Figure S1, related to Table 1

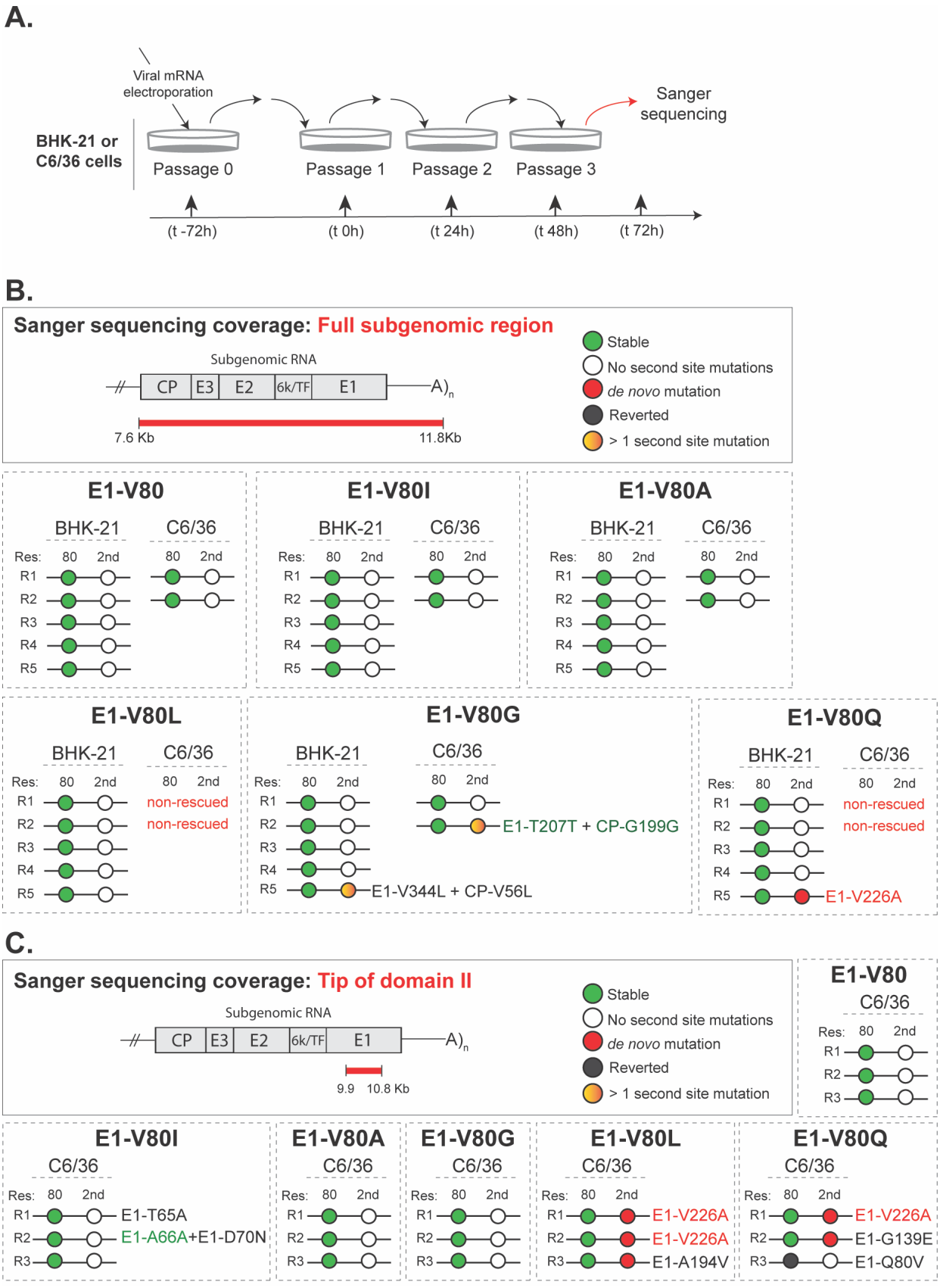

Figure S1. E1-V80X stable variants in BHK-21. Related to Table 1. A. Schematic of the *in vitro* evolution approach. E1-V80X variants were generated by electroporation of viral mRNA into BHK-21 or C6/36 cells. Each E1-V80X mutant was then passaged three times in either cell lines, viral RNA extracted, PCR amplicons were generated and samples were Sanger sequenced. Sanger sequencing coverages: (i) Full sub-genomic region (complete structural polyprotein, ~7.6 to 11.8 Kb) or (ii) tip of domain II (~9.9 to 10.8 Kb). Each virus was generated, passaged five independent times, and the subgenomic region was sequenced to full coverage in at least two of the independent repetitions. Data is organized in results with full subgenomic sanger sequence coverage (B) or partial sanger sequence coverage (C). Sequence stability and acquisition of second site mutations for each mutant bearing specific E1-V80X substitution is indicated. R1 to R5 represent independent biological replicates of electroporation and viral passaging in either BHK-21 or C6/36 cells. Mutations at position 80 and second site mutations are indicated. Mutations E1-A129V/E/M and E1-V226A are indicated in blue and red, respectively. Synonymous mutations are indicated in green. Non-rescued, indicate absence of infectious virus after electroporation and passaging.

