Structural and interaction studies of the NS4B integral membrane protein from dengue virus

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2017

Lee, L. T. (2017). Structural and interaction studies of the NS4B integral membrane protein from dengue virus. Doctoral thesis, Nanyang Technological University, Singapore.

<http://hdl.handle.net/10356/72869>

<https://doi.org/10.32657/10356/72869>

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Structural and interaction studies of the NS4B integral membrane protein from Dengue Virus

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School of Biological Sciences
2017

Structural and interaction studies of the NS4B integral membrane protein from Dengue Virus

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A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Doctor of Philosophy
2017

Acknowledgements

First, I would like to extend my sincere gratitude to both my supervisors, Professor Julien Lescar and Professor Pei Yong Shi, for giving me the opportunity to pursue a PhD degree. Julien for the valuable guidance and advice he has given me throughout my PhD. Pei Yong for his support and encouragements over the years. Without both of them, my project and scientific career would not have progressed this far. I would also like to thank my direct manager and mentor, Dr Christian Noble, for his advice and guidance, and also all the assistance given to me.

My acknowledgements to all my collaborators, Professor Susana Geifman Shochat, Dr Congbao Kang, Dr Yin-Hoe Yau, for helping me in my projects. To all my colleagues in NITD and JL lab, a very big thank you for all their support, guidance, and constructive discussions. Thank you for providing me with such wonderful experiences and memories in the laboratory. To all the people in PPP, thank you for producing the constructs for me, and allow my work to progress so much faster. Lastly, I would like to thank my family for going through my PhD journey with me. I would not have completed this journey, without their unwavering support, both emotionally and financially. The last two years has been very fruitful; I have gotten married to my lovely wife, gotten our home, and gotten our precious son. Now it's time to get my degree.

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Abbreviations

Abbreviation	Full name
-RNA	Negative-strand RNA
+RNA	Positive-strand RNA
2x YT	2x Yeast tryptone
AcrB	Acridine resistance protein B
ADME	Absorption, distribution, metabolism, and excretion
ATCC	American Type Culture Collection
ATF6	Activating transcription factor 6
BEVS	Baculo-virus expression system
bRIL	Cytochrome b ₅₆₂ RIL
C	Capsid
CD	Circular dichroism
CDC	Centers for Disease Control and Prevention
CM	Convolute membrane
CNIBR	China Novartis Institutes for BioMedical Research
co-IP	Co-immunoprecipitation
CPM	N-[4-(7-diethylamino-4-methyl-3-coumarinyl)phenyl]maleimide
cryoEM	Cryo-electron microscopy
CV	Column volume
CYMAL 6	6-Cyclohexyl-1-hexyl- β -D-maltoside
DAA	Direct-acting antivirals
DALY	Disability-adjusted life-years
DAPI	4', 6-diamidino-2-phenylindole
DDM	<i>n</i> -dodecyl- β -D-maltopyranoside
DM	<i>n</i> -Decyl- β -D-maltopyranoside
DENV	Dengue Virus
DF	Dengue fever
DHF	Dengue haemorrhagic fever
DLS	Dynamic light scattering
DMEM	Dulbecco modified Eagle medium
dsRNA	Double-stranded RNA
DSS	Dengue shock syndrome
E / Env	Envelope
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ELF1A	Eukaryotic translation elongation factor 1A
EIF4A3	Eukaryotic Translation Initiation Factor 4A3
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy

EMDB	Electron Microscopy Data Bank
ER	Endoplasmic reticulum
ET	Electron tomography
FASN	Fatty acid synthase
FBS	Fetal bovine serum
FRET	Fluorescence resonance energy transfer
<i>g</i>	Gravitation force
GFP	Green fluorescence protein
GluCl	Glutamate-gated chloride channel
h	Hours
HCV	Hepatitis C virus
HDA	Host directed antivirals
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HRP	Horse radish peroxidase
HTS	High-throughput screen
IgG	Immunoglobulin gamma
IMAC	Immobilized-metal affinity chromatography
IP	Immunoprecipitation
IPTG	Isopropyl- β -D-1-thiogalactopyranoside
IRE-XBP1	Inositol-requiring protein - X-box binding protein
IV	Invaginated vesicles
JEV	Japanese Encephalitis Virus
K_D	Equilibrium dissociation constant
kDa	Kilo-daltons
KRT8	Keratin 8
KUNV	Kunjin Virus
LCP	Lipidic cubic phase
LDAO	Lauryldimethylamine-oxide
LDS	Lithium dodecyl sulfate
LIC	Ligation independent cloning
mAb	Monoclonal antibody
MALDI-TOF	Matrix-assisted laser desorption/ionization – time of flight
MES	2-(N-morpholino)ethanesulfonic acid
MME	Monomethyl ether
MOH	Ministry of Health, Singapore
MOI	Multiplicity of infections
MS	Mass spectrometry
Mtase	Methyltransferase
MWCO	Molecular weight cut-off

NEA	National Environmental Agency, Singapore
NI	Nucleoside/nucleotide inhibitor
NIBR	Novartis Institutes for BioMedical Research
Ni-NTA	Nickel - nitrilotriacetic acid
NITD	Novartis Institute for Tropical Diseases
NM	n-Nonyl- β -D-Maltopyranoside
NMR	Nuclear magnetic resonance
NNI	Non-nucleoside inhibitors
NS	Non-structural
NTPase	Nucleotide triphosphatase
OD	Optical Density
OG	<i>n</i> -octyl- β -D-glucopyranside
OmpF	Outer membrane protein F precursor
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cells
p.i	Post infection
p.t	Post transfection
P2X4	P2X purinoceptor 4 receptor
pAb	Polyclonal antibody
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PERK	Protein kinase RNA-like endoplasmic reticulum kinase
PGK1	Phosphoglycerate kinase 1
PK	Pharmaco-kinetics
PLA	Proximity of ligation assay
PPI	Protein-protein interactions
PPP	Protein Production Platform
PrM	Pre-membrane
RC	Replication complex
RdRp	RNA-dependent RNA polymerase
rER	Rough endoplasmic reticulum
Rluc	<i>Renilla</i> luciferase
rpm	Revolutions per minute
SAR	Structure–activity relationship
scFv	Single chain variable fragment
SDS	Sodium dodecyl sulphate
SEC	Size exclusion chromatography
SEC-MALS	Size exclusion chromatography - multi-angle static light scattering
SILAC	Stable isotope labeling with amino acids in cell culture
SMA	Styrene maleic acid

SMALP	SMA lipid particles
SPA	Scintillation proximity assay
SPR	Surface plasmon resonance
ssRNA	Single-stranded RNA
T4L	T4 lysozyme
TCEP	Tris-(2-carboxyethyl)-phosphine
TEV	Tobacco etch virus
TMD	Transmembrane domains
TNK2	Tyrosine kinase non receptor 2
Tris	Tris(hydroxymethyl)aminomethane
Ube2i	SUMO-conjugating enzyme Ube2i
UPR	Unfolded protein response
UTR	Untranslated regions
v/v	Volume per volume
VCP	Valosin-containing protein
Ve	Elution volume
VLP	Viral-like particle
w/v	Weight per volume
WB	Western Blot
WCL	Whole cell lysate
WHO	World Health Organization
WNV	West Nile Virus
WT	Wild type
Y2H	Yeast two-hybrid
YFV	Yellow Fever Virus
ZV	Zika Virus

Abstract

Dengue Virus (DENV) is a member from the *Flavivirus* genus of positive-stranded RNA (+RNA) viruses, which includes several other major human pathogenic viruses. Disease manifestation of DENV infection ranges from a self-limiting dengue fever (DF) to life threatening forms - dengue haemorrhagic fever (DHF) or dengue shock syndrome (DSS). Due to a combination of urbanisation, globalisation, and climate change over the recent decades, DENV has emerged as the major arbovirus causing diseases in the world. It is now estimated that 40% of the world population is at risk of dengue. However, there is currently no drug available for dengue treatment, and the approved Dengvaxia vaccine has only approximately 60% efficacy. Therefore, there is an urgent need to develop effective anti-dengue therapies. Unfortunately, drug discovery is hampered by the need to target all four serotypes simultaneously, as well as by our still relatively poor understanding of dengue disease progression and pathogenesis. Non-structural protein (NS) 4B from DENV is an essential player during viral replication, and has been identified by several groups as a potential target for drug development. However, its intrinsic membrane association and non-enzymatic nature has impeded progress in our understanding of NS4B. In this project, we aimed to better understand the biological roles of NS4B and also to assist drug development of anti-dengue compound through inhibition of NS4B activities. This goal was approached using three strategies, 1) to overexpress and crystallise NS4B for structural determination; 2) to map the NS4B-NS3 interaction; and 3) to identify new host factors interacting with NS4B during viral replication.

Structural determination of NS4B - various recombinant NS4B protein constructs were successfully expressed, purified to monodispersity and the yield were sufficient for

crystallisation studies. Crystals were obtained using vapour diffusion, antibody-assisted crystallisation and lipidic cubic phase (LCP) methods. However, most of the crystals were not reproducible or did not diffract. Only a few crystals produced weak diffractions, but were not sufficient for solving the NS4B structure. Structural determination by coupling cryo-electron microscopy (cryo-EM) with NS4B purified using styrene maleic acid (SMA) is the most promising technique at the moment, but more experiment to optimise sample preparation for cryo-EM is needed.

