

## Supplementary information

### Irreversible furin cleavage site exposure renders immature tick-borne flaviviruses fully infectious

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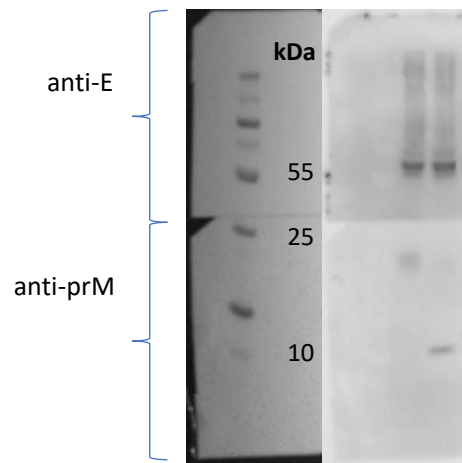
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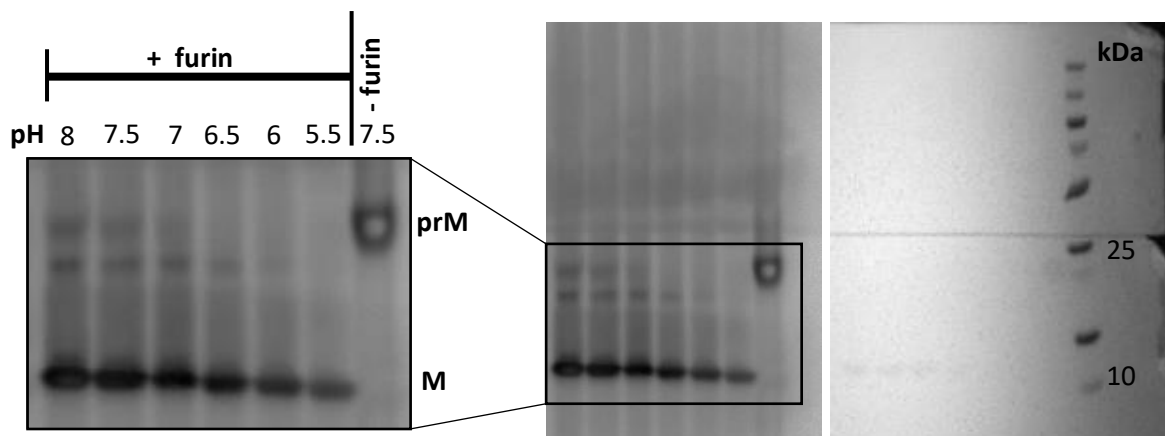
