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Structure and flexibility of Non-structural proteins 3 and -5 of Dengue- and Zika viruses in solution

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Abstract

Dengue- (DENV) and Zika viruses (ZIKV) rely on their non-structural protein 5 (NS5) including a methyl-transferase (MTase) and a RNA-dependent RNA polymerase (RdRp) for capping and synthesis of the viral RNA, and the non-structural protein 3 (NS3) with its protease and helicase domain for polyprotein processing, unwinding dsRNA proceeding replication, and NTPase/RTPase activities. Accumulation of data for DENV- and ZIKV NS3 and NS5 in solution during recent years provides information about their overall shape, substrate-induced alterations, oligomeric forms and flexibility, with the latter being essential for domain-domain crosstalk. The importance and differences of the linker regions that connect the two domains of NS3 or NS5 are highlighted in particular with respect to the different DENV serotypes (DENV-1 to -4) as well as to the sequence diversities between the DENV and ZIKV proteins. Novel mutants of the French Polynesia ZIKV NS3 linker presented, identify critical residues in protein stability and enzymatic activity.

1. Introduction

DENV, consisting of four antigenically distinct serotypes (DENV-1 to -4), and ZIKV belong to the mosquito-transmitted flaviviruses (*Flaviviridae* family), which contain over 90 different viruses, including the most important arthropod-transmitted human viral pathogens such as yellow fever (YFV), West Nile (WNV), Japanese encephalitis (JEV) and tick-borne encephalitis (TBEV) viruses (Pierson and Diamond, 2013). Both are single-stranded positive-sense RNA viruses, whose genome translates into a single polyprotein that is subsequently cleaved by host-cell proteases and viral NS2B/NS3 protease into three structural (C, prM/M, and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5).

The replication of the viral genome takes place in the ER-membrane associated replication complexes (RC), including NS- and host proteins (Fernandez-Garcia et al., 2009; Mackenzie, 2005; Welsch et al., 2009). NS3 and NS5 reside within the functional RC-center and were considered as promising targets for the development of antiviral inhibitors (Lim et al., 2015).

The 70 kDa NS3 possesses protease activity for polyprotein cleavage, helicase activity for unwinding dsRNA proceeding replication, and NTPase/RTPase activities essential for genome capping (Luo et al., 2008a). The N-terminal domain of NS3 forms the protease, which is connected by a 10-residues linker to the helicase (Fig. 1A). The protease is a chymotrypsin-like serine protease that participates in the proteolysis of the polyprotein, to release the individual viral proteins from a long polyprotein. The protease requires the central domain of NS2B as a cofactor for proper folding. The presence of residues 49 to 67 of NS2B is sufficient to stabilize the protease domain (Erbel et al., 2006; Luo et al., 2008a), but the residues 49 – 96 are essential for enzymatic activity and stability (Clum et al., 1997). The C-terminal helicase possesses NTPase-dependent RNA helicase activity, which may unwind the double-stranded RNA during RNA replication using

1 the energy supplied by the NTP hydrolysis (Lindenbach et al., 2007; Luo et al., 2015; Warren et
2 al., 1993). Besides the unwinding activity, the helicase has also been suggested to play a role in
3 RNA-capping. It catalyzes the first RNA-capping reaction termed RNA triphosphatase activity,
4 where the γ -phosphate, located at the 5'-end of newly synthesized RNA, is hydrolyzed to create a
5 diphosphate RNA, which will be then directed to the MTase of NS5 to complete RNA-capping
6 (Ferron et al., 2012; Wengler and Wengler, 1993).

7 The 100 kDa DENV and ZIKV NS5s harbor multiple enzymatic activities: RdRp activity
8 required for genome replication and MTase/guanylyltransferase (GTase) activities for genome
9 capping (Egloff et al., 2002; Issur et al., 2009) (Fig. 2A). The cap, a 7-methylguanosine (m⁷G)
10 moiety linked to the first nucleotide of the transcript via a 5'-5' triphosphate bridge, has the roles
11 to protect viral mRNA from degradation by 5' exoribonucleases, to confer stability to mRNAs and
12 to ensure their efficient recognition by eukaryotic translation initiation factor 4E (eIF4E) for
13 translation (Decroly et al., 2012; Liu and Kiledjian, 2006). The MTase of flaviviral NS5 has been
14 shown to contain both N-7 and 2'O methylation activities to form the Type 1 cap (m⁷Gppp-N_{m2}'O)
15 (Dong et al., 2010). The RNA GTase function is attributed to the N-terminal segment of the MTase
16 domain (Fig. 2A) and it has been described that NS3 stimulates this activity (Issur et al., 2009).
17 The MTase forms the N-terminal domain of NS5 and contains the N-terminal subdomain, the core
18 MTase subdomain, and the C-terminal subdomain. The N-terminal subdomain includes the GTP-
19 binding site, while the core MTase subdomain is responsible for S-adenosyl-methionine (SAM)-
20 binding and catalytic activity (Egloff et al., 2002; Yap et al., 2010; Zhao et al., 2015c). The MTase
21 is connected via a 10-residues linker to the C-terminal RdRp (Fig. 2A), which contains the
22 canonical polymerase subdomains called the *fingers*, *palm*, and *thumb* (Davidson, 2009; Yap et al.,
23 2007; Zhao et al., 2015b). The *fingers* and *thumb* subdomains interconnect via the N-terminal

region of the RdRp and through loops protruding from the *fingers* subdomain to encircle the active site on the *palm* subdomain, forming a closed structure (Yap et al., 2007). Two tunnels that run perpendicular to each other allow access of the single-stranded RNA template to the active site on the *palm* subdomain and diffusion of dNTPs to the active site.