Figure S2, related to Table 1

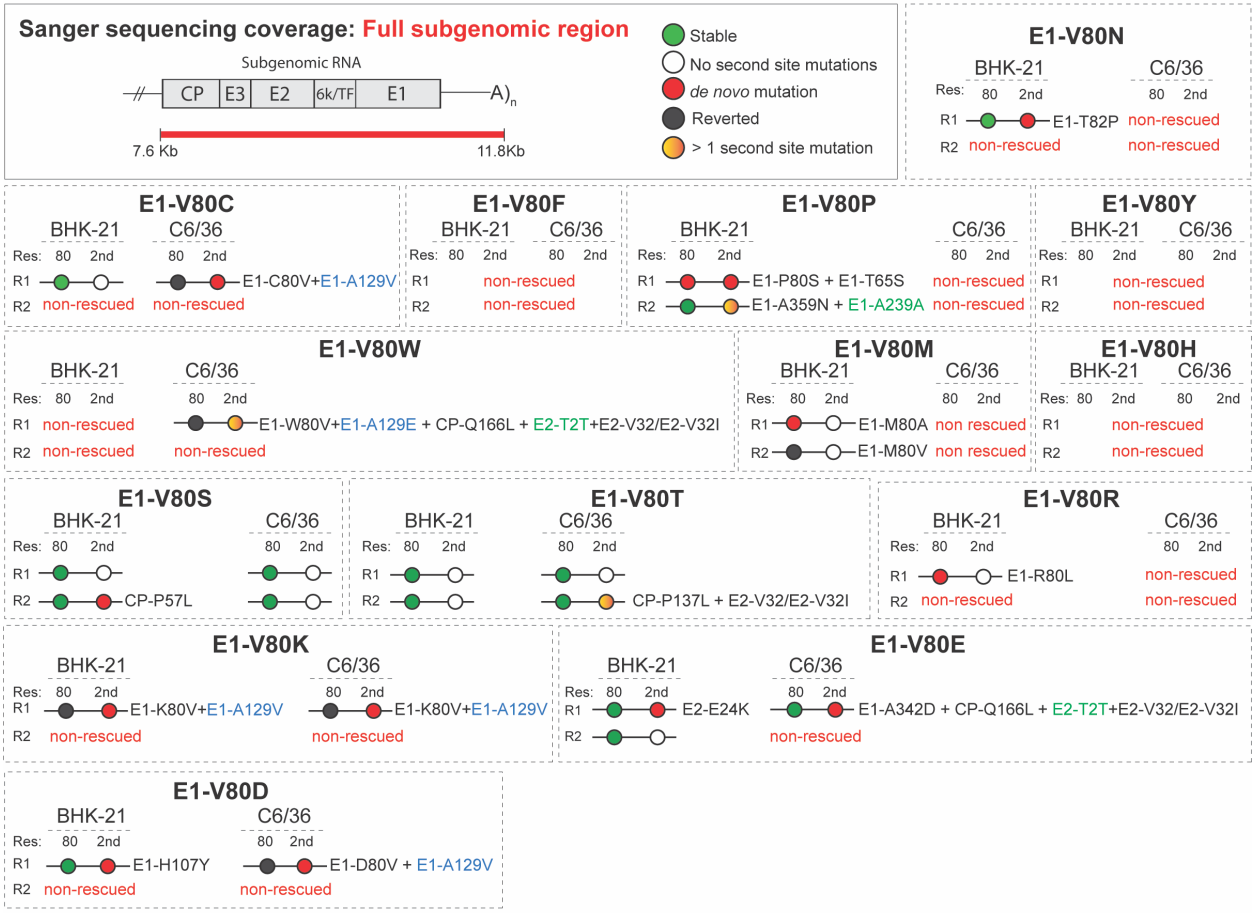

Figure S2. E1-V80X unstable variants in BHK-21. Full subgenomic region coverage. Related to Table 1. Schematic showing the Sanger sequencing coverage for each experiment after three passages in either BHK-21 or C6/36 cells are shown. Full sub-genomic region (complete structural polyprotein, ~7.6 to 11.8 Kb). Each virus was generated, passaged three independent times, and the subgenomic region was sequenced to full coverage. Sequence stability and acquisition of second site mutations for each mutant bearing specific E1-V80X substitution is indicated. R1 to R2 represent two independent biological replicates of electroporation and viral passaging. Mutations at position 80 and second site mutations are indicated. Mutations E1-A129V/E are indicated in blue. Synonymous mutations are indicated in green. Non-rescued, indicate absence of infectious virus after electroporation and passaging.