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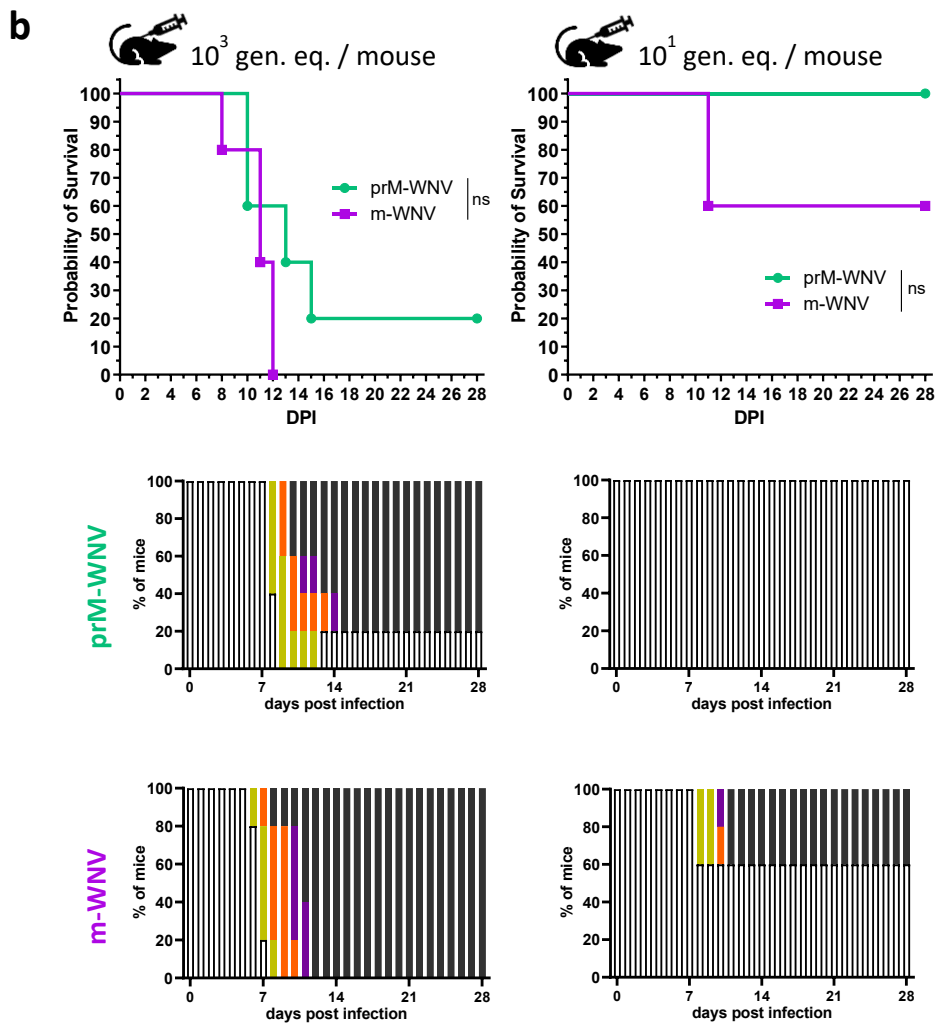
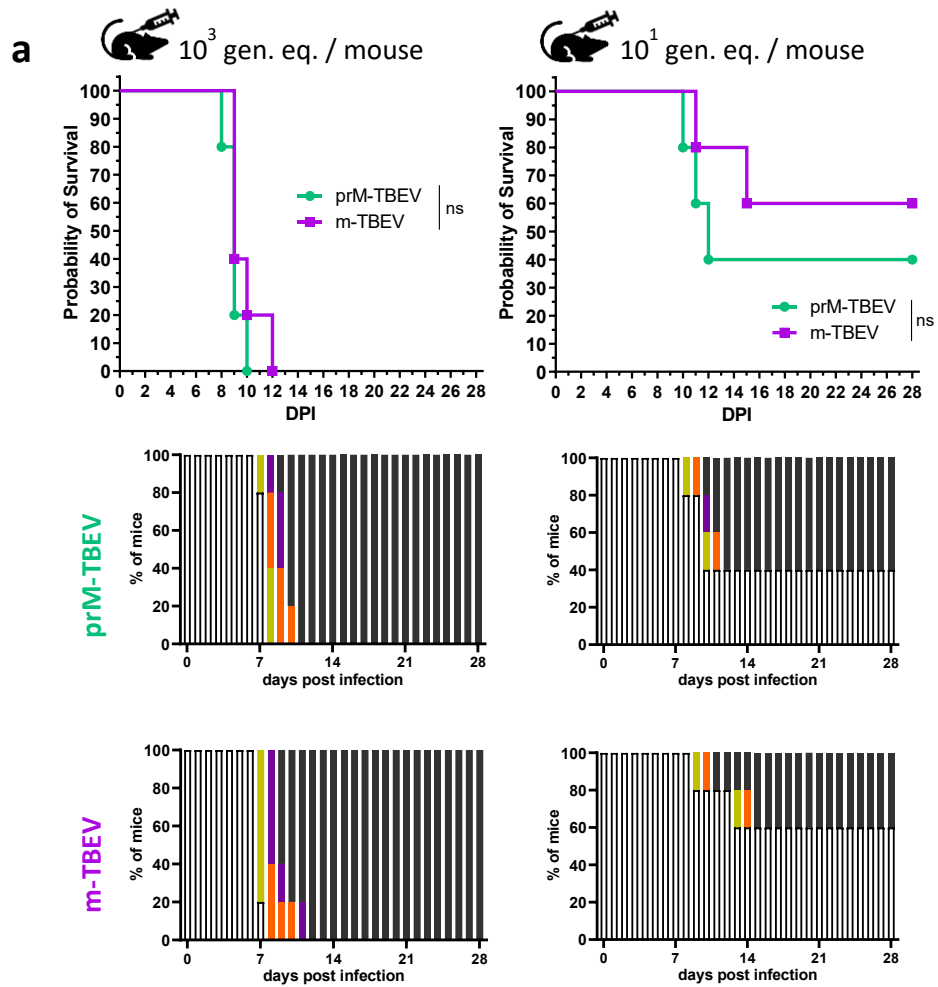
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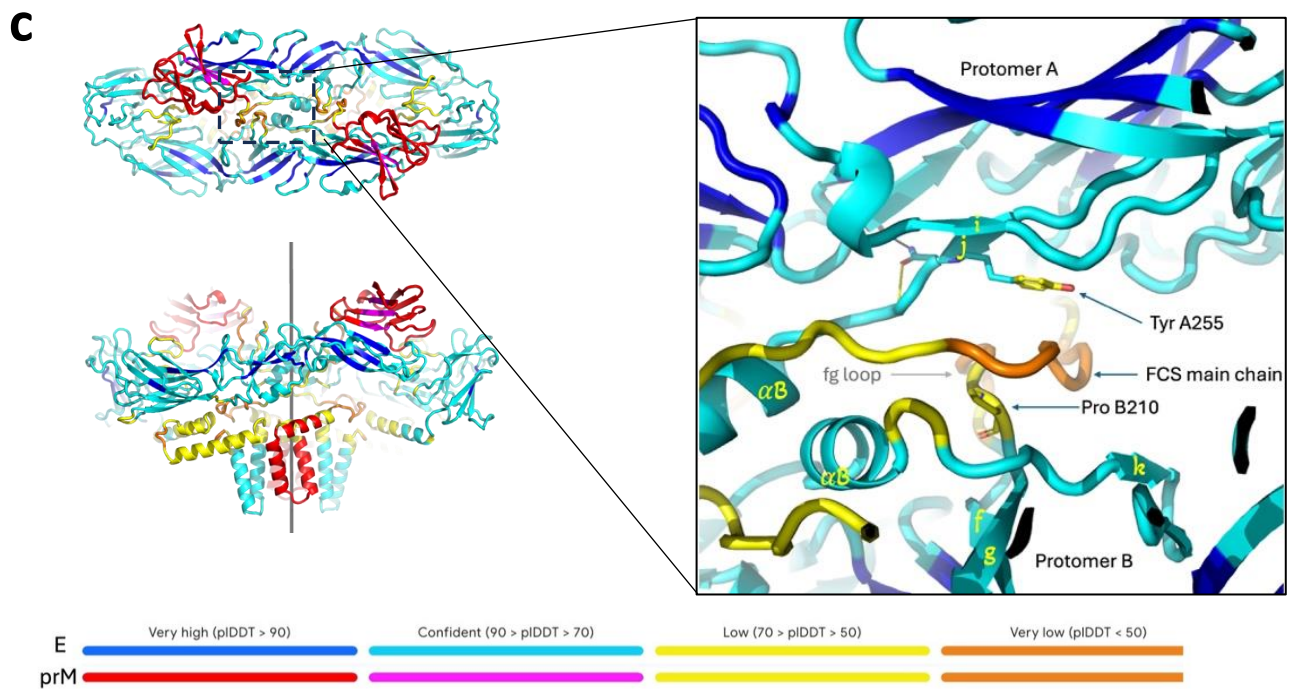
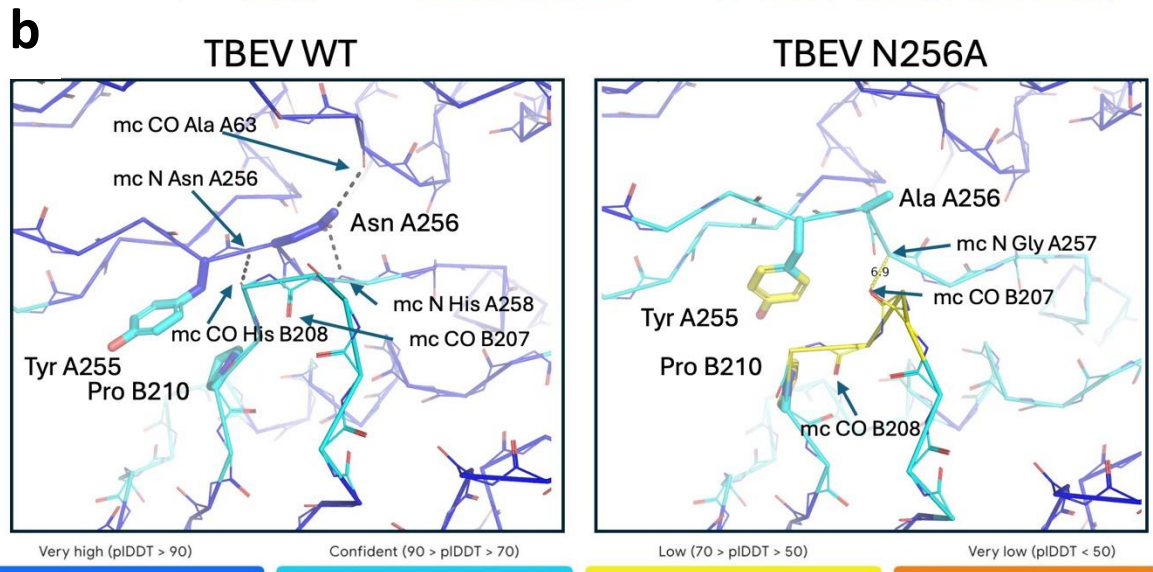
