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### Structural study of the C-terminal domain of non-structural protein 1 from Japanese encephalitis virus

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14 Running Head: Structure of NS1 from Japanese encephalitis virus

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#### 18 Abstract

19 Japanese encephalitis virus (JEV) is a mosquito-transmitted Flavivirus that is closely related to other emerging viral pathogens including dengue, West Nile (WNV) 20 and Zika viruses. JEV infection can result in meningitis and encephalitis, which in 21 22 severe cases cause permanent brain damage and death. JEV occurs predominantly in rural areas throughout Southeast Asia, the Pacific islands and the Far East, causing 23 around 68,000 cases worldwide each year. In this study, we present a 2.1 Å resolution 24 crystal structure of the C-terminal β-ladder domain of JEV non-structural protein 1 (NS1-25 26 C). The surface charge distribution of JEV NS1-C is similar to WNV and ZIKV but differs 27 form DENV. Analysis of the JEV NS1-C structure, with in silico molecular dynamics simulation and experimental solution small angle X-ray scattering, indicates extensive 28 loop flexibility on the exterior of the protein. This, together with the surface charge 29 distribution, indicates flexibility influences the protein-protein interactions that govern 30 31 pathogenicity. These factors also affect the interaction of NS1 with the monoclonal 32 antibody, 22NS1, which is protective against West Nile virus infection. Liposome and 33 heparin binding assays indicate that only the N-terminal region of NS1 mediates interaction with membranes, and that sulfate binding sites common to NS1 structures 34 are not glycosaminoglycan binding interfaces. This study highlights several differences 35 between flavivirus NS1 proteins and contributes to our understanding of their structure-36 37 pathogenic function relationships.

JEV is a major cause of viral encephalitis in Asia. Despite extensive vaccination, 39 40 epidemics still occur. Non-structural protein 1 (NS1) plays a role in viral replication and, because it is secreted, it can exhibit a wide range of interations with host proteins. NS1 41 42 sequence and protein folds are conserved within the Flavivirus genus, but variations in NS1 protein-protein interactions among viruses likely contribute to differences in 43 pathogenesis. Here, we compared characteristics of the the C-terminal β-ladder domain 44 of NS1 between flaviviruses including surface charge, loop flexibility, epitope cross-45 46 reactivity, membrane adherence, and glycosaminoglycan binding. These structural 47 features are central to NS1 functionality and may provide insight into the development of diagnostic tests and therapeutics. 48

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#### 49 Introduction

50 JEV is a positive-sense single strand RNA virus with a 10.9 kb genome, which is translated into a polyprotein consisting of three structural proteins (capsid, membrane, 51 52 and envelope protein (E)), and seven non-structural proteins ((NS)1, NS2A, NS2B, 53 NS3, NS4, NS4B, and NS5). Flavivirus NS1 is a multifunctional glycoprotein that has drawn attention because of its importance in viral replication, immune modulation, and 54 immune evasion. Mutagenesis and trans-complementation assays have established 55 56 that flavivirus NS1 is essential for RNA replication (1-5) and co-localizes with the 57 replication complex (2). Transcomplementation suppressor mutagenesis studies indicate that YFV NS1 interacts with NS4A (6) and WNV NS1 interacts with NS4B (7). 58 WNV NS1 forms a physical complex with NS4B based on coimmunoprecipitation 59 experiments (7). NS1 has been described as a complement fixing antigen (8-11), and 60 DENV NS1 binds to complement pathway components C1s, C4, C4b (12, 13) whereas 61 62 WNV NS1 also can interact with factor H (14) to which protect infected cells from 63 complement-dependent clearance. NS1 also may interfere with the dsRNA sensor Toll-64 like receptor 3 (TLR-3) (15) to escape host pathogen recognition receptor detection. 65 DENV NS1 can induce inflammatory cytokine production, endothelial cell permeability, and changes to the glycocalyx (16) possibly through interactions with TLR-4, all of 66 which appear to contribute to the development of severe dengue (17, 18). Although 67 68 direct interactions between JEV NS1 and TLR-4 have not been evaluated, it may play a 69 role in JEV pathogenesis, becasue deletion of the TLR-4 gene enhances resistance to JEV (19). 70

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71 A signal sequence at the C-terminus of E protein translocates NS1 to the 72 endoplasmic reticulum (ER) where it undergoes cleavage and posttranslational modification (20). There are two characterized forms of NS1: a membrane-associated 73 dimer (~49 kDa per monomer), found on ER surface and the plasma membrane, and a 74 secreted hexamer (52- 55 kDa per monomer) (20). The mass of the two NS1 forms are 75 76 different due to differential glycosylation. Structures of full-length WNV (21, 22), DENV 77 (22), and ZIKV NS1 (23, 24) proteins have been reported. Most NS1 proteins contain six conserved disulfide bonds. NS1 shares a conserved N-linked glycosylation site at 78 79 Asn 207. YFV, DENV, WNV, and JEV share a second glycosylation site at Asn 130, and most of the JE serogroup NS1 proteins have a third glycosylation site at Asn175 linked 80 to high-mannose carbohydrate, but this is not present in JEV NS1 iteself (20, 25-27). 81 82 The NS1 monomer of WNV, DENV and ZIKV contains 3 domains: β-roll (amino acid residues 1-29), wing (38-151), and  $\beta$ -ladder domains (181-352) (22-24). NS1 forms a 83 84 homodimer by extending the  $\beta$ -ladder domain and connecting at  $\beta$ -roll domain forming a 85 cross shape protein. One face of the dimer comprises of the protruding  $\beta$ -roll and part of the wing domain. The hydrophobic surface of the  $\beta$ -roll and wing domains may mediate 86 the interaction with the cell membrane (22) via a number of amino acid residues 87 identified from ZIKV, including 28, 115, 118, 123, and 160-163 (23, 24). The opposite 88 89 side is composed of loops linking the surface  $\beta$ -strands of the ladder domain. This region is a potential host protein interacting surface due to its hydrophilicity. Three NS1 90 dimers can assemble to form a hexameric pore, which can act as a lipid depot (22, 28). 91 expression on the infected cell surface may 92 DENV NS1 occur via a glycosylphosphatidylinositol (GPI) anchor, for which a hydrophobic carboxy-terminal 93

GPI-addition signal peptide at the N-terminus of NS2A is required (29-31). Soluble NS1
also binds to uninfected cell membranes via glycosaminoglycans (GAGs), primarily
heparan sulfate and chondroitin sulphate E (32).

97 Secreted NS1 is used as diagnostic marker for flavivirus infection, as it is found in the blood at early stages (33, 34). Alternatively, detection of anti-NS1 IgM and IgG 98 99 can be used (34, 35). Immunization of NS1 in mice or passive transfer of anti-NS1 100 antibodies can confer protective effects against flavivirus challenge (34, 36-39). 101 However, some anti-DENV NS1 antibodies reportedly are autoreactive and bind to host 102 extracellular matrix components, platelets, and endothelial cells (8, 20), which may have pathogenic consequences. Flavivirus NS1 transferred with blood meal was found to 103 104 enhance viral infection in mosquitoes by downregulating mosquito midgut immune 105 genes (40).

Most of our knowledge of JEV NS1 has been inferred from studies of DENV and 106 107 WNV NS1. Although the protein sequences are highly conserved (Fig 1) and the DENV, 108 WNV, and ZIKV NS1 structures display the same protein fold, there are important 109 differences. For example, polyclonal antibodies raised against DENV NS1 in mice were shown to cross-react with proteins on epithelial cell: ATPase, protein disulfde 110 isomerase, vimentin, and heat shock protein 60. The cross-reactive epitope was 111 mapped to amino acid residues 311-330 on DENV NS1 (41) (Fig 1). Although JEV NS1 112 shares these conserved epitopes, antibodies against JEV NS1 did not react to any of 113 114 these host cell targets (41). NS1 alone was shown to cause endothelial leakage in DENV, but this was not detected in WNV, consistent with the non-vascular leakage 115 phenotype of WNV disease (42). Similar to WNV, other encephalitic flaviviruses 116

including JEV may vary in their NS1-endothelium interactions. As another example,
WNV NS1 binds the alternative complement pathway regulator, factor H, whereas JEV
NS1 does not (8).

