Genome-wide mutagenesis of dengue virus reveals plasticity of the NS1 protein and enables generation of infectious tagged reporter viruses

Nicholas S. Eyre [a,b,*,1], Stephen M. Johnson [a,b,1,2], Auda A. Eltahla [c], Maria Aloi [a,b,3], Amanda L. Aloia [d], Christopher A. McDevitt [a], Rowena A. Bull [c], Michael R. Beard [a,b,*]

[a] Research Centre for Infectious Diseases, School of Biological Sciences, University of Adelaide, Adelaide, Australia

[b] Centre for Cancer Biology, SA Pathology, Adelaide, Australia

[c] Viral Immunology Systems Program (VISP), School of Medical Sciences, The Kirby Institute, UNSW, Sydney, Australia

[d] Cell Screen SA, Flinders Centre for Innovation in Cancer, Flinders University, Bedford Park, Australia.

[*] Address correspondence to: nicholas.eyre@adelaide.edu.au (N.E.) and michael.beard@adelaide.edu.au (M.B.)

[1] N.S.E. and S.M.J. contributed equally to this work.

[2] Current address: The Australian Wine Research Institute, Glen Osmond, South Australia, Australia.

[3] Current address: Department of Microbiology, Monash University, Clayton, Victoria, Australia

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ABSTRACT

Dengue virus (DENV) is a major global pathogen that causes significant morbidity and mortality in tropical and sub-tropical areas worldwide. An improved understanding of the regions within the DENV genome and its encoded proteins that are required for the virus replication cycle will expedite development of urgently required therapeutics and vaccines. We subjected an infectious DENV genome to unbiased insertional mutagenesis and employed next-generation sequencing to identify sites that tolerate 15-nucleotide insertions during the virus replication cycle in hepatic cell culture. This revealed that regions within capsid, NS1 and the 3'UTR were most tolerant of insertions. In contrast, prM- and NS2A-encoding regions were largely intolerant of insertions. Notably, the multifunctional NS1 protein readily tolerated insertions in regions within the *Wing*, *connector* and *β-ladder* domains with minimal effects on viral RNA replication and infectious virus production. Using this information we generated infectious reporter viruses, including a variant encoding the APEX2 electron microscopy tag in NS1 that uniquely enabled high resolution imaging of its localization to the surface and interior of viral replication vesicles. Additionally, we generated a tagged virus bearing an mScarlet fluorescent protein insertion in NS1 that, despite an impact on fitness, enabled live cell imaging of NS1 localization and traffic in infected cells. Overall, this genome-wide profile of DENV genome flexibility may be further dissected and exploited in reporter virus generation and antiviral strategies.

IMPORTANCE

Regions of genetic flexibility in viral genomes can be exploited in generation of reporter virus tools and should arguably be avoided in antiviral drug and vaccine design. Here we subjected the DENV genome to high-throughput insertional mutagenesis to identify regions of genetic flexibility and enable tagged reporter virus generation. In particular, the viral NS1

protein displayed remarkable tolerance of small insertions. This genetic flexibility enabled generation of several novel NS1-tagged reporter viruses, including an 'APEX2'-tagged virus that we employed in high resolution imaging of NS1 localization in infected cells by electron microscopy. For the first time this analysis revealed the localization of NS1 within viral replication factories known as 'vesicle packets' (VPs), in addition to its acknowledged localization to the luminal surface of these VPs. Together this genetic profile of DENV may be further refined and exploited in identification of antiviral targets and generation of reporter virus tools.

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INTRODUCTION

Dengue virus is a mosquito-borne flavivirus that causes approximately 100 million symptomatic infections and 25,000 deaths each year (1). No antiviral drugs are available and the only approved vaccine is partially limited in efficacy, is only available in certain countries and is not recommended for young children or the elderly (2). Accordingly, there remains an urgent need for development of safe and effective antiviral therapies and vaccines; challenges that are made more difficult by significant gaps in our understanding of the precise functions of the individual viral proteins and their domains.

Following entry into susceptible cells via clathrin-dependent endocytosis, the plus-strand RNA viral genome of DENV is released into the cytosol and translated by the host ribosome machinery at the rough endoplasmic reticulum (ER). The encoded polyprotein is then proteolytically cleaved co- and post-translationally by host and viral proteases to liberate the individual structural proteins capsid, prM and E and the non-structural (NS) proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5. The NS proteins are essential for replication of the viral genome via a negative-strand RNA intermediate in virus-induced membranous organelles know as replication factories (3). Specifically, viral RNA replication mediated by the RNA-dependent RNA-polymerase NS5 is thought to take place within invaginations of the ER membrane known as vesicle packets (VPs), while pores in these VPs may enable exchange of metabolites and export of newly synthesised genomes for encapsidation (4). Accordingly, virus particle assembly is thought to take place in close proximity to VP pores and virus particles can be observed by electron microscopy (EM) in ordered arrays that are encased by ‘virion bags’ at these sites (4). The other major morphotype of DENV-induced membrane rearrangements are convoluted membranes (CMs), which are drastic rearrangements of ER membranes that are thought to serve polyprotein expression and maturation and may also serve as membrane reservoirs for further VP biogenesis (4, 5).

While NS4A may be principally required for these rearrangements (6), other NS proteins are also likely to be involved. NS proteins, such as NS1, NS2A and NS3, also play essential roles in virus particle production, possibly by co-ordinating budding of newly formed nucleocapsids into the ER lumen and their envelopment with membranes that are enriched with the structural proteins prM and E (7-10). The conventional secretory pathway is then exploited in the release of virions, which involves a number of post-translational modifications of structural proteins that confer infectivity to virus particles, including cleavage of prM by the host protease furin in the *trans*-Golgi network (TGN) (11).

Reverse genetics analyses have been essential for our current understanding of the DENV replication cycle and the functions of the individual viral proteins and RNA elements. However, these studies are laborious and time consuming and are therefore typically limited to the analysis of discrete regions of a viral genome and/or encoded protein in a single study. One approach to overcome this bottleneck involves the combination of high-throughput random mutagenesis of a cloned viral genome with next generation sequencing (NGS) to quantify the degree to which regions within viral genomes tolerate mutations in cell culture models of viral replication. This approach has recently been employed to provide global maps of genetic flexibility for viruses such as hepatitis C virus (HCV), influenza A virus (IAV) and measles virus (MeV) and how this genetic flexibility or inflexibility relates to functions of viral proteins and their targeting by host immune responses (12-16). Here, we have combined transposon-mediated random insertional mutagenesis and NGS to generate a global map of genetic flexibility for a cloned DENV serotype 2 genome (DENV-2; strain 16881). We reveal for the first time that capsid- and NS1-encoding regions and the 3'UTR display the greatest overall genetic flexibility. In particular, NS1 was highly tolerant of insertions in regions surrounding N-glycosylation sites in the *Wing* and *β-ladder* domains and in a region surrounding a site in the second *connector* domain that is N-glycosylated for several other

flaviviruses. In contrast other regions such as those encoding prM and NS2A were highly intolerant of insertions. Building on these insights, we then generated a panel of infectious epitope- and reporter-tagged DENV-2 isolates, including a variant encoding the APEX2 EM reporter in NS1 that enabled high resolution imaging of NS1 at the membrane and interior of VPs and, less strongly, at the Golgi and modified ER membranes. We also generated a novel DENV2 derivative encoding an mScarlet fluorescent protein insertion in NS1 that enabled visualization of NS1-mScarlet localization and traffic in infected cells and revealed that intense juxtanuclear NS1 foci are relatively static while small and weakly fluorescent structures frequently display rapid, long-range, bidirectional traffic. Together, our data provide new insights into the localization and traffic of NS1 and provide a resource that may be exploited in future generation of tagged reporter viruses and in development of antiviral strategies directed towards genetically inflexible regions of the DENV genome and encoded proteins.

RESULTS

High-throughput transposon mutagenesis coupled to NGS reveals regions of genetic flexibility within the DENV-2 genome. We subjected a cloned DENV-2 genome (strain 16681) to random transposon mutagenesis using *Mu* transposase to generate a mutant pool comprised of approximately 250,000 plasmid clones. The transposon body encoding a kanamycin resistance cassette was then excised and the plasmid pool re-ligated to generate the final mutant pool comprised of a similar number of plasmid clones, each bearing a single 15-nucleotide (nt) insertion of which 10-nt are transposon-derived (5'-TGCGGCCGCA-3') and 5-nt are duplicated from the target site. When inserted in a coding region of the genome, this results in a 5 amino acid insertion whose sequence is dependent on the frame and insertion site (C-G-R-I/M/T/N/K/S/R, L/M/V-R-P-H/Q or X-A-A-A). From this mutant

plasmid pool we generated *in vitro* transcribed viral RNA, referred to as Pool 0: 'Input', for large scale electroporation of Huh-7.5 hepatoma cells. Following culture of electroporated cells for 6 days, total cell RNA was extracted from remaining cell monolayers (Pool 1: 'Replication-competent'), and cell culture supernatants were collected, clarified and applied to naïve cells. Infected target cells were then cultured for 2 days before extraction of total cell RNA to generate Pool 2: 'Infectious' (Fig. 1A). Immunofluorescence microscopy analysis of parallel cell cultures confirmed robust DENV-2 replication in these cultures and an expected delay in replication and spread of the mutant pool as compared to wildtype DENV-2 (not shown). Total RNA from each pool was then subjected to RT-PCR using DENV-specific primers that covered the genome in six overlapping fragments. In parallel, this PCR was also performed to determine insertion distribution in the initial mutant plasmid pool. NGS libraries for each pool were prepared using Nextera XT (Illumina) and sequenced on the NextSeq500 platform. Sequence reads were mapped to the reference DENV-2 genome and the frequency of transposon insertions at each position was measured for each pool (Fig. 1 and Dataset S1). This revealed that insertions were generally evenly distributed across the DENV-2 genome in both the mutant plasmid pool (not shown),and the mutant DENV-2 RNA library, Pool 0, used for transfection (Figs. 1B), with the exception of 'hotspots' for insertions in the NS2A-encoding region that may relate to disruption of cryptic bacterial promoter-driven expression or activity of toxic genes that otherwise limit propagation of flavivirus clones in bacteria (17). In total 74.2, 71.4, 59.2 and 63.4 million reads were generated for the plasmid pool, Pool 0, Pool 1 and Pool 2, respectively, while digital counting of transposon insertions revealed 7867, 4700, 1122 and 1003 unique insertions in the DENV2 genome for these respective pools.