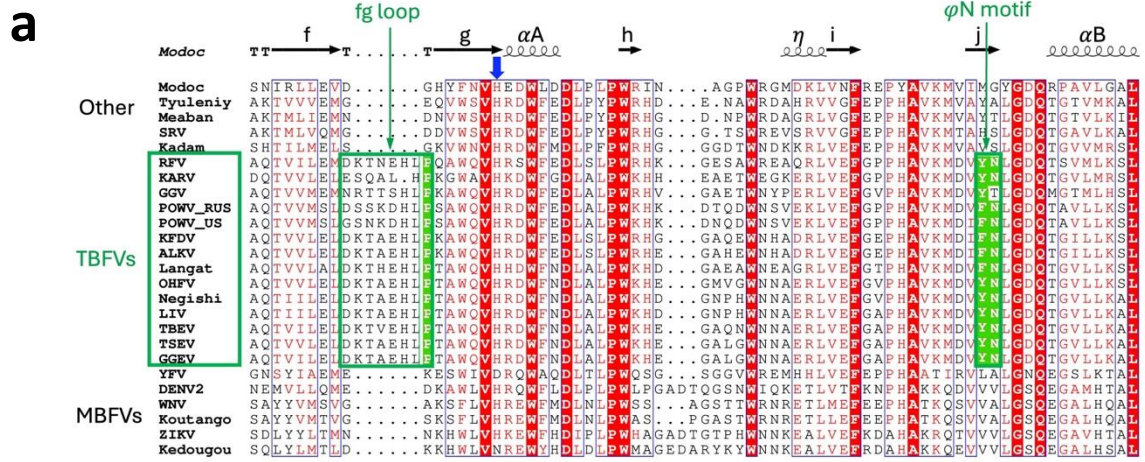
**Supplementary Fig. 1** | Unmodified western blot image corresponding to Fig. 1b. Western blot membrane was divided into two parts to enable separate incubation with different primary and secondary antibodies. Image was acquired with a GE Amersham imager 680.



