# LETTER

# Evolutionary enhancement of Zika virus infectivity in *Aedes aegypti* mosquitoes

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Zika virus (ZIKV) remained obscure until the recent explosive outbreaks in French Polynesia (2013–2014) and South America (2015–2016)[1–3]. Phylogenetic studies have shown that ZIKV has evolved into African and Asian lineages. The Asian lineage of ZIKV was responsible for the recent epidemics in the Americas[1,3]. However, the underlying mechanisms through which ZIKV rapidly and explosively spread from Asia to the Americas are unclear. Non-structural protein 1 (NS1) facilitates flavivirus acquisition by mosquitoes from an infected mammalian host and subsequently enhances viral prevalence in mosquitoes[4]. Here we show that NS1 antigenaemia determines ZIKV infectivity in its mosquito vector *Aedes aegypti*, which acquires ZIKV via a blood meal. Clinical isolates from the most recent outbreak in the Americas were much more infectious in mosquitoes than the FSS13025 strain, which was isolated in Cambodia in 2010. Further analyses showed that these epidemic strains have higher NS1 antigenaemia than the FSS13025 strain because of an alanine-to-valine amino acid substitution at residue 188 in NS1. ZIKV infectivity was enhanced by this amino acid substitution in the ZIKV FSS13025 strain in mosquitoes that acquired ZIKV from a viraemic C57BL/6 mouse deficient in type I and II interferon (IFN) receptors (AG6 mouse). Our results reveal that ZIKV evolved to acquire a spontaneous mutation in its NS1 protein, resulting in increased NS1 antigenaemia. Enhancement of NS1 antigenaemia in infected hosts promotes ZIKV infectivity and prevalence in mosquitoes, which could have facilitated transmission during recent ZIKV epidemics.

Zika virus (ZIKV), a mosquito-borne virus belonging to the genus flavivirus of the family *Flaviviridae*, is transmitted to humans by *Aedes* mosquito species[1,5]. Several neurological complications, such as Guillain–Barré syndrome in adults[6] and microcephaly in newborn babies[7], have been associated with ZIKV infection. Before 2007, cases of ZIKV infection were detected only sporadically[1,8]. The first reported mild ZIKV outbreak occurred in 2007 on Yap Island in Western Pacific Micronesia[9]. In 2013, a larger ZIKV epidemic was recorded in French Polynesia and other Southern Pacific islands[2,10]. In 2015, ZIKV emerged in the Americas for the first time, rapidly spreading to 20 countries or territories[3]. Phylogenetic studies have revealed that ZIKV has evolved into African and Asian clusters[1,3]. Sequence analyses have shown that the ZIKV strains that caused the earlier outbreaks in Micronesia (2007) and French Polynesia (2013–2014) and later outbreaks in the Americas (2015–2016) all belong to the Asian clade[1], suggesting that increased infectivity of the Asian ZIKV lineage might have contributed to the recent epidemic.

Evolutionary increases in the infectivity of mosquito-borne viruses within their vectors results in high epidemic potential[11,12]. We speculate that the adaptability and infectivity of ZIKV within its mosquito vectors
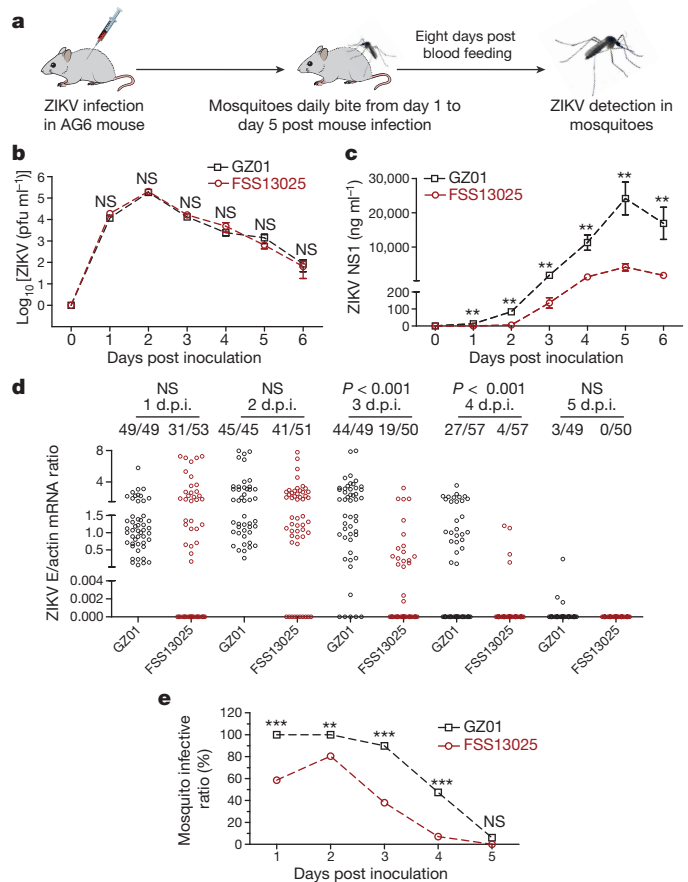


**Figure 1 | Comparing the infectivity of ZIKV isolates of Asian lineage in mosquitoes. a**, Schematic representation of the study design. Separated groups of AG6 mice were intradermally infected with $1 \times 10^4$ p.f.u. of the GZ01 and FSS13025 strains. **b**, Detection of the ZIKV load in the blood plasma by plaque assay ($n = 14$ mice per group pooled from 5 independent biological replicates). **c**, ZIKV NS1 measurement by enzyme-linked immunosorbent assay (ELISA; $n = 6$ mice per group pooled from 3 independent biological replicates). **d, e**, Comparison of the infectivity of two ZIKV isolates in *A. aegypti* ($n = 6$ mice per group pooled from 3 independent biological replicates). The number at the top of each column represents infected number/total number. Each dot represents a mosquito (**d**). The data are represented as the percentage of mosquito infections (**e**). d.p.i., days post inoculation. Data are mean ± s.e.m. (**b, c**). $P$ values were determined by two-tailed Mann–Whitney test (**b–d**) or two-sided Fisher's exact test (**e**). **$P < 0.01$, ***$P < 0.001$, NS, not significant.

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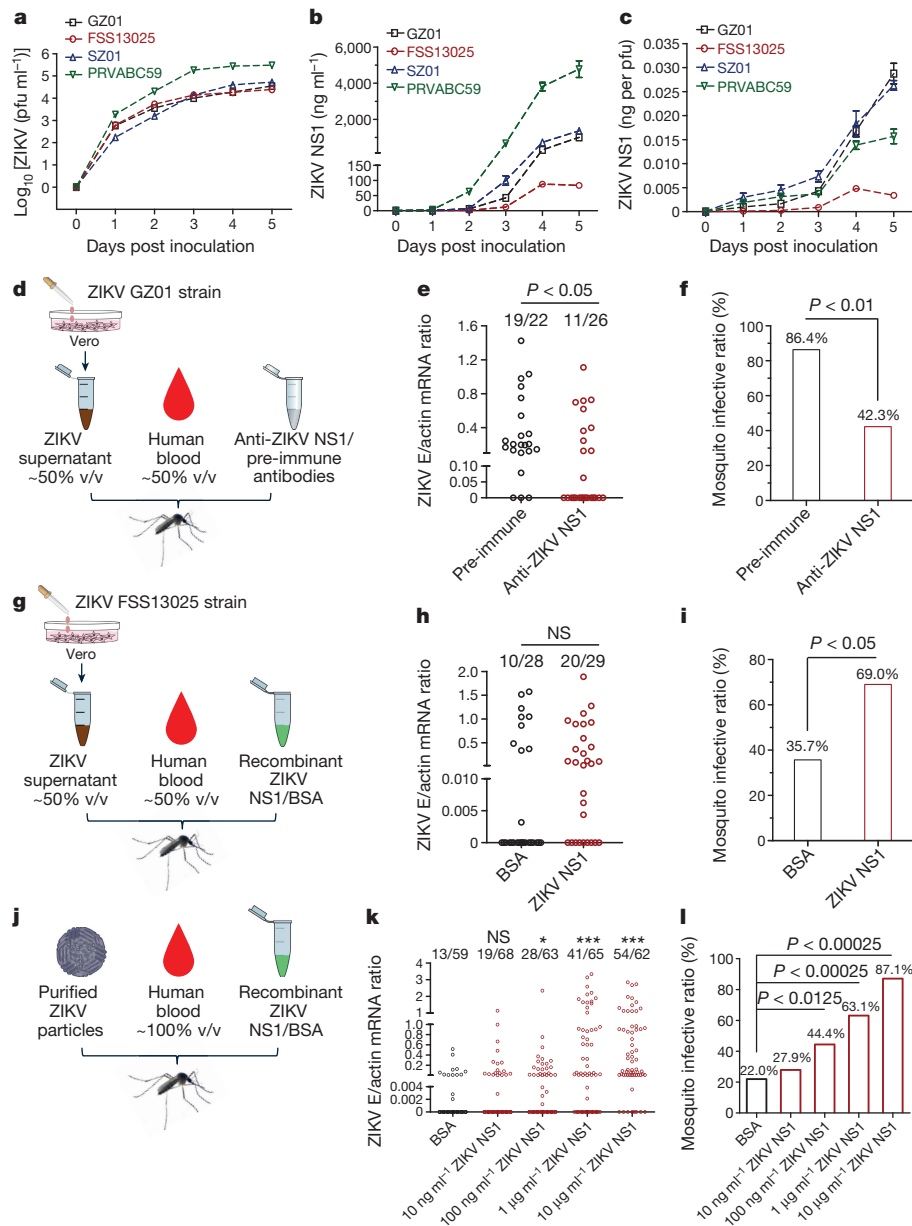*These authors contributed equally to this work.