We compared genome insertion counts in the initial input mutant pool (Pool 0; Fig. 1B) with those capable of viral RNA replication (Pool 1; Fig. 1C) and those capable of viral

RNA replication and infectious virus production (Pool 2; Fig. 1D). This revealed that capsid- and NS1-encoding regions and the 3'UTR were most tolerant of 15-nt insertions, whereas other regions such as prM- and NS2A-encoding regions were largely intolerant of these insertions. Fig. 1E shows insertions that were most strongly selected against (cyan peaks), and insertion mutants capable of being sustained during viral RNA replication but not infectious virus production (blue and magenta peaks). All other peaks, and particularly yellow and red peaks, represent insertion mutants that are viable throughout the entire viral life cycle. We also assessed the frequency of insertions in replication-competent and infectious pools (Pools 1 and 2, respectively) as a percentage of those counted in the initial input pool (Pool 0) and this normalised view also highlighted sites in capsid- and NS1-encoding regions that were most tolerant of insertions and, in particular, the high sensitivity of prM- and NS2A-encoding regions to insertions (Fig. 2). In this context, the encoded amino acid sequences of the 25 most-tolerated insertions are displayed in Dataset S1. More detailed analysis is clearly required before firm conclusions can be drawn as to which frame-dependent transposon-encoded peptide sequences are better tolerated at different sites within the viral proteins. Nevertheless, of the 25 most-tolerated insertions, 60% (15/25) encoded C-G-R-I/M/T/N/K/S/R insertions, 28% (7/25) encoded X-A-A-A insertions and only 12% (3/25) encoded L/M/V-R-P-H/Q insertions (Dataset S1, column 'N'), highlighting that the impact of a 5 a.a. insertion at a given site can be strongly influenced by factors such as the charge, hydrophobicity and bulkiness of the inserted peptide. Given the high degree of overlap between Pool 1 and Pool 2, it is likely that spread of infectious virus during the 6 day culture period following electroporation of the mutant library strongly contributed to representation of infectious mutant viruses in Pool 1. This possibility is also consistent with the unexpected selection against regions encoding structural proteins that are not required for viral RNA replication in Pool 1 (Figs. 1C and 2B).

It was also apparent that peaks representing tolerated mutations were often observed near the termini of viral proteins, for example at the C-terminus of capsid, E, NS1 and NS2B proteins, at the N-terminus of NS3 and at both N- and C-termini of NS4B (Fig. 1E and Fig. 2B). Provided that the sites of DENV polyprotein cleavage are not disrupted by these insertions, the propensity of viral proteins to better tolerate insertions at their termini is consistent with a reduced likelihood of such insertions to disrupt the overall structure and in-turn interactions and functions of these viral proteins. Furthermore, for the coding portion of the genome, regions that were most tolerant of insertions were generally less strongly conserved between different DENV serotypes and other flaviviruses, although these regions were not characterized by unusually long stretches of sequence variation when the amino acid sequences of different flaviviruses were aligned and compared (Fig. S1). Given that compact viral genomes rarely maintain non-functional or non-essential sequences, regions that are highly tolerant of insertions may play roles in immune evasion, pathogenesis or virus-host interactions that are not re-capitulated in Huh-7.5 cell culture.

DENV NS1 protein is highly tolerant of small insertions in regions surrounding N-glycosylation sites. Of the DENV proteins, the multifunctional NS1 protein showed the greatest tolerance to transposon insertions in the context of the complete infectious virus replication cycle. Closer examination of the sites that tolerated insertions, with respect to the recently solved crystal structure of NS1 (18), revealed that sites within the second half of the *Wing* domain, in the second *connector* and in the N-terminal region of the *β-ladder* domain were most tolerant of transposon insertions, while sites near the C-terminus of the *β-ladder* were moderately tolerant of insertions (Fig. 3A). In contrast, the N-terminal *β-roll* domain, the first half of the *Wing* domain and the first *connector* that separates these domains were highly sensitive to transposon insertions, consistent with a recent alanine scanning

mutagenesis study that identified several replication-lethal mutations in these domains, including C4A, W8A, Y32A, C55A and R62A (8). Notably, the regions that were most tolerant of insertions were clustered in close proximity to the N-glycosylation sites in the *Wing* and *β-ladder* domains, Asn-130 and Asn-207, respectively, and surrounding a site in the second *connector* domain (Gln-175) that is a glycosylated Asn residue for several other flaviviruses, including West Nile virus (WNV), Saint Louis encephalitis virus (SLEV) and Murray Valley encephalitis virus (MVEV) (19). As N-glycosylation of NS1 is likely to be important to its functions as a secreted hexameric lipoparticle that modulates immune responses and causes vascular leakage, insertions in the regions surrounding these N-glycosylation sites may impact upon functions of secreted NS1 in immunomodulation and vascular leakage that are not recapitulated in hepatoma cell culture.

To better understand the location of tolerated transposon insertions as they relate to the three-dimensional structure of NS1 dimers, we visualised the locations of the 10 most tolerated insertion sites (Pool 2 / Pool 0) in the crystal structure of DENV-2 NS1 dimer (PDB: 4O6B), provided that at least two positions in the given codon preceded tolerated insertions (Fig. 3B). This approach, focussing on sites that tolerated insertions in at least two alternative codon positions and hence resulted in alternative encoded peptide insertions (C-G-R-I/M/T/N/K/S/R, L/M/V-R-P-H/Q or X-A-A-A), was employed to highlight sites that were broadly tolerant of small insertions. This revealed that the sites most tolerant of insertions were predicted to be solvent-exposed and distant from the lipid bilayer-interacting inner hydrophobic face of the *β-roll* domain and adjacent '*greasy finger*' loop (18). The two residues that preceded the most tolerated insertions were Lys-174 (~550-fold enrichment from input) and Thr-126 (~93-fold enrichment from input) and these sites were flanked by several other top-ranking tolerated insertion sites, including Glu-174 (~81-fold), Asn-130 (~75-fold), Ser-125 (~64-fold), Ser-128 (~58-fold) and Glu-127 (~44-fold).

Another region in the DENV-2 genome that was broadly tolerant of insertions was the capsid-encoding region, although the degree to which insertions were tolerated was considerably lower than that of NS1 (see Fig. 2). Capsid is the least conserved of the flavivirus proteins, although its general structural properties and charge distribution are well-conserved. Structural studies of this highly basic protein have shown that the capsid monomer contains four α-helices (α1 to α4), while the dimer has an asymmetric charge distribution such that the highly basic α4-α4' region may interact with viral RNA while the hydrophobic cleft, comprised of α1-α1' and α2-α2' regions, on the opposite side of the molecule forms an apolar surface that is predicted to interact with membranes (20, 21). Examination of the location of tolerated insertions revealed that the C-terminus of capsid towards the end of the α4 region and, in particular, the region immediately downstream of the NS2B/3 cleavage site and adjacent to the transmembrane anchor were most tolerant of insertions (Fig. 4). Additionally the region surrounding the α1 region was broadly and moderately tolerant of insertions, consistent with the flexibility of this region implied by the differing orientations of DENV and WNV α1 and mutational studies demonstrating that substitutions of uncharged amino acids in the α1 helix and α1-α2 connecting loop do not impair DENV propagation (22, 23). In contrast, the N-terminus of capsid and the α2 and α3 regions were more sensitive to insertions. Taken together, the high tolerance of capsid for insertions is consistent with it being the least conserved of the flavivirus proteins.