2. Crystallographic perspective of entire NS3 and NS5

In case of ZIKV, the atomic structures of the protease domain from the Brazilian ZIKV (ZIKV-B) (Lei et al., 2016) and East African strain MR 766 ZIKV (ZIKV-MR) (Zhang et al., 2016), the helicase from the French Polynesia ZIKV (ZIKV-FP) (Jain et al., 2016) as well as the ZIKV-MR (Tian et al., 2016) and MTase from ZIKV-MR (Coloma et al., 2016; Coutard et al., 2017; Zhang et al., 2017) were determined. However, the atomic structure of the entire ZIKV NS3 is not described yet. In comparison, the atomic structures of DENV-4 NS2B₁₈NS3, consisting of full-length NS3 and a short stretch of NS2B (residues 49-66), revealed two different protease-helicase arrangements, termed conformation I (PDB ID: 2VBC (Luo et al., 2008a); 2WZQ (Luo et al., 2010)) and conformation II (PDB ID: 2WHX (Luo et al., 2010)) (Fig. 1B). Compared to conformation I, the protease in conformation II rotates by 161° relative to the helicase (Fig. 1B), allowing the binding of ADP-Mn²⁺ to the helicase (Luo et al., 2010).

The DENV-3 NS5 structure (PDB ID: 4V0Q (Zhao et al., 2015b)), including residues 6-895 (NS5₆₋₈₉₅), adopts a compact shape with a total length of 8.7 nm, and an MTase domain, which is located above the *fingers* subdomain of the RdRp, such that its GTP-binding- and the SAM-binding sites are oriented away from the inter-domain interface (Zhao et al., 2015b) (Fig. 2B). This assembly arrangement of MTase and RdRp is proposed to be established by four residues (263–266, DENV-3), forming a short 3₁₀-helix (Zhao et al., 2015b).

1 In case of ZIKV, the atomic structures of NS5 from ZIKV-MR (PDB IDs: 5TFR, 5U0B)
2 (Upadhyay et al., 2017; Zhao et al., 2017) showed two molecules of NS5 inside the asymmetric
3 unit forming a dimer, where the MTase domains are oriented relative to each other. In comparison
4 to DENV-4 NS5, the MTase of ZIKV NS5 undergoes a relative rotation of about 100° to its RdRp,
5 shielding the rim of the NTP entry channel (Fig. 2C). This arrangement exposes residue E196 of
6 the ZIKV MTase and R681 of the RdRp at one side and makes both residues accessible to interact
7 with the respective residues of a second NS5 to form a dimer.

8 9 **3. DENV- and ZIKV NS3 in solution**

10 Small angle X-ray scattering (SAXS) studies of DENV- and ZIKV NS3, and their individual
11 protease and helicase domains revealed that all of these proteins are monomeric in solution. Both
12 DENV- and ZIKV NS3 are elongated in solution (Pan et al., 2017; Saw et al., 2017). These studies
13 demonstrated that the entire two-domain enzymes are flexible, and that the flexibility is mainly
14 caused by the linker region of NS3 connecting the two domains. An EOM (ensemble optimization
15 method) analysis showed that the protease of NS3 adopts a random movement with respect to the
16 helicase (Pan et al., 2017; Saw et al., 2017) (Fig. 1D). Considering that the protease of NS3 is able
17 to cleave in *cis* at the NS2A-NS2B junction and in *trans* at the NS3-NS4A junction (Falgout et al.,
18 1991; Preugschat et al., 1990), the two-domain NS3 should adopt a flexible movement, so that the
19 protease can reorient and extend itself to carry out its proteolytic function.

20 In addition, conformational changes in macromolecules upon substrate-binding and catalysis
21 are common features of enzymatic mechanisms and required for optimal enzyme turnover
22 (Eisenmesser et al., 2005; Hammes, 2002). Recent SAXS data of DENV-2 NS2B₁₈NS3 in the
23 presence of MgATP and its analogue MgAMP-PNP did not show any change in the overall

dimensions of the enzyme, whereby MgADP-binding led to a slight decrease of the R_g -value (Pan et al., 2017). Interestingly, the solution X-ray studies demonstrated that RNA induced a significant change in DENV-2 NS3 (Pan et al., 2017). Models calculated based on X-ray scattering data of RNA-free and RNA-bound NS3 indicated different movements of the protease with respect to the helicase in the absence or presence of RNA (Fig. 1D). One of the plausible models of the RNA-free form revealed large translational movements of the protease when compared to the crystal structure (PDB ID: 2VBC (Luo et al., 2008a)), while in case of the RNA-bound form, its protease domain had largely rotational movement. Previously, Luo et al. (2008b) described that binding of ssRNA to the helicase triggered a slight conformational change within the helicase and changed the phosphate-loop (P-loop) into the NTP-bound conformation (Luo et al., 2008b). Further analysis of the DENV-4 NS2B₁₈NS3 crystallographic structure that adopted conformation II (Luo et al., 2010) identified a few interactions among the protease, linker region and the P-loop of the helicase (Luo et al., 2010; Pan et al., 2017). Therefore, the interaction of RNA and NS3 may induce a conformational change in the P-loop of the helicase and thereby affecting ATP hydrolysis, followed by a cascade of conformational changes to the linker and the protease, which undergoes substantial conformational alterations required for optimal catalysis of bound RNA.