Figure S3, related to Table 1

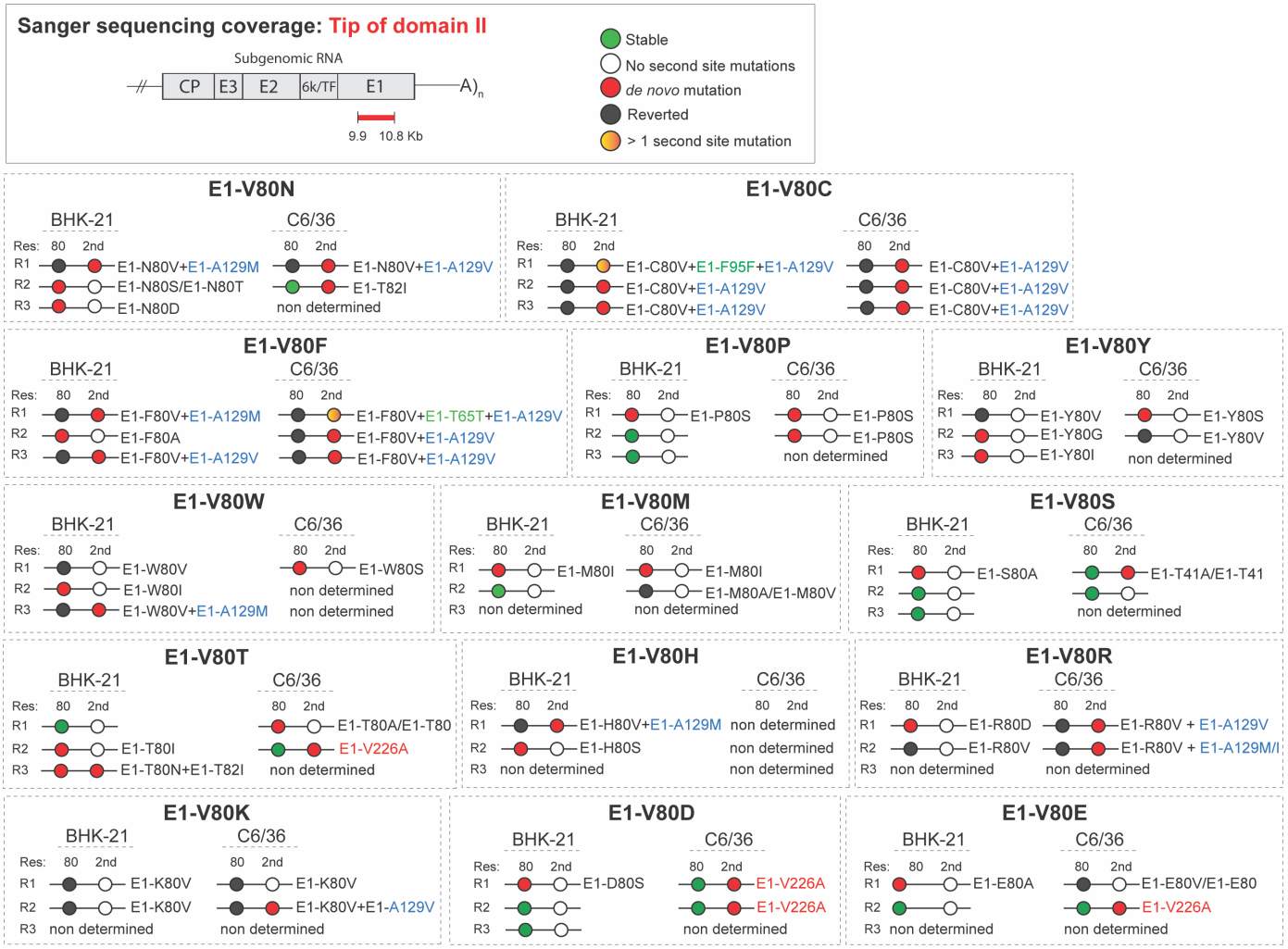

Figure S3. E1-V80X unstable variants in BHK-21. Partial coverage, tip of domain II. Related to Table 1. Sanger sequencing coverages: tip of domain II (~9.9 to 10.8 Kb). Each virus was generated, passaged three independent times, and the tip of domain II was sequenced. Sequence stability and acquisition of second site mutations for each mutant bearing specific E1-V80X substitution is indicated. R1 to R3 represent three independent biological replicates. Mutations E1-A129V/E/M and E1-V226A are indicated in blue and red, respectively. Synonymous mutations are indicated in green.