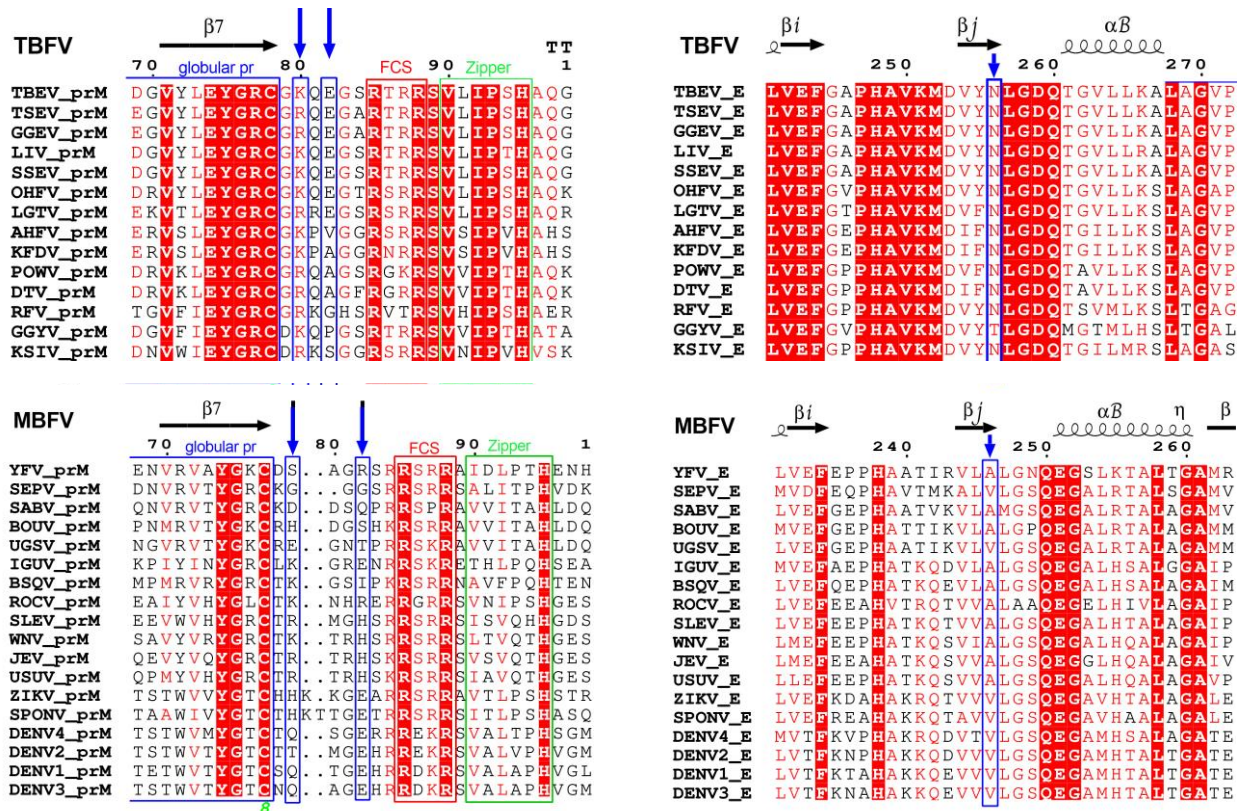
**Supplementary Fig. 2** | pH dependence of furin cleavage of prM-TBEV. Western blot analysis showed efficient furin cleavage over the entire pH range from 5.5 to 8.