4. Differences of DENV- and ZIKV NS3 linker residues

The sequence comparison of the linker residues of ZIKV NS3s from the French Polynesia, Brazil and Singapore lineages represents the identity of the RREEETPVECF-linker sequence (Fig. 1E). While comparing with the linker region of NS3s of DENV-1 to -4, all of them show a significant difference in their amino acid content. A closer look to this region inside DENV-4 NS3 (PDB ID: 2WHX) (Luo et al., 2010) highlights that the linker residues E169 and G172 have

interactions with K88 and W83 of the protease, respectively, whereby the linker residues E173 and D179 form hydrogen bonds with the helicase residues R202 and I182, respectively. R202 belongs to the P-loop region that stabilizes the transition state during ATP hydrolysis. Therefore, any changes in the linker residues will directly affect ATP hydrolysis as well as the flexibility between the two enzyme domains.

To gain further insights into the interaction between linker and the two domains in ZIKV-FP NS3, the model of the full length ZIKV NS3 was created by aligning the protease (PDB ID: 5LC0) (Lei et al., 2016) and helicase (PDB ID: 5GJB) (Tian et al., 2016) domains of ZIKV NS3 according to the position in the DENV-4 NS3 structure (PDB ID: 2WHX) (Luo et al., 2010), and the linker region was built and energy minimized (Saw et al., 2017). This model revealed that the ZIKV NS3 linker residues T174 and P175 form hydrogen bonds ~~interaction~~ with W83 and Y68 of the protease domain, respectively, and V176 interacts with the P-loop residue R203 of the helicase (Fig. 1E). While the interactions with protease residue W83 and helicase residue R203 are preserved in both, the ZIKV NS3 model shows two interactions of the linker with the protease and one with the helicase. In comparison, the DENV NS3 linker is connected via two interactions, each to the protease and helicase domain, implicating a slightly higher flexibility for ZIKV than DENV NS3 as evidenced by the flexibility analysis of the experimental data (Saw et al., 2017).

5. Importance of ZIKV NS3 linker residues for protein stability

5.1 Generation of ZIKV NS3 linker mutants and analysis of their protein stability

In order to investigate the importance of the conserved ZIKV NS3 linker residues, the linker mutants NS2B₁₈NS3_{T174A}, NS2B₁₈NS3_{V176A}, NS2B₁₈NS3_{E177A}, NS2B₁₈NS3_{C178A}, NS2B₁₈NS3_{F179A} and NS2B₁₈NS3_{F179I}, each including the residues 49 to 67 of NS2B, were

generated and purified using same strategies as stated in Saw et al. (2017). In brief, the mutant constructs were cloned using the primers listed in Table 1 and the positions of the mutated amino acids are underlined. The pET32b plasmid encoded ZIKV-FP NS2B₁₈NS3 (Saw et al., 2017) was used as the template to clone all the mutants. The mutations were confirmed by DNA sequencing (Axil Scientific, Singapore), and the plasmids with the confirmed mutated sequences were transformed into *E. coli* BL21-CodonPlus (DE3)-RIL cells (Stratagene, USA) for protein production. To purify recombinant ZIKV-FP NS3 mutants, Ni-NTA affinity chromatography was employed as the first step of purification. The fractions containing the respective ZIKV-FP NS3 mutants eluted at an imidazole concentration of 75-100 mM using an imidazole gradient from 20 mM to 500 mM. The Trx-His₆-tag of the recombinant NS3 mutants was cleaved overnight and separated from the soluble NS3 mutants by size exclusion chromatography (SEC) (Superdex™ 200 Increase 10/300 column).

The SEC chromatogram of NS2B₁₈NS3_{T174A}, NS2B₁₈NS3_{V176A}, NS2B₁₈NS3_{E177A}, and NS2B₁₈NS3_{C178A} revealed that all mutants eluted later compared to the wild-type (WT) protein (Fig. 3A). When applied onto an SDS gel, NS2B₁₈NS3_{T174A} and NS2B₁₈NS3_{V176A} showed a band corresponding to the 70 kDa NS3 mutant and a band at 22 kDa, indicating degradation of both mutants (Fig. 3B). In comparison, complete degradation was observed for NS2B₁₈NS3_{E177A} and NS2B₁₈NS3_{C178A} (Fig. 3B). Although the same wet weight of cells was used for the purification of all four mutants, the peak intensities of the elution profiles as well as the band intensities of the SDS gels reflect either a reduction in production of NS2B₁₈NS3_{E177A} and NS2B₁₈NS3_{C178A}, and/or their solubility. These data demonstrate that expression, stability, and/or solubility of NS2B₁₈NS3_{E177A} and NS2B₁₈NS3_{C178A} are strongly affected. In contrast, stable ZIKV-FP NS2B₁₈NS3_{F179A} and NS2B₁₈NS3_{F179I} mutants could be purified to high purity as presented in the

1 SDS gel (Fig. 3C, *inset*) and these recombinant mutants (top 15% of the eluted peaks; Fig. 3C)
2 were used for SAXS studies.