Mapping NS4B-NS3 interaction - the NS4B-NS3 interaction was mapped using a combination of biochemical, biophysical and genetic approaches. We mapped the interaction to subdomains 2 and 3 of NS3 helicase (NS3hel) and the cytoplasmic loop of NS4B. 12 residues on the NS4B cytoplasmic loop putatively involved in mediating the interaction were identified, and further genetic analysis using alanine substitutions, demonstrated that Q134, G140 and N144 are important for DENV replication, possibly due to their involvement in interacting with NS3hel. Q134 was subsequently confirmed by other authors to be involved in mediating NS4B-NS3 interaction. Co-crystallisation of NS3hel domain with synthesised NS4B cytoplasmic loops was explored, but only apo-NS3hel crystals were obtained.

Identify new host factors interacting with NS4B - we identified 106 host proteins that interact with NS4B during DENV replication using a novel approach of coupling quantitative stable isotope labeling with amino acids in cell culture mass spectrometry (SILAC MS/MS) with immunoprecipitation (IP). Subsequent short interfering RNA (siRNA) validation experiments identified Sec61 α as an important host factor for DENV replication, and biochemical validation demonstrated the association of Sec61 α with NS4B during viral replication. Finally, we demonstrated that inhibition of the

Sec61 translocation complex (to which Sec61 α is a component of) using a natural product, decatransin, led to potent inhibition of DENV replication.

In summary, our results provide important materials for further studies of NS4B, including inhibitor design.

Chapter 1 Introduction

1.1 The Dengue Epidemic – A serious global threat

Dengue Virus (DENV) is a member of the *Flavivirus* genus that includes several other major human pathogens such as Yellow Fever Virus (YFV), West Nile Virus (WNV), Kunjin Virus (KUNV), Japanese Encephalitis Virus (JEV), and Zika Virus (ZV) (CDC, 2016; T.C Pierson & Diamond, 2013). There are four evolutionarily related serotypes but antigenically distinct members within the DENV species – DENV type 1 to type 4 (DENV1 to DENV4) (CDC, 2016; T.C Pierson & Diamond, 2013; WHO, 2016). DENV is the causative agent of DF, which provokes a broad spectrum of disease symptoms - from asymptomatic or mild fever to more severe and potentially life-threatening forms –DHF and DSS (CDC, 2016; WHO, 2016). DHF and DSS, also collectively termed as severe dengue, are mainly caused by secondary infection with another DENV serotype mediated by antibody-dependent enhancement (ADE), although primary infections can lead to severe dengue as well [refer to (Halstead, 2014) for a comprehensive review].

In recent decades, dengue has been spreading across the globe unabated; it has now become the most widely spread disease caused by an arbovirus in the world (CDC, 2016; WHO, 2012). Traditionally, dengue was only prevalent in tropical and sub-tropical regions across Asia, Africa and South America, where its main transmission vector – *Aedes aegypti* and *Aedes albopictus* mosquitoes reside (Gubler, 2011). However, a combination of rapid urbanisation, increased air travel due to globalisation, and climate change due to global warming (termed the ‘unholy’ trinity), has led to the spread of *Aedes* mosquitoes to new territories (Gubler, 2011). This has exacerbated the spread of dengue within traditionally endemic countries, and also caused outbreaks in

temperate climate countries such as France, Japan, USA and Australia (Gubler, 2012; Radke et al., 2012; Rezza, 2014; Schaffner & Mathis, 2014). Consequently, together with increased reporting and surveillance, the global incidence of dengue has increased some thirty-fold since the 1950s, imposing heavy disease and economic burden on the affected countries (WHO, 2012). It is now estimated that 40% of the world's population is at risk of dengue. In 2010, it was estimated that almost 393 million infections occurred globally, of which approximately 91 – 93 million were symptomatic cases, while the rest were asymptomatic or unreported (Figure 1.1) (Bhatt et al., 2013).

A

B

Source: WHO

Figure 1.1. The distribution and estimated dengue cases around the world. (A) Cartogram showing the estimated global distribution of dengue. Tropical and subtropical countries are the most adversely affected regions. **(B)** The estimated

numbers of asymptomatic and symptomatic dengue cases around the world. [Adapted from (Bhatt et al., 2013)].

In two recent studies published by Stanaway *et al*, the disability-adjusted life-years (DALY) for dengue in 2013 was estimated to be 1.14 million, while the economic burden was approximately US\$8.9 billion (Shepard, Undurraga, Halasa, & Stanaway, 2016; Stanaway et al., 2016). Despite the gravity of the situation, there is currently no anti-viral available for treatment, and vector control remains the only effective avenue to reduce the spread of dengue (CDC, 2016; WHO, 2012). Although a new vaccine – Dengvaxia® – produced by Sanofi S.A has been approved in several countries recently, including Singapore, some concerns regarding its efficacy and safety have surfaced (discussed in section 1.4.1). Therefore, there is still an urgent need to develop an effective anti-dengue treatment to curb the spreading epidemic.

1.1.1 The Dengue epidemic in Singapore

Dengue has been plaguing Singapore ever since the first reported dengue outbreak in 1960, where there were 88 hospitalisation cases (Chan, 1972). Significant amounts of resources and efforts have been employed to combat dengue in Singapore over the past five decades, primarily focusing on active surveillance, vector control and public education (NEA, 2017). However, despite having one of the most well-established dengue surveillance and prevention program in the world that has succeeded in lowering the *Aedes* mosquito population, dengue incidences continue to rise rapidly over the years, and reached a record high of 22,077 cases reported in 2013 (MOH, 2017).

Figure 1.2. The number of reported dengue cases in Singapore for the past decade.
(Source: Ministry of Health, Singapore)

This rapid increase has been attributed to three factors, 1) decline in herd immunity; 2) population growth; and 3) climate change (Ooi, Goh, & Gubler, 2006; Struchiner, Rocklov, Wilder-Smith, & Massad, 2015). The herd immunity in Singapore was lowered due to the implementation of effective vector control measures in the 1970 – 1980's, and this resulted in the re-emergence of dengue in the 1990 and 2000. A comparison between school going children in Singapore and Thailand revealed a seroprevalence rate of only 6.7% and 71% respectively (Ooi et al., 2006). In the same period, Singapore underwent rapid urbanisation and the population doubled in size. It was estimated by Struchiner *et al.* that population growth contributed to 86% of the observed increase in dengue cases from 1974 to 2011. Urbanisation increased the number of habitats for *Aedes* mosquitoes to breed, while the higher population density

increased opportunities for mosquitoes to feed and thus accelerate the transmission of dengue. Lastly, it was estimated, from the same study, that Singapore's mean annual temperature increased by approximately 2°C due to global climate change, and this led to the acceleration of *Aedes* mosquito maturation cycle, while shortening the incubation periods for DENV, which accelerates disease transmission (Struchiner et al., 2015). This temperature increase was estimated to contribute to 14% of the increase in dengue incidence in Singapore (Struchiner et al., 2015). It was estimated that dengue has caused an economic cost of US\$0.85-1.15 billion between 2000 - 2010, and an estimated disease burden of 9 – 14 DALYs per 100, 000 habitants (Carrasco et al., 2011). Therefore, to tackle this worsening dengue problem, the Singapore government has been investing significant amounts of money into research programs such as the TCR Flagship STOP Dengue programme, to fund development of better vector control measures; faster diagnostic tools; vaccine development; and development of anti-dengue antiviral compounds (Leo, 2012).

1.2 Dengue molecular biology

1.2.1 Dengue viral life cycle

DENV is an enveloped virus with a +RNA genome of about 11 kb (Lindenbach, Murray, Thiel, & Rice, 2013). Virions enter host cells, mainly immune cells, such as dendritic cells, via several types of cell surface receptors using the clathrin mediated endocytosis pathway, and viral RNA is released into the cytoplasm upon acidification of carrier endosomes (Perera-Lecoin, Meertens, Carnec, & Amara, 2013). The viral RNA acts as mRNA that is directly translated into viral polyproteins, in large quantities, onto the ER membrane by the host translation machinery. The polyproteins are then

cleaved into individual proteins by NS2B/NS3 viral protease and host proteases. The processed viral membrane proteins, namely NS4A and NS4B and recruited host factors such as fatty acid synthase (FASN), induce extensive rearrangements to the rough endoplasmic reticulum (rER) membrane forming ultra-structures called invaginated vesicles (IV, also known as vesicle packets) and convoluted membranes (CM) (Heaton et al., 2010; Kaufusi, Kelley, Yanagihara, & Nerurkar, 2014; Welsch et al., 2009). These ultra-structures facilitate the formation of viral replication complexes (RC) required for RNA replication. Newly synthesised +RNA are packaged into capsid (C) proteins to form the ribonucleocapsids. The ribonucleocapsids are transported into the ER lumen where viral assembly occurs, forming immature virions. The immature virions are transported into and through the Golgi network, where several stages of virus maturation, such as furin cleavage and pH dependent maturation, take place. Finally, mature virions are transported and released into the extra-cellular space at neutral pH via the Golgi secretory network. Refer to (Mukhopadhyay, Kuhn, & Rossmann, 2005; Screaton, Mongkolsapaya, Yacoub, & Roberts, 2015) for a detailed review of the various stages of the flavivirus life cycle.