The influence of host cell type and species on the sensitivity of DENV-2 to 15-nt transposon insertions. To investigate how host cell type and species impacts upon the tolerance of DENV-2 to transposon-derived 15-nt insertions, the infectious virus-containing cell culture supernatant from Huh-7.5 cells that were transfected with RNA transcripts for the mutant DENV-2 RNA library was applied in parallel to naïve mammalian cell lines Huh-7.5

and Vero cells and the insect C6/36 cell line. At 48 hours post-infection RNA was then extracted from these cells and subjected to RT-PCR for Illumina sequencing (Fig. 5A). The frequency of 15-nt transposon insertions was measured and compared for DENV-2 genomes recovered from infected Huh-7.5, Vero and C6/36 cells (Fig. 5B-D), employing an overlay of the respective maps to highlight which transposon insertions are differentially tolerated in the alternative host cell types (Fig. 5E). Despite the appreciable differences in infection rates for revealed by parallel immunofluorescent staining of infected cells (Fig. 5F), our analysis revealed very close overlap between regions that displayed tolerance of insertions in the DENV-2 genome in these highly divergent host cells (Fig. 5E). However, subtle but clear differences were apparent (see Dataset S1), including modest increases in the frequency of tolerated transposon insertions in the 3'-UTR and sites within E for C6/36 host cells as compared to Huh-7.5 and Vero host cells (see yellow peaks for Fig. 5E). In contrast, C6/36 host cells were associated with modest decreases in the frequency of tolerated insertions in regions surrounding the Asn-207 glycosylation site in NS1 and in the C-terminus of NS2B (see blue peaks for Fig. 5E). Taken together these experiments indicate that, following initial selection in Huh-7.5 human hepatoma cells, host cell selective pressures do not dramatically alter regions of genetic flexibility within the DENV-2 genome.

Generation of tagged reporter viruses to study the DENV replication cycle and viral protein localization and interactions. Epitope- and reporter-tagged viruses are valuable tools to study viral protein localization and interactions in the context of productive viral infections. However, their development is challenging and requires prediction of sites that may tolerate insertions and empirical testing of candidate tagged viruses. Guided by our transposon mutational profile of sites that tolerate 15-nt (5 a.a.) insertions in the DENV2 genome, we generated and tested a panel of epitope- and reporter-tagged DENV-2 constructs,

specifically focussing on the NS1 and capsid proteins given their high degree of tolerance of insertions and their essential but incompletely understood functions in the viral replication cycle (Fig. 5A). We found that the region at the C-terminus of the capsid pre-cursor and adjacent to the transmembrane anchor region ('CAPmem'), following Gly-103, could readily tolerate small epitope tags. This included the recently developed NanoBiT luciferase complementation component SmBiT (11 a.a.) for use in studying protein-protein interactions in living cells (24), and the split fluorescent protein component GFP11 (16 a.a.) for use in live cell imaging in cells expressing the complementary GFP 1-10 fragment (25). Tolerance of this site for insertions was influenced by the overall charge of the inserted peptide as incorporation of the smaller but highly charged FLAG tag at this site severely attenuated viral replication and spread (results not shown). We also generated tagged viruses encoding FLAG or GFP11 epitope tag insertions within NS1 between Lys-174 and Gln-175 (Fig. 6A). Similarly, we generated virus constructs bearing insertions of the larger APEX2 EM tag (26, 27) or the extremely bright NanoLuc (NLuc) luciferase reporter (28) at this same site in NS1.

Western blot analysis of Huh-7.5 cells transfected with the respective tagged DENV-2 or wildtype DENV-2 RNA transcripts revealed ready detection of NS1 for all virus constructs and expected increased molecular weights of tagged NS1 proteins (Fig. 6B). We also noted a moderate increase in the levels of tagged NS1 protein that may reflect minor effects of tag insertions at this site on NS1 stability. Importantly, however, analysis of NS1 protein levels in cell culture supernatants revealed no major tag-associated defects in NS1 secretion (Fig. 6B), although further analysis is required to definitively clarify whether the efficiency and kinetics of NS1 secretion are altered. Analysis of the replicative fitness of these tagged viruses compared to wildtype DENV-2 revealed that DENV2-NS1-FLAG displayed wildtype levels of viral replication and spread, as determined by automated immunofluorescence analysis (Fig. 6C), and wildtype levels of infectious virus production (Fig. 6D). Similarly,

310 viruses bearing GFP11 or SmBiT insertions adjacent to the capsid membrane anchor or a
311 GFP11 insertion in NS1 displayed robust replication and spread and infectious virus
312 production that was only moderately impaired compared to wildtype DENV2 (Fig. 6C-D).
313 While viruses encoding larger APEX2 (783-nt, ~28 kDa) or NLuc (516-nt, ~19 kDa)
314 insertions in NS1 were appreciably attenuated compared to wildtype DENV-2, they
315 nonetheless displayed robust levels of viral replication and infectious virus production (Fig.
316 6C-D), supporting their utility in detailed analysis of NS1 localization, using DENV2-NS1-
317 APEX2, and highly sensitive and simple monitoring of protein levels, using DENV2-NS1-
318 NLuc (see below). Interestingly, the tolerance of insertions at this site in NS1 appears to be
319 highly dependent on the sequence and/or structure of the inserted protein as several other
320 reporter protein insertions at this site were not tolerated, including those of green fluorescent
321 protein (GFP) and the self-labelling SNAP-tag (results not shown).

322 Given the potential for tag insertions to alter NS1 conformation, glycosylation,
323 secretion and localization, we further investigated these properties for our panel of epitope-
324 and reporter tagged DENV-2 derivatives. Firstly, in our hands non-reducing, non-denaturing
325 conditions were required for reactivity of the anti-NS1 mAb 4G4 towards secreted DENV-2
326 NS1 (Fig. 7A), with results suggesting that the native epitope recognised by this antibody is
327 similarly retained for all NS1-tagged viruses. Furthermore, we assessed whether *N*-
328 glycosylation of NS1 is affected by the presence of tag insertions in NS1 by digesting cell
329 culture supernatants with PNGase F prior to Western blotting (Fig. 7B). This revealed a
330 similar shift in the apparent molecular weight of wildtype and tagged NS1 proteins,
331 indicating no major defects in *N*-glycosylation of NS1-tagged variants. Given the potential
332 for cell type- and/or species-specific effects on the replicative fitness of these tagged viruses,
333 we also compared infectious virus production for wildtype and tagged viruses following viral
334 RNA transfection of *Aedes albopictus*-derived C6/36 cells and African green monkey-derived

335 Vero cells (Fig. 7C-D). Consistent with observations in Huh-7.5 cells (Fig. 6D), in both
336 C6/36 and Vero cells DENV2-NS1-FLAG produced wildtype levels of infectious virus
337 production, other small insertions in capsid (SmBiT and GFP11) and NS1 (GFP11) were
338 associated with moderate impairment of infectious virus levels and larger insertions in NS1
339 (APEX2 and NLuc) were associated with more marked reductions in infectious virus levels
340 (Fig. 7C-D). Importantly all tagged viruses outlined in Fig. 6 displayed unaltered localization
341 of NS1 and capsid proteins in Huh-7.5 cells and minimal changes to the degree of their
342 colocalization with dsRNA, a marker of viral replication factories (Fig. 8). Taken together,
343 this panel of infectious tagged viruses, and future tagged viruses that are rationally generated
344 using the insertional mutation map of DENV-2 as a guide, will serve as important tools to
345 study viral protein localization, traffic and interactions in the context of a productive viral
346 infection.
347
348 **Sensitive luminescence-based monitoring of intracellular and secreted NS1 protein**
349 **levels.** We next explored the utility of the DENV2-NS1-NLuc virus in monitoring
350 intracellular and secreted NS1 protein levels during viral infection in cell culture. For this,
351 Huh-7.5 cells in 96-well plates were infected with serial dilutions of DENV2-NS1-NLuc,
352 washed and returned to culture for 48 h prior to determination of extracellular and
353 intracellular NS1-associated NLuc activity (Fig. 9A). This revealed simple and extremely
354 sensitive detection of NS1-NLuc levels over a large dynamic range (~4 orders of magnitude).
355 As expected, extracellular NS1-NLuc was also strongly detected with levels closely reflecting
356 those of intracellular NS1-NLuc. Furthermore, we confirmed the utility of this luminescent
357 virus in antiviral drug testing by measuring the impact of the recently identified pan-flaviviral
358 inhibitor Nanchangmycin on viral infection and replication (29). Consistent with that study,
359 Nanchangmycin pre-treatment for 1 hour was associated with dose-dependent inhibition of

360 virus encoded NS1-NLuc, measured in both intracellular and extracellular samples at 48 h.p.i.
361 (Fig. 9B). Taken together, DENV2-NS1-NLuc enables sensitive monitoring of NS1 protein
362 levels during viral infection and may be well-suited to high-throughput screening.
363
364 **High resolution imaging of NS1 localization using APEX EM and STED super-**
365 **resolution microscopy.** Early studies using immuno-EM revealed the localization of NS1 to
366 vesicle packets in DENV-infected cells and its co-localization with dsRNA at these sites (30),
367 consistent with the essential role of NS1 in viral genome replication. Furthermore, recent
368 studies demonstrated co-localization and interaction of NS1 with capsid and E proteins at
369 putative viral assembly sites and/or assembled virions (8). However, traditional antibody-
370 based approaches are limited in their ability to examine protein localization at high resolution
371 in the context of clear visualization of cellular membranes and, accordingly, many details
372 about the exact nature of NS1 localization remain unclear. We therefore examined NS1
373 localization by APEX EM, which allows high resolution imaging of tagged protein
374 localization by EM while maintaining excellent ultrastructural preservation (26, 27). For this,
375 Huh-7.5 cells were infected with untagged DENV-2 or DENV2-NS1-APEX2 (MOI: ~0.01)
376 or mock-infected and returned to culture for 4 days prior to fixation, labelling with
377 DAB/H_2O_2 and processing for EM. Brightfield microscopy following DAB/H_2O_2 labelling
378 confirmed strong and specific staining of NS1 that was unique to DENV2-NS1-APEX2-
379 infected cells (Fig. 10A). Samples were subsequently labelled with electron-dense OsO_4,
380 which stains the osmiophilic DAB polymer, and further processed for EM. As observed in
381 standard EM analysis of DENV-infected cells, untagged virus infection was associated with
382 typical induction of CMs and VPs that were clearly but not intensely stained (Figs. 10B-i and
383 10B-ii respectively), and were absent in mock-infected cells (not shown). In contrast, in cells
384 that were infected with DENV2-NS1-APEX2, intense staining of NS1-APEX2 could be