**Figure 2 | Differences in NS1 secretion determine ZIKV infectivity in mosquitoes. a–c**, NS1 secretability in ZIKV-infected Vero cells. Vero cells were infected with 0.01 multiplicity of infection (MOI) viruses. **c**, NS1 quantity normalized against average viral titre (ng per p.f.u.). The data were pooled from three independent biological replicates. **d–f**, Immunoblockade of NS1 in GZ01-infected supernatants reduced ZIKV acquisition. Either 10 μl of mouse ZIKV NS1 antisera or 10 μl pre-immune sera was mixed with supernatant from ZIKV GZ01-infected Vero cells (500 μl) and fresh human blood (500 μl) for *in vitro* membrane feeding of *A. aegypti*. The data were pooled from two independent biological replicates. **g–i**, Addition of NS1 to FSS13025-infected supernatant increased mosquito infection. Either 10 μg purified NS1 or 10 μg BSA was incubated with fresh human blood (500 μl) and supernatant from ZIKV FSS13025-infected Vero cells (500 μl) to feed

*A. aegypti*. The data were pooled from two independent biological replicates. **j–l**, Assessment of the threshold NS1 concentration that enhances ZIKV acquisition. Serial dilutions of purified ZIKV NS1 (10 μl) were mixed with purified GZ01 ZIKV virions (10 μl) and fresh human blood (1,000 μl) for *A. aegypti* feeding. The data were pooled from four independent biological replicates. A final concentration of $1 \times 10^5$ p.f.u. ml$^{-1}$ ZIKV was used for mosquito oral infection (**d, g, j**). The number at the top of each column represents infected number/total number. Each dot represents a mosquito (**e, h, k**). Data are mean ± s.e.m. (**a–c**). *P* values were determined by two-tailed Mann–Whitney test (**e, h, k**) or two-sided Fisher's exact test (**f, i, l**), and adjusted using Bonferroni correction to account for multiple comparisons (**k, l**). The *P* value represents a comparison between BSA and other groups (**k, l**). *$P < 0.0125$, ***$P < 0.00025$, NS, not significant.

might have evolved over time, contributing to the prevalence and spread of the virus from Asia to the Americas. To test this hypothesis, we compared the viral infectivity of two clinical ZIKV isolates of the Asian lineage, GZ01 (isolated in 2016[13]) and FSS13025 (isolated in 2010[14,15]), in the primary urban mosquito vector *A. aegypti*. Phylogenetic analysis showed that these two strains represent the clades corresponding to the epidemic strains of ZIKV that are prevalent in the Americas (2015–2016) and Southeastern Asia (2007–2012) (Extended Data Fig. 1).

Thus, we exploited a 'host–mosquito' acquisition model, in which mosquitoes acquire ZIKV from a viraemic AG6 mouse[4,16,17], to assess the infectivity of these ZIKV strains (Fig. 1a). Both strains exhibited similar replication kinetics in the blood of infected AG6 mice (Fig. 1b). However, the serum level of secreted NS1 was much lower in mice infected with the FSS13025 strain than the GZ01 strain (Fig. 1c). The infected mice were subjected to daily biting by the Rockefeller laboratory strain of *A. aegypti* from days 1 to 5 after mouse infection

(Fig. 1a). Infection prevalence was substantially lower in mosquitoes that fed on FSS13025-infected mice than in those that fed on GZ01-infected mice (Fig. 1d, e).

Secretion of NS1 into the serum of an infected host is a common property of many flaviviruses[4,18–21]. Our previous study demonstrated that circulating NS1 acquired by mosquitoes together with virions when mosquitoes feed on a viraemic mammalian host enhances viral infectivity[4]. NS1 antigenaemia plays a key role in maintaining the flavivirus life cycle[4]. We hypothesize that NS1 antigenaemia is responsible for the difference in mosquito infectivity between the GZ01 and FSS13025 strains. We therefore infected Vero cells (a monkey kidney cell line) with the American isolates GZ01 and PRVABC59[22], the SZ01 strain representing the French Polynesian clade[23], and the FSS13025 strain. The three post-epidemic strains showed higher NS1 secretion than the FSS13025 strain (Fig. 2a–c), even when NS1 amount was normalized to viral titre (Fig. 2c).

To determine whether NS1 secretability correlates with ZIKV infectivity in mosquitoes, we generated a mouse NS1 polyclonal antibody (Extended Data Fig. 2a). Neutralization of NS1 by antibody treatment reduced the mosquito infection prevalence of the GZ01 strain (Fig. 2d–f). The mouse NS1 antibodies did not neutralize ZIKV virions (Extended Data Fig. 3). We also tested whether the addition of NS1 protein could enhance ZIKV infectivity. A recombinant GZ01 NS1 protein was expressed and purified using a *Drosophila* S2 expression system (Extended Data Fig. 2b). The presence of NS1 increased the infectivity of the FSS13025 strain in mosquitoes (Fig. 2g–i). We next assessed the threshold concentration of NS1 that can effectively enhance ZIKV acquisition. Compared to bovine serum albumin (BSA), the presence of 100 ng ml$^{-1}$ or higher concentrations of NS1 substantially increased ZIKV prevalence in *A. aegypti* (Fig. 2j–l). To identify the role of animal NS1 antigenaemia in ZIKV infectivity in mosquitoes, we allowed mosquitoes to feed on viraemic AG6 mice (Extended Data Fig. 4a). The NS1 antibody treatment reduced the quantity of circulating NS1 in mouse serum (Extended Data Fig. 4b) but did not influence GZ01 ZIKV replication in AG6 mice (Extended Data Fig. 4c). The infection ratios of fed *A. aegypti* were reduced as a result of the antibody-mediated neutralization of NS1 in mice (Extended Data Fig. 4d, e).

Considering the difference in secretion of NS1 between the GZ01 and FSS13025 strains (Figs 1c, 2a–c), we next attempted to identify the amino acids that are critical for NS1 secretion. We focused on the endoplasmic reticulum transmembrane region of pre-membrane (prM), the full length of envelope (E), and NS1 (prM-E-NS1)[24] (Fig. 3a). There are only two sites that vary between the GZ01 and FSS13025 prM-E-NS1 proteins: residue 473 in the E protein (Val/FSS13025 to Met/GZ01) and residue 188 in the NS1 protein (Ala/FSS13025 to Val/GZ01) (Fig. 3a). We therefore individually subcloned the *prM-E-NS1* regions from the GZ01 and FSS13025 strains and subsequently transfected these segments into 293T cells. Ectopic expression of the GZ01 prM-E-NS1 protein, but not the FSS13025 protein, led to abundant NS1 secretion in the supernatants of transfected cells (Fig. 3b). Mutation from valine to methionine at residue 473 of the E protein in the FSS13025 prM-E-NS1 protein did not improve NS1 secretion; however, the alanine-to-valine substitution at residue 188 of NS1 rendered NS1 secretable (Fig. 3b), demonstrating that this single mutation determines NS1 secretability. Residue 188 is located within the interface between two NS1 monomers[25] (Extended Data Fig. 5a). Nonetheless, the alanine-to-valine substitution at position 188 does not influence NS1 dimerization (Extended Data Fig. 5b), suggesting that the mechanism by which the single mutation affects NS1 remains to be identified.

We next performed an alignment of the NS1 proteins from the Asian clade of ZIKV. In ZIKV isolates collected before 2012, residue 188 of the NS1 protein was alanine. However, this residue was mutated to valine in all isolates collected after 2013 (Extended Data Fig. 6), suggesting that the Asian lineage of ZIKV evolved to acquire NS1 secretability when it spread from southeastern Asia to the Southern Pacific islands around 2013. Correspondingly, Singapore reported local ZIKV transmission in
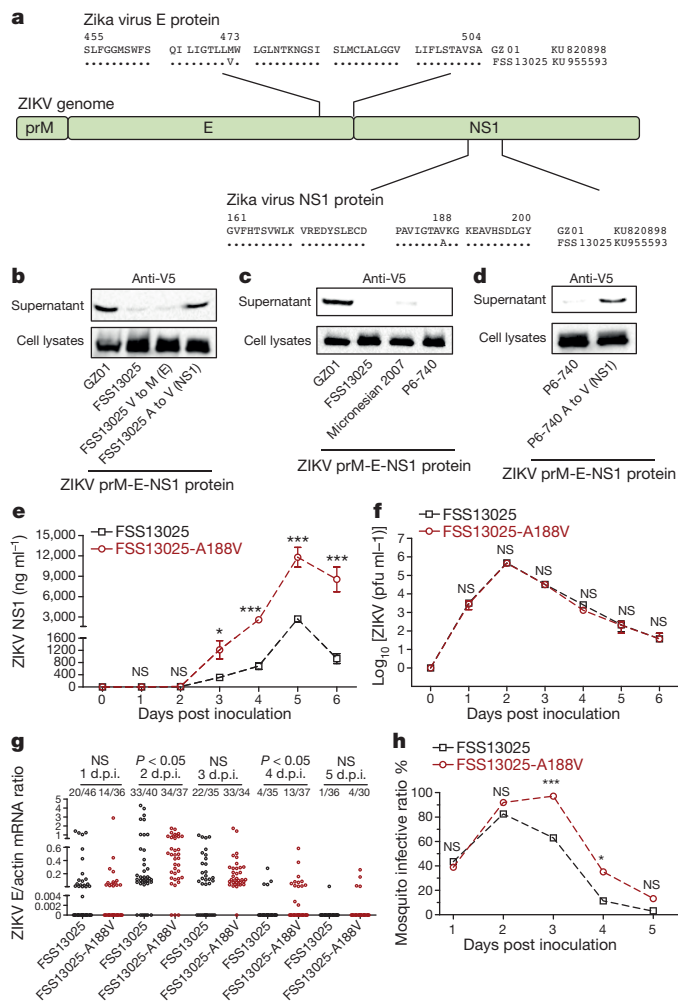


**Figure 3 | A single amino acid residue in NS1 determines its secretability in the Asian lineage of ZIKV. a**, Comparison of the *prM-E-NS1* sequences from GZ01 and FSS13025 isolates. **b**, Detection of NS1 secretion in ectopically expressed prM-E-NS1 proteins from the subcloned and mutated GZ01 and FSS13025 strains. **c**, Detection of NS1 secretion in ectopically expressed prM-E-NS1 proteins from ZIKV Asian strains. **d**, Mutation of alanine to valine in the NS1 protein conferred NS1 secretability on the prM-E-NS1 proteins of a Malaysia P6-740 strain. **b–d**, The experiments were reproduced three times with similar results. For gel source data, see Supplementary Fig. 1. **e**, Measurement of ZIKV NS1 antigenaemia by ELISA ($n = 10$ mice per group pooled from 5 independent biological replicates). **f**, Detection of the ZIKV load in the mouse blood by plaque assay ($n = 8$ mice per group pooled from 4 independent biological replicates). **g**, **h**, Comparison of mosquito infectivity (**g**) and infective ratio (**h**) of ZIKV strains ($n = 6$ mice per group pooled from 3 independent biological replicates). The number at the top of each column represents infected number/total number. Each dot represents a mosquito (**g**). AG6 mice were intradermally inoculated with $1 \times 10^4$ p.f.u. of the FSS13025 and FSS13025-A188V strains. Data are mean ± s.e.m. (**e**, **f**). *P* values were determined by two-tailed Mann–Whitney test (**e–g**) or two-sided Fisher's exact test (**h**). *$P < 0.05$, ***$P < 0.001$, NS, not significant.