3 4 5.2 SAXS experiments of ZIKV NS3 linker mutants

5 SAXS data of ZIKV-FP NS2B₁₈NS3_{F179A} (2.5 mg/ml) and NS2B₁₈NS3_{F179I} (6.0 mg/ml) were
6 recorded using the instruments and parameters stated in Table 2. After subtracting the scattering
7 of the buffer from the protein data, the SAXS patterns of both mutants were normalized with
8 protein concentrations as well as the incoming intensity (Fig. 3D). The primary data analysis was
9 performed using the program PRIMUS (Konarev et al., 2006; Konarev et al., 2003). The Guinier
10 plots at low angle appeared linear and confirmed good data quality with no indication of protein
11 aggregation (Fig. 3D, *right inset*). From the Guinier approximation, the derived radius of gyration
12 (R_g) for NS2B₁₈NS3_{F179A} and NS2B₁₈NS3_{F179I} was 3.38 ± 0.06 nm and 3.31 ± 0.05 nm, respectively.
13 The overall structural parameters are recorded in Table 2. The $P(r)$ function showed a single peak
14 with a small tail (Fig. 3E), and the maximum particle dimension (D_{max}) of 11.0 ± 1 nm for both
15 mutants, indicating that both mutants have an elongated conformation in solution as described for
16 the WT enzyme. The R_g -values extracted from the $P(r)$ function were 3.48 ± 0.02 nm and $3.43 \pm$
17 0.02 nm for ZIKV-FP NS2B₁₈NS3_{F179A} and NS2B₁₈NS3_{F179I}, respectively, which were in
18 agreement with the R_g -values extracted from the Guinier region (Table 2).

19 The SAXS-data were further analyzed to access the potential flexibility of the protein. The
20 normalized Kratky plot provides the information about the conformational behavior of an
21 examined particle (Durand et al., 2010). In comparison to the globular lysozyme, the normalized
22 Kratky plot of ZIKV-FP NS2B₁₈NS3_{F179A} and NS2B₁₈NS3_{F179I} exhibited a broad bell-shaped

profile shifted toward right (Fig. 3F), indicating the presence of motion as well as being extended. The profile is comparable to that of WT ZIKV-FP NS2B₁₈NS3 (Fig. 3F) (Saw et al., 2017).

5.3 Enzymatic characterization of ZIKV NS3 mutant proteins

ATP hydrolysis provides the energy to power the translocation and the unwinding process of NS3. To study whether the substitution of F179 to alanine or isoleucine may alter the enzymatic traits, ATP hydrolysis measurements of WT ZIKV-FP NS2B₁₈NS3, NS2B₁₈NS3_{F179A} and NS2B₁₈NS3_{F179I} were performed in accordance to the protocol described in Saw et al. (2017). In brief, in this assay cleaved ATP was constantly regenerated by an enzymatic reaction, while the consumption of NADH was measured spectroscopically at 340 nm. The change in absorbance was measured for 250 s in 2 s intervals at 37 °C after adding 5 µg of proteins. The enzymatic data were collected with ATP-concentration between 25 to 2000 µM in triplicate. Its activity was derived by fitting the linear section of the slope from the averaged data. The Michaelis constant (K_m) and the maximum rate (V_{max}) of NS2B₁₈NS3 were determined using the program Origin Pro 9.0 (OriginLab Corporation).

The ATP hydrolysis activities of WT ZIKV-FP NS2B₁₈NS3, NS2B₁₈NS3_{F179A} and NS2B₁₈NS3_{F179I} in 2 mM ATP were determined to be 474.5 ± 7.8 , 405.9 ± 2.64 and 472.7 ± 4.75 U/mM of protein, respectively (Fig. 3G). To further characterize the enzymatic parameters of NS2B₁₈NS3_{F179A}, the Michaelis-constant (K_m) of 94.02 ± 7.5 µM and the catalytic turnover number (k_{cat}) of 6.90 ± 0.22 s⁻¹ were determined (Fig. 3H, Table 3), resulting in a catalytic efficiency of (k_{cat}/K_m) of 7.33×10^4 M⁻¹·s⁻¹. For NS2B₁₈NS3_{F179I}, a K_m of 108.08 ± 8.8 µM and a k_{cat} of 7.04 ± 0.27 s⁻¹ were determined (Fig. 3H, Table 3), with a k_{cat}/K_m of 6.51×10^4 M⁻¹·s⁻¹. When compared to WT NS2B₁₈NS3, mutant NS2B₁₈NS3_{F179A} has a lower k_{cat} value, indicating lower substrate

conversion than the WT enzyme ($7.35 \pm 0.19 \text{ s}^{-1}$; Saw et al., 2017). Even though the substrate affinity is similar to WT NS2B₁₈NS3 ($86.3 \pm 8.4 \text{ }\mu\text{M}$; Saw et al., 2017), the catalytic efficiency of this mutant is lower ($8.50 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$). ZIKV NS2B₁₈NS3_{F179I} showed a k_{cat} value similar to WT but has a higher K_m when compared to both WT- and NS2B₁₈NS3_{F179A} (Table 3), indicating a lower affinity for the substrate. The catalytic efficiency of ZIKV-FP NS2B₁₈NS3_{F179I} is also lower than the WT enzyme. In summary, the mutational studies revealed that the specific ZIKV NS3 linker residues T174, V176, E177, and C178 are important for stability of the enzyme, identifying this ZIKV NS3 linker sequence as a novel drug target. Mutating the specific ZIKV NS3 linker residue F179 resulted in a stable and elongated protein in solution, with slight alterations in enzyme kinetics when compared to its WT counterpart.