385 observed in clusters of VPs, with somewhat diffuse staining localized to the outer membrane
386 of VPs and intense, tightly localized staining of punctae that were asymmetrically distributed
387 within VPs (Fig. 10B-iv). We also observed clear but less intense staining of ER membranes
388 that in many instances were proximal to lipid droplets (Fig. 10B; iv). This approach also
389 revealed clear staining of NS1-APEX2 in the Golgi (Fig. 10B; iii). While we could not
390 clearly discern virus particles in these cells, we observed occasional examples of intensely
391 stained clusters adjacent to VPs (Fig. 10B; v) and NS1-APEX2-positive highly ordered arrays
392 (Fig. 10B; vi) that may be relevant to the function(s) of NS1 in virus particle assembly and
393 secretion (8). Although we observed no unanticipated effects of the APEX2 insertion on NS1
394 protein processing (Fig. 6B), secretion (Figs. 6B and 7A), glycosylation (Fig. 7B) or
395 localization (as determined by confocal microscopy; Fig. 8A), given the reduced fitness of
396 the DENV2-NS1-APEX2 virus further studies using complementary imaging techniques are
397 required to support our findings regarding NS1 localization using APEX EM.

398 In this context, to complement the APEX EM imaging analysis of NS1 localization
399 we also applied the DENV2-NS1-FLAG recombinant virus, which encodes a single FLAG
400 tag within NS1, to STimulated Emission Depletion (STED) super-resolution imaging of the
401 localization of FLAG-tagged NS1 and dsRNA, given that this virus displayed wildtype levels
402 of replication and infectious virus production and enables particularly strong and specific
403 labelling of NS1-FLAG, with anti-FLAG antibodies, in combination with anti-dsRNA
404 labelling. While NS1-FLAG and dsRNA displayed near-complete co-localization when
405 imaged by standard laser scanning confocal microscopy (Fig. 8A), even when image
406 deconvolution was applied (Fig. 11A), STED imaging in combination with deconvolution
407 revealed that dsRNA foci were frequently immediately adjacent to NS1 foci, which displayed
408 a more reticular localization pattern compared to the intense and largely discrete dsRNA foci
409 (Fig. 11B). Taken together, these experiments clarify the localization of NS1 with respect to

410 VPs and provide powerful new tools to further examine NS1 localization as it relates to its
411 roles viral RNA replication, virus particle production and as a secreted mediator of immune
412 evasion and pathogenesis.
413
414 **Live cell imaging of mScarlet-tagged NS1.** Although the GFP11-tagged viruses, DENV2-
415 CAPmem-GFP11 and DENV2-NS1-GFP11, displayed robust viral replication and infectious
416 virus production, we could not reliably detect strong GFP11-associated fluorescence upon
417 complementation with heterologously expressed GFP(1-10) (Eyre NS, Beard MR;
418 unpublished results). This may be attributable to inefficient fluorescence complementation
419 and/or inaccessibility of the incorporated GFP11 peptides to GFP(1-10); obstacles that may
420 be overcome by development of analogous viruses bearing multiple tandem repeats of
421 GFP11, targeting of GFP(1-10) to the ER lumen and/or application of newly developed split
422 fluorescent protein systems based on GFP11/GFP(1-10) with improved complementation
423 efficiencies (25, 31). Therefore, while our GFP11-tagged viruses provide important proof-of-
424 concept that capsid and NS1 proteins can tolerate small functional insertions designed for
425 fluorescent imaging, further optimization is required to improve their utility in demanding
426 applications such as live cell imaging. We therefore developed a novel DENV-2 construct
427 featuring an extremely bright and monomeric red fluorescent protein, mScarlet (32), inserted
428 within NS1 (Fig. 12A). This recombinant virus supported infectious virus production and
429 dictated strong NS1-associated fluorescence (Fig. 12B), despite appreciable attenuation
430 compared to wildtype DENV-2 (Fig. 12C). We next investigated NS1 localization and traffic
431 by live cell imaging analysis of Huh-7.5 cells transfected with DENV2-NS1-mScarlet RNA
432 transcripts. At 6 days post-transfection NS1-mScarlet fluorescence was observed throughout
433 the entire culture (Fig. 12D), while imaging at high magnification revealed characteristic
434 localization of NS1-mScarlet to large, intensely labelled juxtanuclear foci and smaller,

435 weakly fluorescent foci throughout the cytoplasm (Fig. 12E). Live cell imaging over 5 minute
436 periods revealed that the intensely labelled NS1-mScarlet foci were largely static (Fig. 12E,
437 cyan arrows and Movie S1), while a minority of small and weakly fluorescent foci displayed
438 rapid, long-range bidirectional traffic that is characteristic of microtubule-dependent transport
439 (Fig. 12E, cyan arrowheads and Movie S1). Importantly, similar NS1-mScarlet localization
440 and trafficking patterns were observed for cells infected with DENV2-NS1-mScarlet,
441 including in longer-term (~45 minute) live cell imaging acquisitions (Movie S2). Taken
442 together these results and the DENV2-NS1-mScarlet virus provide the basis for more detailed
443 analysis of NS1 localization and traffic in live infected cells and indicate that intense NS1
444 foci that likely represent replication complexes are relatively static, while weakly fluorescent
445 NS1 foci that may be involved in other aspects of the viral replication cycle can display rapid
446 long-range traffic. Further characterization of the DENV2-NS1-mScarlet recombinant virus
447 and development of adapted variants that closely mirror the replication, infectious virus
448 production and NS1 secretion kinetics of wildtype DENV2 will enable detailed analysis of
449 NS1 localization, traffic and interactions with relevant viral and host cell factors during a
450 productive infection.
451
452 **DISCUSSION**
453 Reverse genetics studies have contributed greatly to our understanding of the DENV
454 replication cycle and the functions of the individual viral proteins and genetic elements.
455 However, these studies are highly laborious and are typically limited to analysis of discrete
456 regions of individual viral proteins. Our study employed random transposon mutagenesis and
457 high-throughput sequencing to provide a global overview of regions of genetic flexibility
458 within the DENV-2 genome and its encoded proteins. This data provides a resource
459 identifying genetically flexible regions that should arguably be avoided in antiviral and

460 vaccine development strategies, as these sites may be prone to escape mutations with minimal
461 impact on viral replicative fitness. Conversely, sites that are broadly tolerant of insertions can
462 be exploited in the generation of infectious epitope- and reporter-tagged viruses. These
463 viruses can be employed in advanced applications such as high-resolution imaging and
464 interrogation of protein-protein interactions.

465 Our transposon mutagenic profile of DENV2 genetic flexibility indicates that the
466 regions encoding prM, NS2A and NS4A are highly intolerant of 15-nt insertions. For NS2A,
467 an eight-transmembrane protein with essential roles in viral RNA replication and infectious
468 virus production, our observations of intolerance to insertions are consistent with recent
469 alanine scanning mutagenesis studies that have revealed numerous mutations, particularly in
470 transmembrane regions, that are lethal to viral RNA replication or infectious virus production
471 (9, 10). Similarly, recent site-directed mutagenesis studies of NS4A, which is essential for
472 DENV-induced membrane rearrangements, have identified numerous conserved residues that
473 are required for its oligomerization and viral RNA replication (33, 34). However, the lack of
474 detection of replication-competent (Pool 1) viruses bearing insertions in the prM-encoding
475 region was more surprising as this region has no known involvement in viral RNA
476 replication. Whilst it is possible that spread of infectious virus in Pool 1 has masked the
477 presence of replication-competent prM mutants, it is also possible that insertion mutations
478 within this region have unexpected dominant-negative effects on viral replication. Further
479 studies are required to explore the apparent impact of prM mutations on DENV replication.