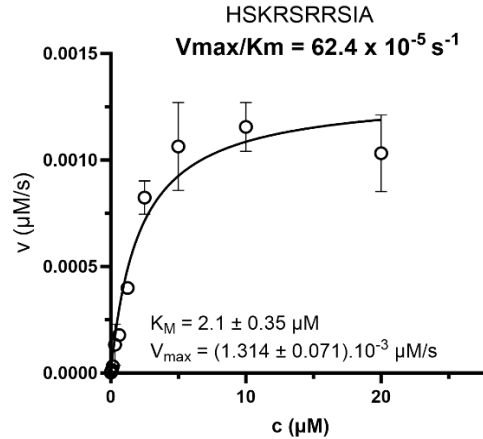
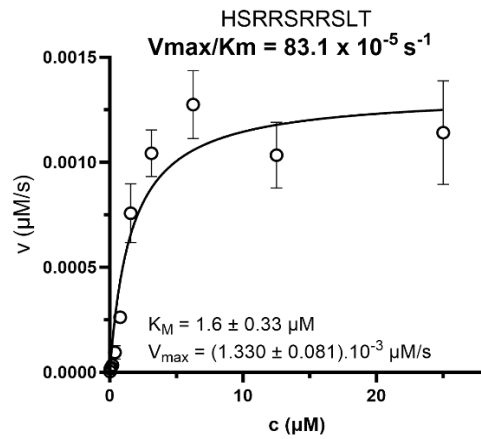
**Supplementary Fig. 3 | Comparison of the pathogenicity of prM-TBEV/m-TBEV and prM-WNV/m-WNV in a mouse model.** **a**, Four groups of adult BALB/c mice ( $n = 5$ , 5) were subcutaneously infected with two different doses ( $10^1$  and  $10^3$  genome equivalents per mouse) of prM-TBEV (two groups) 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.