Figure 1.3. The life cycle of DENV in the human host. [Adapted and modified from: Screaton *et al.*, (2015) and Shi, (2014)].

1.2.2 Dengue genome and protein functions

The viral RNA is made up of one large open reading frame (ORF) that is sandwiched between 5' and 3' untranslated regions (UTR) (T.C Pierson & Diamond, 2013). The ORF is translated as a single large polyprotein of approximately 370 kDa, which is cleaved either during or after translation by host proteases or DENV protease (NS2B-NS3) into individual proteins (Figure 2) (T.C Pierson & Diamond, 2013). There are three structural proteins that are required for viral particle assembly – capsid (C); pre-membrane (prM); envelope (E) protein, and also seven NS proteins and are required for viral replication and modulation of host defences – NS1; NS2A; NS2B; NS3; NS4A; NS4B; NS5 (Lindenbach *et al.*, 2013).

Figure 1.4. Schematics of DENV genome and polyprotein organization. Coloured arrows indicate the regions that are cleaved by host or DENV NS3 proteases. [Modified from: (Umareddy et al., 2007)]

Dengue NS proteins can be classified into enzymatic and non-enzymatic proteins. The enzymatic proteins, NS2B-NS3 protease - helicase, and NS5 methyltransferase - polymerase, have been extensively studied, because they are potentially good drug targets, and are the main enzymes within the RC for synthesising nascent RNA during RNA replication (Klema, Padmanabhan, & Choi, 2015; Lim et al., 2013). The NS3 protein is known to function as a protease (with NS2B as a co-factor), a helicase, as well as a nucleotide triphosphatase (NTPase) (Lescar et al., 2008; H. Li, Clum, You, Ebner, & Padmanabhan, 1999). NS5 is a bi-functional protein with methyltransferase (MTase) and RNA-dependent RNA polymerase (RdRp) activities (Ackermann & Padmanabhan, 2001; M. P. Egloff, Benarroch, Selisko, Romette, & Canard, 2002). NS1 is a non-enzymatic glycoprotein, and has been reported to participate in viral replication, as well as inhibiting innate and adaptive immunity (Muller & Young, 2013). The other NS proteins (NS2A, 2B, 4A, 4B) are transmembrane proteins involved in

RNA replication and/or innate immune response control (Leung et al., 2008; Miller, Sparacio, & Bartenschlager, 2006; Munoz-Jordan et al., 2005; Roosendaal, Westaway, Khromykh, & Mackenzie, 2006; Xie, Gayen, Kang, Yuan, & Shi, 2013). Their intrinsic membrane association makes them less amenable to biochemical studies, and therefore little is known about their functions. However, these membrane proteins, especially NS4B, have emerged as attractive drug targets for development of anti-flavivirus therapy. The main features and functions of the DENV proteins are summarized on Table 1.1.

Table 1.1. Summary of the main features and functions of DENV structural and NS proteins. References for in-depth reviews are indicated on the right panel.

Protein	Size (kDa)	Main feature(s) and function(s)	Reference for in-depth review
Capsid	12	<ul style="list-style-type: none"> • Nucleocapsid protein • Hydrophobic domain mediates membrane association 	Oliveira, Mohana-Borges, de Alencastro, & Horta, 2017
PrM	22	<ul style="list-style-type: none"> • Involved in nucleocapsid dissociation • Prevents premature fusion to host cell membranes before virus release • Cleaved to form mature M protein prior to virus release 	L. Li et al., 2008
Envelope	53	<ul style="list-style-type: none"> • Mediates viral attachment and fusion • Major antigenic determinant 	T. C. Pierson & Diamond, 2012
NS1	46	<ul style="list-style-type: none"> • 2 forms - ER-anchored or secreted • Involved in RNA replication, activate innate immune system, contributes to pathogenesis • Part of viral replication complex 	Watterson, Modhiran, & Young, 2016
NS2A	22	<ul style="list-style-type: none"> • Membrane protein • Involved in RNA replication, viral assembly and release • Part of viral replication complex 	Xie et al., 2013; Xie, Zou, Puttikhunt, Yuan, & Shi, 2015
NS2B	14	<ul style="list-style-type: none"> • Membrane protein, Co-factor for Dengue protease • Involved in mediating membrane permeability; possibly as a viroporin • Part of viral replication complex 	Luo, Vasudevan, & Lescar, 2015
NS3	69	<ul style="list-style-type: none"> • Multi-function protein – protease, helicase, NTPase • Involved in polyprotein processing, RNA replication, regulates host immune response • Part of viral replication complex 	Luo, Vasudevan, & Lescar, 2015
NS4A	16	<ul style="list-style-type: none"> • Membrane protein • Involved in ER membrane remodeling, formation of viral replication complex 	Miller, Kastner, Krijnse-Locker, Buhler, & Bartenschlager, 2007
NS4B	29	<ul style="list-style-type: none"> • Membrane protein • Involved in RNA replication, suppression of Interferon β, γ signaling • Part of viral replication complex 	Xie, Zou, Wang, & Shi, 2015
NS5	105	<ul style="list-style-type: none"> • Multi-function protein – methyl-transferase, RNA-dependent RNA polymerase • Involved in regulation of RNA synthesis, RNA cap formation, blocking Interferon signaling • Part of viral replication complex 	Lim, Noble, & Shi, 2015

1.2.3 Host immune response to dengue infection and dengue pathogenesis

During natural dengue infections, DENV are released into the epidermis of the host via a mosquito's saliva during a blood meal. Within the epidermis, the released DENV can infect and replicate in resident immune cells, such as Langerhan cells. As replication proceeds, more immune cells becomes infected. The DENV then spread into the lymphatic system, when the infected immune cells migrate into the lymph nodes. At the lymph nodes, other immune cells such as monocytes and macrophages become targets for infection. Eventually, the infecting DENV spread to the whole body through the lymphatic system and entering blood circulation, leading to viremia. In responses to viral infection, the infected immune cells will start producing and secreting interferons, as part of a nature host defence mechanism to fight pathogen infections, in this case dengue viral infection. The secreted interferons serve to disrupt viral replication within the cell, alert and protect surrounding uninfected cells, as well as to activate host innate and adaptive immune responses (Martina, Koraka, & Osterhaus, 2009; Scitable, 2017). In the adaptive immune response, both humoral and cell-mediated immunity are activated. Initial activation of the humoral immunity leads to the production of IgM antibodies by naïve B cells to fight infection, and subsequently some B cells switch class to produce IgG antibodies. A subset of B cells eventually turn into memory B cells, which provide life-long protection against the same DENV serotype (CDC, 2017). In the other branch of the adaptive immune response involving cell-mediated immunity, both cytotoxic ($CD8^+$) T cells and helper ($CD4^+$) T cells are activated. $CD8^+$ T cells are involved the direct killing of infected cells via direct cytotoxicity and the production of pro-inflammatory cytokines such as $IFN-\gamma$ and $TNF\alpha$ (Weiskopf & Sette, 2014). While $CD4^+$ T cells indirectly fight DENV infections

by facilitating B cell and CD8⁺ T cell activation and promote the generation of immune memory responses. At the same time, CD4⁺ T cells secrete pro-inflammatory cytokines to limit infection (Weiskopf & Sette, 2014). The anti-viral activities of the adaptive immune response is enhanced by the activation of the innate complement pathway, leading to neutralization and clearance of the infecting DENV (Scitabile, 2017).

Severe dengue is associated with high viral load, vascular permeability, plasma leakage and haemorrhage in patients, and progression to severe dengue has been heavily linked to heterotypic secondary infection (Guzman, Gubler, Izquierdo, Martinez, & Halstead, 2016). Currently, the full picture of the exact mechanisms on how a portion of secondary infections progress to severe dengue remains poorly understood and an area under intense debate and research. Over the years, several key pieces of this puzzle has been identified, these includes 1) host immune (humoral and T-cell) responses; 2) viral determinants; and 3) host determinants.

Host immune responses – A well-known mechanism involving the humoral response is ADE. During primary infection by one DENV serotype, high titre of serotype-specific neutralising antibodies are produced, which provides life-long immunity to that serotype (Halstead, 2014). At the same time, these antibodies also provide short-term heterotypic protection against other serotypes due to cross-reactivity, but the neutralising power wanes over time. It is believed that during a secondary heterotypic infection years later, these antibodies will bind to and facilitate viral entry into immune cells expressing Fcγ receptors instead inactivating the virus due to sub-neutralising abilities, thus enhancing viral infection. This mechanism is believed to increase viral load and rapid disease onset during early infection, which in turn lead to an increased in the production of pro-inflammatory and vasoactive cytokines by the host immune

system and lead to severe dengue due to increased vascular permeability (Guzman et al., 2016; Halstead, 2014). This phenomenon is a major concern for dengue vaccine development, prompting the need to develop a tetravalent vaccine against all serotypes. Another phenomenon associated with the host immune response is termed the ‘original antigenic sin’ (Rothman, 2011). In this phenomenon, it is postulated that pre-existing memory T cells produced after a primary infection are cross-reactive against the new infecting DENV serotype during secondary infections, albeit with low avidity. However, there is a preferential activation and expansion of these low avidity T cells over homotypic naïve T cells that have higher avidity by the host immune system. This results in ineffective viral clearance, resulting in an over-reactive immune response to clear the infecting DENV and leading to the overproduction of pro-inflammatory and vasoactive cytokines such as TNF- α and IFN- γ causing a ‘cytokine storm’ that is believed to cause vascular leakage (Rothman, 2011).