480 Overall, the regions encoding capsid, NS1 and the 3'-UTR were most tolerant of 15-
481 nt insertions. As discussed above, capsid is the least conserved of all flavivirus proteins,
482 although its charge distribution is strongly conserved and structural features are highly
483 similar for capsid proteins of different flaviviruses (22). Accordingly, its relatively high
484 tolerance for 15-nt insertions is not unexpected, especially for insertions that minimally alter

its overall charge. In this context, none of the possible insertions encode negatively charged peptides (C-G-R-I/M/T/N/K/S/R, L/M/V-R-P-H/Q or X-A-A-A). Similarly, Region I of the 3'UTR, where transposon insertions are most tolerated, is the most variable of all DENV nucleotide sequences with various deletions and point mutations identified in mosquito cell-adapted populations (35, 36). Although moderately conserved across flavivirus species, the NS1 protein was remarkably tolerant of 15-nt insertions. Largely consistent with a recent mutagenesis study (8), we found that the N-terminal *β-roll*, which contains a di-amino acid motif that may mediate interaction with NS4B and ER membrane association (37), the N-terminal half of the *Wing* domain and the C-terminal half of the *β-ladder* domain were highly sensitive to insertions. In contrast, relatively broad regions surrounding the N-glycosylation sites in the *Wing* (N130) and *β-ladder* (N207) domains and sites in close proximity to an N-glycosylation site in the *connector* domain of other flaviviruses (N175) were highly tolerant of insertions. Examination of the location of these sites in the crystal structure of the NS1 dimer demonstrated that they were all solvent-exposed and relatively distant from the putative ER membrane-associating *β-roll* and *greasy finger* loop of the *β-ladder* (18). Likewise, in the context of the secreted NS1 hexamer these regions of genetic flexibility are distant from the lipoparticle interior and central lipid core that are contained by six copies of the *β-roll* (18). Accordingly, we hypothesise that the regions of high tolerance of insertions identified in our study are not required for functions of NS1 in viral RNA replication or infectious virus production but, rather, may be required for extracellular functions of NS1 in immune evasion and pathogenesis that are not recapitulated in hepatoma cell culture. If so, such replication-competent and infectious mutants that lack the immune evasion and vascular permeability functions of wildtype NS1 may potentially be combined and exploited in future attenuated vaccine strategies. In this context a live-attenuated Zika virus (ZIKV) strain with engineered mutations to abolish NS1 glycosylation has very recently been shown to protect

510 mice against virus-induced placental damage and fetal demise (38). Alternatively or
511 additionally, regions of genetic flexibility in NS1 may be inherently flexible to skew humoral
512 immune responses towards these epitopes and away from regions that are susceptible to
513 function-neutralising antibody responses and/or facilitate rapid viral adaptation in alternative
514 host species. Such roles have been suggested for genetically flexible regions of influenza A
515 virus (IAV) NS1 protein in similar high-throughput insertional mutagenesis studies (13).

516 Although our mutant library did not achieve saturation, it nonetheless enabled
517 generation of a comprehensive profile of regions in the DENV-2 genome that are broadly
518 tolerant of small (15-nt) insertions and non-essential for viral RNA replication and infectious
519 virus production in hepatoma cells. In this context, sequencing analysis of the initial mutant
520 DENV-2 RNA pool indicated that ~44% of all possible insertions were present. However,
521 additional transposon insertions frequently emerged in sequencing analysis of the replication-
522 competent and infectious virus pools (Pools 1 and 2, respectively), such that the percentage of
523 all possible unique transposon insertions in the DENV-2 genome in our study rose to ~49%.
524 Future studies of this nature involving near-saturation mutagenesis will help to resolve gaps
525 in our mutational profile of DENV-2 but will not likely alter the overall appearance of this
526 profile. Furthermore, additional approaches are required to better delineate regions that are
527 essential to viral RNA replication and those that are required uniquely for infectious virus
528 production. For example, blockade of infectious virus spread or parallel comparison of
529 genetic flexibility in an analogous subgenomic replicon could resolve regions in the NS
530 proteins that are required for replication versus those required for infectious virus production.

531 As detailed above, another application of our mutational profile of DENV2 is in the
532 rational generation of epitope- and reporter-tagged viruses. While the viability of such viruses
533 is predictably dependent on the size and structure of the insertion, our mutational profile
534 nonetheless provides a valuable resource for prediction of sites that may tolerate insertions

for advanced imaging, proteomics and molecular applications. For example, our APEX EM analysis indicates that NS1 is localized to both the luminal membrane of VPs and in discrete clusters within VPs, in contrast to longstanding models of replication complexes with respect to VPs, which depict NS1 as an exclusively luminal viral protein. Furthermore, live cell imaging studies using mScarlet-tagged NS1 revealed that intensely labelled NS1 foci, which may reflect clusters of vesicle packets, are relatively static. In contrast, small and less intensely labelled NS1 foci infrequently display rapid bi-directional traffic. Although further investigations are required, including confirmation that NS1 secretion is not perturbed by the mScarlet insertion, these motile foci may reflect pools of NS1 that are involved in other functions of NS1 such as virus assembly and as a secreted mediator of vascular damage and immune evasion. Further studies using the NS1-tagged viruses developed in this study and additional variations of these tagged viruses may help to further resolve the localization, traffic, interactions and functions of this enigmatic multifunctional viral protein.

The transposon mutagenesis-coupled high-throughput sequencing approach applied to DENV-2 here also provides a basis for numerous additional extensions that could rapidly improve our understanding of flavivirus- and host cell-specific functions of viral proteins and genetic elements. For example, this approach could be readily applied to other DENV serotypes, related flaviviruses or alternative cell types to unveil flavivirus- and host cell type- and species-specific differences in the functions and interactions of viral proteins. In this context, our analysis of cell type- and/or species-specific determinants of genetic flexibility indicate that the diverse host cells that we examined do not impart markedly different selective pressures that alter the relative fitness of viable transposon mutants. Nevertheless, it is possible that continued passage of the transposon mutant pool in these different cell types may further unveil regions of the DENV-2 genome that are differentially susceptible to host cell-specific selective pressures. In regards to flavivirus-specific determinants of viral

560 replicative fitness, Fulton *et al* very recently reported the impact of high throughput
561 transposon mutagenesis of a cloned ZIKV genome on viral replication and infectious virus
562 production (39). Largely consistent with our findings for DENV-2, Fulton *et al* demonstrated
563 that NS1 and the structural genes of ZIKV displayed the greatest overall flexibility, although
564 key differences in the mutational maps of DENV-2 and ZIKV were apparent. For example,
565 the flexibility of prM appeared much higher for ZIKV compared to DENV-2 prM, while
566 regions surrounding *N*-glycosylation sites of DENV-2 NS1 displayed greater apparent
567 flexibility compared to the corresponding regions of ZIKV NS1. Further studies are required
568 to define the commonalities and differences in sites of genetic flexibility between different
569 flaviviruses and DENV serotypes and the impact of different host cell types and species on
570 this flexibility. In a further refinement of the transposon mutagenesis approach, several recent
571 studies have combined random point mutagenesis with NGS-based analysis of viral fitness to
572 provide comprehensive profiles of the impact of all possible amino acid substitutions in a
573 viral protein with respect to analysis of corresponding high-resolution protein structures
574 (reviewed in (15)). This approach could similarly be applied to great effect for DENV and
575 related flaviviruses. In summary, we propose that variations of the high throughput
576 mutagenesis-coupled genetic profiling approach applied here, in conjunction with increased
577 availability of high-resolution viral protein structural information, may rapidly increase our
578 understanding of the DENV replication cycle and expedite the development of urgently
579 required antiviral therapies and vaccines.
580
581 ## MATERIALS AND METHODS
582 ### Cell culture
583 Huh-7.5 cells (40) were generously provided by Charles M. Rice (Rockefeller University,
584 New York, USA) and were maintained as described previously (41). C6/36 cells, derived

585 from *Aedes albopictus* mosquitoes, were generously provided by Jillian M. Carr (Flinders
586 University, Adelaide, Australia) and were cultured at 28°C in a 5% CO_2 atmosphere in Basal
587 Medium Eagle supplemented with MEM non-essential amino acids, sodium pyruvate,
588 GlutaMAX, Penicillin Streptomycin and 10% FBS. Vero cells were generously provided by
589 Jillian M. Carr (Flinders University, Adelaide, Australia) and were cultured as described
590 previously (42). All cell culture media and additives were purchased from Thermo Fisher
591 Scientific.
592
593 **Antibodies and chemicals**
594 Mouse anti-NS1 mAb 4G4 and mouse anti-dsRNA mAb 3G1 (IgM) were generously
595 provided by Roy Hall (University of Queensland, Brisbane, Australia) (43, 44). Mouse anti-
596 capsid mAb 6F3.1 was kindly provided by John Aaskov (Queensland University of
597 Technology, Brisbane, Australia) (45). Mouse anti-β-actin mAb (AC-74) was purchased from
598 Sigma-Aldrich. Rabbit anti-FLAG mAb (D6W5B) was purchased from Cell Signalling
599 Technologies. AlexaFluor 488-, 555- and 647-conjugated secondary antibodies and HRP-
600 conjugated secondary antibodies were purchased from Thermo Fisher Scientific.
601 Nanchangmycin was purchased from Selleck Chemicals and dissolved in DMSO to 10 mM,
602 aliquoted and stored at -80°C.
603
604 **Viruses and plasmids**
605 Plasmid pFK-DVs containing a full-length DENV-2 genome (strain 16681) was generously
606 provide by Ralf Bartenschlager (University of Heidelberg, Heidelberg, Germany) (46). Exact
607 details about reporter- and epitope-tagged virus generation are available upon request. To
608 initiate viral RNA replication, DENV plasmids were linearized with *Xba*I before use as
609 templates in *in vitro* transcription reactions using an mMessage mMachine SP6 Transcription

610 kit (Thermo Fisher Scientific) and transfection of viral RNA into Huh-7.5 cells by
611 electroporation or transfection with DMRIE-C Reagent (Thermo Fisher Scientific), as
612 described previously (41). Virus infectivity was measured by focus-forming assay. Briefly,
613 Huh-7.5 cells were seeded at 2×10^4 cells/well into 96-well plates and returned to culture
614 overnight prior to inoculation with 40 µl/well of 10-fold serial dilutions of virus-containing
615 cell culture supernatants. Following infection for 3 h at 37°C/5%CO_2, cells were washed once
616 with PBS and returned to culture in fresh media for 72 h prior to fixation and
617 immunofluorescent labelling with anti-capsid antibody. Clusters (foci) of infected cells were
618 then enumerated and virus infectivity expressed as focus-forming units per millilitre
619 (FFU/ml).
620
621 **Generation of the DENV insertional mutant library**
622 Plasmid pFK-DVs was mutagenized using the Mutation Generation System (Thermo Fisher
623 Scientific), as per manufacturer's recommendations. Three independent *in vitro* transposon
624 insertion reactions were performed using 500 ng of plasmid per reaction, pooled and
625 transformed into XL-10 Gold Ultracompetent Cells (Agilent Technologies). Transformants
626 were plated onto 15-cm plates with LB agar containing ampicillin and kanamycin and grown
627 for 18 h at 37°C. Bacterial colonies (~2.5×10^5) were then scraped and pooled before
628 extraction of plasmid DNA using a NucleoBond Xtra Midi kit (Macherey-Nagel). Plasmid
629 DNA was then digested with *Not*I-HF (New England Biolabs) to remove the transposon
630 body, gel extracted and re-ligated using T4 DNA ligase (Promega). Approximately 150 ng of
631 ligated plasmid was then transformed into XL-10 Gold cells, as above, and cells were plated
632 onto LB agar plates containing ampicillin and cultured grown for 18 h at 37°C. Plasmid DNA
633 was extracted from pooled colonies (~2.5×10^5 colonies), as above, and verified by
634 diagnostic restriction digest (not shown).