NS1' is an extended form of NS1 with 52 extra amino acids from the NS2A N-120 121 terminus, generated by a -1 ribosomal frameshift (43). It is specific to the JE serogroup of flaviviruses. NS1' was found in dimeric form (monomer molecular mass around 58 122 kDa), detected in both cell lysate and culture media (44, 45), and suggested to play a 123 124 role in neuroinvasiveness; selectively abolishing NS1' production reduces WNV 125 mortality in mice (43, 46). NS1' co-localized with viral RNA replication complex, and can substitute for NS1 in cells (45). However, there is a discrepancy between the results of 126 in vitro and in vivo studies. WNV NS1' provides an advantage only in in vivo studies 127 (47). There is also variation of NS1' involvement in replication among different viruses. 128 Whereas WNV NS1' does not contribute to viral replication in vitro, JEV NS1' mutants 129 have less infectivity in a cell model (47, 48). Therefore, the role of NS1' in the JEV life 130 131 cycle and pathogenesis remains unclear.

Here, we report the crystal structure of the C-terminal domain (amino acids 172-352) of JEV NS1 and compare it with published DENV, WNV, and ZIKV NS1 structures. Our findings reveal a diversity in protein surface charges. Furthermore, the solution conformation of the protein was examined by small-angle X-ray scattering (SAXS) and molecular dynamics (MD) simulations along with analysis of cell membrane association. Importantly, we define a cross-reactive epitope on NS1 using an antibody that shows protective activity against WNV infection. Our study shows the common and contrasting

- 139 features of flavivirus NS1 structure, which contributes to our knowledge of the molecular
- basis of multiple NS1 functions.

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#### 142 **Results**

143 Structure of C-terminal domain of JEV NS1 and NS1'. The crystal structure of the C-terminal region of JEV NS1 determined at 2.1 Å resolution is similar by fold to all 144 previously solved flavivirus NS1 structures (Fig. 2a). The electron density is visible for 145 residues 177-352, whereas the first 5 residues at the N-terminus are not visible. The 146 147 monomer consists of 10 β-strands on one side and 4 helices and unstructured loops on 148 the other side. Between each  $\beta$ -strand are  $\beta$ -turns and short loops, apart from  $\beta$ 4 and  $\beta$ 5 which are separated by a long unstructured loop (residues 218-273) (**Fig. 2a-b**). The 149 protein contains four conserved disulfide bonds (C179-C229, C280-C329, C291-C312, 150 and C313-C316) and hydrogen bonds between  $\beta$ -strands and loops. JEV NS1-C forms 151 152 20  $\beta$ -strands oriented in a head-to-head arrangement in the dimer, as do ZIKV, WNV, 153 and DENV NS1 with the dimer length of 9.65 nm at its widest point (Fig. 2c). The dimer interface is created by 21 residues from each monomer with an average distance of 2.9 154 155 Å (Tables 1 and 2). Eight of these interface residues are conserved among flavivirus NS1 (Table 1, score 7-9). The dimer is connected by 12 hydrogen bonds. When 156 comparing the hydrogen bonding network at the dimer interface of the C-terminal 157 domains of ZIKV (PDB ID 5IY3), WNV (4OIE), and DENV NS1 (4OIG), there are 6 158 common residues with the same bond arrangement: Thr (JEV, ZIKV, WNV)/Ala (DENV) 159 186, Val (JEV, ZIKV, WNV)/lle (DENV) 188, Thr (JEV, WNV)/Ser (ZIKV, DENV) 228, 160 His 254, and Thr 230 -Trp 232 (Tables 1, 2, 3, and Fig. 3). In addition, we solved the 161 162 structure of JEV NS1'-C, which is distinguished from NS1 by an extra 52 amino acids from the C terminus of the protein, to 2.6 Å resolution. The structure revealed the same 163 protein fold and dimer orientation. However, it showed only 2 extra amino acids in 164

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comparison with the C-terminal domain of JEV NS1 (0.348 Å Cα RMSD) (data not
 shown). The C-terminus is disordered, so the electron density is not visible.

Solution model of JEV NS1-C dimer. The dimeric nature of JEV NS1-C was 167 confirmed by SAXS studies performed on the protein in solution. The SAXS profiles 168 calculated from the monomer and dimer of JEV NS1-C crystal structure were compared 169 170 with the JEV NS1-C experimental SAXS data (Fig. 4a). A monomer of JEV NS1-C 171 yielded a poor fit to the experimental data with  $\chi$  of 14.11, whereas a dimer provided an 172 improved fit with  $\chi$  of 4.02. The radius of gyration of 27.02 Å was obtained from Guinier analysis, which agrees with the value extracted from the pair distribution function, 27.08 173 Å. The pair distribution function of JEV NS1-C shows characteristics of a lengthy ovoid 174 175 particle with the maximum intra-particle distance (D<sub>max</sub>) of 94.1 Å, similar to the widest point of JEV NS1-C dimer crystal structure (96.5 Å) (Fig. 2c and 4b). The calculated 176 molecular mass was 45.5 kDa, corresponding to the dimeric form of C-terminus NS1. 177 178 An averaged ab initio model was generated at 30 Å resolution with good similarity 179 agreement (normal spatial discrepancy (NSD) =  $0.513 \pm 0.016$ ) and compared with the 180 JEV NS1-C dimer crystal structure (Fig 4c). The structures are well-matched although there is an extra region of mass near the dimer interface in the SAXS model (labelled M, 181 Fig. 4c). This feature also is seen in the SAXS model of WNV, suggesting the NS1 182 crystal structures of JEV and WNV may not fully represent the structure of the protein in 183 solution (21). Analysis of the crystallographic atomic mean-square displacements or B-184 185 factors in our JEV NS1-C crystal structure indicates that surface regions of loop 218-272, particularly sub-loop 235-237, have high conformational freedom within the crystal 186 187 lattice (Fig. 4d-e). A 40 nanosecond all atom molecular dynamics (MD) simulation of the

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JEV NS1-C dimer at 37°C confirmed that movement of this loop is unrestrained in both monomers (**Fig. 4d-e**). We hypothesized that the apparent extra region of mass observed in the JEV NS1-C and WNV SAXS structures could be accounted for by the dynamic nature of the loop 218-272 and the resulting expansion of volume in the solution structures. To model JEV NS1-C behaviour in solution more accurately we created a pool of possible structures with varying loop 218-272 conformations and compared them with our SAXS data. Using this approach, we improved the fit to the

experimental SAXS data from x of 4.02 to 1.48 (Fig 4a).

Comparison of JEV NS1-C with other flavivirus NS1-C structures. JEV NS1-196 C has the same fold as ZIKV (2.2 Å resolution), WNV (2.6 Å resolution), DENV (3 Å 197 198 resolution), and superposition gives Ca RMSD closest to WNV NS1 (1.162 Å for ZIKV, 0.959 Å for WNV, and 1.333 Å for DENV) (Fig. 2c). The structural superimposition 199 showed low positional conservation only at the N-terminus, C-terminus, and  $\beta$ -turns. 200 The electrostatic surface potential maps of known NS1-C domains (ZIKV, WNV, and 201 202 DENV) showed symmetric patterns consistent with homodimers. On the  $\beta$ -ladder surface, all displayed neutral charge in the central regions flanked by negatively 203 204 charged regions (Fig. 5). This negatively charged region is small in DENV, larger in WNV, and expanded diagonally from the top left to bottom right in JEV and ZIKV. 205 Adjacent to it, toward the ends, are small positively charged pockets that are seen 206 207 clearly only in JEV and ZIKV, and the tips of all NS1-C have mixed charge. The loop 208 surface is more variable than the ladder surface. DENV has a distinct positively charged 209 central region, whereas JEV and WNV have negative charge in their central area. ZIKV is different, as the middle region displays both positive and negative charge. The 210

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211 adjacent area has positively charged pockets in all NS1 structures (Fig. 5, Table 4). 212 Three pockets are found in WNV and DENV, whereas ZIKV has only pockets 1 and 2, 213 and JEV has pockets 1 and 3. The residues building the positively charged pockets are conserved in pocket 1 and partially conserved in pocket 2, pocket 3, and front pocket on 214 215 ladder surface (Fig. 5b, Table 4). 216 Cell membrane interaction via GAGs determination. Sulfate molecules were 217

found on the surface of JEV NS1-C (Fig. 6, Table 5) similar to ZIKV (PBD ID 5K6K), WNV (406C), and DENV (40IG). Moreover, they are distributed near the positively 218 219 charged pockets. Hence, it is possible that this positively charged area is the binding site of negatively charged ligands. Sulfate containing molecules, such as GAGs, which 220 221 are involved in NS1-dependent membrane attachment (32), could interact here. To test 222 if the interaction with GAGs occurs via sulfate binding sites at the C-terminus, JEV NS1-C binding to heparin agarose beads was analysed. However, the 20 kDa JEV NS1-C 223 224 was found only in the flow-through and wash fractions (Fig. 7a) indicating that it did not 225 interact efficiently with heparin. The interaction of heparan sulfate, chondroitin sulfate, and dermatan sulfate polymers with JEV NS1-C was investigated by protein thermal 226 shift assay. No JEV NS1-C stabilizing effect was observed for any of GAG polymers 227 tested even at high concentration of GAG (100 μM). The absence of GAG binding was 228 229 consistent with the pull-down experiments.

