVO	CT	TR	TL
LGTV	SRC	THLE	NRDF	VIT	GVO	CT	TR	TL
USUV	FN	CLGM	NRDF	LE	GVS	GAT	WV	DL
WNV	FN	CLGM	NRDF	LE	GVS	GAT	WV	DL
ZIKV	IR	CG	VSN	RDF	VE	GMS	GG	TW

	80	90	100	110	120	130	140
TBEV	AAR	CPT	MGP	ATL	AEE	HGG	TV
LIV	AAR	CPT	MGP	ATL	AEE	HGG	TV
LGTV	AAR	CPT	MGP	ATL	AEE	HGG	TV
USUV	VSN	CPT	G	AHNP	KRA	EDTY	VCK
WNV	RAA	CPT	G	AHNE	KRAD	PAF	VCK
ZIKV	DSR	CPT	G	EAL	YLD	KQSD	TQY

	150	160	170	180	190	200	
TBEV	V	KVE	PH	TG	DY	VAA	NE
LIV	V	KVE	PH	TG	DY	VAA	NE
LGTV	V	KVE	PH	TG	DY	VAA	NE
USUV	V	G	F	I	H	G	S
WNV	V	A	L	S	V	H	G
ZIKV	I	M	S	V	H	G	S

	210	220	230	240	250	260	270
TBEV	T	V	H	L	P	T	A
LIV	T	V	H	L	P	T	A
LGTV	T	V	H	L	P	T	A
USUV	V	G	F	I	H	G	S
WNV	V	A	L	S	V	H	G
ZIKV	I	M	S	V	H	G	S

	280	290	300	310	320	330	340
TBEV	P	V	A	H	I	E	G
LIV	P	V	A	H	I	E	G
LGTV	P	V	A	H	I	E	G
USUV	V	G	F	I	H	G	S
WNV	V	A	L	S	V	H	G
ZIKV	I	M	S	V	H	G	S

	350	360	370	380	390	400	
TBEV	P	V	A	H	I	E	G
LIV	P	V	A	H	I	E	G
LGTV	P	V	A	H	I	E	G
USUV	P	V	A	H	I	E	G
WNV	P	V	A	H	I	E	G
ZIKV	P	V	A	H	I	E	G

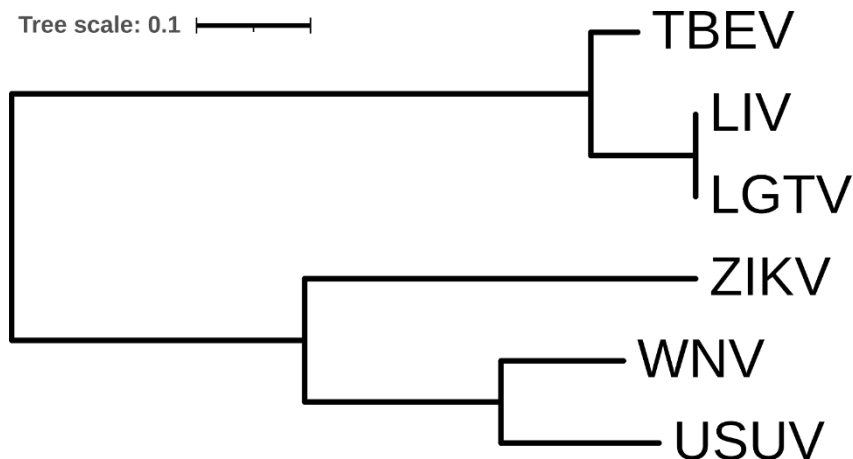
  

	410	420	430	440	450	460	470
TBEV	Q	X	T	R	K	G	E
LIV	Q	X	T	R	K	G	E
LGTV	Q	X	T	R	K	G	E
USUV	I	T	I	K	S	A	Q
WNV	T	I	T	L	R	G	A
ZIKV	E	A	T	V	R	G	A