Figure S4, related to Figure 1 and 4

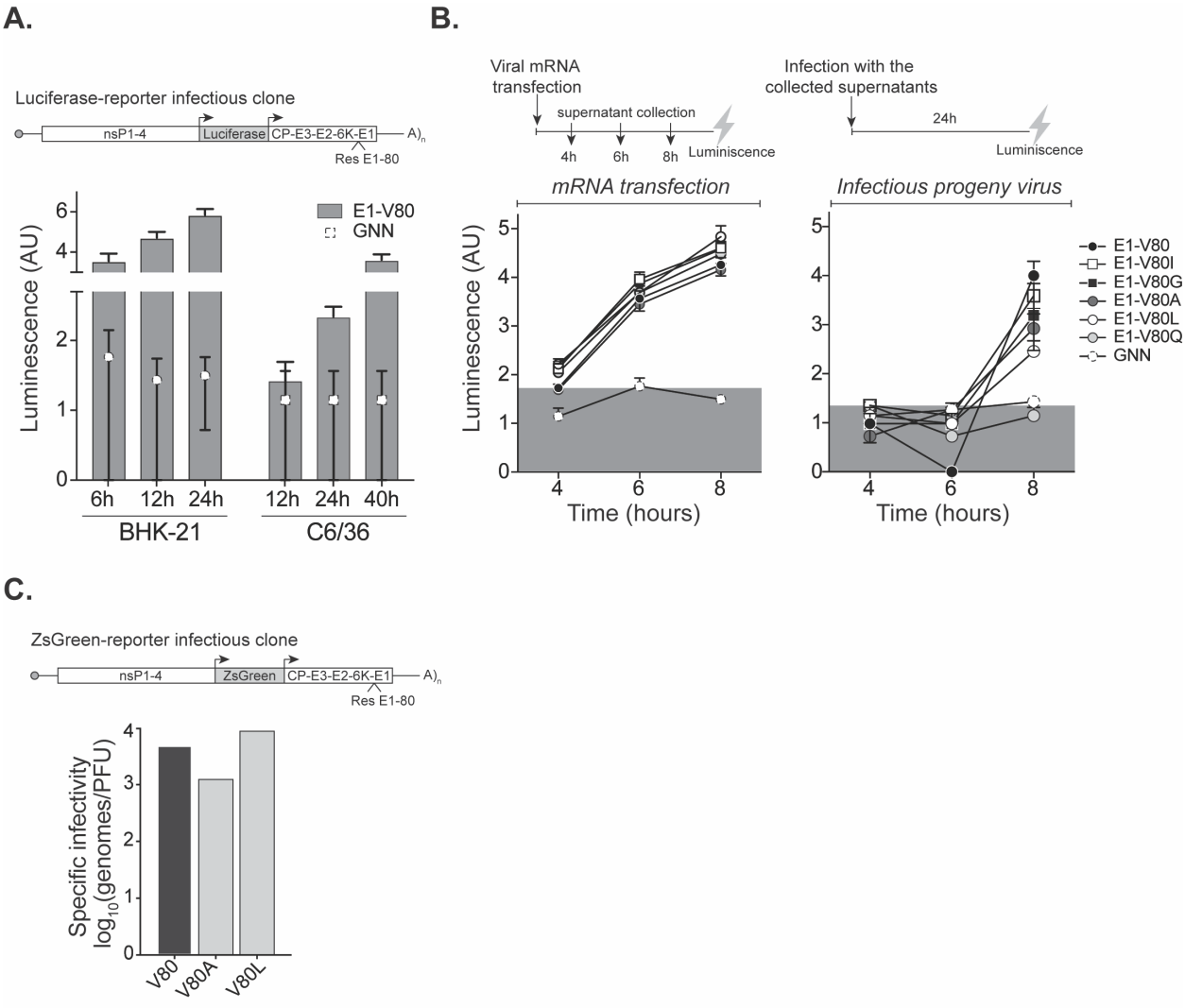

Figure S4. Analysis of the CHIKV reporter viruses. Related to Figure 1 and 4. (A) Top panel: Schematic representation of the luciferase reporter virus. The Luciferase cassette is depicted in gray. The circle indicates the cap, each arrow indicates the sub-genomic promoter, and the $A)_n$ indicates the poly-A tail. Lower panel: Replication rate of CHIKV E1-V80 in either BHK-21 or C6/36 cells as measured by Luciferase activity. BHK-21 or C6/36 cells were transfected with mRNA from CHIKV E1-V80 or with a non-replicative nsP4 active site polymerase mutant (GNN), and luciferase activity was measured at the indicated time points. We used a GNN control to set the basal levels of noise. The mean and SEM are shown. Data represent two independent experiments, each with internal duplicates. (B) Left panel: BHK-21 cells were transfected with mRNA from the different reporter viruses, and luciferase activity was measured at the indicated

time points. Right panel: Generation of infectious progeny virus was monitored. Supernatants of the time points 4, 6 and 8 hours post transfection were used to infect BHK-21 cells. Generation of infectious progeny virus was addressed by the presence of luminescence at 24 hpi. GNN mutant was used as non-replicative control. The mean and SEM are shown. Data represent three independent experiments, each with internal duplicates, ns, $p > 0.05$; two-way ANOVA with Bonferroni post-hoc test. (C) Top panel: Schematic representation of the ZsGreen reporter virus. The ZsGreen cassette is depicted in gray. The circle indicates the cap, each arrow indicates the sub-genomic promoter, and the $A)_n$ indicates the poly-A tail. Lower panel: Specific infectivity of the ZsGreen purified stocks. Specific infectivity expressed as number of genomes over infectious particles (genomes/PFU) was determined by plaque assay on Vero cells and by quantification of the number of genomes by RT-qPCR.

Figure S5, related to Figure 1

Figure S5. Sequence conservation of position 80 in E1 glycoprotein within the alphavirus genus. Related to Figure 1. Upper panel: Sequence logo showing the probability of each possible amino acid within the region comprising residues 62 to 99 generated from a sequence alignment of 13 representative members of the alphavirus genus (see materials and methods). Positions comprising the *bc-loop, β-strand c* and the fusion loop are indicated. Residue E1-80 is highlighted in bold. Lower panel: CHIKV IOL and SINV sequence alignment. Position E1-80 and E1-65 are highlighted in bold (* = fully conserved residues, : and . = partially conserved residues).