Viral determinants –High titres of NS1 and anti-NS1 antibodies has been observed in DSS/DHF patients, and therefore it is speculated that these two observations may contribute disease progression. The NS1 protein possesses a conserved motif that is also conserved in host proteins, and the production of anti-NS1 antibodies lead to autoimmunity (Amorim, Alves, Boscardin, & Ferreira, 2014). Anti-NS1 antibodies have been reported to bind protein disulphide isomerase on platelets which disrupts platelet aggregation and increase bleeding tendencies (H. J. Cheng et al., 2009). Binding to prothrombin and thrombin has also been observed, resulting in the disruption of host coagulation function (Lin et al., 2012). It is also hypothesised that the auto-antibodies could bind to endothelial cells leading to tissue damage and plasma leakage by activating the complement pathway, but this hypothesis requires further

validation because vascular permeability was reported to alleviate rapidly in patients recovering from severe dengue (Amorim et al., 2014). NS1 is also involved in modulating the innate host response by interacting with C1s, C4 and C4b binding protein of complement pathway leading to evasion of the host immune response by inhibiting complement-dependent neutralization, and promote viral replication (Avirutnan et al., 2010; Avirutnan et al., 2011). More recently, it was reported by Modhiran *et al* that NS1 is involved increased pro-inflammatory cytokine production through the activation of mouse macrophages and human peripheral blood mononuclear cells (PBMC) by Toll-like receptor 4, and blockage of this receptor inhibited vascular leakage *in vitro* and *in vivo*. The study also reported that NS1 could disrupt endothelial cell integrity *in vitro* (Modhiran *et al.*, 2015). Another study reported that injected of purified NS1 induced the production of pro-inflammatory cytokines in mice, and a sublethal DENV-2 challenge together with of NS1 in mice led to lethal consequences due to vascular leakage. The observation could be reversed by immunising the mice with anti-NS1 mAb or serum from mice that are immune to NS1. *In vitro*, addition of NS1 led to increased permeability in endothelial cells (Beatty et al., 2015). Therefore, these recently two studies provided strong evidences that NS1 might play an important role in disrupting endothelial cell integrity and causing vascular leakage during progression to severe dengue.

Another possible viral determinant is the intrinsic DENV virulence. It has been suggested that the degree of virulence – the ability to infect hosts and cause severe dengue - varies between DENV serotypes and genotypes, and infection by a more ‘virulent’ strain increases the chance of developing severe dengue (Rico-Hesse, 2003, 2007). This hypothesis is exemplified by observations that majority of severe dengue

cases were caused by secondary DENV-2 infections (Watts et al., 1999). It was also observed that within the DENV-2 serotype, the American genotype was less virulent and do not cause DHF even during secondary infections, while the Southeast Asian genotype is more virulent and is capable of causing DHF (Cologna & Rico-Hesse, 2003). It was uncovered by Rico-Hesse et al. that nucleotides differences in the 5'-3' UTR and an amino acid difference at position 390 on the E proteins between both genotypes were the main contributing factor for a higher rate of replication by the Southeast Asian genotype compared to the American genotype in monocytes and dendritic cells. A N390D mutation and the replacement of 5'-3' UTR sequence of the Southeast Asian genotype with that of the American genotype led to reduced replication rate in dendritic cells (Cologna & Rico-Hesse, 2003). In addition, investigations by Anderson *et al* revealed that difference in virulence could be attributed to the ability of the Southeast Asian genotype to replicate more efficiently within the mosquito vector, which leads to faster disease dissemination by the Aedes mosquitoes (Anderson & Rico-Hesse, 2006). These two observations provides a possible explanation on why the Southeast Asian genotype is more infectious and has the ability to cause DHF, while the American genotype could not.

Host determinants – Apart from the host immune statues, host determinants such as patient genetics and age were postulated to play a role disease progression.

Genetic variations within hosts has reported to increase susceptibility or confer resistance to severe dengue [see (Kalichamy Alagarasu, 2016) for a comprehensive list]. For example, hosts with certain human leukocyte antigens (HLA) variants such as HLA-A*0203 or HLA-DQ1 were more prone to DF, while host having HLA-A*24 or HLA-A*03 were associated with higher susceptibility to DHF. On the hand, host

with HLA-A-33 or HLA-B-18 were protected from DHF. Furthermore, hosts that possess a Vitamin D3 receptor variant at position 352 was reported to be resistance to DF, while hosts with single nucleotide polymorphism causes a R131H substitution on the Fcγ receptor IIa gene confers protection to DHF (K. Alagarasu et al., 2012; Loke et al., 2002).

The age of infected patients also have important implication in dengue disease progression. Young children were observed to be more susceptibility to DHF during secondary infections compared to adults with the same exposure, which might be contributed by greater capillary fragility in children (Guzman et al., 2016). While elders with pre-existing medical conditions such as diabetes and health diseases, were reported to be more susceptible to severe dengue (Guzman et al., 2016).

1.3 RNA replication – A concerted process involving viral proteins and host factors

1.3.1 Virus induced ultra-structures and the viral RC

Formation of membranous ultra-structures during viral replication is a hallmark of all +RNA viruses (Shulla & Randall, 2016). Flaviviruses in particular are well-known for extensively remodelling different host organelle membranes to facilitate efficient viral RNA replication by 1) spatially partitioning RNA replication from viral particle assembly to prevent possible steric clashes; 2) allowing storage of essential metabolites within the ultra-structure; 3) providing protection from host RNA sensing in the cytoplasm (Paul & Bartenschlager, 2013). Using electron tomography (ET) and 3D reconstruction techniques, Welsch *et al.* were able to visualise the architecture of CM and IV induced by DENV during infection (Welsch et al., 2009). Both structures were

visualised to be formed from a single layer of ER derived membrane, and are interconnected with each other within the ER membrane network. The CM was found to be unstructured, and may serve as a storage site for metabolites and viral proteins required for RNA replication, because several viral proteins were detected within the CM, while double-stranded RNA (dsRNA), a common marker for RNA replication) was absent. The IV, on the other hand, is a porous invagination into the ER lumen due to negative membrane curvatures induced by the viral membrane remodelling proteins – NS4A and NS4B. The invaginations are approximately 90 nm in diameter and were found to be connected to the cytoplasm through a 10 nm opening (Figure 1.5). The opening is believed to serve as a channel for transporting metabolites required for RNA replication into the IV from the cytoplasm. Replicase proteins (NS3 and NS5) and dsRNA, which form part of the viral RC, were detected within the IV found in both infected mammalian and mosquito cells. This finding suggested that IV is the site housing the viral RC and where viral RNA replication takes place (Welsch et al., 2009).

Figure 1.5. Transmission electron micrograph and corresponding 3D reconstruction of ET of IV, and CM induced during DENV infection. [Adapted from (Chatel-Chaix & Bartenschlager, 2014)]

The RC is a multi-component structure, consisting of viral proteins, host proteins, and the RNA template, assembled for the replication of viral RNA (Klema et al., 2015; Paul & Bartenschlager, 2013). Using colocalisation with dsRNA as a marker for RNA replication, all the NS proteins have been reported to play a role in RNA replication (Klema et al., 2015; Paul & Bartenschlager, 2013, 2015). However, the exact stoichiometry and architecture of the Dengue RC still remains unknown. Elucidating the RC architecture would greatly enhance our understanding on DENV biology and pathogenesis, and will also benefit drug discovery efforts.

1.3.2 Viral protein functions and protein-protein interactions (PPI) within the RC

The two enzymatic viral proteins, NS3 and NS5 form the replicase complex and are the most important viral components of the RC because both proteins perform all the enzymatic activities required for RNA replication (Klema et al., 2015). The NS3hel domain contains conserved RNA binding motifs on subdomains 1 and 2, and RNA binding channel on subdomain 3 that are important for RNA binding (Luo et al., 2015). At the same time, NS3hel has ATPase, NTPase and helicase activities that are important for dsRNA unwinding during RNA replication (Luo et al., 2015). NS3hel also has RNA 5'triphosphatase activity that is required for RNA cap formation after nascent +RNA synthesis (Luo et al., 2015). The NS5 MTase domain is responsible for maintaining the stability of newly synthesised +RNA via cap formation and sequential methylation at the N-7 and 2'-O positions of the cap structure (m⁷GpppAm-RNA) (M. P. Egloff et al., 2002). The MTase domain also plays a role for regulating NS5 RdRp activity to ensure efficient *de novo* initiation and elongation phases (Potisopon et al., 2016). The NS5 RdRp is the main enzyme involved in the synthesis of new RNA during RNA replication (Klema et al., 2015). It is responsible for the initial synthesis of a negative-sense RNA (-RNA) genome using viral +RNA genome as a template to form a dsRNA intermediate. The -RNA genome is subsequently used as a template for the synthesis of additional +RNA genome. An asymmetric replication occurs and approximately 10 – 100 times more +RNA than -RNA is synthesised by NS5 RdRp during replication (Cleaves, Ryan, & Schlesinger, 1981).