6. DENV NS5 is extended and flexible in solution

Solution SAXS of the full length 900 amino acids NS5 from DENV-1 to -4 described monomeric NS5 in which the MTase and RdRp ensemble is extended, with maximum particle dimensions between 11.0 nm to 11.5 nm (Saw et al., 2015). EOM calculations using SAXS data, wherein the flexibility of the MTase with respect to the RdRp was considered, revealed that DENV NS5 has an ensemble of bent and elongated conformations (Saw et al., 2015). These studies demonstrated the flexible nature of DENV NS5 with the MTase existing in several different conformations in solution (Fig. 2D), which is vital for its catalytic function and also for the preferential interaction with RdRp and helicase of NS3 during replication as described for the presence of capped-RNA below.

The SAXS data demonstrated also a more compact shape profile of DENV-4 NS5 compared to NS5s of the other serotypes. Inserting the genetically engineered linker from DENV-4 into

DENV-3 NS5 and *vice versa* reflected the importance of the 10-residue linker of NS5 in flexibility. Hydrogen/deuterium exchange mass spectrometry of NS5 from DENV-3 and -4 highlighted the higher deuterium-exchange in the linker region of DENV-4 compared to DENV-3 NS5, which may reflect a degree of dynamism that enables DENV-4 NS5 to become slightly more compact in the conformational interplay of complex and extended formation during catalysis (Saw et al., 2015). A variety of single, double, triple and quadruple mutants of DENV-4 NS5 combined with solution X-ray scattering experiments unravelled that the residues K271, S266 and T267 are important for the compactness of DENV-4 NS5 and therefore, may be critical for the regulation of virus replication (Subramanian Manimekalai et al., 2016). Furthermore, quantitative characterization of the flexibility of these DENV-4 NS5 linker mutants using EOM revealed that these mutants possess a similar conformational distribution like DENV-3 NS5, confirming that these residues in the linker region cause higher compactness of DENV-4 NS5. As the linker region of NS5 is postulated to contribute to conformational regulation in order to achieve optimum viral replication (Zhao et al., 2015a), DENV-4 NS5 may have evolved an amino acid composition of its linker region, providing optimal conformation(s) for catalysis and ensemble formation inside the replication machinery of DENV-4.

The inter- and intra-ensemble formation and crosstalk require structural alterations and dynamic properties of the enzymes involved. Interestingly, solution studies showed that the MTase is more flexible compared to the compact RdRp domain (Saw et al., 2015). Such flexibility maybe essential for the multi-functional and sequential steps of mRNA capping inside the MTase. Recent studies of DENV-2 NS5 bound to GpppAGUUGUU or m7-GpppAGUUGUU compared with the RNA-free DENV-2 NS5 confirmed that the enzyme undergoes structural alterations due to substrate-binding, leading to a more extended enzyme complex (Saw et al., 2015). The observed

overall size increase of the capped-RNA bound form of DENV-2 NS5 suggests that either the MTase domain and/or the RdRp domain might undergo conformational changes that allow the entire NS5 to take up a more open or extended conformation necessary for the elongation step after the initiation/transition phase. Such conformational changes are inherent to the NS5 during RNA synthesis. These alterations would also provide the conformations needed for binding and/or dissociation of the helicase domain of NS3 during the sequential steps of RNA replication.

Interestingly, crystallographic structures of individual domains (Egloff et al., 2002; Yap et al., 2010), DENV-3 NS5 (Zhao et al., 2015c) or SAXS data of DENV-2 NS5 (Saw et al., 2015) showed that no significant changes occur due to the binding of smaller substrates GTP, GDP, S-adenosyl-methionine (SAM) or S-adenosyl-homocysteine (SAH).

7. Oligomeric form of ZIKV NS5s and its implications for the RC

Previous solution X-ray data of the ZIKV-FP NS5 demonstrated that in contrast to the monomeric DENV NS5, the ZIKV-FP NS5 is dimeric at a concentration of 1 mg/ml and becomes a mixture of dimers and tetramers at 1.5 - 2.5 mg/ml. The presented dimeric state of ZIKV-FP NS5 in solution, which fits well to the crystallographic arrangement of two neighboring MTase domains (PDB ID: 5TFR), may provide a platform to speculate that the proposed dimeric ZIKV-FP NS5 orients to two single NS3 proteins, which are bound to the membrane-embedded NS2B (Fig. 4). Hereby, subdomain III of the helicase and the *thumb* subdomain of the RdRp would provide the interacting epitopes as proposed for the DENV NS3-NS5 interaction, enabling long term coordinated synthesis of positive- and negative-strand RNA inside the RC (Tay et al., 2015). A dimeric arrangement of NS5 inside the NS3-NS5 ensemble may allow cooperative effects to occur for maximum catalysis of the four enzymes inside the NS3-NS5 complex.