August 2016. The Singaporean strains stemmed from an ancestral node of the Asian lineage[26]. In all ZIKV isolates from Singapore, residue 188 of the NS1 protein was a valine (Extended Data Fig. 6), suggesting that the alanine-to-valine substitution may have contributed to the recent ZIKV epidemics. Therefore, we synthesized the *prM-E-NS1* genes from a Malaysia P6-740 strain isolated in 1966 and a Micronesian 2007 strain and ectopically expressed these genes in 293T cells. Compared to the secretion of NS1 in the supernatants of GZ01 *prM-E-NS1*-transfected cells, the NS1 protein was hardly detectable in the supernatants of these
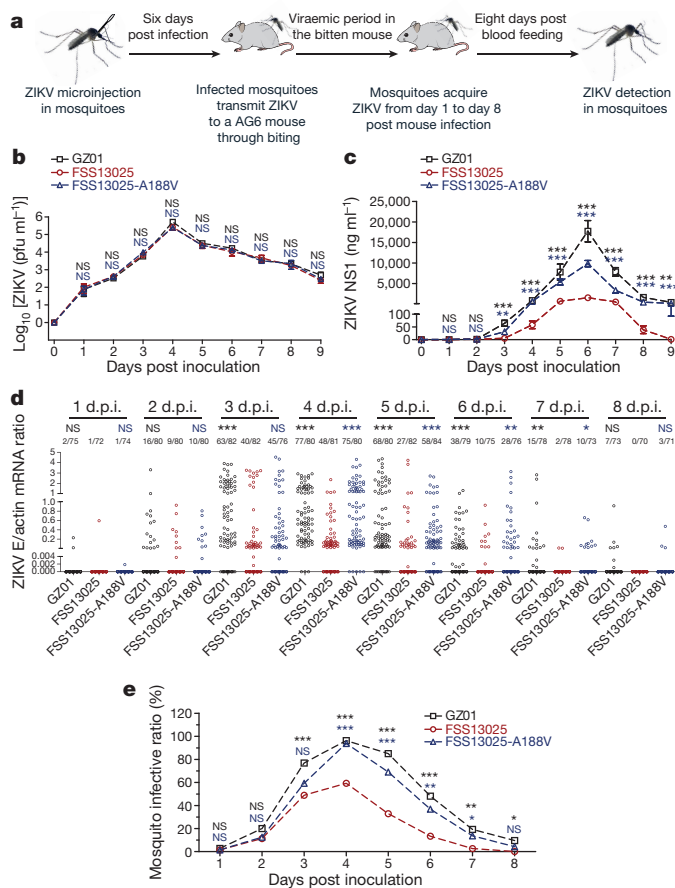
**Figure 4 | A single amino acid substitution influences the prevalence of mosquito infection during the mosquito–host–mosquito life cycle. a**, Schematic representation of the study design. **b**, Detection of ZIKV viraemia by plaque assay ($n = 12$ mice per group pooled from 4 independent biological replicates). **c**, Measurements of ZIKV NS1 antigenaemia by ELISA ($n = 12$ mice per group pooled from 4 independent biological replicates). **d**, **e**, Comparison of mosquito infectivity (**d**) and infective ratio (**e**) of ZIKV strains ($n = 9$ mice per group pooled from 3 independent biological replicates). The number at the top of each column represents infected number/total number. Each dot represents a mosquito (**d**). Data are mean ± s.e.m. (**b**, **c**). $P$ values were determined by two-tailed Mann–Whitney test (**b–d**) or two-sided Fisher's exact test (**e**), and adjusted using Bonferroni correction to account for multiple comparisons (**b–e**). The black $P$ value represents a comparison between GZ01 and FSS13025. The blue $P$ value represents a comparison between FSS13025-A188V and FSS13025. *$P < 0.025$, **$P < 0.005$, ***$P < 0.0005$, NS, not significant.

pre-epidemic ZIKV *prM-E-NS1*-expressing cells (Fig. 3c). However, the alanine-to-valine mutation in the NS1 protein of the P6-740 strain conferred NS1 secretability (Fig. 3d).

Next, we created the alanine-to-valine mutation at residue 188 of the NS1 protein in an infectious clone of the FSS13025 strain (FSS13025-A188V)[15]. The substitution substantially increased NS1 secretion in the supernatant of infected Vero cells (Extended Data Fig. 7a–c). The FSS13025-A188V strain showed higher ZIKV infectivity than the wild-type virus in mosquitoes (Extended Data Fig. 7d, e). In the mouse–mosquito acquisition model, NS1 antigenaemia was increased in the serum of FSS13025-A188V ZIKV-infected mice (Fig. 3e). Mutation of residue 188 of NS1 did not influence viral infectivity or dissemination kinetics in infected AG6 mouse blood (Fig. 3f). However, the substitution enhanced ZIKV transmission from mice to mosquitoes (Fig. 3g, h). Moreover, neutralization of NS1 with mouse ZIKV NS1 antibodies effectively reduced the prevalence of FSS13025-A188V ZIKV in mosquitoes (Extended Data Fig. 8a–d), confirming that the valine at residue

188 of NS1 has a critical role in antigenaemia, which is essential for ZIKV acquisition by mosquitoes.

We next investigated whether NS1-mediated enhancement of ZIKV infectivity influenced the prevalence of ZIKV-carrying mosquitoes using a mosquito–mouse–mosquito transmission cycle. We thoracically inoculated equal titres (5 plaque-forming units (p.f.u.)) of the GZ01, FSS13025 and FSS13025-A188V mutant strains into female *A. aegypti* mosquitoes. Six days after virus inoculation, two infected mosquitoes were allowed to feed on AG6 mice (Fig. 4a). The mosquito-bitten animals developed high-titre viraemia for all three ZIKV strains (Fig. 4b). Replication of both the GZ01 and FSS13025-A188V ZIKV strains, but not the FSS13025 strain, resulted in efficient NS1 antigenaemia (Fig. 4c). Subsequently, the infected mice were subjected to daily biting by naive *A. aegypti* from days 1 to 8 after mouse infection (Fig. 4a). Through a complete transmission cycle, the FSS13025-A188V strain showed a substantially higher prevalence of mosquito infection than did the FSS13025 strain (Fig. 4d, e), demonstrating that the valine at residue 188 is required for the generation of NS1 antigenaemia, which enhances ZIKV transmissibility from hosts to vectors.

ZIKV independently separated into the African and Asian lineages. We found that residue 188 in all ZIKV African strains was valine (Extended Data Fig. 9a). The Uganda MR766-infected Vero cells abundantly secreted NS1 (Extended Data Fig. 9b–d), implying that infection with African ZIKV might produce high NS1 antigenaemia. ZIKV has two ecologically distinct transmission cycles: a sylvatic cycle (mosquitoes to non-human primates) and a human cycle (mosquitoes to humans)[27]. Knowledge of ZIKV before 2012 was almost exclusively centred on the enzootic sylvatic cycle in Africa[27,28]. A mosquito survey in Africa showed that ZIKV was detected in mosquitoes collected in multiple surveyed landscapes, except for indoor locations within villages[28]. Therefore, we hypothesize that the presence of a valine at residue 188 of NS1 might bestow on the African lineage of ZIKV a highly efficient enzootic or epizootic transmission cycle[27,28]. Other unknown factor(s) might prevent African ZIKV from efficiently establishing a human infection cycle. However, the Asian strains may have evolved to target humans with high infectivity; indeed, one such strain caused a medium-scale epidemic in Micronesia in 2007[1,9]. The alanine-to-valine substitution at position 188 further improved ZIKV transmission efficiency from humans to mosquitoes and thus increased its prevalence in mosquitoes. Our data offer a potential explanation for the recent re-emergence of ZIKV, and suggest that co-evolution of arboviruses with their hosts or vectors contributes to their long-term existence and occasional widespread re-emergence.

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**Supplementary Information** is available in the online version of the paper.

## METHODS

**Ethics statement.** Human blood for mosquito feeding was taken from healthy donors who provided written informed consent. The collection of human blood samples was approved by the local ethics committee at Tsinghua University.