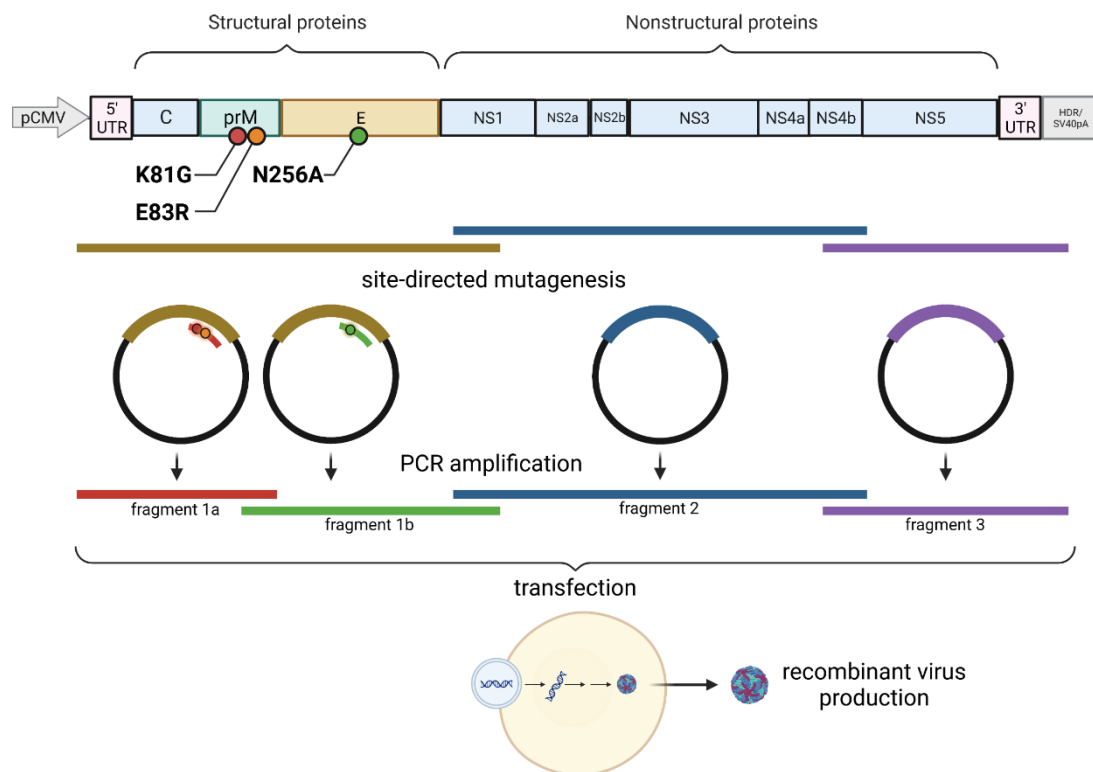
  

	480	490
TBEV	P	T
LIV	P	T
LGTV	P	T
USUV	R	S
WNV	R	S
ZIKV	G	S

Tree scale: 0.1



**Supplementary Fig. 7** | Multiple sequence alignment of flavivirus E proteins with highlighted TBEV residue E243, USUV residue E241 (green), and TBEV position N256 (orange) mutated in rTBEV. The percentage of equivalent residues was calculated considering the physicochemical properties. Fully conserved residues are shown on a black background, and residues in black frames indicate that over 70% of the residues have similar properties. The alignment was created using Clustal Omega, and then graphically processed with ESPript 3.0. The numbering reflects the position within the alignment. The highlighted residues were numbered and used in the study based on the position of the respective residue in the amino acid sequence for a particular virus. The phylogenetic tree was based on amino acid multiple sequence alignment (E protein) using MAFFT, and then constructed using the neighbor-joining method with JTT substitution model. Visualization and graphic processing were performed with the iTOL online tool.



**Supplementary Fig. 8** | Scheme representing the site-directed mutagenesis approach using infectious subgenomic amplicons. Mutated sites in the prM (K81G, E83R) and E (N256A) proteins were introduced using modified primers, and are highlighted. Overall, four amplified DNA fragments with overlapping ends were transfected into BHK-21 cells, followed by harvesting of the recombinant virus.

**Supplementary Table 1** | Primers used to generate overlapping amplicons of TBEV fragments to produce recombinant virus rTBEV.

<b>Primer ID</b>	<b>Sequence (5' - 3')</b>
<b>mut_prM_site_1+2_FWa</b>	GGGGGTCAGCGTGGCTCACGGACAAGGCGC
<b>mut_prM_site_1+2FWb</b>	GGGGGTCAGCGTGGCTCACGGACAAGG
<b>mut_prM_site_1+2_R</b>	GCCACGCTGACCCCCACAGCGTCCATACTCC
<b>mut_Env_site_3_FW</b>	TGTACGCACTCGGAGACCAGACTGG
<b>mut_Env_site_3_R</b>	TCTCCGAGTGCGTACACATCCATC