Figure S6, related to Figure 2

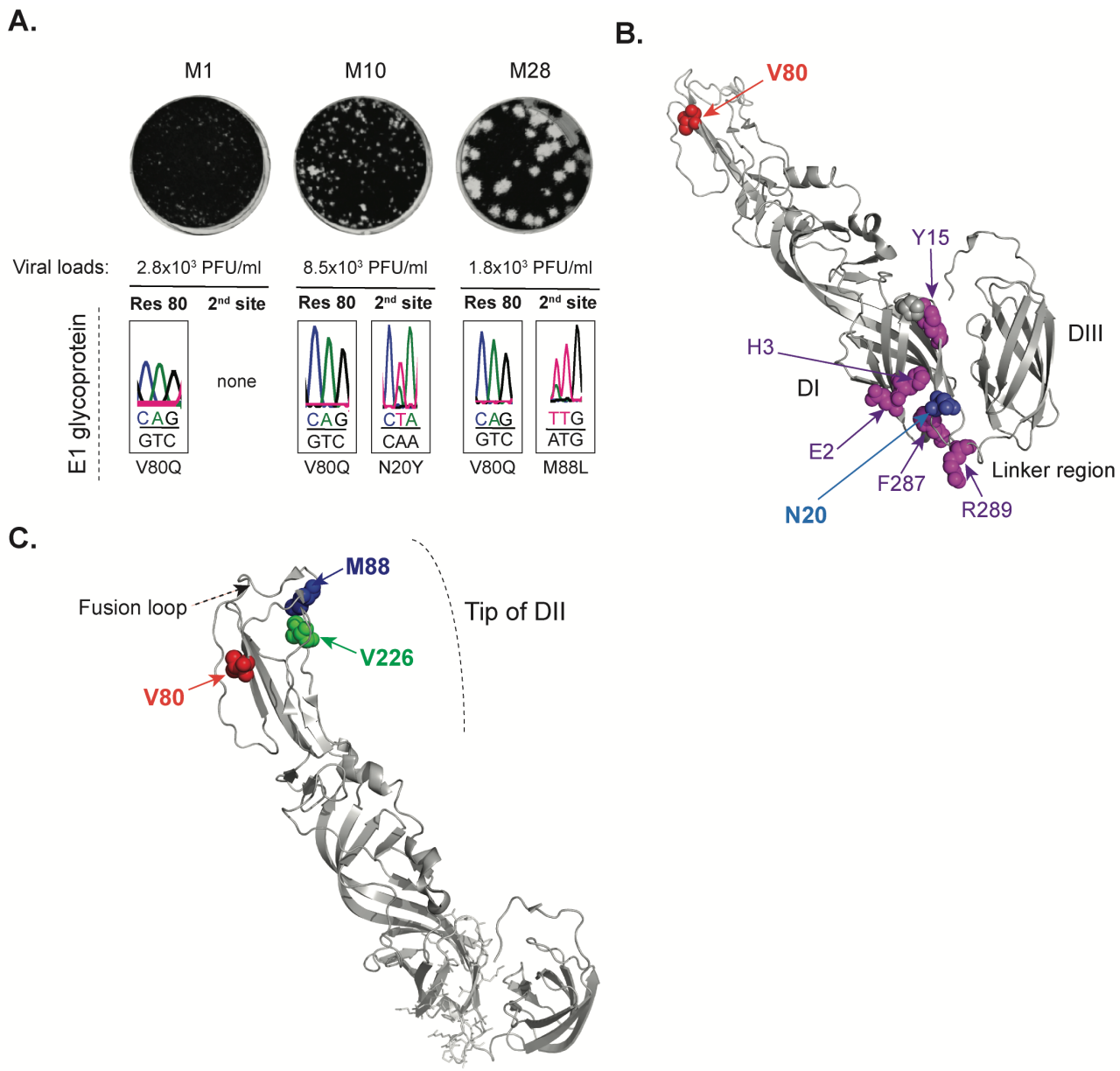

Figure S6. E1-V80Q with second-site mutation and reverted plaque phenotype. Related to Figure 2. (A) Upper panel. Plaque phenotype analysis of viruses isolated from E1-V80Q infected mosquitoes. A representative plaque phenotype of a E1-V80Q infected mosquitoes is depicted in M1. M10 and M28 states for two mutant variants that reverted their plaque phenotype during the course of infection in *Ae. aegypti* mosquitoes. Lower panel. Chromatograms showing the stability at position 80 and the acquisition of second site mutations. Sanger sequence coverage: complete structural polyprotein region (~7.6 to 11.8 Kb). Viral loads of the infected mosquitoes are also

shown. (B) Ribbon representation of E1 glycoprotein (PDBID: 3N42) highlighting position N20 is shown. Residues described in the domain I-domain III (DI-DIII) linker interaction network with the E1 core trimer are depicted in magenta (Zheng et al., 2011). Residue V80 is highlighted in red. (C) Ribbon representation of E1 glycoprotein (PDBID: 3N42) highlighting position 88 is shown. Position 88, 80 and 226 are depicted in blue, red and green, respectively.