**Mice, mosquitoes, cells and viruses.** AG6 mice were donated by the Institute Pasteur of Shanghai, Chinese Academy of Sciences. The mice were bred and maintained under a specific pathogen-free animal facility at Tsinghua University. Groups of age- and sex-matched AG6 mice, 6–8 weeks of age, were used for the animal studies[4]. All experiments were approved by and performed under the guidelines of the Experimental Animal Welfare and Ethics Committee of Tsinghua University. *A. aegypti* (Rockefeller strain) were reared in a low-temperature, illuminated incubator (Model 818, Thermo Electron Corporation) at 28 °C and 80% humidity according to standard rearing procedures[29]. The ZIKV GZ01 (*KU820898*), PRVABC59 (*KU501215*), SZ01 (*KU866423*), MR766 (*NC_012532*) and FSS13025 (*KU955593*)[15] strains were grown in Vero cells for blood meals[4]. The *Drosophila melanogaster* S2 cell line was cultured in Schneider's medium with 10% heat-inactivated fetal bovine serum and 1% antibiotic–antimycotic (15240-062, Invitrogen). The Vero cells and 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% heat-inactivated fetal bovine serum (16000-044, Gibco). For ZIKV infection, the Vero cells were maintained in VP-SFM medium (11681-020, Gibco). The Vero and 293T cell lines were purchased from the ATCC (CCL81, CRL-3216). The *Drosophila* S2 cell line was sourced from a *Drosophila* expression system of Invitrogen (R690-07). None of these cell lines were found in the database of commonly misidentified cell lines maintained by ICLAC and NCBI Biosample. All these cell lines were authenticated by ATCC or Invitrogen and do not have mycoplasma contamination. ZIKV was titred by a plaque formation (p.f.u.) assay[29].

**Antibodies.** The ZIKV *NS1* genes were amplified from the cDNA of infected cells and cloned into a pET-28a (+) expression vector (69864, Millipore). The cloning primers are presented in Extended Data Table 1. Recombinant NS1 proteins were expressed in the *Escherichia coli* BL21 DE3 strain in an insoluble form in inclusion bodies. The proteins were then solubilized by 8 M urea and purified with a TALON Purification Kit (635515, Clontech). Mouse antisera were produced via inoculation with recombinant ZIKV NS1 with three boosts. Polyclonal antibodies were purified from the immunized antisera using protein A/G agarose (20423, Thermo). The anti-V5-HRP antibody for tag detection was purchased from Invitrogen (R96125, Thermo).

**Expression of prM-E-NS1 protein in human cells.** The *prM-E-NS1* regions (base pairs 820–3,438 in GZ01 *KU820898*; 927–3,545 in FSS13025 *KU955593*) were amplified from cDNA of infected cells. The *prM-E-NS1* genes from the P6-740 strain (base pairs 820–3,438, *HQ234499*) and the Micronesian 2007 strain (base pairs 820–3,438, *EU545988*) were commercially synthesized (Genescript). All the genes were subcloned into a pcDNA3.1/V5-His/TOPO DNA vector (K4800-01, Invitrogen), and subsequently transfected using X-treme Gene HP DNA transfection reagent (06366236001, Roche) for ectopic expression in 293T cells. The amount of NS1 in the supernatants of the 293T cells was measured 48 h after transfection. The cloning primers are shown in Extended Data Table 1.

**ZIKV NS1 protein generation in a *Drosophila* expression system.** The ZIKV *NS1* gene was cloned into a pMT/BiP/V5-His A vector (V4130-20, Invitrogen) for expression in *Drosophila* S2 cells. The cloning primers are shown in Extended Data Table 1. The procedure for ZIKV NS1 expression and purification in a *Drosophila* expression system has been described previously[4,29].

**ZIKV NS1 detection by ELISA.** Secretion of ZIKV NS1 into Vero cell supernatant and mouse serum was measured using a ZIKV NS1 Antigen ELISA Kit (ZIKV-NS1-EK, BioFront MonoTrace). The experiment was performed according to the kit's manual. Samples were diluted to the range of quantification of the kit using the sample dilution buffer provided in the kit. Standard materials for the generation of a standard curve were also provided in the kit. The measured amount of ZIKV NS1 matched the linearity in the assay. Optical density (OD) was measured at 450 nm with an ELISA reader (Varioskan Flash Multimode Reader, Thermo Scientific). The detection limit of NS1 protein by ELISA is 0.1 ng ml$^{-1}$.

**Thoracic inoculation of ZIKV in mosquitoes.** Detailed procedures for microinjection in mosquitoes have been described previously[4,29]. In brief, female *A. aegypti* mosquitoes were anaesthetized on a cold tray, and subsequently a certain titre (5 p.f.u.) of ZIKV was microinjected into the mosquito thoraxes. The infected mosquitoes were used for ZIKV transmission to AG6 mice by a blood meal.

**Membrane blood feeding.** Fresh human blood was placed in heparin-coated tubes (367884, BD Vacutainer) and centrifuged at 1,000*g* and 4 °C for 10 min to separate the plasma from the blood cells. The plasma was collected and heat-inactivated at 55 °C for 60 min. The separated blood cells were washed three times with PBS to remove the anticoagulant. The cells were then resuspended in the heat-inactivated plasma. The donor sera were negative for the E and NS1 proteins of JEV, DENV and ZIKV. Either purified proteins or antibodies were mixed with the ZIKV-infected supernatant and human blood for mosquito oral feeding via a Hemotek system (6W1, Hemotek Limited). Engorged female mosquitoes were transferred into new containers and maintained under standard conditions for an additional 8 days. The mosquitoes were subsequently killed for further analysis.

**Mosquito feeding on infected mice.** Eight to ten female *A. aegypti* mosquitoes were separated into a netting-covered container for blood feeding. The mosquitoes were starved for 24 h before engorgement. ZIKV-infected AG6 mice were anaesthetized and placed on top of the container. The mosquitoes were allowed to feed on the mice for 30 min in the dark. After being anaesthetized using ice, the engorged mosquitoes were transferred to new containers and maintained under standard conditions for an additional 8 days. The mosquitoes were subsequently killed for further analysis.

**Purification of infectious ZIKV virions.** The purification of flaviviral infectious virions has been described previously[4]. In brief, supernatant from GZ01-infected Vero cells was collected 5 days after infection. Cell fragments were removed by centrifugation at 25,000*g* and 4 °C for 20 min. Subsequently, the supernatant was carefully transferred into a clean centrifuge tube and centrifuged at 25,000*g* and 4 °C for an additional 6 h to pellet the virions. The precipitated virions were washed twice and then solubilized in VP-SFM medium (11681-020, Gibco). Insoluble material was removed by an extra centrifugation step at 12,000*g* and 4 °C for 2 min. The virions in the VP-SFM medium were aliquoted and stored at −80 °C.

**Viral genome quantification by TaqMan qPCR.** Total RNA was isolated from homogenized mosquitoes using an RNeasy Mini Kit (74106, Qiagen) and reverse-transcribed into cDNA using an iScript cDNA synthesis kit (170-8890, Bio-Rad). Viral genomes were quantified via TaqMan qPCR amplification of ZIKV genes, normalized against *A. aegypti actin* (*AAEL011197*). The detection limit of ZIKV E/Actin mRNA ratio is 0.001. The primers and probes used for this analysis are shown in Extended Data Table 1.

**Determination of the virus titre in infected mice by plaque assay.** Blood samples were collected from the tail veins of infected mice in 0.4% sodium citrate and were centrifuged for 5 min at 6,000*g* and 4 °C to isolate plasma. The presence of infectious viral particles in the plasma was determined using a plaque assay[4]. The detection limit in the plaque assay is 10 p.f.u. ml$^{-1}$ in cell supernatant, and 100 p.f.u. ml$^{-1}$ in serum.

**RNA transcription, transfection and virus recovery.** The pFLZIKV-FSS13025 and pFLZIKV-FSS13025-A188V[15] plasmids were amplified in *E. coli* Top10 (C404010, Invitrogen) and purified using MaxiPrep PLUS (12965, Qiagen). For *in vitro* transcription, 10 μg plasmid was linearized with the restriction enzyme ClaI. A mMESSAGE mMACHINE T7 kit (AM1344, Ambion) was used for *in vitro* transcription of RNA in a 20 μl reaction with an additional 1 μl of 30 mM GTP solution. The RNA was precipitated with lithium chloride and quantified by spectrophotometry. Subsequently, the RNA was transfected into Vero cells seeded in a T175 flask with DMEM medium and incubated at 37 °C and 5% $CO_2$ using Lipofectamine 3000 reagent (L3000015, Invitrogen). Recombinant viruses in cell culture media were harvested 4 days after transfection, stored in aliquots at −80 °C, and subjected to further investigation.

**Statistics.** Animals were randomly allocated into different groups. Mosquitoes that died before measurement were excluded from analysis. The investigators were not blinded to allocation during the experiments or to the outcome assessment. No statistical methods were used to predetermine sample size. Descriptive statistics have been provided in the figure legends. Kruskal–Wallis analysis of variance was conducted to detect any significant variation among replicates. If no significant variation was detected, the results were pooled for further comparison. Given the nature of the experiments and the types of samples, differences between continuous variables were assessed with a non-parametric Mann–Whitney test. Differences in mosquito infective rates were analysed using Fisher's exact test. *P* values were adjusted using the Bonferroni correction to account for multiple comparisons. All analyses were performed using GraphPad Prism statistical software.

**Data availability.** Source Data for the main and Extended Data Figures are provided in the online version of this paper. All other data are available from the corresponding authors upon reasonable request.