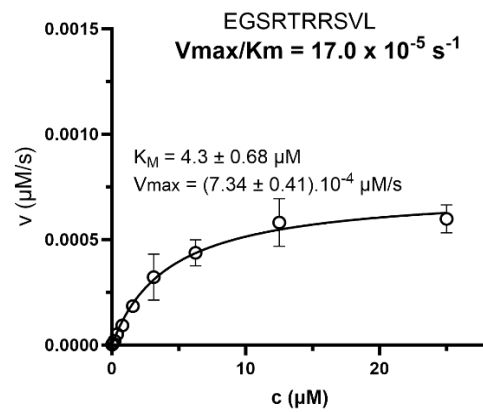
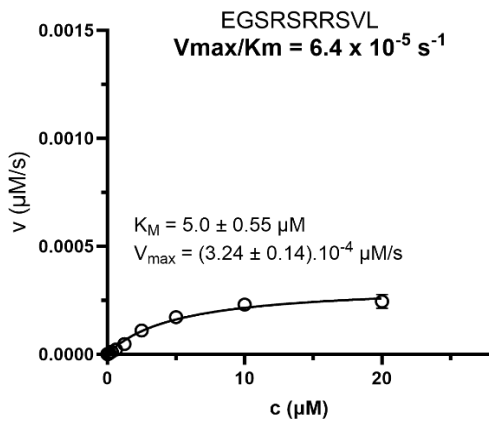
**Supplementary Fig. 4 | (prM/E)<sub>2</sub> dimer interface.** **a**, Amino acid sequence alignment of E from flaviviruses that infect vertebrates, highlighting in green two key TBFV-specific motifs identified here: the conserved proline in the fg loop insertion and the “ $\varphi$ N” where  $\varphi$  stands for tyrosine or phenylalanine. Note that the conserved asparagine in this motif is replaced by threonine in one instance, whose side chain could also make a hydrogen bond with the main chain. A vertical blue arrow points to position 216 (TBEV numbering) in the  $\alpha$ A- $\alpha$ B groove, believed to play a role in unzipping via repulsion with prM His95. The alignment shows that His216 is not conserved across all flaviviruses, indicating that other residues may contribute to unzipping for those viruses. **b**, AlphaFold3 prediction of a wild type sE dimer (left) and a N256A mutant sE dimer (right). The two chains of the sE dimer are labeled A and B and are displayed as C $\alpha$  trace superposed with main chain atoms as lines color coded by atoms, with oxygen red, nitrogen blue, and carbon atoms according to AlphaFold confidence in the prediction (pLDDT score, as indicated at the bottom by the colored bars). Dotted lines indicate hydrogen bonds. Note that the prediction for the wt sE dimer recapitulates the inter-chain interactions observed experimentally, but not the N256A mutant: in the absence of the hydrogen bond made by the Asn256 side chain, the main chain nitrogen at this position is not in the right geometry for the inter-chain hydrogen bond, and the fg loop re-organizes in a different conformation, and the CH/ interaction between Tyr255 and Pro210 broken. mc CO= main chain carbonyl. Mc N=main chain amine. **c**, AlphaFold3 prediction of a wild type sE dimer. The pLDDT color code is represented differently for prM and E, to distinguish the two in the left panel. A vertical bar in the bottom-left panel represents the molecular 2-fold axis of the (prM/E)<sub>2</sub> dimer. The inset (right panel) shows that the AF3 prediction places the main chain of the linker in between the side chains of Tyr255 and Pro210 of the two E protomers facing each other. The relevant  $\beta$ -strands and  $\alpha$ -helices in protein E are labeled in yellow.