An interaction of JEV NS1-C with lipids common to cell membranes was tested 230 231 using a liposome binding assay. JEV NS1-C did not associate with liposomes at either pH 7.5 and 5.5 (Fig. 7b). As full-length NS1 can bind liposomes (22, 49), it appears that 232 233 the NS1 C-terminus is not responsible for membrane binding. We note that the

hydrophobic residues of β-roll and wing domains have been suggested to play a role in
 membrane binding (22-24).

JEV NS1-C and JEV NS1'-C complexed with 22NS1 Fab. Comparison of the 236 22NS1 antibody epitope of WNV-NS1-C with JEV NS1-C showed that 9 of 16 residues 237 (Trp232, Ser239, Tyr260, Lys261, Thr262, Glu289, Arg294, Arg314, and Ser315) are 238 239 conserved between the two viruses (Fig. 1) (36). Indeed, 22NS1 mAb cross-reacts with 240 JEV NS1-C and NS1'-C protein, which was confirmed by Western Blotting analysis (Fig. 241 8, lower left inset) and size exclusion chromatography (Fig. 8). JEV NS1-C and 22NS1 242 Fab alone eluted at retention time of 7.9 and 8.3 min, respectively. JEV NS1-C incubated with 22NS1 Fab eluted faster at a retention time of 6.9 min corresponding to 243 244 complex formation with a small amount of free 22NS1 Fab fragments left. The eluted 245 fraction was analyzed by SDS-PAGE and 2 peaks representing JEV NS1-C and 22NS1 (~25 kDa) were identified. This confirms that NS1 and 22NS1 mAb interact in solution 246 247 (Fig. 8, lower right inset). The incubation also generates a small peak at retention time 248 of 6.1 min. This may represent a higher order oligomer of JEV NS1-C, which recruits 249 multiple 22NS1 monomers into a complex which higher hydrodynamic radius than the 2:2 complex observed at 6.9 min. In support of this idea, we note the absence of the 250 NS1-C species eluting at ~7.4 min in the complex chromatogram. The protein-Fab 251 252 complex also was analysed by SAXS. The complex experimental profile was compared to the WNV NS1-C-22NS1 complex (PDB ID 40II) calculated SAXS scattering profile 253 254 (Fig. 9a and c). The complex (40II) however, gave a poor fit to the experimental SAXS data with  $\chi$  of 6.82. Guinier analysis gave the radius of gyration of 52.89 ± 0.34 Å, which 255 coincides with 52.50 Å extracted from the pair distribution function. The pair distribution 256

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257	function of the complex has multiple peaks which signify the multi-domain geometric
258	shape with D <sub>max</sub> of 154.9 Å ( <b>Fig. 9b</b> ). The calculated molecular mass was 149.96 kDa.
259	An averaged ab initio model was generated at 30 Å resolution. The Fab part of WNV
260	complex (4OII) did not fit into the SAXS envelope and shifted from the positions in 4OII
261	model, whereas the WNV NS1-C dimer fit well (Fig. 9c-e) indicating flexibility of Fab
262	epitope in solution. We generated a pseudo-atomic model of the JEV NS1-C antibody
263	complex by replacing the WNV-NS1-C with JEV NS1-C and optimizing the position of
264	the Fab molecules. This model with the 2 Fab molecules shifted away from its primary
265	location in 4OII model had better fit to the SAXS data (χ of 6.82 to 3.09; Fig. 9a, 9c-e).
266	Both JEV NS1-C and NS1'-C are able to cross-interact with the protective WNV 22NS1
267	mAb in which JEV NS1-C interact with some flexibility.

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269 Flavivirus NS1 proteins have generated much interest because of their multiple 270 functions in viral replication, cell signaling, and immune evasion. Since 2014, the structures of nine NS1 proteins have been solved (21-24, 50). These proteins were 271 expressed in bacterial or insect cell expression systems. Here, we expressed JEV NS1 272 273 C-terminus in E. coli after the failure of several attempts to express full-length JEV NS1 274 in E. coli, insect cells, and mammalian cells. We describe the first structure of E. coli expressed JEV NS1 C-terminus, which when compared to other NS1 structures as well 275 as JEV NS1'-C, shows a high degree of structural conservation. As expected for NS1', 276 the same fold could explain the similar functions in vitro of NS1 and NS1'. However, the 277 278 specific role of the extra amino acids is not clear yet, although WNV lacking the NS1' 279 form are less neuroinvasive (43).

The availability of WNV, DENV, and ZIKV NS1 structures has allowed us to 280 281 assess similarity and differences which may be relevant to their functional behavior. All 282 NS1 proteins are dimeric in crystallo, even though the recombinant protein contains only the C-terminal domain (21, 23). The molecular mass and low resolution model 283 generated from SAXS data confirm the dimeric nature of the isolated C-terminal domain 284 in solution. In contrast to previous work, which suggested that the  $\beta$ -roll domain is 285 286 responsible for dimerization (49), we propose that 6 common residues which form hydrogen bonds at the dimer interface of all NS1 structures mediate dimer formation 287 288 (49). In principle, inhibition of dimer formation by interposing a ligand at this site could 289 facilitate anti-flavivirus drug development.

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291 However, when considering the full-length flavivirus NS1 protein structure (22-24), the 292 ladder face of the C-terminal domain is positioned underneath the β-roll domain. The Nterminus protects the central region of the ladder face from the environment. Besides 293 294 that, the  $\beta$ -roll domain is contained by a hydrophobic region that is suspected to interact 295 with the cell membrane or form a lipid cargo pore in NS1 hexamer making it harder for 296 the ladder face to make an interaction. This model conflicts with a suggestion that the  $\beta$ ladder might bind to the complement control protein domain (sushi domain) of 297 complement proteins (51). In comparison, the loop face in the JEV NS1-C is fully 298 exposed with its diverse surface charge when compared to ZIKV, WNV, and DENV. 299 300 Particularly, DENV has the most distinct positive central area whereas the rest are Journal of Virology 301 negatively charged. Positively charged pockets found on the loop face of the NS1 302 crystal structure could mediate anionic ligand binding. Moreover, the pockets, especially 303 pocket 1, are composed of conserved sequences and are found in all known NS1 304 structures. The presence or absence of each pocket in NS1 from different flaviviruses 305 306 307 308

may confer upon the individual NS1 proteins the ability to interact with different target proteins or ligands in a virus-specific manner. Sulfate molecules distributed on the NS1 surface agree with previous findings for DENV and ZIKV and indicate the potential for anionic ligand interaction. We thought that NS1 might interact with uninfected cell membranes via these sulfate binding sites (32), but further experiments confirmed that 309 310 JEV NS1-C cannot bind efficiently to heparin, heparan sulfate, chondroitin sulfate, or dermatan sulfate polymers. Thus, the sulfate binding sites are not GAG binding 311 312 interfaces and could represent a crystallographic artifact. Moreover, JEV NS1-C cannot

Both faces of the JEV NS1-C dimer display electrostatic surface charge diversity.

bind to liposomes. Our results also suggest that NS1 C-terminal is not responsible for binding to the cell membrane through GAGs. Instead, cell membrane interactions may occur at the  $\beta$ -roll and wing domains, as was suggested previously (22-24).