Other than remodeling of ER membrane during the formation of IV and CM, and colocalisation with dsRNA, how or whether the membrane-bound NS proteins play a direct role in RNA synthesis is unclear. Nevertheless, they are believed to be important

for anchoring the viral replicase proteins and RNA template to the ER membrane within the IV (Figure 1.6) (Klema et al., 2015; Paul & Bartenschlager, 2013). Dimeric NS1 was found to be important for RC formation and infectious WNV clones lacking NS1 gene were not able to form a functional RC (Youn, Ambrose, Mackenzie, & Diamond, 2013). However, since the RC sits at the cytoplasmic side, while dimeric NS1 is located in the ER lumen, the NS1 might facilitate the formation of viral RC by interacting with NS4B that lies on the cytoplasmic side of the ER lumen (Youn et al., 2012). NS2A of KUNV was reported to interact with the 3' UTR of viral RNA (Mackenzie, Khromykh, Jones, & Westaway, 1998). NS2B is required for the proper functioning of the NS3 protease and is tightly associated with the NS3 protease domain, and might act as a tether for the RC to the ER membrane (Westaway, Mackenzie, Kenney, Jones, & Khromykh, 1997) (J. Zou, Lee le, et al., 2015). NS4A is an important driver for ER membrane remodelling during the formation of IV (Miller et al., 2007). Proper cleavage of the 2K peptide downstream of NS4A was found to be important for NS4A function, as NS4A with uncleaved 2K peptide was unable to induce membrane remodelling. NS4B is involved in the dissociation of NS3hel from single-stranded RNA (ssRNA) during RNA replication (Umareddy, Chao, Sampath, Gu, & Vasudevan, 2006). This interaction was further characterised in this thesis (discussed in Chapter 3), where we observed binding between subdomains 2 and 3 of NS3 and the cytoplasmic loop of NS4B.

Figure 1.6. Proposed model of the possible architecture of dengue viral proteins within the RC. [Adapted from (Shi, 2014)]

1.3.3 Recruitment of host factors is required for efficient RNA replication

RNA replication is a highly complex and tightly regulated process and ten viral proteins alone are not sufficient to accomplish this demanding task (Fernandez-Garcia, Mazzon, Jacobs, & Amara, 2009). Apart from the viral proteins mentioned in section 1.3.2, the recruitment of host proteins (also known as host factors) is essential for proper RNA replication. For example, unknown host factors present in host cell lysates were required for NS5 to utilise dsRNA as a template for RNA replication *in vitro* (Raviprakash, Sinha, Hayes, & Porter, 1998). Therefore, viruses have evolved ways to recruit or co-opt host factors or hijack host cell pathways for the formation of virus induced ultra-structures and the RC, for efficient RNA replication and for evasion of the host immune defences (Nagy & Pogany, 2011; Paul & Bartenschlager, 2015). Targeting these host factors or pathways could serve as potential approach for development of host directed antivirals (to be discussed in section 1.4.2).

Besides using traditional genetic and biochemical methods to identify individual interacting host factors, the use of genome wide screen technologies such as RNAi, proteomics and more recently CRISPR-Cas9 screens have identified a long list of proteins that are important for facilitating DENV and other flaviviral RNA replication (Chiu, Hannemann, Heesom, Matthews, & Davidson, 2014; Kakumani et al., 2013; Savidis et al., 2016). Only a few selected examples are presented in this report. Please refer to (Krishnan & Garcia-Blanco, 2014; Pastorino, Nougairede, Wurtz, Gould, & de Lamballerie, 2010) for a more comprehensive list. These proteins can be mainly grouped into 4 different categories, 1) cell cytoskeleton network protein, 2) stress response protein; 3) host translational machinery protein; 4) protein involved in metabolic process.

Cell cytoskeleton protein - JEV NS3 has been reported to associate with microtubules and Tumor Susceptibility Gene 101 protein (TSG101) in the viral CM. Association with microtubules might be important for transportation of viral proteins during the formation of CM and association with TSG101 might be required for regulating microtubule stability (Chiou et al., 2003). In another study, the intermediate filament protein - Vimentin, was reported to directly interact and colocalise with NS4A in the DENV RC. Vimentin is an important host factor to assist NS4A in the reorganisation of ER membrane and also for tethering the DENV RC to the ER membrane (Teo & Chu, 2014).

Stress response protein – UPR is an evolutionarily conserved stress response mechanism to maintain homeostasis during accumulation of unfolded or misfolded protein in the ER lumen. This pathway involves transient inhibition of protein synthesis, increased synthesis of chaperones involved in protein folding and transport, increased

degradation of misfolded proteins, including ER-associated degradation (ERAD) and autophagy. If these mechanisms cannot resolve the protein-folding defects, cell apoptosis is triggered (M. Wang & Kaufman, 2014; Zhang & Wang, 2012). In the context of viral infections, large amounts of viral proteins are synthesised in infected cells, leading to the accumulation of unfolded or misfolded proteins, which in turn activates UPR (Zhang & Wang, 2012). DENV infection was reported by Peña *et al.* to usurp the unfolded protein response (UPR) pathway in a time dependent fashion to adapt the infected cells to viral infection (Figure 1.7) (Pena & Harris, 2011). During early infection, the Protein kinase RNA-like endoplasmic reticulum kinase (PERK) arm of UPR is activated to halt host translation and free up metabolites for viral replication. During the mid-phase of infection, the Inositol-requiring protein - X-box binding protein (IRE-XBP1) arm of UPR is hijacked, 1) for transportation of viral polyprotein into the ER lumen; 2) for reactivation of the halted host translation; 3) for prevention of apoptosis. During late stage of infection, the Activating transcription factor-6 (ATF6) arm of UPR is activated and might be required for to sustain UPR which prolongs viral life cycle within the infected cells. In another study, DENV has been reported to make use of the autophagy pathway for the generation of ATP to facilitate RNA replication (Heaton & Randall, 2010). This was achieved by inducing autophagy leading to lipid metabolism of lipid droplets to release free fatty acids that are transported to the mitochondria for ATP generation via β -oxidation.

Figure 1.7. Mechanism of UPR pathway modulation by DENV-2 proposed by Peña *et al.* Arrows indicate activation or induction by DENV-2, and T-lines and X indicate inhibition. [Adaption from (Pena & Harris, 2011)]

Host translational machinery protein - The eukaryotic translation elongation factor 1A (eEF1A) has been found to interact with NS3, NS5, and dsRNA in both WNV and DENV infected cells through colocalisation and co-immunoprecipitation (co-IP) assays (Davis, Blackwell, Shi, & Brinton, 2007). More recently, upregulation eEF1A was reported to be important for protecting JEV NS5 and NS3 from proteosomal degradation, and eEF1A positively regulates viral replication by stabilising JEV RC (Z. Chen *et al.*, 2016).

Protein involved in metabolic process – DENV NS3 was reported to recruit fatty acid synthase (FASN) to synthesise lipids required to produce new membranes for expansion of the membrane surface area of the ER during membrane remodelling, to avoid reduction of functional surface area within the ER which could affect proper functions (Heaton *et al.*, 2010).

1.3.4 Targeting RC components or their interactions as an anti-dengue therapeutic strategy

RNA replication of DENV and other related flavivirus is a well-orchestrated process involving viral and host factors. It is a multi-step process that involves a myriad of interactions - protein-protein, RNA-RNA, RNA-protein and protein-lipid. Apart from targeting the individual viral or host protein within the RC, targeting or disrupting interactions between the different players involved in viral replication constitutes a promising approach for the development of anti-viral therapy. To achieve this endeavour, new interactions can also be identified using genome-wide approaches to complete the RC puzzle. Further characterisation of these interactions can be performed: for example to map the regions involved in mediating the interaction for the production of truncated constructs for co-crystal structure determination. Having a co-crystal structure of the complex will greatly facilitate designing of such PPI inhibitors.

1.4 Drug discovery and vaccine development for dengue treatment and prevention

Given the severity of the global dengue epidemic, development of a vaccine and anti-dengue therapy has been a major focus of several pharmaceutical companies and academic laboratories. However, despite several decades of research efforts, no anti-dengue compound has reached the clinics. Vaccine development has been more fruitful: Dengvaxia has recently been approved for use in several countries, but some efficacy and safety issues have surfaced. Amongst other factors, the slow progress can be attributed to three main challenges (Kok, 2016; C.G Noble & Shi, 2013): 1) the need

to target all serotypes (with approximately 65% amino acid sequence identity) simultaneously due to the ADE phenomenon (discussed in section 1.2.3); 2) the lack of understanding on the disease progression and pathogenesis of dengue infection; and 3) lack of suitable small animal model for efficacy studies. With the increasing incidence and spread of dengue globally, there is an urgent need to develop better vaccines and effective anti-dengue compounds.