**Supplementary Fig. 5 | Sequence alignment of flavivirus prM and E proteins showing the regions where mutations were introduced in this study (solid blue arrows).** On the left, in TBFVs, the residue at prM position 80 is consistently basic, whereas in MBFVs, this position is highly variable. In contrast, position 82 never contains a basic residue and often features a negatively charged amino acid. Upstream of this region is the globular pr domain, while downstream are the furin cleavage site (FCS, framed in red) and the zipper region (framed in green). On the right, the alignment of E proteins highlights the mutation at position 256 (TBEV numbering) within  $\beta$ -strand j (solid blue arrow), and also includes the  $\alpha$ B helix region.

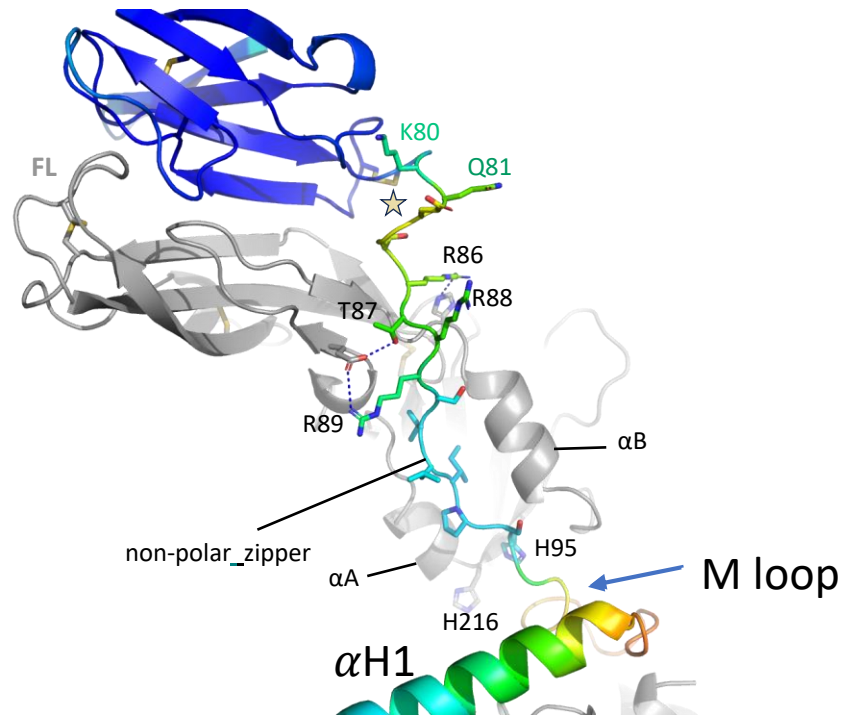


Mosquito-borne

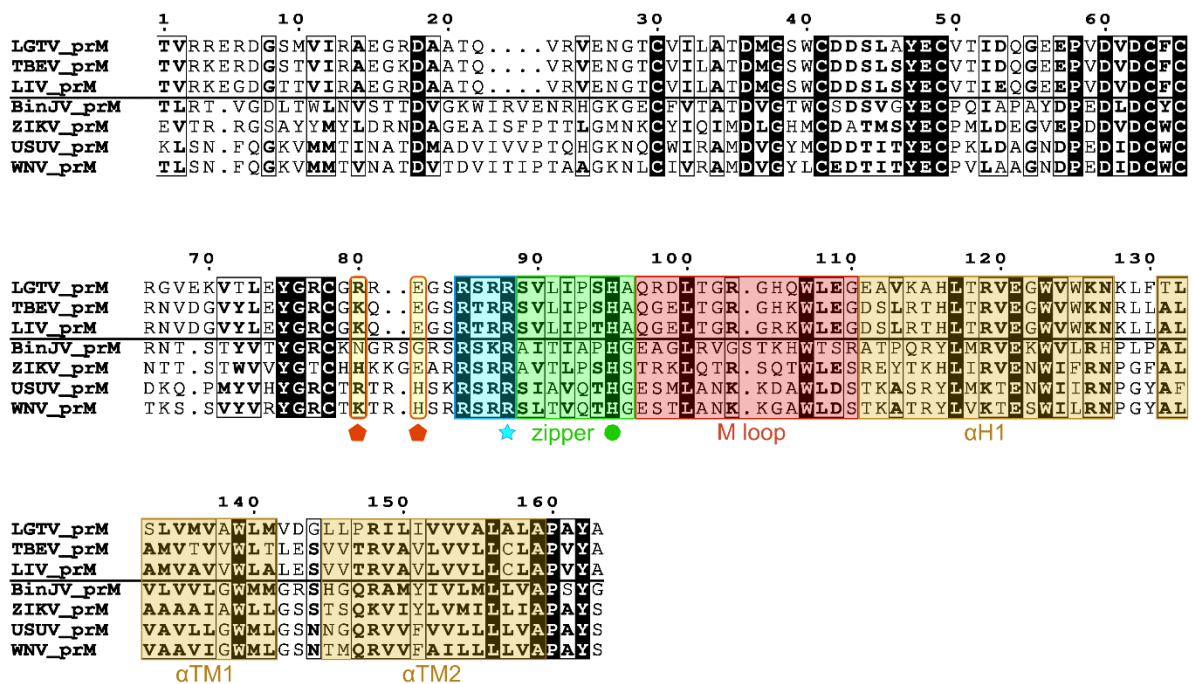


Tick-borne

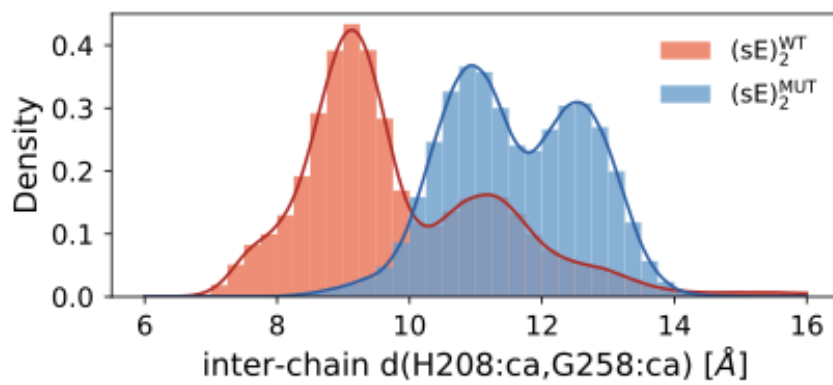
**Supplementary Fig. 6 | Determination of  $K_m/V_{max}$  values.** The substrate-dose-dependent initial velocity of cleavage was measured at pH 7.0 in buffer (25 mM acetic acid, 25 mM MES, 25 mM glycine, 1 mM  $\text{CaCl}_2$ ) for each peptide substrate, in triplicate. Reactions were started by addition 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.



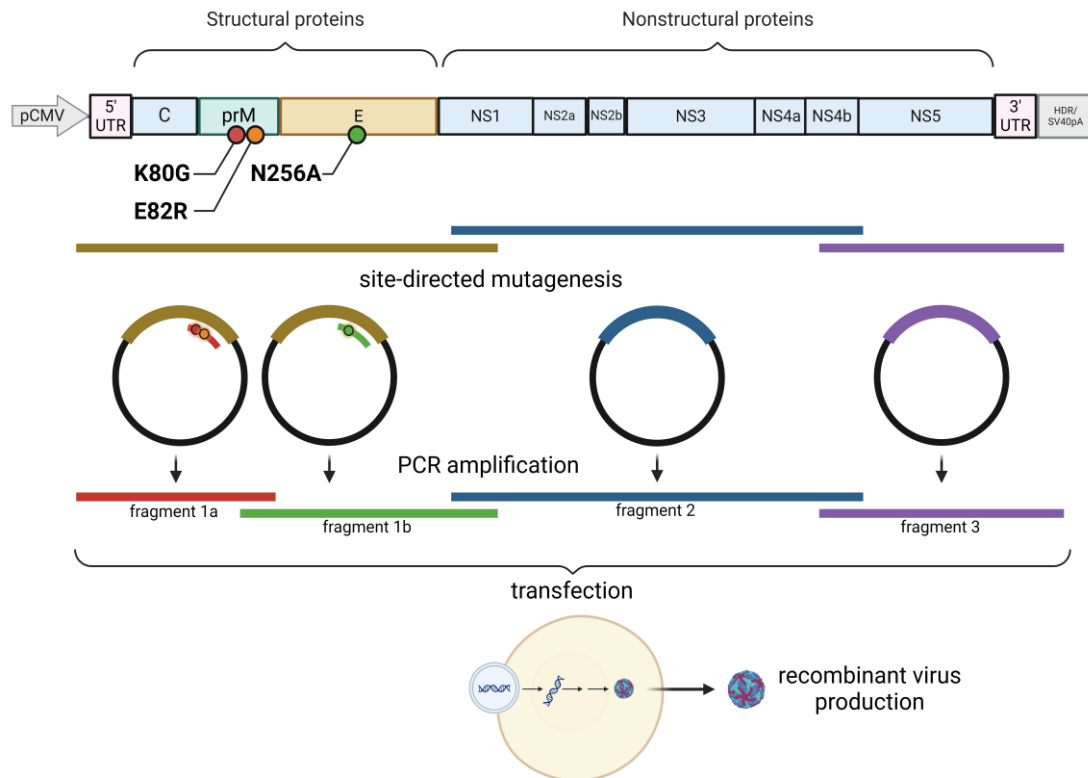
**Supplementary Fig. 7 | The prM/E interfaces in TBEV.** Detailed view of the TBEV prM/E protein complex in the zipper region, highlighting interactions along the cleavage site. The E protein is shown in grey, and the prM structure is coloured according to AlphaFold prediction accuracy (blue – high confidence, red – low confidence). The star marks the last disulfide bond at the end of the globular part of prM. The AF3 predictions are shown color-coded by pLDDT (>90 blue, < 90 cyan >70, < 70 yellow > 50, <50 orange).