B-factor and MD analyses suggest that loop 218-272 is conformationally 316 dynamic. Although the B-factors are high for this region, the X-ray structure does not 317 318 319 loop. Interestingly, the 22NS1 epitope forms part of this loop (Trp232, Gly235, Ile236, 320 Leu237, Ser239, Asp240, Asn253, Try260, Lys261, and Thr262). Binding with antibody may stabilize the loop as seen in the WNV NS1-C-22NS1 complex (4OII). We suspect 321 that the dynamic 218-272 loop may harbor distinct protein-protein interaction functions, 322 323 a phenomenon which was found independently in WNV (21). NS1 from other 324 flaviviruses may share this characteristic. Taken together, the models agree that the membrane-associated NS1 dimer orients with the N-terminus facing the endoplasmic 325 326 reticulum or cell membrane and the loop facing outward (21, 22, 24) making an interacting interface, and likely mediating the biological functions of the protein. 327 Therefore, the loop domain could be a candidate for sturture based drug targeting. 328

The anti-WNV NS1 mAb, 22NS1, is protective in mice and did not cross-react with DENV-2 (36). We demonstrated that this mAb can cross-react with the more closely related JEV NS1-C at the same epitope, but with some conformational flexibility. This finding agrees with our MD result showing elasticity in the epitope loop, which may affect the antibody-NS1 structure in solution. Even though the JEV NS1'-C has extra amino acids at the C-terminus, JEV NS1'-C can interact with WNV 22NS1 mAb indicating the C-terminal tail does not obstruct the binding surface of 22NS1. The C-tail

may then locate at the side flanking the dimer. The presence of NS1' is a shared characteristic of JE serocomplex viruses, and NS1' may have specific properties that contributes to the propensity of JE serogroup viruses to cause encephalitis.

339 Despite Flavivirus NS1 proteins having a conserved protein fold, these proteins differ in their charge distribution, which may enable unique interactions with host 340 341 proteins (8, 41). The fact that WNV 22NS1 mAb interacts with JEV NS1 is consistent with close similarity of charge distribution of WNV and JEV NS1. This similarity also 342 343 extends to ZIKV. Overall, these results provide structural details that aid NS1 function 344 determination and highlight both similarities and contrasts among NS1 othologs, which may be a productive avenue for developing common diagnostic and therapeutic 345 346 strategies against this important group of Flavivirus diseases.

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#### 347 Materials and methods

348 Protein expression and refolding. JEV strain SA14 (GenBank: M55506) was used as a template. Synthetic DNA optimized for expression in E. coli of JEV NS1 was 349 acquired from Life Technologies. To create JEV NS1-C (amino acid residues 172-352) 350 the target sequences were cloned into pET303 at Xbal/Xhol cloning site by using 351 352 forward primer 5' gctctagaatgCGTGAAGAAAGCACCGATGAATGTGAT 3', reverse 353 primer 5' ccg ctcgagTTATGCATCAACCTGGCTACGAACCAG 3'. Synthetic JEVNS1' was purchased from GenScript (Piscataway, NJ, USA). The full-length NS1' was the 354 NS1 sequence with 156 additional nucleotides. The frameshift sequence was manually 355 added by insertion of thymine at position 3561 as a result of -1 ribosomal frameshifting. 356 357 JEV NS1'-C were generated from the synthetic JEVNS1' by using forward primer 5' 358 gctctagaatgCGTGAAGAAAGCACCGATGAATGTGAT 3'. 5' reverse primer 359 ccgctcgagTTAATGCAGATGATAACCCCATGCATctg 3'. Proteins were expressed in E. 360 coli by autoinduction and refolded by using method modified from previously described 361 in Edeling et al, 2014. The theoretical molecular weight of JEV NS1-C and JEV NS1'-C are 20.54 kDa and 25.98 kDa. Protein yield and purity were analysed by SDS-PAGE. 362

Protein crystallization and data collection. JEV NS1-C (~6 mg/ml) and JEV NS1'-C (5-7 mg/ml) were screened using commercial crystallization screens. Successful conditions were optimised by hanging drop method. Needle crystals of C-*JEV*NS1 were produced from 1 M Ammonium sulphate and 0.1 M MES pH 5.5. The crystals were flash frozen in reservoir buffer added with 20-25% ethylene glycol. The JEV NS1' -C also crystallized in needle form in 1 M Ammonium sulphate and 5% propanol. The JEV NS1'-C was cryo-protected in reservoir buffer with 20% glycerol.

370 X-ray data were collected at cryogenic temperature, wavelength of 0.98 nm, at 371 beamline PROXIMA 1 at Soleil synchrotron, France and at beamline I02 at Diamond 372 Light Source, UK. Data reduction was carried out by XDS programs (52) or iMosflm (53). The protein structure was determined by molecular replacement using the 373 structure of WNV NS1 C-terminal domain (PDB: 4OIE, sequence identity >70%) as a 374 375 starting model by MOLREP (54) in the CCP4 program suit. The structure was refined by 376 REFMAC5 (55) and built in COOT (56). Data collection and refinement statistics are shown in Table 6 The JEV NS1-C refinement statistic of Ramachandran plot is 95.98% 377 favoured and 0% outliers. MolProbity score is 1.6. The JEV NS1'-C refinement statistic 378 of Ramachandran plot is 94.89% favoured and 0% outliers. MolProbity score is 1.84. 379

Protein structure analysis. Assembly analysis was performed by program PISA (57). Conservation scores of residues on protein structures was given by Consurf (58) using 21 homologous sequences. The input homologous sequences of NS1 C-terminus were searched by the program and existing NS1 structure sequences were added manually. Electrostatic surface maps were generated by using PDB2PQR (59) to convert PDB files into PQR files and Adaptive Poisson-Boltzmann Solver (APBS) for electrostatics calculations (60) without pKa prediction.

JEV NS1 C-terminus-22NS1 complex formation. Complex formation was confirmed by Western blot analysis, with 22NS1 (36) and goat anti-mouse IgG-HRP (Santa Cruz Biotechnology, sc-2055) used as primary and secondary antibodies, respectively. Purified JEV NS1 C-terminus and 22NS1 fragment antigen-binding (FAb) (prepared from 22NS1 IgG mAb using Pierce<sup>™</sup> Fab Preparation Kit, Cat No. 44985) were mixed overnight at 4°C at 1:1 ratio JEV NS1-C to 22NS1 and purified by Agilent

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Bio SEC-3 4.6 300 or GE Superdex 200 10 300 GL. Eluted fractions were analyse by
SDS-PAGE.

SAXS data collection and processing. The JEV NS1-C at a concentration of 395 3.4 mg/ml and JEV NS1-C-22NS1 Fab complex at concentration of 3 mg/ml in TBS 396 buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl) were analyzed with SEC-SAXS on 397 398 beamline SWING at Soleil synchrotron, France. Samples were loaded onto an Agilent 399 BioSEC-3 4.6/300 column at a flow rate of 0.25 ml/min, 15°C. Data were collected at a distance of 1.8 m and X-ray wavelength of 1 Å. Data processing was conducted in 400 PRIMUS (61). Comparison of scattering profile was done in FoXS (62). Ab initio model 401 was the average from 10 (JEV NS1-C) or 20 (protein complex) independent model 402 403 calculations with (protein complex) or without symmetry (JEV NS1-C) using DAMMIF (63). The model was averaged with DAMAVER (64) and refined with DAMMIN (65). The 404 low resolution model surface representation was created in CHIMERA (66) using 405 406 'molmap' command. The molecular mass was calculated from Porod volume (67). 407 Molecular dynamics (MD) simulations were performed using GROMACS 4.6.5 and GROMOS96 54A7 force field in a cubic box solvated with single point charge-E water 408 molecules on JEV NS1-C dimers. A neutral charge was introduced at 150 mM NaCl. 409 The distance between JEV NS1-C dimers and the box edge was set to 10 Å. Long 410 range interactions were defined using the particle mesh Ewald algorithm and other non-411 bonded interactions were restricted to 10 Å. An energy minimization was performed 412 413 using the steepest descent algorithm followed by a 100 ps NVT ensemble at 310 K and a 200 ps NPT ensemble at 310 K and 1 bar. Production MD was performed at 310 K 414 and 1 bar for 40 ns. Ca displacement was calculated with the GROMACS RMSF 415

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function. Torsion angle MD was performed with CNS at 100,000 K for 37.5 ps with sampling every 7.5 fs in eight separate simulations. The best structure was found with FoXS using experimental data over data range 0.017 < q < 0.25 Å<sup>-1</sup> and was refined with another eight separate 7.5 ps simulations and energy minimization in GROMACS using the procedure described above. Models again were compared with FoXS. Freeing loop 214-243 gave a fit with experimental data of 1.66. Expanding the flexible region to 218-272 allowed us to improve the fit to 1.48.