Figure S7, related to Figure 3

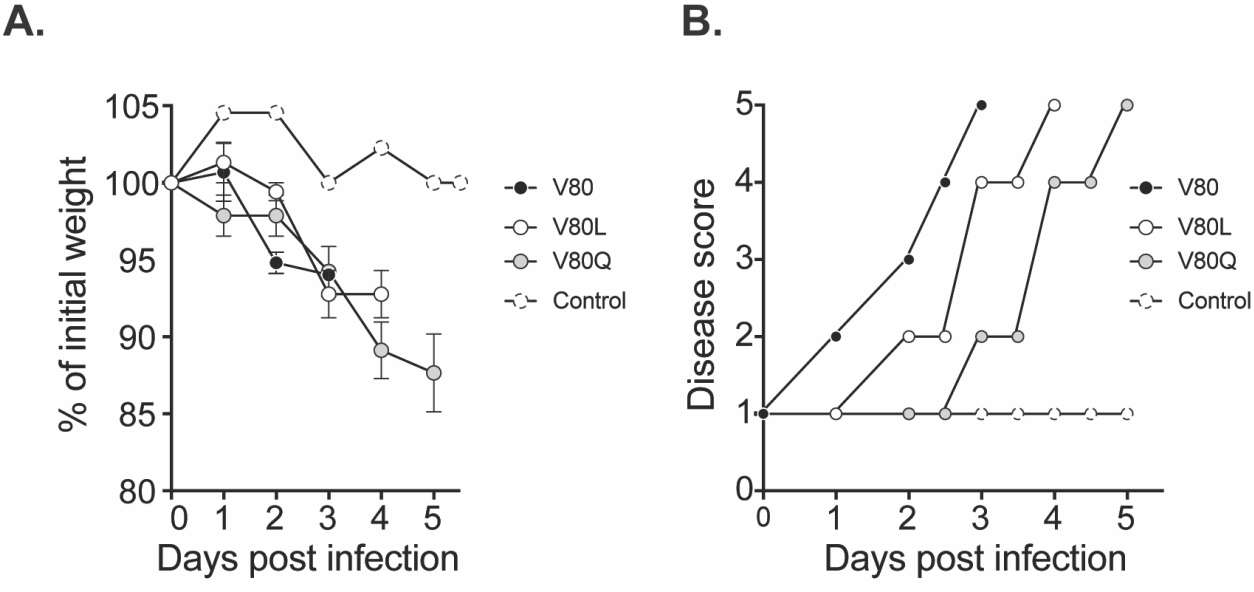

Figure S7. Related to Figure 3. Body weight and disease scores over the course of the infection. (A) Body weight. *Ifnar*$^{-/-}$ mice were inoculated via footpad injection with 1000 PFU of E1-V80, E1-V80L, E1-V80Q and carrier control. Mice were monitored twice a day for signs of infection. Data represent mean and standard deviation (N=4). (B) Disease score. Infected mice were monitored and inspected for signs of infection twice a day. We stablished a 1 to 5 disease score, where: 1 = normal; 2 = Inflammation in the site of inoculation; 3 = Inflammation in the site of inoculation and less dynamic; 4 = Hind limb inflammation, difficult to walk and hunched back; 5 = dead or moribund.

