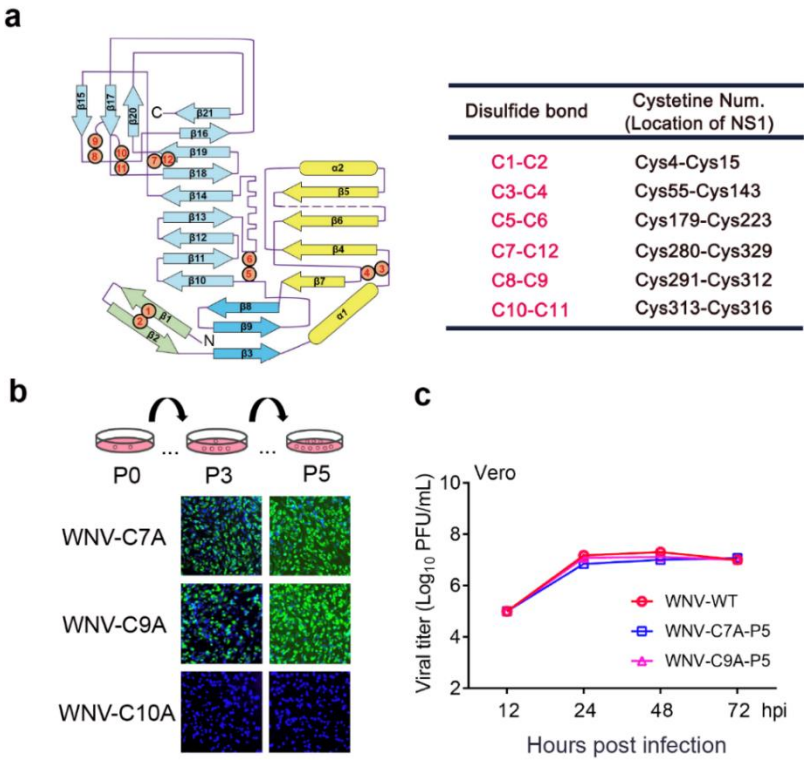
Supplementary Information

Flavivirus Attenuation via Targeting the Specific Disulfide Bond of NS1 Protein to Promote Its Degradation

Table of Contents

Supplementary Figure 1.



Disulfide bond	Cystetine Num. (Location of NS1)
C1-C2	Cys4-Cys15
C3-C4	Cys55-Cys143
C5-C6	Cys179-Cys223
C7-C12	Cys280-Cys329
C8-C9	Cys291-Cys312
C10-C11	Cys313-Cys316

Fig. S1 Serial passaging of the rescued viruses and validation of reverse mutations

a, Topology diagram for NS1 monomer, the corresponding cysteines positions and their pairing for disulfide bonds were shown.

b, Supernatants from rescued WNV-C7A, -C9A and -C10A viruses were serially passaged in Vero cells for 5 rounds. IFA detection of the E protein using 4G2 antibody at different passages.

c, Vero cells were infected with either WNV-WT, WNV-C7A-P5 or WNV-C9A-P5 at an MOI of 5. The supernatants were harvested at the indicated time points, and viral titers were determined by plaque assay.

Supplementary Figure 2.