29. Liu, Y. *et al.* Transmission-blocking antibodies against mosquito C-type lectins for dengue prevention. *PLoS Pathog.* **10**, e1003931 (2014).
30. Brown, W. C. *et al.* Extended surface for membrane association in Zika virus NS1 structure. *Nat. Struct. Mol. Biol.* **23**, 865–867 (2016).
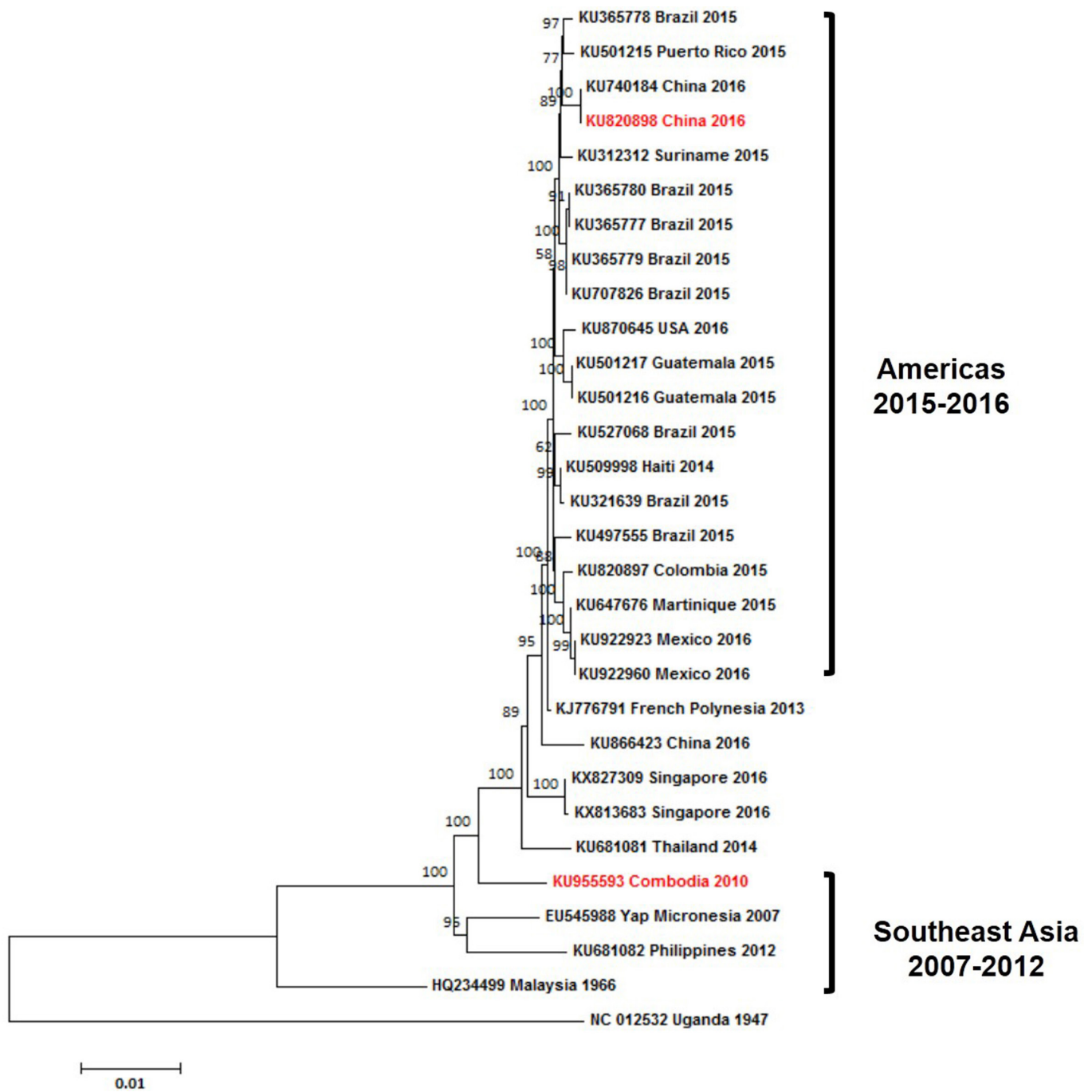
**Extended Data Figure 1 | Phylogenetic analysis of ZIKV isolates in the Asian lineage.** The tree was constructed using the neighbour-joining method based on the alignment of ZIKV sequences in the Asian lineage. The bootstrap values of 2,000 replicates are indicated on the branch nodes.
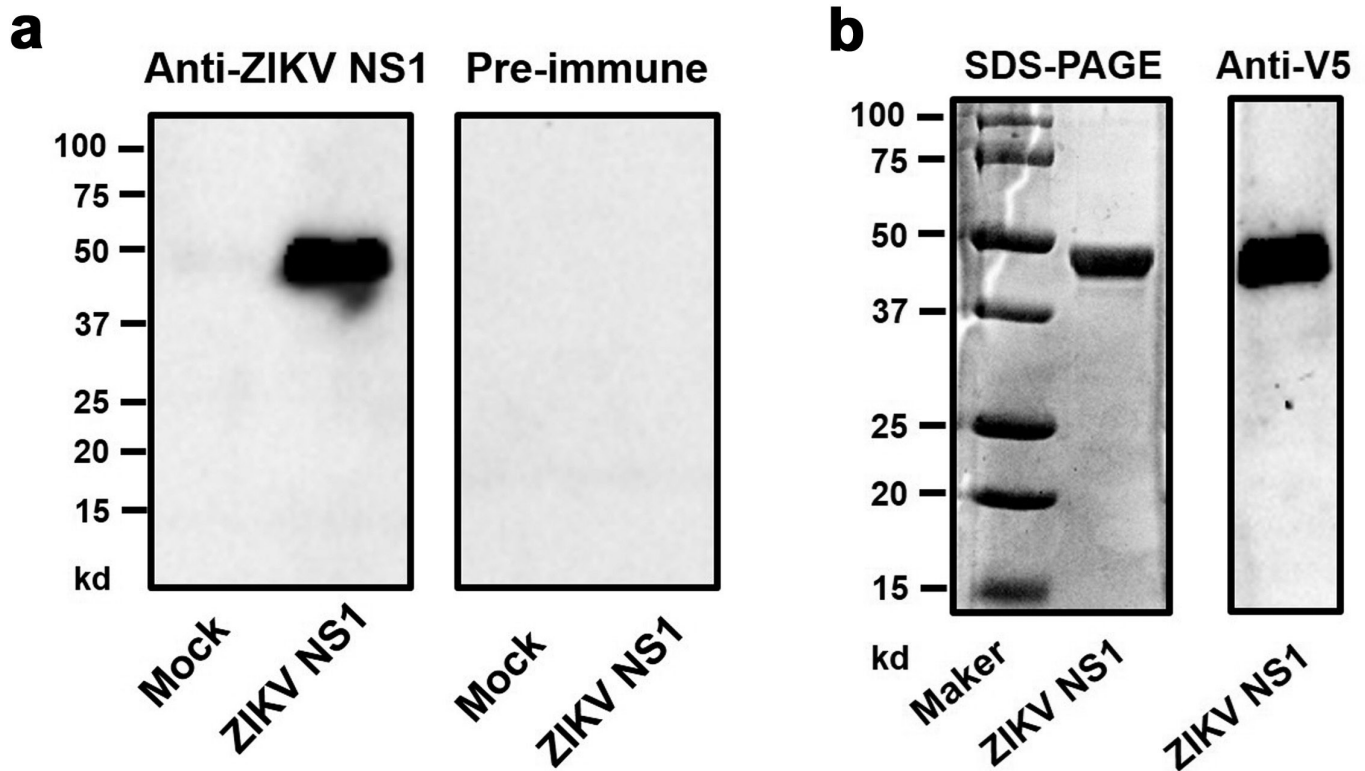
**Extended Data Figure 2 | Generation of mouse anti-ZIKV NS1 polyclonal antibody and purification of the GZ01 ZIKV NS1 protein from *Drosophila* S2 cells. a**, Generation of mouse anti-ZIKV NS1 polyclonal antibody by immunization with purified GZ01 ZIKV NS1 recombinant protein expressed in *E. coli*. The antibody was validated by probing an S2-expressed ZIKV NS1 recombinant protein. The same samples probed by mouse pre-immune antibody served as a negative control. **b**, Purification of the GZ01 ZIKV NS1 protein from *Drosophila* S2 cells. Full-length *NS1* was cloned into the pMT/BiP/V5-His A expression vector. Recombinant ZIKV NS1 protein was expressed in *Drosophila* S2 cells and purified using a cobalt-His column (left). Protein quality was evaluated by western blotting with an anti-V5 antibody (right). **a**, **b**, The experiments were reproduced three times with similar results. For gel source data, see Supplementary Fig. 1.
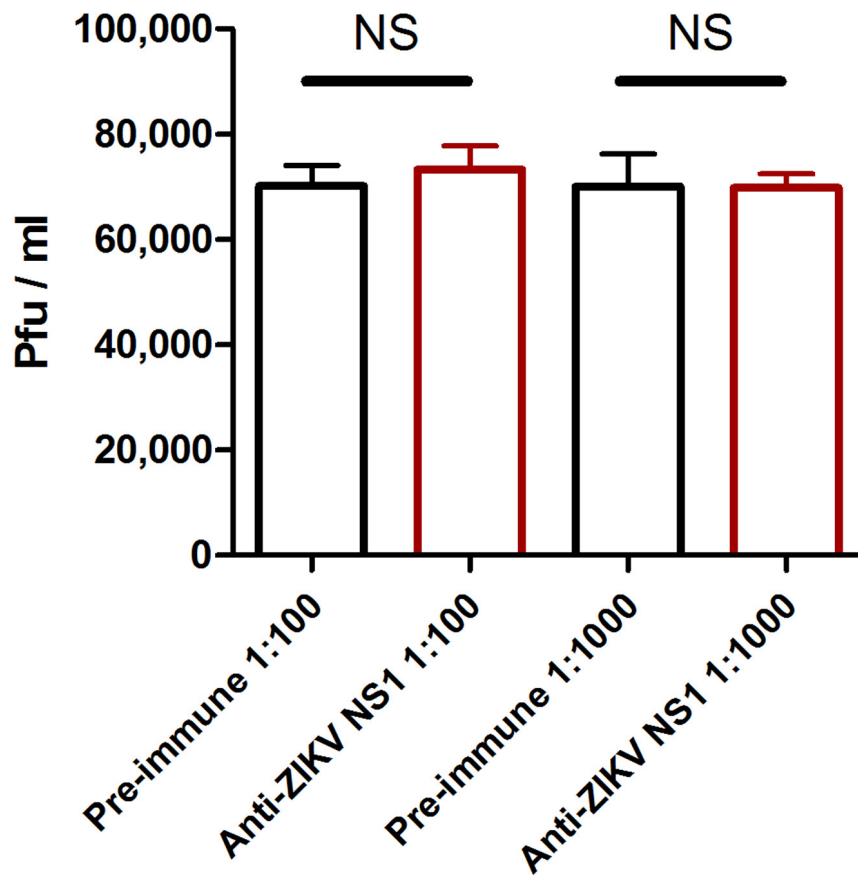
**Extended Data Figure 3 | Anti-ZIKV NS1 antibodies did not neutralize ZIKV virions.** We premixed two dilutions of mouse ZIKV NS1 polyclonal antisera (1:100 and 1:1,000) with purified ZIKV GZ01 infectious virions. The same amount of pre-immune serum served as a negative control. After a 30-min incubation, the infectivity of the antibody–virion mixture was determined by plaque assay on Vero cells. The values in the graph represent the mean ± s.e.m. *P* values were determined by two-tailed Mann–Whitney test. The data were combined from three biological repeats. NS, not significant.
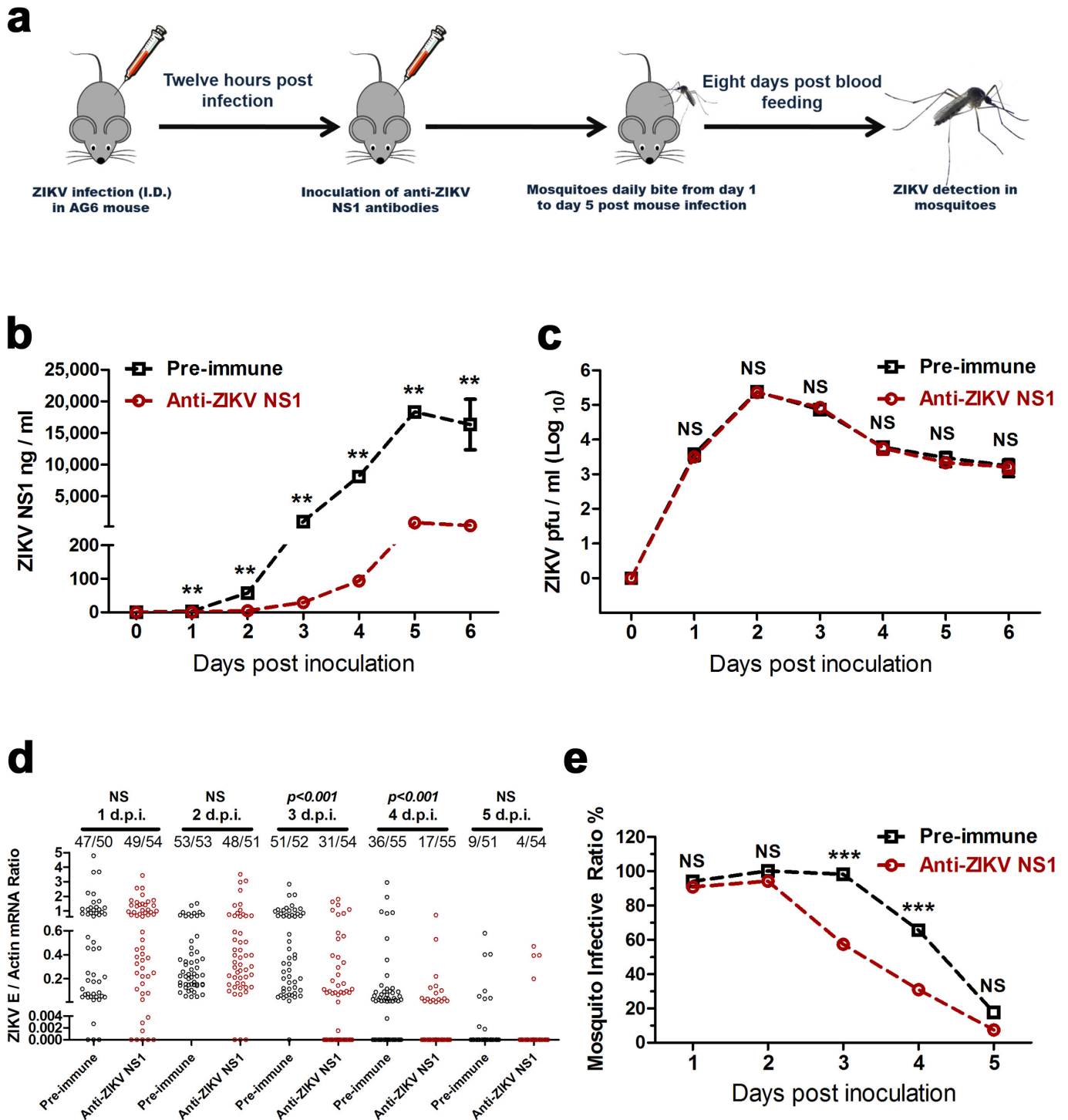
**Extended Data Figure 4 | Passive transfer of ZIKV NS1 antibodies into infected AG6 mice prevented ZIKV acquisition by *A. aegypti*.**
**a**, Schematic representation of the study design. AG6 mice were intradermally infected with $1 \times 10^4$ p.f.u. of the ZIKV GZ01 strain. Subsequently, 100 μl of a mouse ZIKV NS1 antibody was intraperitoneally inoculated into the mice 12 h after infection. The same amount of pre-immune serum was inoculated as a mock control. After 12 h of antibody dissemination, the infected mice were subjected to daily biting by female *A. aegypti* from days 1 to 5 after mouse infection. The mouse-blood-fed mosquitoes were reared for an additional 8 days for ZIKV detection. **b**, ZIKV NS1 measurement by ELISA ($n = 6$ mice per group pooled from 3 independent biological replicates). Mouse serum was collected to quantify ZIKV NS1 protein from days 0 to 6 after mouse infection.