635

636 **Mutant Virus Library Passage and Analysis of Transposon Insertion Frequency by RT-**
637 **PCR and Illumina Sequencing**

638 Huh-7.5 cells were electroporated with *in vitro*-transcribed transposon mutant DENV library
639 RNA. For this, thirteen electroporations were performed with 4×10^6 cells and 10 µg of RNA
640 per electroporation using 0.4 cm cuvettes and a Gene Pulser Xcell Electroporation System
641 (Bio-Rad) to deliver a single pulse (270 V, 100 ohms, 970 µF). Cells were then resuspended
642 in complete media, pooled and plated into thirteen 75 cm^2 flasks and returned to culture.
643 Virus replication and spread was monitored in parallel cultures by immunofluorescence and
644 at 6 days post-electroporation, when ~50% of cells were infected, cell culture supernatants
645 were collected, cleared by centrifugation and diluted in an equal volume of fresh media
646 before applying to naïve target cells, seeded the previous day into thirteen 75 cm^2 flasks at
647 1.6×10^6 cells per flask. Total RNA was isolated from electroporated cell monolayers (6 d
648 post-electroporation) and infected cell monolayers (2 d post-infection) using TRIzol (Thermo
649 Fisher Scientific) as per manufacturer's instructions. This cellular RNA (Pool 1 and Pool 2)
650 and the 'input' RNA used for electroporation (Pool 0) were reverse-transcribed into cDNA
651 using Superscript III (Thermo Fisher Scientific) and the oligonucleotide DV2NGS6R (see
652 below) as per manufacturer's instructions. Alternatively, the infectious virus-containing
653 supernatant from mutant library-electroporated Huh-7.5 cells (see above) was diluted (1:3) in
654 appropriate media and applied to naïve Huh-7.5, Vero or C6/36 cells that had been seeded the
655 previous day into 75 cm^2 flasks (three flasks per cell type with 1.2×10^6 cells/flask). As
656 above, at 2 days post-infection total RNA was extracted from these cells and used to prepare
657 cDNA. In both instances, this cDNA then served as template for PCR to amplify the entire
658 genome in six overlapping fragments using Q5 High-Fidelity DNA polymerase (New
659 England Biolabs) and the six following oligonucleotide pairs: DV2NGS1F 5'-

AGTTGTTAGTCTACGTGGACCG-3' and DV2NGS1R 5'-CGAATGGAGGTTCTGCTTCTATGT-3'; DV2NGS2F 5'-GCAGAAACACAACATGGAACAATAG-3' and DV2NGS2R 5'-CCTAAGGCTAACGCATCAGTC-3'; DV2NGS3F 5'-TGTCCTTTAGAGACCTGGGAAG-3' and DV2NGS3R 5'-ATGTCAGTTGTAACCACGAAGTCC-3'; DV2NGS4F 5'-CAGCAAGTATAGCAGCTAGAGGA-3' and NGS4R 5'-TTTCCCTTCTGGTGTGACCATG-3'; DV2NGS5F 5'-CTCAAGTATTGATGATGAGGACTACATG-3' and DV2NGS5R 5'-ACTTGTGTCCAATCATTCCATCC-3'; DV2NGS6F 5'-CCGCAGGATGGGATACAAGA-3' and DV2NGS6R 5'-AGAACCTGTTGATTCAACAGCAC-3'. PCR products were then gel-extracted, quantified using a Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific) and combined in equimolar amounts for each pool. Samples were then processed using a Nextera XT Library Preparation Kit (Illumina) and sequenced using a NextSeq500 (Illumina) and 150-nt paired-end reads. For analysis, trimmed sequencing reads were filtered for sequences containing the transposon-derived insertion sequence (TGCGGCCGCA), the transposon sequence annotated and mapped against the DENV-2 reference sequence (GenBank accession number NC_001474 with minor modifications, as described (46)), using Bowtie 2 (47). The locations of the annotated transposon insertions were then identified from the alignments and counted using Geneious version 8 software (48). Original sequencing files are accessible from the NCBI Sequence Reads Archive (SRA) under the series record PRJNA400339.

Immunofluorescence Microscopy, Immunoblotting and Luciferase Assays

684 Immunofluorescent labelling was performed as described (41). Widefield fluorescence
685 microscopy for infectivity assays was performed using a Nikon TiE inverted fluorescent
686 microscope system. Confocal fluorescence microscopy was performed using a Zeiss LSM
687 700 confocal microscope system equipped with a 60× NA 1.4 water-immersion objective.
688 Images were processed using NIS Elements AR v.3.22 (Nikon) and Photoshop 6.0 (Adobe)
689 software. For STED super-resolution imaging, Huh-7.5 cells were cultured overnight on
690 coverslips (18mm round #1.5 glass) that were pre-coated with 0.2% gelatin. Cells were then
691 infected with DENV2-NS1-FLAG (M.O.I.: ~0.1) and returned to culture for 48 h prior to
692 fixation (ice-cold methanol:acetone [1:1], 5 min), washing, blocking (5% BSA in PBS for 30
693 min at room-temperature) and labelling for 1 h at room temperature with rabbit anti-FLAG
694 mAb (D6W5B; Cell Signalling Technologies diluted 1:200) and anti-dsRNA (mAb 3G1.1
695 hybridoma supernatant diluted 1:5) diluted in PBS/1% BSA. Samples were then washed
696 twice with PBS before incubation for 1 h at 4°C with Alexa Fluor 488-conjugated anti-rabbit
697 IgG and Alexa Fluor 647-conjugated anti-mouse IgG (cross reactive to IgM) antibodies
698 (Thermo Fisher Scientific) diluted 1:200 in 1%BSA/PBS. Samples were then washed three
699 times and mounted with ProLong Gold (Thermo Fisher Scientific). Samples were then
700 imaged using a Leica TCS SP8 STED 3× microscope system (Leica Microsystems) equipped
701 with 592, 660 and 775 nm STED lasers, using a 100x, NA 1.4 oil objective at 4× zoom. For
702 STED, Alexa Fluor 488 labels were excited with a 488 nm wavelength of a pulsed white light
703 (WL) laser (80 MHz) and depleted with a CW 592 STED laser with a maximum power of
704 1500 mW (typically operating at ~30%). Similarly, for STED Alexa Fluor 647 labels were
705 excited with a 647 nm wavelength of a WL laser and depleted with a CW 775 nm STED laser
706 with a maximum power of 1500 mW (typically operating at ~50%). Images were acquired in
707 2D STED mode with settings optimized for maximum gains in lateral resolution. Time gates
708 were 0.5-6 ns. A total of 4 z-sections were acquired (0.142 µm z-steps) for both confocal and

STED channels and a line averaging of 7 was applied. Deconvolution of confocal and STED data (see Fig. 9) was performed using Huygens Professional Deconvolution software (version 14.10; Scientific Volume Imaging) and default settings. Immunoblotting was performed as described (49). Where indicated pre-cleared supernatants were treated with PNGase F (New England Biolabs) for 4 hour at 37°C under non-reducing conditions, as per manufacturer's instructions. Western blots were imaged using a ChemiDoc MP Imaging System (Bio-Rad) and, where applicable, band intensities were quantified using Image Lab software (vers. 5.2.1; Bio-Rad). Assays of NanoLuc (NLuc) activity were performed as described (50). In brief, Huh-7.5 cells were seeded into 96-well plates at 2×10^4 cells per well and cultured overnight prior to infection with DENV2-NS1-NLuc at the indicated M.O.I., with or without pre-treatment with Nanchangmycin for 1 hour at the indicated concentration (or vehicle control; DMSO at 0.1%). Four hours later cells were then washed and returned to culture for 48 h prior to collection of supernatants, washing of monolayers with PBS and lysis using Passive Lysis Buffer (Promega). Supernatant samples were cleared by centrifugation and mixed 1:1 with 2 × Passive Lysis Buffer before measurement of NLuc activity in cell lysates and supernatants using a Nano-Glo Luciferase Assay System (Promega) and a GloMax 20/20 luminometer (Promega).