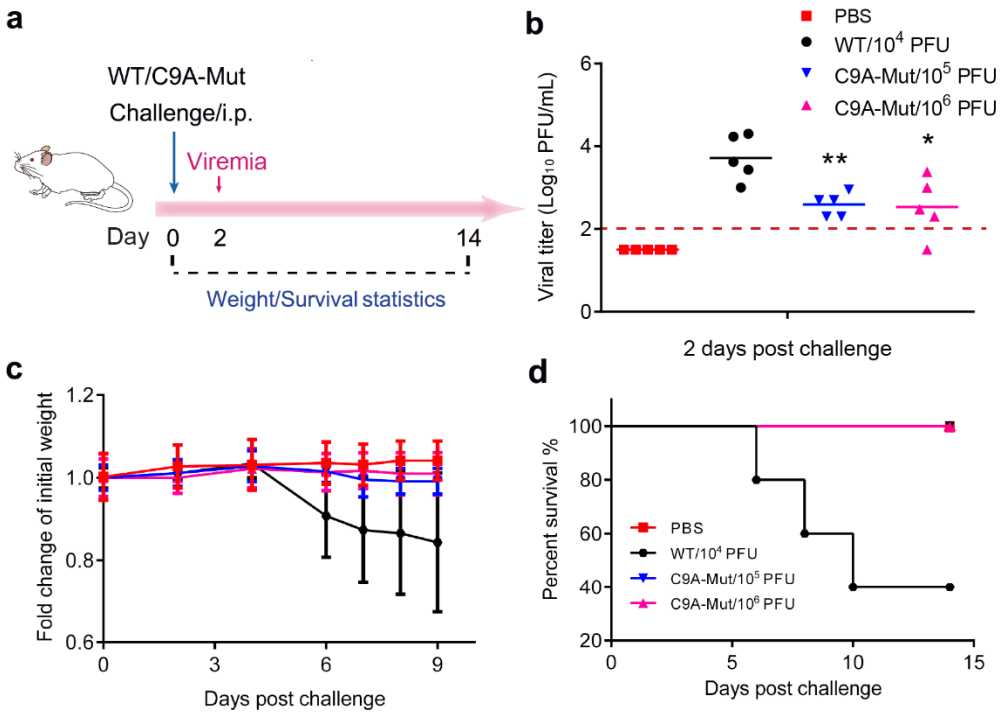


Fig. S2 WNV-C9A-Mut was highly attenuated in BALB/c mice.
a, Groups of 4-week-old BALB/c mice (n=5 per group) were challenged i.p. with PBS, 10^4 PFU of WT WNV, 10^5/10^6 PFU of WNV-C9A-Mut.
b, Viral titers in mice serum at 2 dpi were detected via plaque assay.
c, d, Body weights and survival rates of these mice were monitored after infection.

Supplementary Figure 3.