**c**, Detection of the ZIKV load in the blood of the infected mice ($n = 8$ mice per group pooled from 4 independent biological replicates). The presence of infectious ZIKV particles in blood plasma was determined by plaque assay from days 0 to 6 after mouse infection. **d, e**, Immunoblockade of ZIKV NS1 in infected AG6 mice reduced the infection of fed *A. aegypti* ($n = 6$ mice per group pooled from 3 independent biological replicates). The number of infected mosquitoes relative to the total number of mosquitoes is shown at the top of each column. Each dot represents a mosquito (**d**). The data are represented as the percentage of mosquito infection (**e**). Data are mean ± s.e.m. (**b, c**). *P* values were determined by two-tailed Mann–Whitney test (**b–d**) or two-sided Fisher's exact test (**e**). \*\**P* < 0.01, \*\*\**P* < 0.001, NS, not significant.
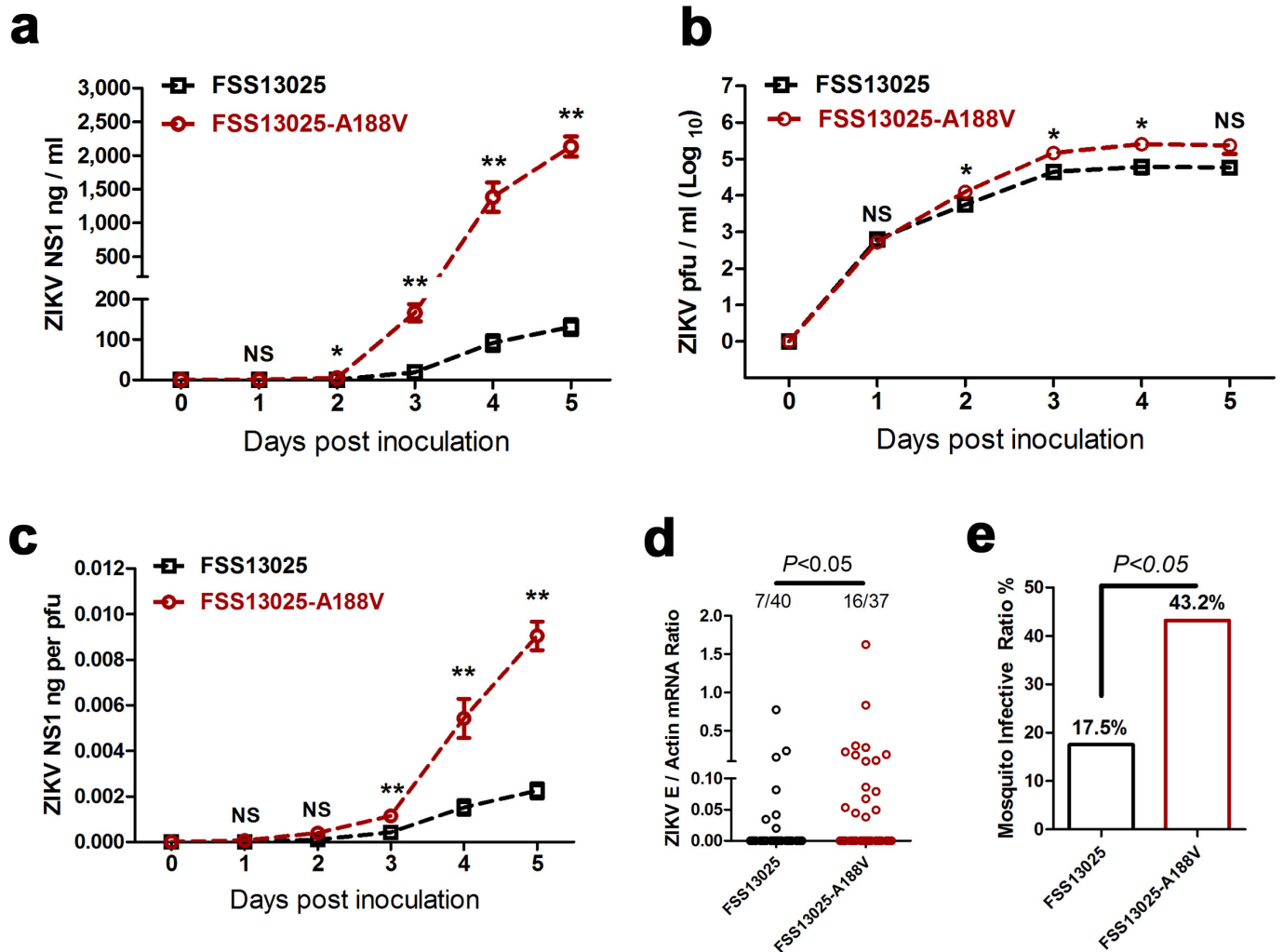
## a