Live Cell Imaging

Live cell imaging was performed as described (51). Briefly, DENV2-NS1-mScarlet RNA-transfected Huh-7.5 cells at 4 d post-transfection were seeded onto 0.2% gelatin-coated coverglass bottom dishes (MatTek) and cultured for 2 d in phenol red-free DMEM containing 10% FBS. Alternatively, Huh-7.5 cells were seeded into these dishes at 1.5×10^5 cells per dish, infected the following day at an M.O.I. of ~0.01 and returned to culture for 3 d. Imaging was performed at 37°C using a Nikon TiE inverted fluorescent microscope system equipped

734 with a heated stage (Okolab), a Plan Apochromat 60× NA 1.4 oil immersion objective
735 (Nikon), BrightLine single-band filter sets (DAPI-5060C-NTE-ZERO, FITC-3540C-NTE-
736 ZERO and TxRed-4040C-NTE-ZERO; Semrock), a Perfect Focus System (Nikon) and a
737 monochrome 12-bit cooled charge-coupled device camera with a maximum resolution of
738 1,280 × 1,024 (DS-Qi1; Nikon). Illumination was provided by an Intensilight C-HGFIE
739 Precentered Fiber Illuminator mercury light source (Nikon). Images were acquired every 1.5
740 s for 5 mins (Movie S1) or every 10 s for 50 mins (Movie S2). Image processing was
741 performed using NIS Elements v 3.22 software (Nikon), as described in the Figure Legends
742 and Supplementary Movie Legends.
743
744 **APEX Electron Microscopy**
745 Huh-7.5 cells were seeded into 150 mm cell culture dishes at 1.5×10^6 cells per dish and
746 cultured overnight before mock-infection or infection with DENV2 or DENV2-NS1-APEX2
747 viruses (MOI: ~0.01), prepared as cell culture supernatants collected from electroporated
748 Huh-7.5 cells. At 4 days post-infection, cells were fixed in EM fixative (1.25%
749 glutaraldehyde, 4% paraformaldehyde, 4% sucrose in PBS [pH: 7.2]) for 30 min at 4°C,
750 washed, stained with DAB/H_2O_2 and processed for electron microscopy as described
751 previously (41). Samples were imaged using a Tecnai G2 Spirit transmission electron
752 microscope (FEI) operating at 100 kV.
753
754 **Molecular Graphics and Sequence Alignments**
755 Molecular graphics were performed on the DENV-2 NS1 crystal structure (Protein Data Bank
756 [PDB] accession number 4O6B) using Pymol version 1.8 molecular visualization system
757 (Schrodinger). Sequence alignments of DENV polyprotein sequences were performed using
758 JalView Desktop software with the ClustalW scoring algorithm. The flavivirus isolates with

759 the following Uniprot KB/Swiss-Prot accession numbers were compared: DENV-1, *P27909*
760 (Brazil/97-11/1997); DENV-2, *P29991* (Thailand/16681-PDK53); DENV-3, *Q6YMS3*
761 (Martinique/1243/1999); DENV-4 *Q2YHF0* (Thailand/0348/1991); West Nile virus *P06935*;
762 Yellow Fever virus, *Q6J3P1* (Ivory Coast/1999); Japanese Encephalitis virus *P27395* (SA-
763 14).
764

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780

781 **FIGURE LEGENDS**

782 **FIG 1.** Insertional mutagenesis of the DENV-2 genome and identification of tolerated
783 insertions. (A) Schematic overview of the transposon mutagenesis-coupled next-generation

784 sequencing (NGS) approach to identify sites within the DENV-2 genome that tolerate small
785 (15-nt) insertions. Briefly, Huh-7.5 cells were transfected by electroporation with *in vitro*-
786 transcribed RNA for the DENV-2 insertional mutant pool (Pool 0). At 6 days post-
787 transfection (Pool 1) virus-containing cell culture supernatants were collected and applied to
788 naïve target cells for an additional 2 days (Pool 2). Total RNA was extracted from transfected
789 and infected cell monolayers (Pools 1 and 2, respectively) and, together with the input mutant
790 RNA library (Pool 0), was used for RT-PCR and NGS-based quantitation of each insertion
791 mutant. Bar graphs displaying the number of reads containing transposon insertions at each
792 nucleotide position are depicted for: (B) the input mutant library (Pool 0); (C) replication-
793 competent viruses (Pool 1) and; (D) infectious viruses. (E) Graphical overlay of insert counts
794 for each pool. The colors assigned to each pool are depicted in the legend shown in the lower
795 panel of (A).
796
797 **FIG 2.** Tolerance of transposon insertions in DENV-2 expressed as a percentage of the initial
798 input mutant RNA pool. (A) Graph depicting the frequency of insertions in the replication-
799 competent pool (Pool 1; P1), expressed as a percentage of the frequency of the corresponding
800 mutants in the initial mutant RNA pool (Pool 0; P0). (B) Similarly, the frequency of
801 insertions in the infectious pool (Pool 2; P2) was expressed as a percentage of the frequency
802 of the corresponding mutants in the initial mutant RNA pool (Pool 0; P0). Nucleotide
803 positions along the x-axis indicate the junctions of the coding sequences of the individual
804 DENV-2 proteins. For clarity the regions encoding the respective DENV-2 proteins are
805 indicated as colored backgrounds. Note that 1 insertion count was added to the raw counts for
806 Pool 0 to enable representation of insertions that only appeared in Pools 1 and/or 2, but not in
807 Pool 0. See also Dataset S1.
808

809 **FIG 3.** The DENV-2 NS1 protein is highly tolerant of insertions. (A) The insertional
810 mutagenesis profile within the NS1-encoding region of the DENV-2 genome was examined
811 with respect to NS1 protein domains. In the NS1 schematic the *β-roll*, *Wing* and *β-ladder*
812 domains are colored blue, yellow and red, respectively, while *connector* sub-domains are
813 depicted in orange and the location of N-glycosylation sites (*N*-glyc) are indicated in green.
814 The graph shows an overlay of the number of raw reads for each transposon insertion site in
815 Pool 0 (cyan), Pool 1 (magenta) and Pool 2 (yellow) and where they overlap, according to the
816 legend to the right of the graph. The *x*-axis shows the DENV-2 nucleotide positions of the
817 NS1-encoding region (nucleotides 2422-3477). (B) The location of the 10 most tolerated
818 transposon insertion sites in the NS1 protein, provided that at least two nucleotides within the
819 given codon preceded tolerated insertions, are shown as green *van der Waals* spheres in a
820 ribbon representation of the DENV NS1 dimer structure (Protein Data Bank [PDB] accession
821 no. 4O6B) (also see Dataset S1). One monomer is shown in gray and the other monomer is
822 colored according to domains as in (A). Insertions at Lys-174, Thr-126 and Ser-204 were
823 highly tolerated. As shown in the perpendicular view (right panel), the most tolerated
824 mutations are relatively distant from the inner hydrophobic face that is pointed downward.
825
826 **FIG 4.** Tolerance of transposon insertions within the capsid-encoding sequence. (A) The
827 insertional mutagenesis profile within the capsid-encoding region of the DENV-2 genome
828 was examined with respect to capsid protein domains. In the capsid schematic the α1, α2, α3
829 and α4 alpha helices are colored blue, green, yellow and red, respectively, while the
830 transmembrane anchor is colored gray. The graph in the upper panel shows the distribution of
831 insertions in the input mutant RNA pool (Pool 0). The graph in the middle panel shows the
832 number of raw sequencing reads for each insertion in the replication competent pool (Pool 1),
833 while the graph in the lower panel shows the number of reads for each insertion in the

834 infectious virus pool (Pool 2). (B) Graphical overlay of the number of raw reads for each
835 transposon insertion site in Pool 0 (cyan), Pool 1 (magenta) and Pool 2 (yellow) and where
836 they overlap, according to the legend below the graph. The *x*-axis shows the DENV-2
837 nucleotide positions of the capsid-encoding region (nucleotides 97-342).
838
839 **FIG 5.** The influence of host cell type on the tolerance of DENV-2 to transposon
840 mutagenesis. (A) Infectious virus-containing cell culture supernatants collected from Huh-7.5
841 cells that were electroporated with the DENV-2 mutant library RNA (see Fig 1A) were
842 applied to naïve Huh-7.5, Vero and C6/36 cells in parallel for 48 hours prior to RNA
843 extraction, RT-PCR and Illumina (NextSeq500) sequencing, according to the depicted
844 schematic diagram. The frequencies of transposon-derived insertions in the DENV-2 genome
845 in infected Huh-7.5 cells (B), Vero cells (C) and C6/36 cells (D) were determined by
846 sequencing. (E) Graphical overlay of normalised insert counts for each pool. The colors
847 assigned to each pool are depicted in the legend shown in the lower panel of (A). (F)
848 Representative immunofluorescent micrographs of parallel cultures of mutant DENV-2-
849 infected cells at 48 h post-infection. Anti-E staining and DAPI counter-stained nuclei are
850 shown in red and blue, respectively. Scale bars are 100 μm.
851
852 **FIG 6.** Characterization of epitope- and reporter-tagged DENV-2 constructs**.** (A) Guided by
853 the insertional map, a panel of tagged viruses were generated that featured the indicated
854 epitope or reporter protein insertions in capsid, adjacent to the membrane anchor
855 ('CAPmem'), or NS1, immediately downstream of Lys-174. (B) Huh7.5 cells were
856 electroporated with *in vitro* transcribed RNA for the indicated DENV-2 constructs and
857 cultured for 4 d prior to Western blot analysis of NS1 protein. Detection of β-actin served as
858 a loading control. Similarly supernatants from these cells were cleared by centrifugation and