1.4.1 Dengue vaccine development

Vaccines for three closely related flaviviruses had been approved for use – several brands of live-attenuated vaccines based on the 17D lineage are available for YFV (WHO, 2013); about 15 vaccine for JEV in the form of inactivated, live attenuated, and live recombinant vaccine (WHO, 2015), and 4 approved TBEV formalin-inactivated virus vaccines (Lehrer & Holbrook, 2011). However, only one dengue vaccine – Dengvaxia - has recently been approved for use, the lag in the development is due to the challenging factors mentioned above. Dengvaxia is a tetravalent, live-attenuated recombinant dengue vaccine based on the yellow fever 17D vaccine backbone where the prM and E proteins from DENV 1-4 have replaced the structural proteins of YFV (Figure 1.8) (Guy, Briand, Lang, Saville, & Jackson, 2015).

Figure 1.8. Viral components of Dengvaxia live attenuated vaccine. The vaccine uses prM and E proteins from all four DENV serotype that are recombinantly inserted into a common YFV backbone. [Modified from: (Whitehead, 2016)]

The overall efficacy of Dengvaxia in patients with symptomatic dengue was almost 60% after completing all 3 recommended doses, and almost 80% efficacy against severe dengue (Pang, Mak, & Gubler, 2017). However, there was lower protection to DENV-1 and -2 infections with an overall efficacy of only 54.7% and 43.0% respectively since these 2 strains are the prevalent strains circulating in Singapore, it limited the applicability of this vaccine. Currently, there are safety concerns with vaccinating seronegative individuals (not infected with dengue prior to vaccination) as vaccination of these individuals only provided an efficacy of 38% (Hadinegoro et al., 2015; HSA, 2016; WHO, 2017). Importantly, vaccination of seronegative subjects might actually lead to severe dengue during a secondary wild-type infection after vaccination (Aguilar, Stollenwerk, & Halstead, 2016; Pang et al., 2017; WHO, 2017). There is also a concern with higher hospitalisation rates in vaccinated children between the ages of 2 – 5 as compared to unvaccinated children (Aguilar et al., 2016; HSA, 2016; WHO, 2017). Finally, a recent study showed that Dengvaxia elicits low long-term immune memory in vaccine participants 5 years after vaccination, and re-vaccination might be required (Velumani et al., 2016). These issues surrounding Dengvaxia signal that there is still an urgent need to develop better vaccines. Fortunately, there are two

vaccine candidates currently in phase 3 clinical trials – DENVax (Takeda), and TV003/TV005 [National Institute of Allergy and Infectious Diseases (NIAID) / Bhutantan Institute] (Pang et al., 2017) (Figure 1.9). However, the efficacy and safety data are still not available. Several other dengue vaccines under development, the progress and properties are summarised on Table 1.2.

Figure 1.9. Viral components of DENVax and TV003/TV005 live attenuated dengue vaccine. [Modified from: (Whitehead, 2016)] **(A)** DENVax vaccine from Takeda. The viruses contain a common attenuated DENV-2 backbone carrying prM and E genes from each of the four serotypes. **(B)** TV003/TV005 vaccine from NIAID and Bhutantan Institute. The vaccine uses full-length DENV-1, -3 and -4 viruses, but a chimeric DENV-2 that inserted into a DENV-4 backbone. The viruses are attenuated by a 30 nucleotide deletion in the 3'UTR region. Components in TV003 and TV005 are the same, except that TV005 contains a higher quantity of DENV-2 than TV003, 10^4 plaque-forming units (pfu) as compared to 10^3 pfu.

Table 1.2. Summary of the dengue vaccine candidate under clinical development
(Modified from: Dengue Vaccine Initiative, 2016)(Initiative, 2016)

Vaccine	Properties	Stage of development
CYD ^f -TDV (Dengvaxia) [Sanofi AS]	<ul style="list-style-type: none"> • Live chimeric virus • Tetravalent • YFV backbone carrying prM and E protein from all four serotypes 	Approved
DENVax (TDV ^g) [Takeda]	<ul style="list-style-type: none"> • Live attenuated virus (LAV) • Tetravalent • DENV-2 backbone carrying prM and E protein from all four serotypes 	Phase III
TV003 [Butantan Institute]	<ul style="list-style-type: none"> • Live attenuated virus • Tetravalent • Full-length DENV-1, -3, -4, and chimeric DENV-2 on a DENV-4 backbone • Attenuation through 30nt deletion at 3' UTR 	Phase III
TV003/TV005 [NIAD ^a , Butantan Institute]	<ul style="list-style-type: none"> • Same as TV003, except TV005 contains higher quantities of DENV-2 	Phase III
TDENV ^g PIV [GSK ^b , WRAIR ^c , Flocruz]	<ul style="list-style-type: none"> • Purified inactivated virus (PIV) • Tetravalent • Formulated with alum or different Adjuvant Systems 	Phase I/II
V180 [Merck Sharp and Dohme]	<ul style="list-style-type: none"> • Subunit vaccine • Tetravalent • Recombinant, truncated E protein (DENV-1 -80E) expressed for all serotypes in <i>Drosophila</i> S2 insect-cell culture 	Phase I
TVDV ^h (DNA) [NMRC ^d]	<ul style="list-style-type: none"> • DNA Vaccine • Tetravalent • Expression of prM and E membrane proteins from all four serotypes • Formulated with and without Vaxfectin adjuvant 	Phase I
TDENV-LAV + TDENV-PIV [WRAIR ^c]	<ul style="list-style-type: none"> • Combination of both LAV and PIV vaccine – to study the synergistic effect of administering 2 dengue vaccine candidates 	Phase I
TV005 [Panacea Biotec]	<ul style="list-style-type: none"> • As described above 	Phase I

^a National Institute of Allergy and Infectious Diseases

^b GlaxoSmithKline

^c Walter Reed Army Institute of Research

^d Naval Medical Research Center

^e Tetravalent dengue vaccine

^f Chimeric dengue vaccine in a yellow fever 17D backbone

^g Tetravalent DENV

^h Tetravalent DNA vaccine

Meanwhile, new novel vaccines are being explored many research groups in the discovery phase. Two such examples are NS1 based vaccine (Liu, Liu, & Cheng, 2016) and 2'-O-MTase mutant vaccine are currently in development (Zust et al., 2011). 2'-O-methylation during the formation of RNA cap on nascent RNA is essential for evasion of host immune response (Dong et al., 2012). Immunisation of 2'-O-MTase mutant virus led to 100% sero-conversion and complete protection against homologous DENV infections *in vivo* (Zust et al., 2013). Using a similar approach, immunisation of 2'-O-MTase mutants from SARS coronavirus was able to reduce pathogenesis in mice, and provided complete protection in lethal mouse model (Menachery et al., 2014).

Another promising approach for DENV vaccine development might be mRNA based vaccines. Two studies were recently published demonstrating the successful use of mRNA vaccine in inducing sterilising immunity against ZV. Richner *et al.* created modified mRNA vaccine using prM and E proteins, which contained mutations that destroyed the conserved fusion-loop epitope (Richner et al., 2017). The vaccine was able to elicit high titers of neutralizing antibodies, while mutations at T76R, Q77E, W101R, and L107R on the fusion loop prevented the formation of cross-reactive antibodies against DENV which would lead to ADE. A second mRNA vaccine against ZV was reported Pardi *et al.*, where they demonstrated that protective immunity in mice and non-human primates could be achieved using prM and E protein encoding mRNA that contained 1-methylpseudouridine substitution for uridine, a modified type 1 cap, and modified 5' and 3' UTR sequences. This vaccine led to high neutralizing antibody production and protected both mice and rhesus macaques in subsequent ZV challenges (Pardi et al., 2017). The success of these two mRNA vaccines demonstrates

that this technique is a promising approach for generating DENV vaccines. However, mRNA vaccine is an emerging technology and several challenges still need to be overcome. For example, vaccine efficacy in animal models may not translate to efficacy in humans, as shown by CV9104, an mRNA vaccine against prostate cancer from CureVac which failed phase II clinical trials, even though the mRNA vaccine demonstrated potent humoral immune activation in their preclinical animal studies (Rausch, Schwentner, Stenzl, & Bedke, 2014). As a new therapeutic modality, gaining regulatory approvals may require a significant amount of time. Lastly, for DENV vaccine development, the challenge of targeting all four DENV serotypes simultaneously with equal immune response still remains. If these challenges could be overcome, mRNA vaccines might replace the traditional virus-based vaccines, as it has been shown to have a good safety profile, and new mRNA vaccines could be designed and produced very rapidly and cheaply, within months as compared to years for traditional virus-based vaccines (DeFrancesco, 2017).

However, even after an efficacious vaccine becomes available, symptomatic dengue may still be a healthcare issue. As seen from precedence of YFV and JEV, despite vaccines being available, there were still approximately 200,000 and 70,000 annual reported cases respectively (Kok, 2016). This is due to poor uptake of the vaccine globally and are only taken as a prophylactic measure by travellers to endemic regions. Therefore, on top of developing better vaccines, the development of efficacious anti-dengue compounds is needed to complement the vaccines.