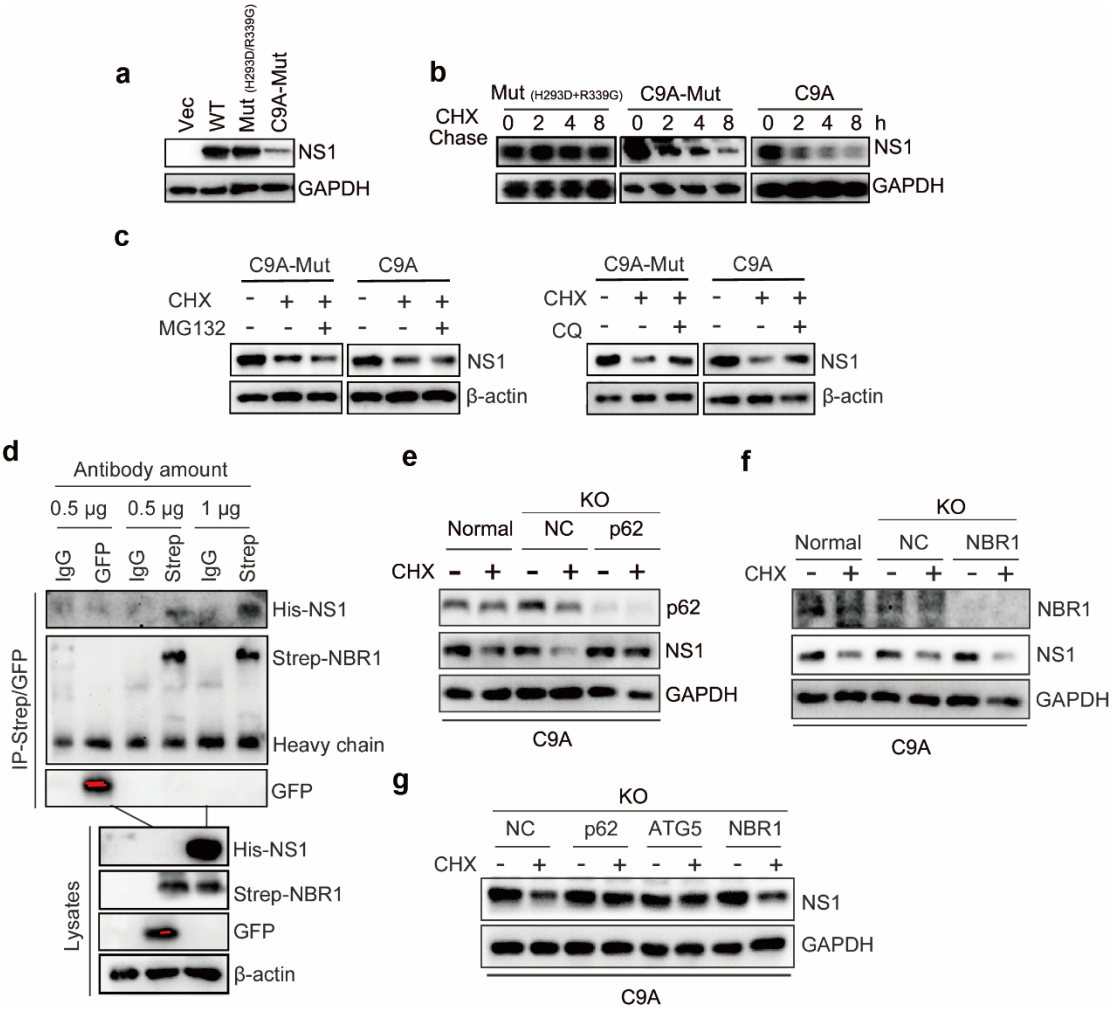


Fig. S3 NS1-C9A or WNV-C9A-Mut did not cause higher levels of autophagy than WT. NBR1 was not the cargo receptor of mediating NS1-C9A selective autophagy.

a, HEK-293T cells were transfected with plasmids expressing HA-tagged NS1-WT, NS1-Mut (H293D/R339G) and NS1-C9A-Mmut for 24 h, then the protein levels were detected by immunoblotting.

b, HEK-293T cells were transfected with HA-tagged NS1-Mut (H293D/R339G), NS1-C9A-Mut and NS1-C9A for 24 h, then the cells were treated with CHX (40 μg/ml) and harvested at the time points as indicated. The protein levels were detected by immunoblotting.

c, HEK-293T cells were transfected with NS1-C9A-Mut or NS1-C9A for 24 h and then treated by CHX (40 μg/ml) along with MG132 (80 μM) or chloroquine (CQ) (40 μM) for 8 h. The cell lysates were then analyzed by immunoblotting analysis.

d, HEK-293T cells were co-transfected with His-NS1-C9A or GFP and strep-tagged NBR1 for 24 h, followed by immunoprecipitation with protein A/G beads. The WCL and IP precipitates were analyzed by immunoblotting with antibodies indicated.

e, His-NS1-C9A protein was transfected into Normal, NC or p62 KO HEK-293T cells for 24 h, followed by CHX (40 μg/ml) treatment for 8 h. Cell lysates were then immunoblotted with indicated antibodies.
f, His-NS1-C9A protein was transfected into Normal, NC or NBR1 KO HEK-293T cells for 24 h, followed by CHX (40 μg/ml) treatment for 8 h. The cell lysates were immunoblotted with indicated antibodies.
g, NC, p62, ATG5 or NBR1 KO HEK-293T cells were transfected with His-tagged NS1-C9A for 24 h. The cell lysates were then analyzed by immunoblotting analysis.