859 subjected to SDS-PAGE under non-reducing, non-denaturing conditions and Western blotting
860 with anti-NS1 antibody. Numbers below NS1 Western blots indicate levels of NS1 protein in
861 whole cell lysates (normalized to β-actin and expressed as a percentage of wildtype levels
862 [%-WT]; upper panel) and extracellular NS1 protein (expressed as a ratio to intracellular NS1
863 bands, with the wildtype ratio set to 1 [Ext:Int]; lower panel). (C) Automated
864 immunofluorescence analysis of the proportion of capsid protein-positive Huh-7.5 cells at 4 d
865 post-electroporation with the indicated DENV-2 RNA transcripts. Data are means + S.D (n =
866 3, for >1500 cells/electroporation). (D) Infectivity titres were determined by focus forming
867 unit (FFU) assays at 24-120 h post-electroporation of Huh-7.5 cells with the indicated
868 DENV-2 RNA transcripts. Data are means + S.D. (n = 3). The dashed line indicates the limit
869 of detection of the assay.
870
871 **FIG 7.** The impact of epitope- and reporter-insertions on NS1 secretion and glycosylation in
872 Huh-7.5 cells and infectious virus production in C6/36 and Vero cell lines. (A) Supernatants
873 were collected at 96 h post-transfection from Huh-7.5 cells transfected with the indicated
874 DENV2 RNA transcripts and cleared of cells and debris by low-speed centrifugation (5,000
875 ×*g*, 5 mins). Samples were subjected to SDS-PAGE under reducing and denaturing
876 conditions (βME/heat, +) or non-reducing, non-denaturing conditions (βME/heat, -), as
877 indicated, and Western blotting was performed using anti-NS1 mAb 4G4. Secreted NS1 was
878 strongly detected under non-denaturing, non-reducing conditions for wildtype and tagged
879 viruses alike. (B) Supernatant samples were also subjected to de-glycosylation using PNGase
880 F under non-denaturing conditions (4 h at 37°C) or mock-treated, before SDS-PAGE under
881 non-reducing, non-denaturing conditions and Western blotting with anti-NS1 mAb 4G4. A
882 similar shift in electrophoretic mobility was observed for wildtype and tagged NS1 proteins
883 following PNGase F treatment. (C-D) Infectious virus production by C6/36 cells and Vero

884 cells transfected with epitope- and reporter-tagged DENV2 RNA transcripts. (C) C6/36 cells
885 and (D) Vero cells were transfected with the indicated DENV2 RNA transcripts and returned
886 to culture before collection of supernatant samples at the indicated time-points (24-120 h).
887 Infectivity of supernatants were determined by focus-forming assay. Data are means + S.D.
888 (n = 3). The dashed line indicates the limit of detection.
889
890 **FIG 8.** Localization of NS1 with respect to dsRNA and capsid is unaltered for epitope- and
891 reporter-tagged DENV-2 viruses. Huh-7.5 cells were transfected with *in vitro*-transcribed
892 RNA for DENV2 and NS1- and capsid-tagged DENV2 derivatives and cultured for 96 h prior
893 to fixation and sequential indirect immunofluorescent labelling using anti-dsRNA (mAb 3G1)
894 and Alexa Fluor 555-conjugated anti-mouse IgM (red), followed by either: (A) anti-NS1
895 (mAb 4G4) and Alexa Fluor 488-conjugated anti-mouse IgG (green) or; (B) anti-capsid
896 (mAb 6F3.1) and Alexa Fluor 488-conjugated anti-mouse IgG (green). Samples were
897 counterstained with DAPI (blue) and analysed by confocal fluorescence microscopy. Yellow
898 in the merged images indicates co-localization. Pearson's co-localization coefficients are
899 indicated in white in the merged images (means ± S.D. for n = 3-5 [stitched] fields of 203 ×
900 203 µm, each containing >10 cells). 'Insets' represent zoomed images of the boxed areas in
901 the 'Merge' panels. Scale bars are 10 µm.
902
903 **FIG 9.** Sensitive monitoring of intracellular and secreted levels of NanoLuc-tagged NS1
904 following infection with DENV2-NS1-NLuc. (A) Huh-7.5 cells in 96-well plates were
905 infected with DENV2-NS1-NLuc at the indicated multiplicity of infection (M.O.I.) for 4 h,
906 washed and returned to culture for 48 h prior to determination of intracellular and
907 extracellular NanoLuc activity. (B) Semi-confluent Huh-7.5 cells in 96-well trays were
908 treated with the indicated concentration of Nanchangmycin for 1 hour, prior to infection with

DENV2-NS1-NLuc (M.O.I.: ~0.05) for 4 h. Cells were then washed and returned to culture for 48 h prior to measurement of intracellular and extracellular NLuc activity. Data are means ± S.D. (n = 4). Results are representative of similar repeat experiments.

FIG 10. APEX electron microscopy analysis of NS1 localization. Huh-7.5 cells were mock-infected or infected with untagged DENV2 or DENV2-NS1-APEX2 (M.O.I. ~0.01) and cultured for 96 h prior to fixation and labelling with DAB/H_2O_2. (A) Light microscopy revealed the presence of DAB polymerization only in DENV2-NS1-APEX2-infected cells (right panel). Scale bars are 100 µm. (B) Samples were then processed for EM. Compared to mock-infected cells (not shown), DENV2-infected cells (upper panels) displayed characteristic virus-induced ER rearrangements including CMs (see 'i') and VPs (see 'ii'). Scale bars are 500, 200, 500 and 100 nm, left-to-right, as indicated. In contrast, DENV2-NS1-APEX2-infected cells revealed discernible DAB deposition in the Golgi apparatus ('iii'), ER membranes (see 'ER' arrowheads in 'iv') and intense diffuse staining of VP membranes and punctate staining of the VP interior (see 'zoom inset'). Scale bars are 1000, 100, 500 and 100 nm, left-to-right, as indicated. Enlarged views are shown to the right of each boxed area. In addition to localization to the surface and interior of VPs (see yellow arrows in 'v'), NS1-APEX2 was also detected in irregular punctae that were adjacent to VPs (see yellow arrowheads in 'v'). Furthermore, NS1-APEX2 was less frequently identified in regular arrays (see cyan arrowheads in 'vi') that featured more diffuse membrane staining and punctate luminal staining. Scale bars for 'v' and 'vi' are 100 nm and 200 nm, respectively. CM, convoluted membranes; mit, mitochondrium; VP, vesicle packet; ER, endoplasmic reticulum; LD, lipid droplet.

933 **FIG 11.** STED super-resolution imaging of NS1 and dsRNA localization in infected cells.
934 Huh-7.5 cells were infected with DENV2-NS1-FLAG (M.O.I.: ~0.1) and returned to culture
935 for 48 h prior to fixation and indirect immunofluorescent labelling using anti-FLAG (Alexa
936 Fluor-488, green) and anti-dsRNA (Alexa Fluor-647; red) as described in Materials and
937 Methods. Samples were then mounted and imaged using a Leica TCS SP8 STED 3×
938 microscope system (Leica Microsystems) equipped with 592, 660 and 775 nm STED lasers
939 using a 100x, NA 1.4 oil objective at 4× zoom. Confocal and STED images (in 2D STED
940 mode) were acquired sequentially, as described in Materials and Methods. Imaging data for
941 both confocal imaging and STED imaging were deconvolved using Huygens Professional
942 Deconvolution software (version 14.10; Scientific Volume Imaging) applying default
943 settings. A single optical section for confocal (A) and STED (B) channels is depicted. Scale
944 bars are 5 µm for main images and 500 nm for 'insets'.
945
946 **FIG 12.** Live cell imaging of mScarlet-tagged NS1 in infected cells. (A) Schematic diagram
947 of the DENV2-NS1-mScarlet construct. (B-C) Infectivity of DENV2-NS1-mScarlet
948 compared to wildtype DENV-2. Huh-7.5 cells were transfected with wildtype DENV-2 or
949 DENV2-NS1-mScarlet RNA transcripts and cell culture supernatants were collected at 24-
950 120 h post-transfection for determination of virus infectivity by focus-forming assay (FFA).
951 (B) The immunofluorescent micrographs show representative images from a FFA of cells
952 infected with undiluted DENV-2 or DENV2-NS1-mScarlet supernatants collected at 120 h
953 post-transfection. Cells were labelled with anti-E antibody (green) and mScarlet-associated
954 epifluorescence was also visualised (red). Scale bars are 100 µm. (C) Quantitation of
955 infectivity by FFA. Data are means + S.D. (n = 3). The dashed line indicates the limit of
956 detection. n.d., not detectable. (D-E) Live cell imaging of Huh-7.5 cells transfected with
957 DENV2-NS1-mScarlet transcripts (6 d post-transfection). (D) Detection of mScarlet

autofluorescence in live Huh-7.5 cells following mock-transfection ('Mock') or transfection DENV2-NS1-mScarlet RNA transcripts. Scale bars are 100 µm. (E) Live cell imaging of NS1-mScarlet localization and traffic. Insets depict examples of intensely fluorescent, relatively static NS1-mScarlet foci (cyan arrows) and weakly fluorescent, highly motile NS1-mScarlet foci (cyan arrowheads). See also Supplementary Movie 1. Scale bars are 10 µm for the main image and 5 µm for insets.

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