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Figure Legends

Figure 1: Multiple sequence alignments of ZIKV- and DENV NS3 proteins. (A) Multiple sequence alignment of NS3 proteins of ZIKV isolated from French Polynesia (GenBank: [KJ776791](#)), Brazil (GenBank: [KU729217](#)), Singapore (GenBank: [KX827309](#)) and NS3 from DENV-1 (GenBank: [EU081230](#)), DENV-2 (GenBank: [GQ398264](#)), DENV-3 (GenBank: [AY662691](#)) and DENV-4 (GenBank: [AY776330](#)). The alignment is colored based on percentage identity. The secondary structures derived from the crystallographic structure of ZIKV protease and helicase (PDB IDs: 5LC0 (Lei et al., 2016) and 5GJB (Tian et al., 2016)) are shown above the sequence alignment. The gap indicates the missing residues in the crystal structure. The secondary structures derived from the crystallographic structure of DENV-4 NS3 (PDB ID: 2VBC (Luo et al., 2008a)) are shown below the sequence alignment. The secondary structures are indicated by a box (α -helices), a line (loops) and an arrow (β -sheets), and colored in *purple* for the protease and in shades of orange for the three subdomains of helicase (*wheat* for subdomain 1, *orange* for subdomain 2 and *dark orange* for subdomain 3). The 10-residues linker region is shown in *red*, solid box. The seven motifs of helicase, namely Walker A, Motif Ia, Walker B, Motif III, Motif IV, Motif V and Motif VI, are shown in *red dashed box*. (B) Cartoon representation of crystal structure of DENV-4 NS3 in two different conformations. In conformation I of NS3 (PDB ID: 2VBC (Luo et al., 2008a)), the protease is situated at the entrance of the ATP-binding site between subdomains 1 and -2 of the helicase. In conformation II (PDB ID: 2WHX (Luo et al., 2010)), the protease is rotated by 161° with respect to the helicase. Conformation II was described to bind ADP-Mn²⁺ (Luo et al., 2010). The overlay of both structures shows a rotated protease with respect to the helicase. (C) Cartoon representation of an ensemble of seven possible orientations for the protease domain (in *shades of purple*) of DENV-4 NS2B₁₈NS3 relative to its helicase domain

(*orange*). The calculated ensemble scattering profiles fit well with experimental SAXS patterns (Pan et al., 2017). (*D*) The complete SREFLEX simulated movement of the protease domain of DENV-4 NS2B₁₈NS3 (*purple*) with respect to its helicase domain (*lemon green*) for the RNA (*blue*) bound form. Vectors are drawn connecting the equivalent residues from the crystal structure to the SREFLEX model. The crystal structure is not shown for clarity. (*E*) Interaction between ZIKV linker residues and protease or helicase domains. Linker residues (₁₆₉RREEETPVECF₁₇₉) in the ZIKV NS3 model generated based on the structure of DENV-4 NS3 (PDB ID: 2WHX). The protease (PDB ID: 5LC0) and helicase (PDB ID: 5GJB) domains of ZIKV were individually docked on to the full length DENV-4 NS3 structure and the linker residues were manually built and energy minimized. The protease and helicase domains are highlighted in *purple* and *orange*, respectively. The linker residues are depicted in *green* and the P-loop is colored in *blue*. The ZIKV NS3 linker residues T174 and P175 interact with W83 and Y68 of the protease, respectively, and the linker residue V176 interacts with the P-loop residue R203 of the helicase. The mutated residues, namely T174, V176, E177, C178, and F179 are shown in *sticks* and highlighted in *red*. (*Right inset*) Multiple sequence alignment of NS3 linker residues of ZIKV isolated from French Polynesia (GenBank: [KJ776791](#)), Brazil (GenBank: [KU729217](#)), Singapore (GenBank: [KX827309](#)) and DENV-1 (GenBank: [EU081230](#)), DENV-2 (GenBank: [GQ398264](#)), DENV-3 (GenBank: [AY662691](#)) and DENV-4 (GenBank: [AY776330](#)). The mutated residues are highlighted in *red* in the sequence.

Figure 2: (A) Multiple sequence alignment of NS5 proteins from ZIKV isolated from French Polynesia (GenBank: [KJ776791](#)), Brazil (GenBank: [KU729217](#)), Singapore (GenBank: [KX827309](#)) and from DENV-1 (GenBank: [EU081230](#)), DENV-2 (GenBank: [GQ398264](#)), DENV-

1 3 (GenBank: [AY662691](#)) and DENV-4 (GenBank: [AY776330](#)). The alignment is colored based
 2 on percentage identity. The secondary structures derived from the crystallographic structure of
 3 ZIKV-FP NS5 (PDB IDs: 5TFR (Upadhyay et al., 2017) are shown above the sequence alignment,
 4 and the secondary structures derived from the crystallographic structure of DENV-3 NS5 (PDB
 5 ID: 4V0Q (Zhao et al., 2015c)) are shown below the sequence alignment. The secondary structures
 6 are indicated by a box (α -helices), a line (loops) and an arrow (β -sheets), and indicated in *blue* for
 7 the MTase (*dark blue* for the N-terminal GTP-binding subdomain, *blue* for core subdomain and
 8 *light blue* for C-terminal domain), and in *magenta* for the RdRp (*magenta* for *fingers* subdomain,
 9 *dark magenta* for *palm* subdomain and *light magenta* for *thumb* subdomain) (Davidson, 2009).
 10 The 10-residue linker region, GTP-binding site, SAM-binding site, proposed protease interacting
 11 site, bNLS/helicase interacting site, a/b NLS and priming loop are highlighted in *red dashed boxes*.
 12 (B) Cartoon representation of the atomic structure of DENV-3 NS5 including residues 6-895 (PDB
 13 ID: 4V0R (Zhao et al., 2015c)). The MTase colored in *blue*, the 10-residues linker (residues 263
 14 to 272) is marked in *black* and the RdRp is colored according to their subdomains, where the
 15 *fingers* subdomain is *red*, the *palm* subdomain is *green*, and the *thumb* subdomain is *violet*. The
 16 proposed protein stretch interacting with NS3 is colored in *yellow*. Two substrates, GTP
 17 (Guanosine triphosphate) and SAH (*S*-adenosyl-homocysteine) inside the MTase are represented
 18 as *sticks* in *green*. (C) Superimposition of the monomeric atomic structure of ZIKV-FP NS5 (PDB
 19 ID: 5TFR) with NS5 of DENV-3 (PDB ID: 4V0R). The MTase and RdRp domains of ZIKV are
 20 colored in *light blue* and *magenta*, respectively. The MTase of DENV-3 NS5 is colored in *blue*.
 21 All MTase structures are represented in *ribbon*, and the RdRp structure is represented as *surface*.
 22 The superimposition shows that the residue R681 in ZIKV-FP NS3 resides on the same face as
 23 residue E196, while the residue E194 in DENV-3 NS5 (equivalent to E196 in ZIKV) is resided on

the opposite face. (D) An ensemble of six representative models (*cartoon representation*) for full length DENV-3 NS5 derived from the EOM calculations by maintaining the original position of RdRp domain (*magenta*), while giving positional freedom to the MTase domain (*blue*) during the calculation. The missing residues, which were re-built, are represented as *grey spheres*.