1.4.2 Dengue drug development

Just like dengue vaccine development, anti-dengue drug development is no trivial task, and is confronted by numerous challenges. The ideal target product profile of the drug candidate is, 1) pan-serotypic; 2) efficacious and fast acting; 3) safe; 4) cost effective, and 5) easy to use (oral dosing). The drug candidate is preferred to have pan-serotype activity as it is common to have 4 serotypes co-circulating within a hyper-endemic region, a pan-serotype antiviral will eliminate the need for an additional diagnostic step which will delay drug intervention. The candidate has to be fast acting, as dengue is an acute disease, with symptoms typically lasting 2 – 7 days (Lim et al., 2013). At the same time, it is hypothesised that high viremia during early infection is correlated with progression to severe dengue, and reduction of viremia by one order of magnitude is believed to be able to reduce the risk of progression to DHF or DSS (NITD target product profile) (Vaughn et al., 2000). Therefore, the drug candidate must be fast acting and reduce viremia quickly within a short therapeutic window. However, this hypothesis remains untested. It is still unknown whether reducing viremia by one log would indeed reduce disease severity. A clinical trial with a promising anti dengue compound would be needed to address this question. Since dengue affects individuals from all age groups, the candidate has to be safe for young children, as well as elderlies (Whitehorn et al., 2014). Dengue is endemic in mostly developing countries; therefore the cost of the candidate must be affordable to these affected populations. Ideally only a single oral dose is required. If administration requires trained medical personnel, it will affect drug uptake rates in rural areas, as clinics or hospitals may be very far away. A summary of the criteria for an ideal drug candidate set by NITD is summarised on Table 1.2.

Table 1.3. Criteria of an ideal anti-dengue compound. (Source: NITD)

Properties	Criteria
MOA	<ul style="list-style-type: none"> • Active against viral or host targets
Clinical efficacy	<ul style="list-style-type: none"> • Rapid reduction of viremia • Active against all 4 serotypes of viruses • Active against primary and secondary dengue infection
Route of administration	<ul style="list-style-type: none"> • Oral
Dosing regimen	<ul style="list-style-type: none"> • Ideally 1 dose. Acceptable for ≤ 3 doses daily for as long as needed to suppress virus production (3-5 days)
Safety	<ul style="list-style-type: none"> • Good safety profile in adult and children with few or no secondary effects.
Chemical Manufacturing Control	<ul style="list-style-type: none"> • Long shelf-life in endemic countries (>3 years) • Low Cost of Goods

Only a short summary on the approaches and challenges involved in anti-dengue drug discovery would be discussed in this thesis; more detailed information can be found in these excellent reviews - (Lescar et al., 2008; Lim et al., 2015; Lim et al., 2013; Luo et al., 2015; C. G. Noble et al., 2010; C. G. Noble & Shi, 2012; C.G Noble & Shi, 2013; Xie, Zou, Wang, et al., 2015).

Anti-dengue inhibitor can be divided into two categories – direct-acting antivirals (DAA) or host directed antivirals (HDA). As their names suggest, DAA are drugs that target viral proteins, while HDA are drugs that target host proteins important for viral replication.

DAA – The multi-functional NS3 (protease – helicase) and NS5 (MTase – RdRp) are ideal targets and are the focus of many drug discovery campaigns. They are the only two viral proteins with known enzymatic activities, and this facilitated development of *in vitro* target-based assays during the drug discovery process. On top of this, NS3 and NS5 form the replicase complex, and targeting them would adversely affect viral RNA replication.

The NS2B-NS3 protease domain was the target of several groups, where either *in vitro* high-throughput screens (HTS) using large small molecule libraries or peptidomimetic approaches were utilised to identify potential protease inhibitors. However, HTS efforts were hindered by poor cell permeability of identified compounds during validation using cell-based screens. While the peptidomimetic approach was hindered by the flat architecture of the protease active site, and due to the preference for positively charged peptide substrates, which are usually not orally bioavailable. Another major impediment is that *in vitro* assays were carried out using various recombinant single-chain NS2B-G₄SG₄-NS3 constructs, which may not be representative of the *in vivo* environment where a full-length NS3 is bound to full-length membrane-anchored NS2B (Luo et al., 2015; C. G. Noble et al., 2010). This issue is evident as the optimal activity of DENV NS2B-NS3 protease is at non-physiological conditions *in vitro* (C. G. Noble et al., 2010).

NS3hel drug discovery were carried out by HTS using helicase unwinding assays, which were either fluorescence resonance energy transfer (FRET)-based or scintillation proximity assay (SPA)-based. Similar to NS2-NS3 protease, the *in vitro* assays do not reflect physiological conditions, as high concentrations of helicase were needed to obtain sufficient activity for HTS readout (C. G. Noble et al., 2010). Also, based on the helicase crystal structures, no suitable binding pockets were identified for small molecule binding. A pan-serotype inhibitor, ST-610, was identified by Byrd *et al.* using DENV infection assay. Resistance mutation was mapped to NS3hel, where an A236T mutation conferred resistance. However, the *in vivo* efficacy of this compound in mice was poor.

NS5 MTase domain has high structural homology with human MTases, thus the number of sites that can be targeted for drug discovery is very limited. Using available MTase structures, a unique hydrophobic pocket next to the SAM site was identified by Lim *et al.* for structure-guided drug design. A selective inhibitor, Compound 10, was identified to inhibit both DENV and WNV MTase activity. However, the compound suffers from poor cell permeability issues.

RdRPs are well validated drug targets (in HIV and HCV), and considered as the most suitable target for drug development (Rawlinson, Pryor, Wright, & Jans, 2006). RdRp inhibitor can be divided into nucleoside/nucleotide inhibitors (NI) or non-nucleoside inhibitors (NNI). NIs are prodrugs that need to be first converted into an active triphosphate by host cell kinases, before being incorporated into RdRp active site and affecting viral replication by terminating RNA elongation or by introducing mutations deleterious to the viral genome (Lim *et al.*, 2013; C. G. Noble *et al.*, 2010). Although efficacious, NIs have complex and unpredictable structure–activity relationship (SAR) due to the complexities involved during prodrug conversion steps, making optimisation of NI potency very challenging (Lim *et al.*, 2013). At the same time, NIs commonly suffer from toxicity issue that are not apparent during *in vitro* studies. For example, NITD-008, an adenosine nucleoside, was efficacious *in vitro* and in mouse model studies, but development was terminated due to toxicity in rats and dogs (Lim *et al.*, 2013). NNI are usually inhibitors that bind to allosteric sites within the RdRp locking the enzyme in an inactive form or by preventing conformational changes required for proper function. NNIs are usually identified by HTS using *in vitro* RdRp assays. A drawback of NNIs is that these compounds are susceptible to viral mutations and may

need to be administered in combination with another anti-dengue compound (Lim et al., 2015).

Balapiravir is a nucleoside analogue prodrug initially developed for the inhibition of HCV polymerase. Unfortunately, development of Balapiravir for long term treatment of chronic HCV was halted due to toxicity involved long term usage (Roberts et al., 2008). Subsequently, Balapiravir was repurposed to treatment of dengue infection after efficacy was observed in cell culture. Since dengue infections are acute, short term treatment with Balapiravir was rationalised as a potentially therapeutic option (Nguyen et al., 2013). However, in a phase II clinical trial conducted by Nguyen *et al.*, Balapiravir failed to achieve the intended outcome of viremia reduction, and did not perform better than placebo. One possible explanation for the mismatch with the observed efficacy *in vitro* over *in vivo* was the reduction in the ability for pre-DENV-infected peripheral blood mononuclear cells (the major cell types involved in natural dengue infection) to convert the prodrug into its active triphosphate form due to cytokine activation upon viral infection. Therefore, in the clinical context, where patients were already infected with DENV, the conversion of Balapiravir into the active form is greatly attenuated, leading to poor viremia reduction (Y. L. Chen et al., 2014).

Host directed anti-virals – As discussed in section 1.3.2, host factors are recruited by viral proteins and play vital roles during viral replication, and these host factors are potential anti-viral targets. Targeting host factors has three main advantages, 1) there is higher resistance barrier as mutation rate in these targets are much lower than that of the viral proteins; 2) HDA will most likely be pan-serotypic, since all DENV serotypes will interact with the target; and 3) the HDA might already be approved for use, and

repurposing the compound for dengue treatment would accelerate clinical trials (Acosta & Bartenschlager, 2016; Krishnan & Garcia-Blanco, 2014). Unfortunately, toxicity is a major concern when targeting host factors, as these factors are important for normal host functions. To date, many HDA blocking different stages of DENV life cycle or targeting host inflammatory responses have been identified, and several have progressed to clinical trials [refer to (Acosta & Bartenschlager, 2016; Krishnan & Garcia-Blanco, 2014) for comprehensive reviews].

Using cell-based assays, two HDAs were discovered by NITD – NITD-451 and NITD-982, which are a RNA translation inhibitor and pyrimidine biosynthesis inhibitor respectively (Q. Y. Wang, Bushell, et al., 2011; Q. Y. Wang, Kondreddi, et al., 2011). NITD-451 exhibited strong *in vitro* and *in vivo* anti-dengue activities, but suffered from *in vivo* toxicity, while NITD-982 demonstrated potent *in vitro* activity, but no *in vivo* activity.