Table S1, related to STAR Methods

Primer #	Name	Sequence
1	CHIKV_E1 V80R Forward	5'-CTGACTACAGCTGTAAG**CG**CTTCACCGGCGTCTAC-3'
2	CHIKV_E1 V80R Reverse	5'-GTAGACGCCGGTGAAG**CG**CTTACAGCTGTAGTCAG-3'
3	CHIKV_E1 V80H Forward	5'-CTGACTACAGCTGTAAG**CA**CTTCACCGGCGTCTAC-3'
4	CHIKV_E1 V80H Reverse	5'-GTAGACGCCGGTGAAG**TG**CTTACAGCTGTAGTCAG-3'
5	CHIKV_E1 V80K Forward	5'-CCTGACTACAGCTGTAAG**AAG**TTCACCGGCGTCTACCC-3'
6	CHIKV_E1 V80K Reverse	5'-GGGTAGACGCCGGTGAA**CTT**CTTACAGCTGTAGTCAGG-3'
7	CHIKV_E1 V80D Forward	5'-GACTACAGCTGTAAGG**AT**TTCACCGGCGTCTACCC-3'
8	CHIKV_E1 V80D Reverse	5'-GGGTAGACGCCGGTGAA**AT**CCTTACAGCTGTAGTC-3'
9	CHIKV_E1 V80E Forward	5'-GACTACAGCTGTAAGG**AG**TTCACCGGCGTCTACCC-3'
10	CHIKV_E1 V80E Reverse	5'-GGGTAGACGCCGGTGAA**CT**CCTTACAGCTGTAGTC-3'
11	CHIKV_E1 V80S Forward	5'-CTGACTACAGCTGTAAG**TC**CTTCACCGGCGTCTAC-3'
12	CHIKV_E1 V80S Reverse	5'-GTAGACGCCGGTGAAG**GA**CTTACAGCTGTAGTCAG-3'
13	CHIKV_E1 V80T Forward	5'-CTGACTACAGCTGTAAG**AC**CTTCACCGGCGTCTAC-3'
14	CHIKV_E1 V80T Reverse	5'-GTAGACGCCGGTGAAG**GT**CTTACAGCTGTAGTCAG-3'
15	CHIKV_E1 V80N Forward	5'-CCTGACTACAGCTGTAAG**AA**CTTCACCGGCGTCTACCC-3'
16	CHIKV_E1 V80N Reverse	5'-GGGTAGACGCCGGTGAAG**TT**CTTACAGCTGTAGTCAGG-3'
17	CHIKV_E1 V80Q Forward	5'-CCTGACTACAGCTGTAAG**CAG**TTCACCGGCGTCTACCC-3'
18	CHIKV_E1 V80Q Reverse	5'-GGGTAGACGCCGGTGAA**CTG**CTTACAGCTGTAGTCAGG-3'
19	CHIKV_E1 V80C Forward	5'-CTGACTACAGCTGTAAG**TG**CTTCACCGGCGTCTAC-3'
20	CHIKV_E1 V80C Reverse	5'-GTAGACGCCGGTGAAG**CA**CTTACAGCTGTAGTCAG-3'
21	CHIKV_E1 V80G Forward	5'-GACTACAGCTGTAAGG**GA**TTCACCGGCGTCTACCC-3'