Figure 3: (A) Comparison of Superdex™ 200 Increase 10/300 column elution diagrams of purified ZIKV-FP NS2B₁₈NS3 (*black*), NS2B₁₈NS3_{T174A} (*red*), NS2B₁₈NS3_{V176A} (*blue*), NS2B₁₈NS3_{E177A} (*cyan*) and NS2B₁₈NS3_{C178A} (*green*). (B) 17% SDS gels of purified ZIKV linker mutants. Protein marker was loaded in the lane marked as *M*. The peak fraction of NS2B₁₈NS3_{T174A} (1), NS2B₁₈NS3_{V176A} (2), NS2B₁₈NS3_{E177A} (5) and NS2B₁₈NS3_{C178A} (3, 4) were applied onto the gel. Degradation of the purified linker mutants was observed on the gels. (C) Elution diagrams of ZIKV NS2B₁₈NS3_{F179A} (*green*) and NS2B₁₈NS3_{F179I} (*magenta*) purified using Superdex™ 200 Increase 10/300 column. The fractions from the shaded region were pooled and concentrated. (Inset) The SDS gels showed the bands of the purified NS2B₁₈NS3_{F179A} (*green*, 1) and NS2B₁₈NS3_{F179I} (*magenta*, 2), which were suitable for SAXS studies. Protein marker was loaded in the lane marked as *M*. (D) SAXS pattern (○) of NS2B₁₈NS3_{F179A} (*green*) at 2.5 mg/ml and NS2B₁₈NS3_{F179I} (*magenta*) at 6.0 mg/ml with a buffer composed of 20 mM Tris/HCl, pH 7.5, 200 mM NaCl and 1 mM DTT. (Right inset) Guinier plots show linearity at the concentrations measured, indicating no aggregation. (E) Comparison of pair-distance distribution function $P(r)$ of ZIKV-FP NS2B₁₈NS3 (*black*) with NS2B₁₈NS3_{F179A} (*green*) and NS2B₁₈NS3_{F179I} (*magenta*). (F) Normalized Kratky plot of ZIKV-FP NS2B₁₈NS3 (●; *black*) compared with NS2B₁₈NS3_{F179A} (○; *green*), NS2B₁₈NS3_{F179I} (○; *magenta*) and the compact globular lysozyme (●; *grey*) with a peak (---; *grey*), representing the theoretical peak and assuming an ideal Guinier region of a globular

particle. The scattering pattern of both mutants exhibits a broad bell-shaped profile shifted toward right with respect to standard globular proteins, similar to WT protein. The SAXS data were collected according to the procedures described in Pan et al. (2017). All the data processing steps were performed using the program package PRIMUS (Konarev et al., 2003). The radius of gyration, R_g was computed using the Guinier approximation (Glatter and Kratky, 1982). The extended scattering pattern using the indirect transform package GNOM (Petoukhov et al., 2012) provides the distance distribution function $p(r)$ and the maximal particle dimension, D_{max} , and R_g . (F) Normalized Kratky plots (Durand et al., 2010; Kikhney and Svergun, 2015) of the mutants were generated by normalizing the scattering intensity $I(q)$ to the forward scattering $I(0)$, and the scattering vector q to the radius of gyration R_g . (G) Comparison of ATP hydrolysis activity of ZIKV-FP NS2B₁₈NS3, NS2B₁₈NS3_{F179A} and NS2B₁₈NS3_{F179I} represented as a bar diagram. The graph indicates that while NS2B₁₈NS3_{F179I} has an ATP hydrolysis activity similar to the WT protein, the activity of NS2B₁₈NS3_{F179A} is about 14% lower than ZIKV-FP NS2B₁₈NS3. (H) The Michaelis-Menten plots of ZIKV-FP NS2B₁₈NS3_{F179A} (*green*) and NS2B₁₈NS3_{F179I} (*magenta*) with their fit lines for the determination of the enzyme kinetic parameters of each protein.

Figure 4: Model of dimeric ZIKV-FP NS5 (PDB ID: 5TFR) (*surface* representation) interacting with two single NS2B₁₈NS3 proteins (*BUNCH* model; *surface* representation) (Saw et al., 2017). For chain A and chain B, the MTase (M) domains of NS5 are shown in *blue*, and the RdRp (R) domains are colored in *magenta*. The protease (P) domain of NS3 is colored in *light blue* with the NS2B cofactor in *green*. The helicase (H) domain of NS3 is shown in *orange*.