Liposome binding assay. Liposome preparation was modified from previous 423 publications (49, 68). Liposomes were prepared from cholesterol (CHOL) (Sigma, 424 C8667) and 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (PC) (Sigma, P4329) at 1:9 425 CHOL to PC (22, 49, 68). CHOL and PC powder were dissolved in chloroform. To 426 achieve total 400 nmol, 40 nmol of CHOL and 360 nmol of PC were mixed together in a 427 2 ml tube, and the lipid mixture was dried under nitrogen gas stream. To hydrate the 428 429 lipid sheets, 50 µl of buffer (50 mM Bis-Tris pH 5.5, 50 mM (NH<sub>4</sub>)<sub>2</sub>S0<sub>4</sub>, 10 % glycerol or 150 mM KCl, 25 mM Tris-HCl pH 7.5, 1 mM DTT, 0.5 mM EDTA.) was added and 430 incubated at room temperature on a shaker for 30 min. Then, the lipid was sonicated 431 with an exponential probe at amplitude 4 for 30 sec with 30 sec interval on a warmed 432 water bath for 5 times. Liposome binding reaction (50 µl) was setup at 400 nmol, 125 433 434 nmol and 25 nmol of total lipid and mixed with 5 µg of protein. The reactions were incubated at 37°C for 45 min. After that, the reactions were centrifuged at 16000 x g for 435 436 30 min at 22°C and the supernatant was transferred to a new tube. The lipid pellet was resuspended in 200 µl buffer and also transferred to a new tube. Liposomes were 437 pelleted again and the supernatant was discarded. The liposome pellet was 438

resuspended in 30 µl of 1x SDS-PAGE sample buffer. Bovine cytochrome bc1 complex,
membrane proteins, was used as positive control in 25 mM phosphate buffer pH 7.5,
100 mM NaCl, 3 mM NaN<sub>3</sub>, 0.015 % DDM buffer. The supernatant and pellet fractions
were analyzed by SDS-PAGE.

Heparin binding assay. Small scale 50 µl column was setup in pipette tip by 443 444 using heparin agarose beads (Affi-Gel heparin gel, BIO-RAD). The binding buffer was 445 20 mM HEPES pH 7.4, 150 mM NaCl, and the elution buffer was 20 mM HEPES pH 7.4 supplemented with 1.5 M and 2 M NaCl. The column was equilibrated with 400 µl of 446 binding buffer. JEV NS1-C (5 µg) was applied to the column and incubated on a roller 447 for 30 min at 4°C. The column was washed with 400 µl binding buffer 3 times before 448 eluted twice with 100 µl of 1.5 M and 2 M NaCl elution buffer. Superoxide Dismutase 3 449 450 (SOD3), which contain heparin binding domain, was used as positive control. Samples 451 from each step: load, flow-through, wash, and elute, were analysed by SDS-PAGE.

452 Differential scanning fluorimetry (DSF). Polymers of heparan sulfate (average 453 molecular weight 30,000), chondroitin sulfate (62% chondroitin 4-sulfate and 33% chondroitin 6-sulfate, average molecular weight 45,400), and dermatan sulfate (average 454 molecular weight 41,000) from Iduron at final concentration of 100, 50, 25, 10, 5, 1, 0.5 455 µM were mixed with JEV NS1-C and Sypro Orange 5000X (Invitrogen) at final 456 457 concentration of 10 µM and 10X, respectively. The reaction volume was 10 µl The experiments were set in 96 well-plates and performed using StepOnePlus™ Real-Time 458 459 PCR Systems (software version 2.3) (Applied Biosystems). The reactions were equilibrated at 25 °C for 2 min followed by increase to 95 °C at 1 °C min<sup>-1</sup>. The 460 461 experiments were performed in three replicates.

#### 462

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#### 478 Author contributions

T.S. and S.V.A. originated and designed the project; T.P. expressed and purified
proteins; T.P. and G.S.A.W performed the experiments; T.P., G.S.A.W., and S.V.A.
undertook data analysis; T.P., G.S.A.W., M.S.D., T.S., L.T., and S.V.A. contributed to
interpretation of data and wrote the manuscript.

483	Data deposition: The atomic coordinates and structure factors have been deposited in
484	the Protein Data Bank, <u>www.pdb.org</u> (PDB ID <b>5019</b> for JEV NS1-C and <b>5036</b> for JEV
485	NS1'-C)

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#### 671 FIGURE LEGENDS

672 Figure 1. Sequence alignment of full-length Flavivirus NS1 produced from Clustal W (1). An asterisk indicates fully conserved residue. A colon indicates 673 conservation between groups of strongly similar properties. A period indicates 674 conservation between groups of weakly similar properties. The amino acid sequences 675 were used for X-ray structure studies: DENV1 U88535 for PDB ID 4OIG, DENV2 676 M84727 for 4O6B, WNV 196835 for 4O6C and 4OIE, ZIKV KU365779 for 5IY3, and 677 ZIKV AY632535 for 5K6K and 5GS6. The 22NS1 light chain epitopes are highlighted in 678 679 red and heavy chain epitopes are in black squares.

Figure 2. The C-terminal domain structure of JEV NS1. (a) Ribbon model of JEV NS1-C monomer. One side is built of 10 β-strands and the opposite is the nonstructured loops. Disulfide bonds are shown in yellow. (b) Topology diagram of JEV NS1-C. Four disulfide bonds are indicated as white spheres. β represent the β-sheet and η represent 310 helix. (c) Superimposed ribbon diagram of NS1-C of JEV (magenta), ZIKV (PDB: 5IY3, blue), WNV (PDB: 4OIE, green), and DENV1 (PDB: 4OIG, gold). Downloaded from http://jvi.asm.org/ on February 21, 2018 by University of Liverpool Library

Figure 3. Dimer interface of JEV NS1-C. (a, b) The surface of 21 residues from one monomer involved in dimer interface is colored in lime green and the surface that form hydrogen bonds are colored in dark green. Similarly, another monomer interfacing surface is in magenta and surface forming hydrogen bonds are in dark magenta. (c) Residues involved in hydrogen formation at the dimer interface are highlighted in lime green and magenta, respectively. Hydrogen bonds are indicated by dashed lines.

693 Figure 4. Solution model of JEV NS1-C dimer. (a) SAXS scattering curve. 694 Experimental scattering curve is shown in black scattering. Scattering profile of JEV NS1-C monomer, dimer, and the best molecular dynamic simulation structure calculated 695 with FoXS are shown in blue, green, and red, respectively. (b) Pair distribution functions 696 (c) Low-resolution model of JEV NS1-C calculated from SAXS profiles docked with the 697 698 crystal structure of the JEV NS1-C dimer. An extra region of mass is labelled with M. (d) 699 RMSF plot of the molecular dynamic simulation at the flexible loop. RMSF values of each monomer are in black and red. Average  $\beta$ -factor of each residue is in grey. (e) The 700 best molecular dynamic simulation structure (red) was superimposed with JEV NS1-C 701 crystal structure (blue). The flexible loop 218-272 shown in yellow. 702

Figure 5. JEV NS1-C compared to other flavivirus NS1-C structures. (a) Electrostatic surface map of NS1-C from JEV, ZIKV, WNV, and DENV. Surface is colored by electrostatic potential from -5 kT/e (red) to 5 kT/e (blue). Positive potential pockets are depicted in dash circles. (b) Surface model color-coded by conservation. The most conserved residues are represented in dark magenta and the most variable residues are represented in dark green.

Figure 6. Sulfate molecules bound to the loop surface of JEV NS1-C. Sulfate
molecules were found not only for JEV NS1, but also in DENV 4OIG, ZIKV 5K6K, and
WNV 4O6C. Thus, it is suspected to be an importance sulfate binding interface.