## b
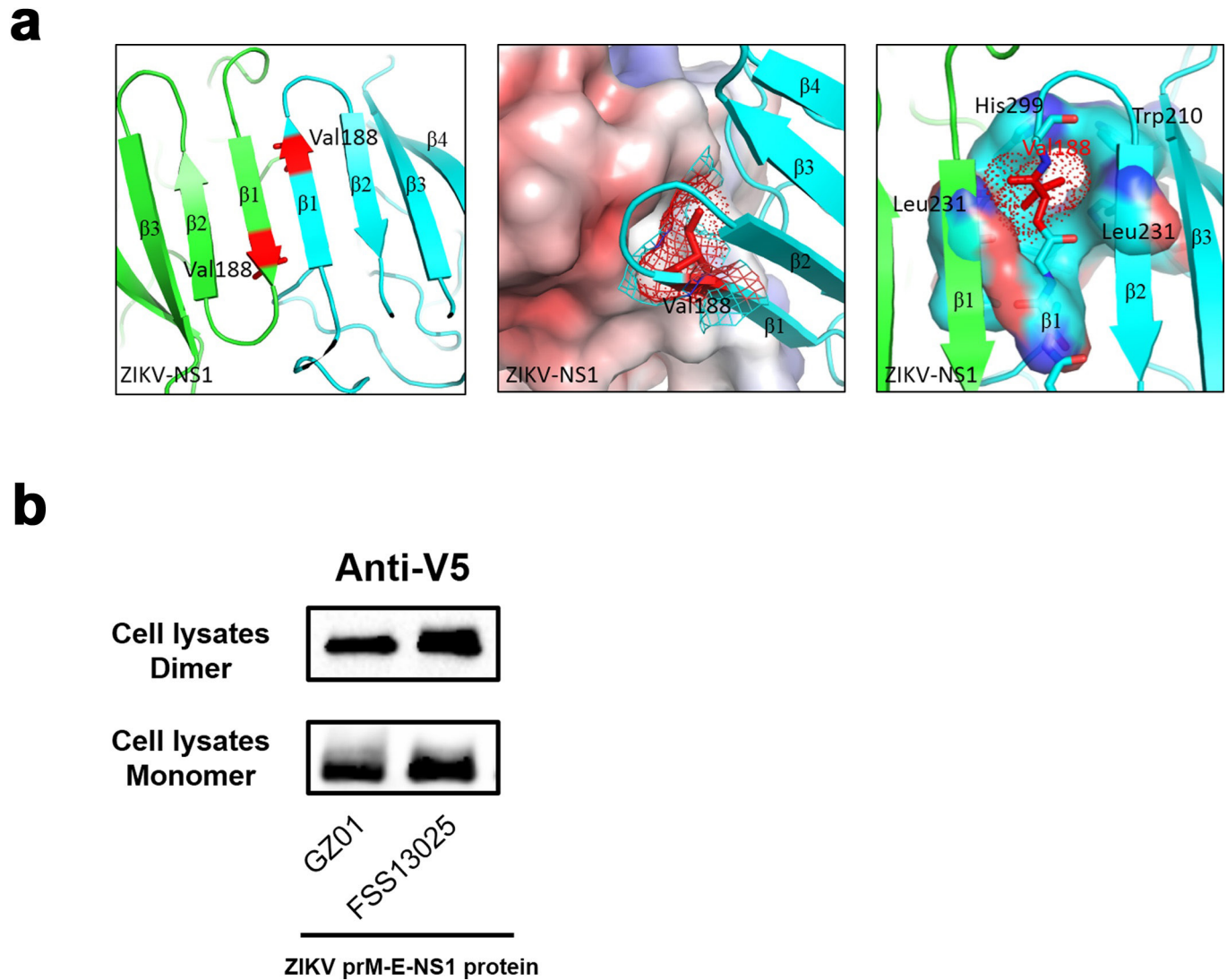


**Extended Data Figure 5 | Modelling the location of residue 188 of ZIKV NS1 and assessing NS1 dimer formation in cell lysates. a**, Modelling the location of residue 188 of ZIKV NS1. Modelling was based on the ZIKV NS1 structure (PDB: 5K6K)[30]. Residue 188 is located within the interface of a NS1 dimer (contoured red), which is formed by hydrophobic interactions. Residue 188 is deep within the hydrophobic pocket formed by Trp210, Leu23, His299 and Leu231. **b**, Assessing NS1 dimer formation in the lysates of cells transfected with GZ01 or FSS13025 *prM-E-NS1* recombinant plasmids. The cell lysates, with or without heating treatment, were used for assessing NS1 monomers and dimers, respectively, by western blotting with an anti-V5 antibody. The experiment was repeated three times with similar results. For gel source data, see Supplementary Fig. 1.

**Extended Data Figure 6 | Sequence alignment of NS1 sequences in ZIKV isolates of the Asian lineage.**
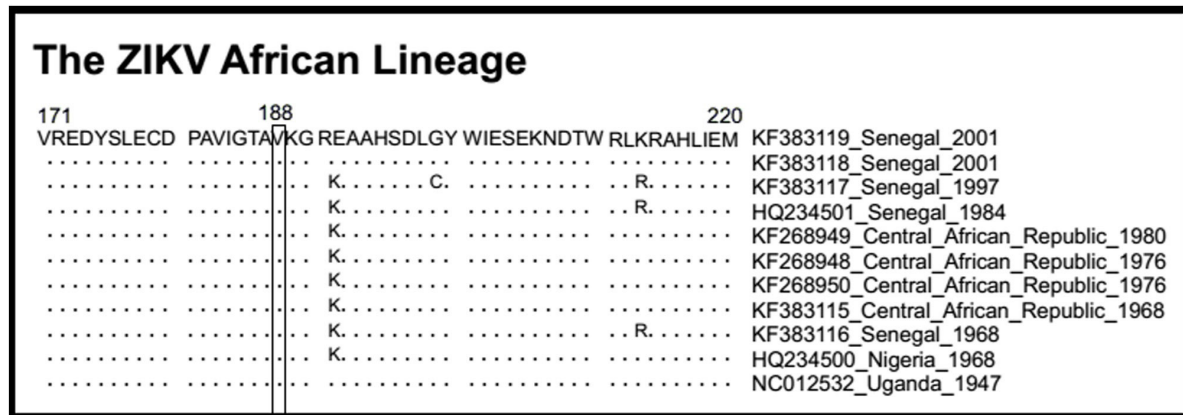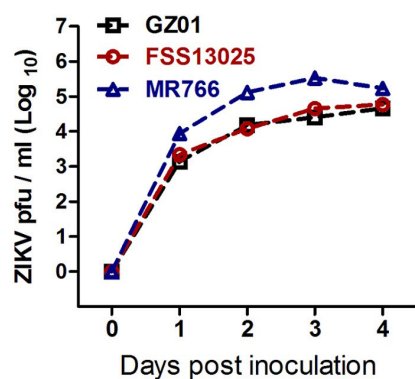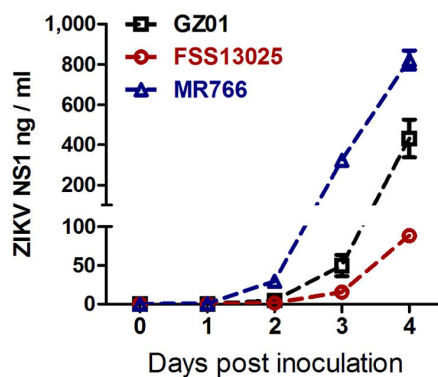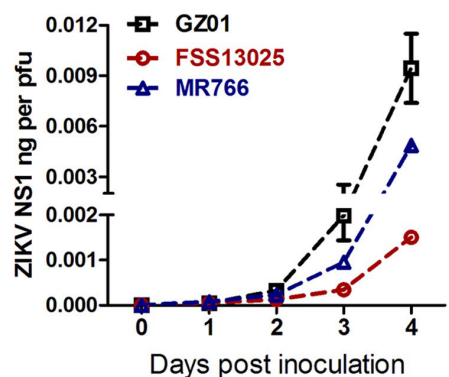
**Extended Data Figure 7 | A single amino acid substitution enhanced NS1 secretability and ZIKV infectivity in mosquitoes. a–c**, NS1 secretability in ZIKV-infected Vero cells. We mutated the single amino acid from alanine to valine at residue 188 in the NS1 protein in an infectious clone of ZIKV Cambodian FSS13025 strain (FSS13025-A188V strain). The FSS13025 or FSS13025-A188V strain (0.01 MOI) was used to infect Vero cells. Supernatants from the infected Vero cells were collected from 0 to 5 days after infection. **a**, The amount of ZIKV NS1 protein was determined by ELISA. **b**, The ZIKV titre was determined by plaque assay. **c**, NS1 quantity normalized against average viral titre (ng per p.f.u.). The data were pooled from three independent biological replicates. **d**, **e**, NS1 secretability determined ZIKV infectivity in mosquitoes. The