1 **Table 1:** Primer list of ZIKV-FP NS3 linker mutants used in this study

Mutant Name	Primers (5'-3')	
NS2B₁₈NS3_{T174A}	F	5'- GAG GAA GAG <u>GCT</u> CCT GTT GAG TGC TTC GAG CCT -3'
	R	5'- CTC AAC AGG <u>AGC</u> CTC TTC CTC CCT CCT CCC TTG-3'
NS2B₁₈NS3_{V176A}	F	5'- GAG ACT CCT <u>GCT</u> GAG TGC TTC GAG CCT TCG ATG-3'
	R	5'- GAA GCA CTC <u>AGC</u> AGG AGT CTC TTC CTC CCT CCT-3'
NS2B₁₈NS3_{E177A}	F	5'- ACT CCT GTT <u>GCG</u> TGC TTC GAG CCT TCG ATG CTG-3'
	R	5'-CTC GAA GCA <u>CGC</u> AAC AGG AGT CTC TTC CTC CCT-3'
NS2B₁₈NS3_{C178A}	F	5'-CCT GTT GAG <u>GCT</u> TTC GAG CCT TCG ATG CTG AAG-3'
	R	5'-AGG CTC GAA <u>AGC</u> CTC AAC AGG AGT CTC TTC CTC-3'
NS2B₁₈NS3_{F179A}	F	5'-GAG ACT CCT GTT GAG TGC <u>GCC</u> GAG CCT TCG ATG-3'
	R	5'-AGG CTC <u>GGC</u> GCA CTC AAC AGG AGT CTC TTC CTC-3'
NS2B₁₈NS3_{F179I}	F	5'-GAG ACT CCT GTT GAG TGC <u>ATC</u> GAG CCT TCG ATG-3'
	R	5'-AGG CTC <u>GAT</u> GCA CTC AAC AGG AGT CTC TTC CTC-3'

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1 **Table 2:** Data collection and scattering derived parameters for NS2B₁₈NS3_{F179A} and
2 NS2B₁₈NS3_{F179I}

Sample details	NS2B ₁₈ NS3 _{F179A}	NS2B ₁₈ NS3 _{F179I}
Organism	Zika Virus French Polynesia (ZIKV-FP)	
GenBank	KJ776791	
Extinction coefficient (A ₂₈₀ , M ⁻¹ cm ⁻¹)	107830	107830
MM from chemical composition (Da)	71272.11	71314.19
Protein concentration (mg ml ⁻¹)	2.5 mg/ml	6.0 mg/ml
Solvent	50 mM Tris/HCl, pH 7.5, 300 mM NaCl, 5% glycerol, 1 mM DTT	
Data collection parameters		
Instrument (source & detector)	Bruker NanoStar equipped with MetalJet eXcillum X-ray source and VÅNTEC-2000 detector	
Beam geometry	100 μm slit	
Wavelength (nm)	0.134	
Sample-to-detector distance (m)	0.67	
q range (nm ⁻¹)	0.16 - 4	
Normalization	To transmitted intensity by glassy carbon	
Monitoring for radiation damage	Data frame-by-frame comparison	
Exposure time (min)	30 (6 frames x 5 min)	
Sample configuration	Vacuum tight quartz capillary	
Sample temperature (K)	288.15	
Software employed		
Primary data reduction	BRUKER SAXS program suite	
Data processing	PRIMUSqt from ATSAS 2.8.2	
Ab initio analysis	DAMMIF from ATSAS 2.8.2	
Validation and averaging	DAMAVAR from ATSAS 2.8.2	
Atomic structure modeling	CRY SOL from ATSAS 2.8.2	
	CORAL from ATSAS 2.8.2	
	EOM via ATSAS online	
3D graphics representations	PyMOL v1.5.0.4 (Delano, 2002)	
Structural parameters		
Guinier analysis		
I(0) (arbitrary unit)	106.61 ± 1.36	78.86 ± 0.97
R _g (nm)	3.38 ± 0.06	3.31 ± 0.05

q_{min} (nm ⁻¹)	0.71	0.79
qR_g max	1.28	1.29
<i>P(r)</i> analysis		
$I(0)$ (arbitrary unit)	106.80 ± 0.81	79.46 ± 0.56
R_g (nm)	3.48 ± 0.02	3.43 ± 0.02
D_{max} (nm)	11.0 ± 1	11.0 ± 1
q range (nm ⁻¹)	0.180 - 1.98	0.184 - 1.98
χ^2 (Total estimate from GNOM)	0.471	0.537
Porod volume (V_p) (nm ³)	~93	~96
MM from V_p ($V_p/1.6$) (kDa)	58.55 ± 5.8	60.26 ± 6.0
DAMMIF excluded volume (V_{ex}) (nm ³)	118	118
MM from <i>excluded volume</i> ($V_{ex}/2$) (kDa)	59 ± 5.9	59 ± 5.9

1

2

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14

Table 3: Kinetic parameters of WT NS2B₁₈NS3 and its mutants

Protein	$k_{cat} \pm SD \text{ (s}^{-1}\text{)}$	$K_m \pm SD \text{ (}\mu\text{M)}$	$k_{cat}/K_m \text{ (M}^{-1}\cdot\text{s}^{-1}\text{)}$
NS2B ₁₈ NS3 _{F179A}	6.90 ± 0.22	94.02 ± 7.5	7.33×10^4
NS2B ₁₈ NS3 _{F179I}	7.04 ± 0.27	108.08 ± 8.8	6.51×10^4
NS2B ₁₈ NS3*	7.35 ± 0.19	83.30 ± 8.4	8.50×10^4

* Data from Saw et al., 2017

Figure 1A

Figures 1B-E

Figure 2A

Figures 2B-D

1
2
3
4
5
6

Figures 3A-F

Figures 3G-H

1

2

Figure 4