Figure 7. Cell membrane interaction via GAG determination. (a) Heparin binding determination. JEV NS1-C was incubated with heparin agarose beads. Total JEV NS1-C loaded to the column is shown in lane 1. Lane 2 is flow-through fraction. Lane 3-4 are wash fraction. The column was eluted with buffer supplemented with 1.5 M

NaCl shown in lane 5. Three independent experiments were conducted. (b) Liposome
binding assay. The experiments were conducted at pH 7.5 (upper) and pH 5.5 (lower).
Supernatant and pellet fractions separated by centrifugation were analysed by SDSPAGE. Lanes 1, 2, and 3, were pellet of 400 nmol, 100 nmol, and 25 nmol reactions,
respectively. Lanes 4, 5, and 6, were supernatant of 400 nmol, 100 nmol, and 25 nmol
reactions, respectively. Three independent experiments were conducted.

Figure 8. JEV NS1-C complexed with 22NS1 Fab. JEV NS1-C was detected by 22NS1 mAb (lower left inset). JEV NS1-C was incubated with 22NS1 Fab at 1:1 molar ratio protein to Fab fragment and the complex formation was analysed on an Agilent BioSEC-3 4.6/300. The lower right panel show SDS-PAGE analysis of each elution fraction.

Figure 9. SAXS analysis of JEV NS1-C-22NS1 Fab complex. (a) SAXS 727 scattering curve. Experimental scattering curve of JEV NS1-C-22NS1 Fab complex is 728 729 shown in black scattering. Calculated scattering profile of WNV NS1-C -22NS1 complex (4OII) is displayed in green, and JEV NS1-C -22NS1 Fab complex manually fit model is 730 731 shown in orange. (b) Pair distribution functions shows multiple peaks signify the multidomain structure. (c) WNV NS1-C-22NS1 complex (4OII). WNV NS1-C is colored in 732 deep sky blue. 22NS1 Fabs are coloured in salmon. (d) WNV NS1-C-22NS1 complex 733 (4OII) fit to the JEV NS1-C -22NS1 Fab complex ab initio model (upper). A pseudo-734 atomic model JEV NS1-C -22NS1 Fab complex are manually fit to the ab initio model 735 (lower). (e) JEV NS1-C-22NS1 Fab complex pseudo-atomic model. JEV NS1-C is 736 coloured in light green. 22NS1 Fab is colored in orchid and another Fab is colored in 737 738 sky blue.

739 Figure 10. JEV NS1 homology model. JEV NS1 full length model created by 740 using SWISS-MODEL Homology Modelling. Dimerization was generated by superimposition of the JEV NS1 homology model to ZIKV NS1 PDB ID 5GS6. (a) 741 Cross-shaped homodimer NS1. One subunit is coloured in grey and another is coloured 742 by domain. β-roll (amino acid residues 1-29) domain is coloured in green, wing (38-151) 743 744 domain is coloured in blue, and β-ladder domains (181-352) is coloured in brown. (b) Side view of NS1. Residues 108-128 of the JEV homology model are indicated in 745 746 magenta. Residues 108-128 are disordered and not visible in DENV 406B, WNV 406C, 747 but they are visible in ZIKV 5GS6 (shown in yellow) and 5K6K. Hydrophobic residues (28, 115, 118, 123, and 160-163) suspected to involve with cell membrane interaction 748 749 are labelled.

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Number	Residue	ASA (Å <sup>2</sup> ) <sup>1</sup>	BSA (Å <sup>2</sup> ) <sup>2</sup>	ΔG (kcal/mol) <sup>3</sup>	Conservation <sup>4</sup>
1	Gly181	23.56	6.81	0.03	3
2	Ala182	91.29	45.68	0.30	1
3	lle184	22.47	16.36	-0.18	5
4	Gly185	40.33	16.05	0.26	7
5	Thr186	37.46	36.73	-0.22	7
6	Ala187	63.58	21.49	0.34	9
7	Val188	64.98	63.40	0.34	6
8	Lys189	181.59	9.98	0.16	8
9	Gly190	63.17	54.83	0.30	5
10	His191	110.70	33.66	0.74	1
11	Trp210	60.01	29.42	0.08	5
12	Glu227	104.51	54.79	0.51	5
13	Thr228	120.12	94.74	0.66	6
14	His229	54.08	52.04	0.90	9
15	Thr230	24.89	21.26	-0.20	9
16	Leu231	48.09	48.09	0.77	8
17	Trp232	95.68	59.18	0.38	5
18	Gly233	39.95	30.60	-0.02	4
19	Asp234	91.67	54.93	0.26	6
20	Asp235	128.72	0.58	-0.01	1
21	His254	13.56	10.75	0.73	8

#### 751 Table 1 JEV NS1 C-terminus dimer interfacing residues

752 <sup>1</sup> ASA= Accessible Surface Area

<sup>2</sup>BSA= Buried Surface Area

 $^{3}\Delta G$ = Solvation energy effect

<sup>1,2,3</sup> Assembly analysis in the program PISA.

<sup>4</sup>Amino acid conservation score are given by Consurf. (9 = conserved and 1 = variable)

758	Number	Structure 1	Distance (Å) <sup>1</sup>	Structure 2
759	1	Gly190 [N]	2.93	lle184 [O]
760	2	Val188 [N]	2.86	Thr186 [O]
761	3	Thr186 [N]	2.89	Val188 [O]
,01	4	His229 [NE2]	2.83	Gly190 [O]
762	5	His254 [NE2]	2.94	Thr228 [O]
763	6	Trp232 [N]	2.96	Thr230 [O]
764	7	lle184 [O]	2.93	Gly190 [N]
765	8	Thr186 [O]	2.86	Val188 [N]
,05	9	Val188 [O]	2.89	Thr186 [N]
766	10	Gly190 [O]	2.83	His229 [NE2]
767	11	Thr228 [O]	2.94	His254 [NE2]
768	12	Thr230 [O]	2.96	Trp232 [N]

#### 757 Table 2 Hydrogen bonds between JEV NS1 C-terminus dimer interfacing residues

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<sup>1</sup> Assembly analysis in the program PISA.

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#### Table 3 Residues forming hydrogen bond at dimer interface compared with

773 existing flavivirus NS1

JEV	ZIKV			WNV	WNV			DENV	
	5k6k	5gs6	5iy3	406d	406c	4oie	406b	4oig	
	Asp1	His1		Asp1	Asp1				
	Val2	Val2		Thr2	Thr2		Ser2		
	Cys4	Cys4		Cys4	Cys4		Cys4		
	Ser5	Ser5							
	Val6	Val6		lle6	Val6		lle6		
	Phe8								
	Ser9								
				Arg10	Arg10				
	Lys11								
	Glu12			Glu12	Glu12				
				Leu13					
	Arg14	Arg14		Arg14	Arg14		Lys14		
	Thr17	Thr17		Ser17	Ser17		Ser17		
	Val19	Val19		Val19	Val19		lle19		
	Phe20	Phe20		Phe20	Phe20				
	lle21	Val21		lle21	lle21		lle21		
	Tyr22	Tyr22							
	Asn23	Asn23		Asn23	Asn23		Asp23		

JEV	ZIKV			WNV			DENV			
	5k6k	5gs6	5iy3	406d	406c	4oie	406b	4oig		
	Asp24	Asp24		Asp24	Asp24					
	Arg31			Arg31	Arg31					
	Tyr32			Tyr32	Tyr32					
	Asp157	Asp157								
							Tyr158			
				Phe160						
	Thr165			Thr165	Thr165					
				Ser181	Ser181					
				Lys182	Lys182			Arg18		
lle184	lle184	lle184	lle184							
								Ser18		
Thr186	Ala186	Ala18								
Val188	lle188	lle188								
	Lys189			Lys189	Lys189			Lys18		
Gly190	Gly190	Gly190	Gly190					Asp19		
		Lys191		Asn191	Asn191					
	192Glu	Glu192								
	193Ala									
	Glu203			Glu203						
	Lys227	Lys227	Lys227							

Σ

JEV	ZIKV			WNV			DENV	
	5k6k	5gs6	5iy3	406d	406c	4oie	406b	4oig
Thr228	Ser228	Ser228	Ser228	Thr228	Thr228	Thr228	Ser228	Ser228
His229	His229	His229	His229					
Thr230								
Trp232								
	Thr233	Thr233	Thr233				Ser233	Ser233
	Asp234	Asp234	Asp234				Asn234	Asn234
His254								

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775 Note: shared residues are shaded in grey.