22	CHIKV_E1 V80G Reverse	5'-GGGTAGACGCCGGTGAA**TC**CCTTACAGCTGTAGTC-3'
23	CHIKV_E1 V80P Forward	5'-CTGACTACAGCTGTAAG**CC**CTTCACCGGCGTCTAC-3'
24	CHIKV_E1 V80P Reverse	5'-GTAGACGCCGGTGAAG**GG**CTTACAGCTGTAGTCAG-3'
25	CHIKV_E1 V80A Forward	5'-GACTACAGCTGTAAGG**CG**TTCACCGGCGTCTACCC-3'
26	CHIKV_E1 V80A Reverse	5'-GGGTAGACGCCGGTGAA**CG**CCTTACAGCTGTAGTC-3'
27	CHIKV_E1 V80L Forward	5'-CTGACTACAGCTGTAAG**C**TCTTCACCGGCGTCTAC-3'
28	CHIKV_E1 V80L Reverse	5'-GTAGACGCCGGTGAAGA**G**CTTACAGCTGTAGTCAG-3'
29	CHIKV_E1 V80M Forward	5'-CCTGACTACAGCTGTAAG**ATG**TTCACCGGCGTCTACCC-3'
30	CHIKV_E1 V80M Reverse	5'-GGGTAGACGCCGGTGAA**CAT**CTTACAGCTGTAGTCAGG-3'
31	CHIKV_E1 V80F Forward	5'-CTGACTACAGCTGTAAG**T**TCTTCACCGGCGTCTAC-3'
32	CHIKV_E1 V80F Reverse	5'-GTAGACGCCGGTGAAGA**A**CTTACAGCTGTAGTCAG-3'
33	CHIKV_E1 V80Y Forward	5'-CCTGACTACAGCTGTAAG**TA**CTTCACCGGCGTCTACC-3'
34	CHIKV_E1 V80Y Reverse	5'-GGGTAGACGCCGGTGAAG**TA**CTTACAGCTGTAGTCAGG-3'
35	CHIKV_E1 V80W Forward	5'-CCTGACTACAGCTGTAAG**TGG**TTCACCGGCGTCTACCC-3'
36	CHIKV_E1 V80W Reverse	5'-GGGTAGACGCCGGTGAA**CCA**CTTACAGCTGTAGTCAGG-3'
37	CHIKV_E1 V80I Forward	5'-CCTGACTACAGCTGTAAG**A**TCTTCACCGGCGTCTACCC-3'
38	CHIKV_E1 V80I Reverse	5'-GGGTAGACGCCGGTGAAGA**T**CTTACAGCTGTAGTCAGG-3'
39	CHIKV_E1 V226A Forward	5'-CTGCAGAGACCGGCTG**C**GGGTACGGTACACGTG-3'
40	CHIKV_E1 V226A Reverse	5'-CACGTGTACCGTACCC**G**CAGCCGGTCTCTGCAG-3'
41	SINV_E1(V80L) Forward	5'-CAGACTATACCTGCAAG**C**TCTTCGGAGGGGTCTACC-3'

42	SINV_E1(V80L) Reverse	5’-GGTAGACCCCTCCGAAGA**G**CTTGCAGGTATAGTCTG-3’

Table S1. Primers used for site directed mutagenesis. Related to STAR Methods.