supernatant, isolated from FSS13025-infected or FSS13025-A188V-infected Vero cells, was premixed with fresh human blood at a 1:1 ratio for an *in vitro* membrane feeding experiment (final titre $5 \times 10^4$ p.f.u. ml$^{-1}$). The fed mosquitoes were killed 8 days after a blood meal to determine the ZIKV load by qPCR. The number of infected mosquitoes relative to the total number of mosquitoes (infected number/total number) is shown at the top of each column. Each dot represents a mosquito (**d**). The data are represented as the percentage of mosquito infections (**e**). The data were pooled from three independent biological replicates. Data are mean ± s.e.m. (**a–c**). *P* values were determined by two-tailed Mann–Whitney test (**a–d**) or two-sided Fisher's exact test (**e**). *$P < 0.05$, **$P < 0.01$, NS, not significant.
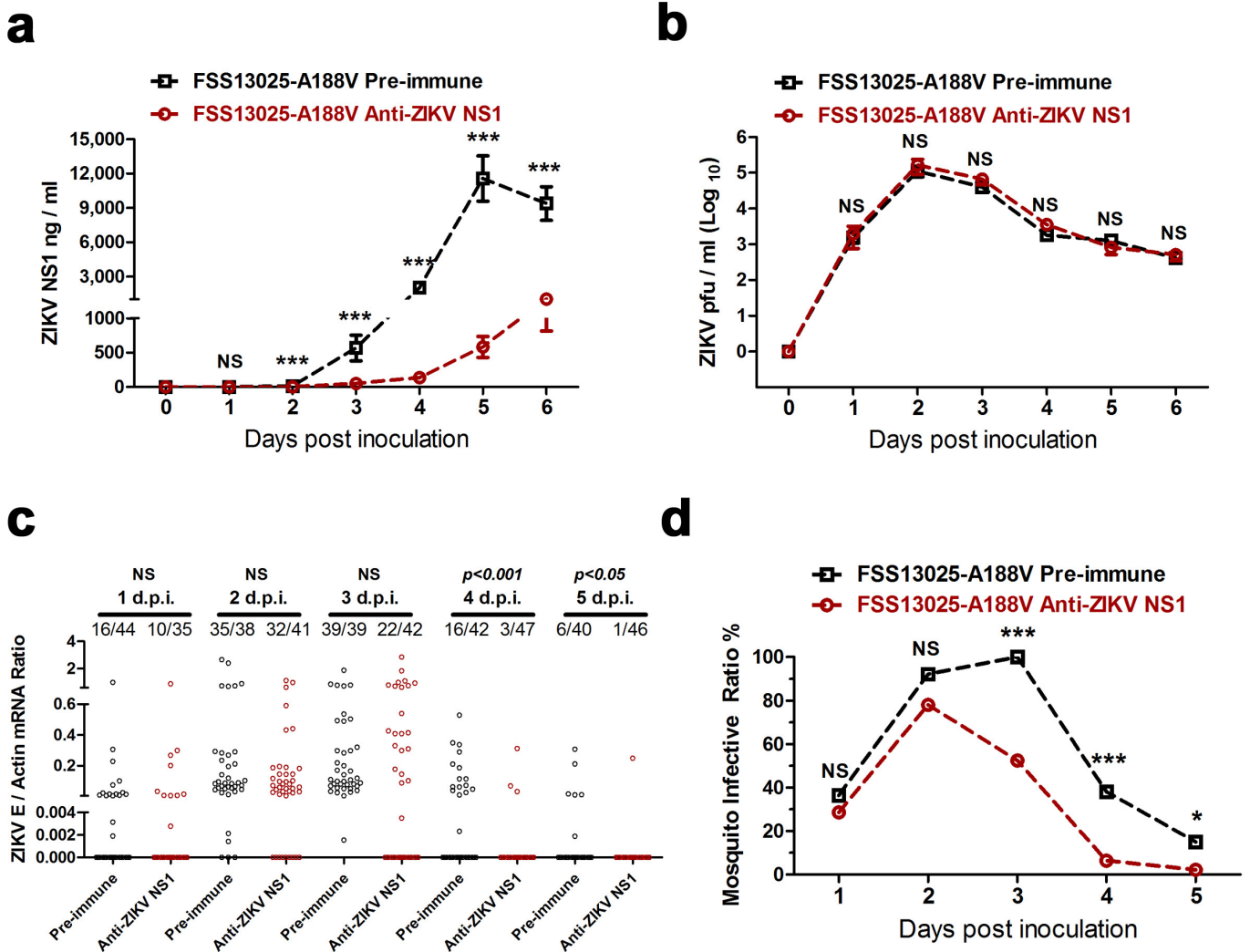
**Extended Data Figure 8 | Neutralization of NS1 by mouse ZIKV NS1 antiserum effectively reduced the prevalence of FSS13025-A188V ZIKV in *A. aegypti*. a, b,** AG6 mice were intradermally inoculated with viruses ($1 \times 10^4$ p.f.u.). ZIKV NS1 was measured using ELISA. Mouse serum was collected to quantify ZIKV NS1 protein from days 0 to 6 after mouse infection (**a**). The viral load of infectious ZIKV particles in the blood plasma of infected AG6 mice was determined using plaque assay (**b**). $n = 10$ mice per group pooled from 4 independent biological replicates. **c, d,** Immunoblockade of NS1 in FSS13025-A188V ZIKV-infected AG6 mice reduced the infection of fed *A. aegypti* ($n = 7$ mice per group pooled from 3 independent biological replicates). The number of infected mosquitoes relative to the total number of mosquitoes is shown at the top of each column. Each dot represents a mosquito (**c**). The data are represented as the percentage of mosquito infection (**d**). Data are mean ± s.e.m. (**a, b**). $P$ values were determined by two-tailed Mann–Whitney test (**a–c**) or two-sided Fisher's exact test (**d**). *$P < 0.05$, ***$P < 0.001$, NS, not significant.

## a



**The ZIKV African Lineage**

```
171                 188                      220
VREDYSLECD PAVIGTAVKG REAAHSDLGY WIESEKNDTW RLKRAHLIEM   KF383119_Senegal_2001
.......... .......... .......... .......... ..........   KF383118_Senegal_2001
.......... ......K... ....C..... .......... ...R......   KF383117_Senegal_1997
.......... ......K... .......... .......... ...R......   HQ234501_Senegal_1984
.......... ......K... .......... .......... ..........   KF268949_Central_African_Republic_1980
.......... ......K... .......... .......... ..........   KF268948_Central_African_Republic_1976
.......... ......K... .......... .......... ..........   KF268950_Central_African_Republic_1976
.......... ......K... .......... .......... ..........   KF383115_Central_African_Republic_1968
.......... ......K... .......... .......... ...R......   KF383116_Senegal_1968
.......... ......K... .......... .......... ..........   HQ234500_Nigeria_1968
.......... .......... .......... .......... ..........   NC012532_Uganda_1947
```

## b



## c



## d



**Extended Data Figure 9 | Sequence alignment of NS1 sequences in African ZIKV isolates, and NS1 secretability in African ZIKV-infected Vero cells. a**, Sequence alignment of NS1 sequences in African ZIKV isolates. **b–d**, NS1 secretability in African ZIKV-infected Vero cells. Vero cells were infected at an MOI of 0.01 with the GZ01, FSS13025 and MR766 strains. Supernatants from the infected Vero cells were collected from 0 to 4 days post-infection. **b**, ZIKV titre was determined by plaque assay. **c**, The amount of ZIKV NS1 protein was determined using ELISA. **d**, NS1 quantity normalized against average viral titre (ng per p.f.u.). The data were pooled from three independent biological replicates. The values in the graph represent the mean $\pm$ s.e.m. (**b–d**).

**Extended Data Table 1 | Primers and probes for gene cloning and qPCR**

| Primers for cloning into pET28a(+) | Upper primer | Lower primer | |
|---|---|---|---|
| *ZIKV NS1* (His tag in both N and C-terminal) | TATCTAGCTAGCGATGTGGGGTGCTCG | GATTCCGCTCGAGTGCAGTCACCATTGA | |

| Primers for cloning into pMT/BiP/V5-His/A | Upper primer | Lower primer | |
|---|---|---|---|
| *ZIKV NS1* (V5 tag in C-terminal) | TCTCGGGGTACCTGATGTGGGGTGCTCG | TATTACGCTCGAGTGCAGTCACCATTGAC | |

| Primers for cloning into pcDNA3.1/V5-His/TOPO | Upper primer | Lower primer | |
|---|---|---|---|
| *Zika prM-E-NS1* (V5 Tag in C-terminal) | TAAGGCGGTACCGCCACCATGCAAAAAGTCATATAC | TATCCGCTCGAGCGTGCAGTCACCATTGA | |
| *FSS13025 V-M mutation ( E )* | ATGTGGTTGGGTCTGAATACAAAGAATGGA | TCCATTCTTTGTATTCAGACCCAACCACAT | |
| *FSS13025 A-V mutation ( NS1 )* | ATTGGAACAGCCGCTAAGGGAAAGGAGGCT | AGCCTCCTTTCCCTTAGCGGCTGTTCCAAT | |
| *P6-740 A-V mutation ( NS1 )* | GTCATAGGAACAGCTGTTAAGGGAAAGGAG | CTCCTTTCCCTTAACAGCTGTTCCTATGAC | |

| The primers for Taqman RT-qPCR | Upper primer | Lower primer | Probe (for Taqman QPCR) |
|---|---|---|---|
| *ZIKV envelope gene* | CCGCTGCCCAACACAAG | CCACTAACGTTCTTTTGCAGACAT | FAM-AGCCTACCTTGACAAGCARTCAGACACTCAA-TAMRA |
| *Aedes aegypti actin* | GAACACCCAGTCCTGCTGACA | TGCGTCATCTTCTCACGGTTAG | FAM-AGGCCCCGCTCAACCCGAAG-TAMRA |
| *Mouse actin* | AGCCATGTACGTAGCCATCCA | TCTCCGGAGTCCATCACAATG | FAM-TGTCCCTGTATGCCTCTGGTCGTACCAC-TAMRA |