**Supplementary Fig. 8 | Multiple sequence alignment of full-length prM proteins from flaviviruses analyzed in this study, with Binjari virus included for comparative context.** The alignment highlights furin cleavage site (blue), zipper region (green) with highly conserved histidine H95 (green circle) and amino acid residues K80 and E82 (orange), which were mutated in rTBEV. The downstream region, consisting of the M loop and the C-terminal helical regions ( $\alpha$ H1,  $\alpha$ TM1 and  $\alpha$ TM2), are shown in red and ochre, respectively. 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 ESPrpt 3.0.



**Supplementary Fig. 9 | Molecular Dynamics simulations of (sE)<sub>2</sub> dimers.** Distributions of distances between the Cα atoms of residues H208 and G258. Raw normalized histograms are represented by bars, kernel density estimations as solid lines; the red and blue colours indicate (sE)<sub>2</sub><sup>WT</sup> and (sE)<sub>2</sub><sup>MUT</sup>, respectively.



**Supplementary Fig. 10 | Scheme representing the site-directed mutagenesis approach using infectious subgenomic amplicons.** Mutated sites in the prM (K80G, E82R) 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. Created in BioRender. Ruzek, D. (2025) <https://BioRender.com/4n4b27g>.

**Supplementary Table 1 |** Primers used to generate overlapping amplicons of TBEV fragments to produce recombinant virus rTBEV. The corresponding sequencing results with sequencing primers are shown below.

| Primer ID                        | Sequence (5' - 3')              |
|----------------------------------|---------------------------------|
| mut_prM_site_1+2_FW <sub>a</sub> | GGGGGTCAGCGTGGCTCACGGACAAGGCGC  |
| mut_prM_site_1+2FW <sub>b</sub>  | GGGGGTCAGCGTGGCTCACGGACAAGG     |
| mut_prM_site_1+2_R               | GCCACGCTGACCCCCACAGCGTCCATACTCC |
| mut_Env_site_3_FW                | TGTACGCACTCGGAGACCAGACTGG       |
| mut_Env_site_3_R                 | TCTCCGAGTGC GTACACATCCATC       |
| TBEV_1A_Fseq                     | TGTCTACGCTTCGCGTTGCACACA        |
| TBEV_2B_Rseq                     | TTCGTTCCGTGTCCACAGCGCA          |
| TBEV_2C_Fseq                     | GGTCATTACTCTGTTGGGGA            |
| TBEV_2D_Rseq                     | ACACATCCATTGAAGGCTTC            |

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ATCGTCATCCCCAACAAAGAT

**Supplementary Table 2** | Details of the Molecular Dynamics simulations. The table reports from left to right: the name of the system, the number of lipids per each species, the number of potassium and

chloride ions in solution, the number of water molecules, the total number of atoms, and the production simulation time.

| System                              | # Lipids |      |      |      |      | # K/Cl ions | # waters | Total # atoms | Production time [ $\mu$ s] |
|-------------------------------------|----------|------|------|------|------|-------------|----------|---------------|----------------------------|
|                                     | POPC     | POPE | POPS | POPI | CHOL |             |          |               |                            |
| (prM-E) <sub>2</sub> <sup>WT</sup>  | 444      | 185  | 37   | 37   | 37   | 258/184     | 67294    | 317505        | 1.0                        |
| (prM-E) <sub>2</sub> <sup>MUT</sup> | 444      | 185  | 37   | 37   | 37   | 257/185     | 67846    | 319141        | 1.0                        |
| (sE) <sub>2</sub> <sup>WT</sup>     | -        | -    | -    | -    | -    | 85/81       | 25308    | 88114         | 1.0                        |
| (sE) <sub>2</sub> <sup>MUT</sup>    | -        | -    | -    | -    | -    | 84/80       | 24793    | 86559         | 1.0                        |