JEV	ZIKV	WNV	DENV	Conservation <sup>1</sup>		
	5IY3	40IE	40IG	CONSELVATION		
Pocket 1						
Gly259	Gly259	Gly259	Gly259	9		
Tyr260	Tyr260	Tyr260	Tyr260	9		
Lys261	Arg261	Lys261	Phe261	1		
			Ala265	1		
Ser292	Gly292	Gly292	Gly292	1		
Lys293	Thr293	His293	Asn293	1		
Arg294	Arg294	Arg294	Arg294	9		
Cys313				9		
Arg314	Arg314	Arg314	Arg314	9		
Ser315	Glu315	Ser315	Ser315	5		
Cys316	Cys316	Cys316	Cys316	9		
Glu334	Glu334	Glu334	Glu334	9		
Pocket 2						
	Thr262	Thr262	Thr262	6		
	Met264	Asn264	Thr264	1		
	Lys265			1		
	Gly295	Gly295	Gly295	9		
	Pro296	Pro296	Pro296	4		
		Gly332		6		
	Met333	Met333	Met333	9		
	Thr351	Asn351	Ser351	3		
Pocket 3						
Gly295				9		
Pro296				4		
Ser297		Ala297	Ser297	9		

## Table 4 Residues forming positively charge pockets compared to existed C-NS1.

JEV	ZIKV	WNV	DENV	Conservation <sup>1</sup>			
	5IY3	40IE	40IG	Conservation			
Val298		Thr298	Leu298	1			
Arg336		Arg336	Arg336	9			
Pro337		Pro337	Pro337	9			
Met339				3			
			Glu340	2			
		Glu342	Glu342	8			
Leu345		Leu345	Leu345	6			
Arg347		Gln347	Lys347	3			

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<sup>1</sup>Amino acid conservation score are given by Consurf. (9 = conserved and 1 = variable)

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## 779 Table 5 Sulphate contact residues from assembly analysis in the program PISA

780 (57)

Area	C-JEV	ZIKV	WNV	C-DENV
Area	C-JEV	5K6K	406C	40IG
	Arg347	Ser342		His309
	Gln349	Glu343		Glu310
	Thr302	Thr302		Lys339
Тір	Ser304	Ser304		
	Lys306	Arg306		
	Thr343			
	Thr344			
Positively charge pockets	Arg294	Arg294		
rosilively charge pockets	Arg314	Arg261		
	Asp235		Gly235	His181
				Lys206
				Thr210
Central				Ser228
				Trp232
				Asn234
				Gly235

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## 784 **Table 6 Data collection and refinement statistics.**

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	JEV NS1-C	JEV NS1'-C
Data collection		
Space group	I212121	I212121
Cell dimensions		
a, b, c (Å)	49.42, 78.24, 163.18	50.32, 77.94, 163.49
a, b, g (°)	90, 90, 90	90, 90, 90
Resolution (Å)	47.3-2.10 (2.21-2.10)	81.75-2.6 (2.72-2.6)
R <sub>merge</sub>	0.103 (0.907)	0.2 (1.413)
R <sub>pim</sub>	0.045 (0.383)	0.141 (1.030)
Ι/σΙ	11.5 (2.3)	7.1 (2.1)
Completeness (%)	99.8 (99.6)	99.9 (99.9)
Redundancy	6.3 (6.5)	5.3 (5.2)
Refinement		
Resolution (Å)	47.3-2.10	81.75-2.6
No. reflections	17944	9719
R <sub>work</sub> / R <sub>free</sub>	0.189/0.228	0.166/0.225
No. atoms	1574	1573
Protein	1398	1418
Sulfate ion	60	60
Ligand	24 (MES)	4 (POL)
Water	92	91
B-factors		
Protein	41.516	38.612
Sulfate ion	90.121	85.148
Ligand	86.736 (MES)	61.722 (POL)
Water	50.187	48.669
R.m.s. deviations		
Bond lengths (Å)	0.016	0.016
Bond angles (°)	1.785	1.741

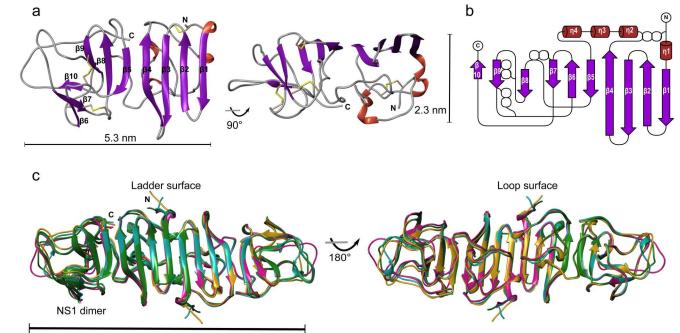
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	· EEEEEEEE *	SSSSSS	REEEAQ	LKKKLK	NNNNNN	DDDEDG	· T T T T T T T S T	WWWW	KKRRKKKQ		EKKAEE	RRRK	A A A A A A	VHHSSSS	LLFFL	GIII	E			Downloaded from http
SSSS	NHHQQ	HHHHHH	NNNNNN	RTTYYH	RRRRRR	PEEPPP	G G G G G G G G G G G G	YYYYY	KKRRFHH	TTTTTT	· QQQQQQQ *	NMVTIT	QKKATA	GGGGGG	PPPPP			)         		://jvi.asm.org/ on Februar
TTTTT	. DEAAVAVA	S S S T S	GGGGG	KRRKK			TTEEHT	EEE		CCCCC	CCCCCC	RRRRRR R	SEESSS	CCCC	TTTTTT		PPPFF			Downloaded from http://jvi.asm.org/ on February 21, 2018 by University of Liverpool Library
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JEV_M55506 WNV_AF196835 ZIKV_KU365779.1 ZIKV_AY632535 DENT1_U88535 DENV2_M84727 DENT3_EF643017 DENT4_AF326573 Clustal Consensus	KIREESTD KVRESNTT KVREDYSL KVREDYSL KLRDSYTQ KLKEKQDV KLREVYTQ	E C D G A I I G T A Y E C D S K I I G T A Y E C D P A V I G T A Y E C D P A V I G T A Y V C D H R L M S A A F C D S K L M S A A	V K Ġ H V A V H S V K N N L A I H S V K G K E A V H S V K G R E A A H S I K D S K A V H A I K D N R A V H A V K D E R A V H A I K D Q K A V H A	200 DL SYWIE SRY DL SYWIE SRL DL GYWIE SEK DL GYWIE SEK DMGYWIE SEK DMGYWIE SAL DMGYWIE SQK DMGYWIE SQK DMGYWIE SSK *:.****
JEV_M55506 WNV_AF196835 ZIKV_KU365779.1 ZIKV_AY632535 DENT1_U88535 DENV2_M84727 DENT3_EF643017 DENT4_AF326573 Clustal Consensus	VKSCTWPE VKSCTWPE MKTCEWPK MKTCEWPK VKTCIWPK VKTCIWPK VKTCTWPK VKTCLWPK	T H T L WG D D V E T H T L WG D G I L S H T L WT D G I E S H T L WT D G V E S H T L WS N G V L S H T L WS N G V L S H T L WS N G V L	E S E L I I P H T E S D L I I P V T E S D L I I P K S E S D L I I P K S E S E M I I P K I E S E M I I P K N E S D M I T P K S E S Q M L I P K S	250 I A G P K S K H N R L A G P R S N H N R L A G P L S H H N T L A G P L S H H N T Y G G P I S Q H N Y L A G P V S Q H N Y L A G P F S Q H N Y Y A G P F S Q H N Y . * * * : * *
JEV_M55506 WNV_AF196835 ZIKV_KU365779.1 ZIKV_AY632535 DENT1_U88535 DENV2_M84727 DENT3_EF643017 DENT4_AF326573 Clustal Consensus	ENGIVLDF EGRVEIDF SEELEIRF SEELEIRF LGKLELDF LGKLEMDF LGKLELDF	D Y C P G T K V T I D Y C P G T T V T L E E C P G T K V H V E E C P G T K V Y V D L C E G T T V V V D F C D G T T V V I N Y C E G T T V V I	TEDCSKRGP SESCGHRGP EETCGTRGP EETCGTRGP DEHCGNRGP TEDCGNRGP TENCGTRGP QEDCDHRGP	300 S V R T T T D S G K I A T R T T T E S G K I S L R S T T A S G R S L R S T T A S G R S L R T T T V T G K S L R T T T V T G K S L R T T T V S G K I S L R T T T A S G K I S L R T T T A S G K I S L R T T T A S G K I S L R T T T A S G K I
JEV_M55506 WNV_AF196835 ZIKV_KU365779.1 ZIKV_AY632535 DENT1_U88535 DENV2_M84727 DENT3_EF643017 DENT4_AF326573 Clustal Consensus	PLRFRTEN PLRYQTDS PLSFRAKD PLSFRAKD PLRFKGED PLRYRGED PLRYMGED	330 G CWY G ME I R P G CWY G ME I R P	V M H D E T T L V Q R H D E K T L V R K E P E S N L V R K E P E S N L V V K E K E E N L V L K E K E E N L V I S E K E E N M V	R S Q V D A Q S Q V N A R S M V T A R S M V T A K S M V S A N S L V T A K S L V S A K S Q V T A

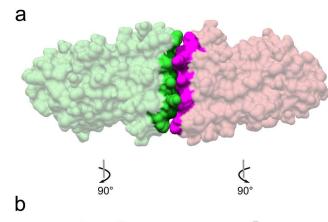
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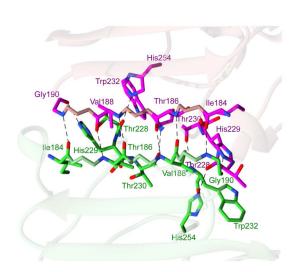
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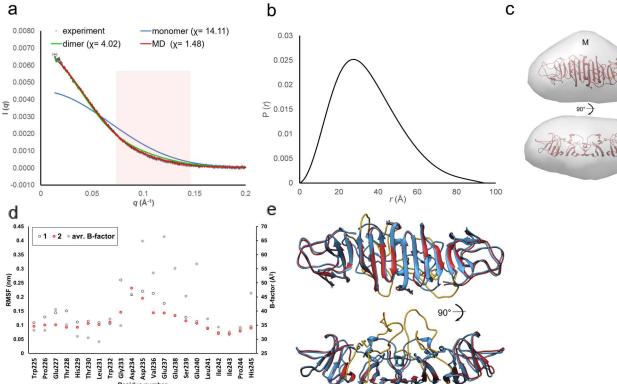


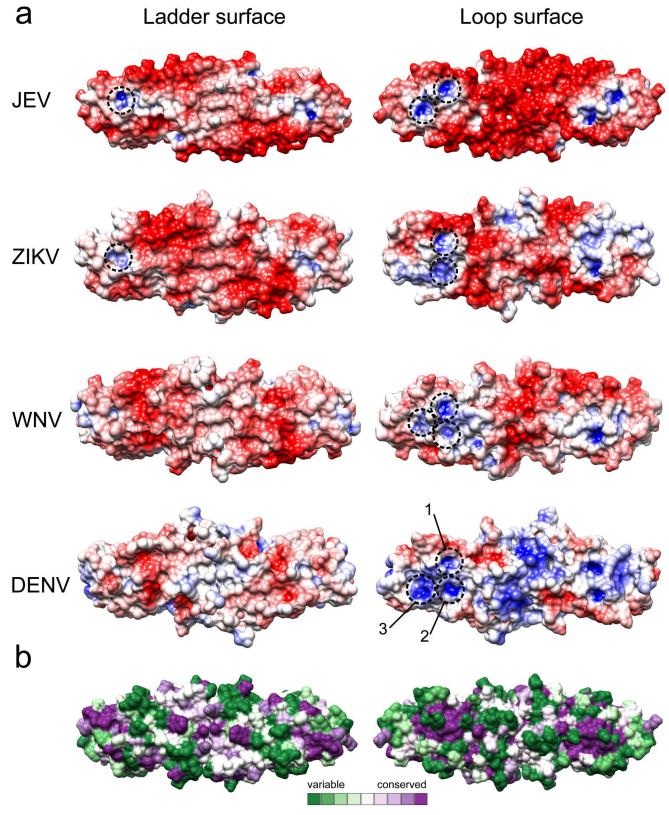


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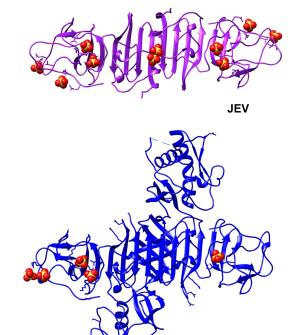
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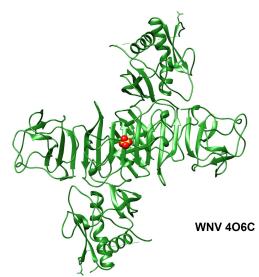


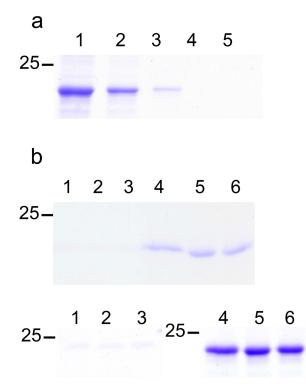
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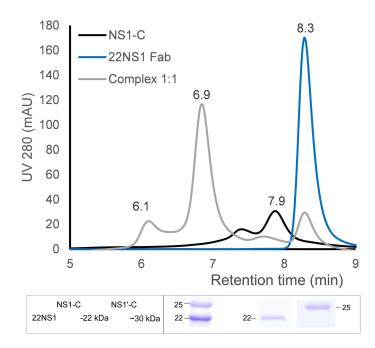


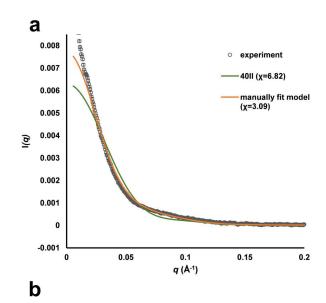
ZIKV 5K6K

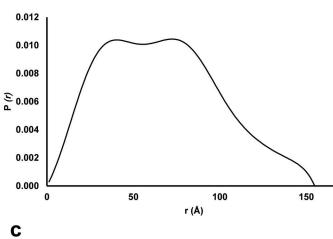


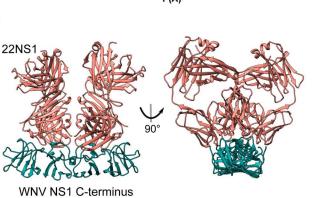


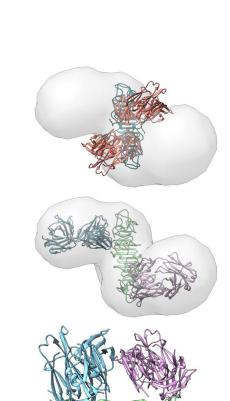






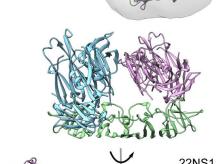


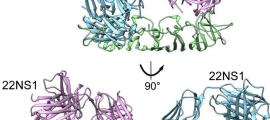




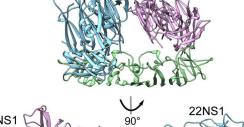
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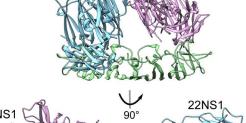
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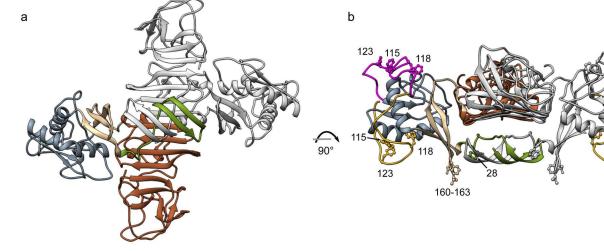


JEV NS1 C-terminus









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