Chloroquine is an approved drug for the treatment of malaria by inhibiting the haeme polymerase in malaria parasites (one of the suggested mechanism of action for its anti-malarial activity). Crystallisation of free haeme released during the digested of haemoglobin by the parasite haeme polymerase has been proposed as a possible mechanism for malaria parasites to circumvent haeme toxicity issues (Slater & Cerami, 1992). Apart from its ability to inhibit the haeme polymerase, chloroquine has been reported to prevent acidification of endosomes by alkalinisation of the cell compartment (Farias, Machado, & da Fonseca, 2013). Endosome acidification is required for membrane fusion and release of DENV particles into the host cell cytoplasm, and this process can be disrupted by alkalinisation of the endosomes (Farias et al., 2013). Inhibition of dengue replication by chloroquine was reported in Vero cells,

and a study in non-human primate demonstrated *in vivo* efficacy of chloroquine against dengue infection, leading to significant reduction in viremia (Farias et al., 2013; Farias, Machado, Muniz, Imbeloni, & da Fonseca, 2015). However, no clinical benefits were observed in two separate clinical trials conducted in Brazil and Vietnam (Borges, Castro, & Fonseca, 2013; Tricou et al., 2010).

Celgosivir, an imino sugar prodrug, was developed for HCV treatment, and later repurposed for anti-dengue clinical trials. It acts on α -glucosidase, an enzyme required for processing viral glycoproteins (Mehta, Zitzmann, Rudd, Block, & Dwek, 1998). Inhibition of α -glucosidase by imino sugars was reported in DENV and other related flavivirus (Chang, Block, & Guo, 2013). It is suggested that imino sugars prevents proper processing of N-linked oligosaccharides on pRM, E and NS1 protein, leading to inhibition of viral maturation (Courageot, Frenkiel, Dos Santos, Deubel, & Despres, 2000; Mehta et al., 1998; Rathore et al., 2011). Celgosivir was demonstrated to have pan-serotype activity against DENV *in vitro* and *in vivo* (Rathore et al., 2011; Watanabe et al., 2012). However, no convincing anti-dengue effects were observed during a phase 1B clinical trial at low dosage (Low et al., 2014). Another clinical trial with more frequent dosing regime is underway after *in vivo* efficacy was observed in mice (Watanabe et al., 2016).

As mentioned in section 1.3.2 FASN is reported by Heaton *et al.* to be recruited by NS3 for remodelling of the ER membrane and formation of IV and CM (Heaton et al., 2010). C75 and cerulenin, both FASN inhibitors, were used to demonstrate its importance during DENV replication. Both inhibitors led to dose-dependent reduction in DENV replication, with C75 producing a two log reduction virus replication (Heaton et al., 2010). This study is a good demonstration that discovery of essential host factors

can lead to rapid identification of existing inhibitors that could be repurposed for anti-dengue treatment.

The pathogenesis of severe dengue involves over-reactive host inflammatory responses leading to symptoms such as vascular leakage, haemorrhage and shock (section 1.2.3). Therefore, it is hypothesized that immunosuppressant such as corticosteroids might be able to prevent disease progression to severe dengue or be used as treatment for severe dengue patients. In a recent clinical trial, Tam *et al.* studied the use of prednisolone during early acute phase of dengue, but the treatment did not reduce disease progression or symptoms (Tam et al., 2012). Several other studies were conducted during the early 1970-1980s using various corticosteroids such as hydrocortisone, dexamethasone, methylprednisolone, and prednisolone based on this hypothesis, but the results were inconclusive [see (Rajapakse, Rodrigo, Maduranga, & Rajapakse, 2014) for a comprehensive list of clinical trials performed]. A systematic review of these early studies conducted by Panpanich *et al* concluded there were no clinical benefits with corticosteroid treatments, instead these treatment could potentially increase the susceptibility of the patients to other infections due to the immunosuppressive effects of the corticosteroids (Panpanich, Sornchai, & Kanjanaratanakorn, 2006). Treatment of dengue using corticosteroid is currently not included in the WHO dengue guidelines.

Besides exploring small molecular drugs, the use of human mAb is another promising therapeutic modality currently being explored for anti-dengue therapy. Majority of these mAbs are isolated from infected patients, and mostly target the E and prM proteins, with several mAbs binding to quaternary epitopes on the E protein [see (Fibriansah & Lok, 2016) for a detailed list]. VIS513 is currently the most advanced

mAb candidate that targets EIII domain of the E protein (Robinson et al., 2015; Visterra, 2017). In preclinical animal studies, VIS513 was found to be effective against all four DENV serotypes, and was able to reduce viremia mouse and non-human primate models (Ong et al., 2017). A phase 1 clinical trial in India early 2018 is planned (Visterra, 2017). Another potential antibody is SIgN-3C-LALA that has been reported to confer pan-serotype protection in mouse models at picomolar concentrations (M. Xu et al., 2017). This mAb was found to be targeting a quaternary epitope between two E protein monomer that is speculated to lock the fusion loop and viral fusion with the endosomal membrane. The authors also reported better in vivo efficacy of SIgN-3C compared to VIS513. Despite significant progress in this area, the cost of development would be very high, thus a high cost of goods for treatment. The therapeutic mAbs would have to be administered intravenously by trained medical professionals, and might not be easily accessible in rural areas. These two factors are against the ideal target product profile for dengue therapeutic, especially for patients in developing countries who are the most adversely affected.

1.5 NS4B – A potential therapeutic target

The DENV NS4B protein is made up of 248 amino acids, with 40% hydrophobic residues, and an approximate molecular mass of 28 kDa. The NS4B membrane topology was first described by Miller *et al.* through a series of biochemical and biophysical assays. NS4B composed of three transmembrane domains (TMD-3, -4, and -5) and has two amphipathic helices at its first N-terminal 100 residues (pTMD-1, and -2) (Figure 1.10A) (Miller et al., 2006). A flexible loop located between TMD-3 and -4, termed “cytoplasmic loop”, is the only part of NS4B exposed to the host cytoplasm,

and is speculated to be important for mediating NS4B interactions with other proteins. A signal peptide located before the N-terminus of NS4B – the 2K peptide - is essential for the correct folding and insertion of NS4B into the ER membrane. This topology was used as reference for all our construct designs reported in this thesis. Very recently, a more detailed topology was determined by our collaborator, Dr Congbao Kang's group, where they employed NMR to study the secondary structure of NS4B in the presence of LMNG micelles (Y. Li, Wong, et al., 2016). From their NMR assignments, NS4B is found to be made up of eleven alpha-helices, and possibly forming five TMDs at alpha-helix-2, -3, -5, -7, and -9 (Figure 1.10B). However, alpha-helix-9 only forms a TMD before NS5 cleavage, and will be flipped to the luminal side of the ER upon NS3 protease cleavage. This result is consistent with Miller *et al.* initial prediction (Miller et al., 2006).

A.

B.

Figure 1.10. Proposed NS4B membrane topology. (A) Membrane topology proposed by Miller *et al.* The reported NS4B functions and interactions are also indicated. [Modified from (Zmurko, Neyts, & Dallmeier, 2015)] (B) Membrane topology resolved by NMR in the presence of LMNG micelles [Adapted from (Y. Li, Wong, et al., 2016)].

A sequence alignment of NS4B from existing DENV-1 to DENV-4 strains shows that there is an 85% sequence similarity, with sequence conservation found in the proposed TMDs and the cytoplasmic loop (Figure 1.11). This high similarity implies strong functional conservation.

Figure 1.11. Sequence alignment of NS4B from 3182 strains of DENV-1 to DENV-4. (Alignment provided by Dr Kim Long Yeo, NITD). The number of strains used is indicated in the parenthesis.

Functionally, NS4B does not have any known enzymatic activities. Nevertheless, important NS4B roles during viral replication have been reported. NS4B has been found to facilitate viral replication through making various PPI with other viral and host proteins; and to suppress the host immune response through various virus-host protein interactions (Figure 1.10A).

Interactions with other viral proteins - NS4B – NS1 interaction in WNV was reported by Youn *et al.*, where defective viral replication due to NS1 mutation after a swap between residues 10 and 11 could be rescued by mutation F86C mutation on NS4B. This interaction was further confirmed via co-IP and MS experiments (Youn *et al.*, 2012). NS4B – NS3 interaction was reported using yeast two-hybrid (Y2H) assay, and further confirmed using pull-down and IP assays. It was observed that association of NS4B with NS3 increases the processivity of the DENV replicase complex by dissociating NS3 from bound ssRNA thus freeing NS3 to bind to and unwind the next dsRNA during RNA replication (Umareddy *et al.*, 2006). NS4B – NS4A interaction was discovered and mapped to residues 40 - 76 in NS4A and 84 – 146 of NS4B. This interaction proved to be important, as alanine substitutions to interacting residues L48, T54 and L60 in NS4A abolished viral replication (J. Zou, Xie, *et al.*, 2015).

Interactions with host factors –Several targets were reported by Khadka *et al.* using high throughput yeast-two hybrid screens – Ube2i (SUMO-conjugating enzyme Ube2i), KRT8 (Keratin 8), PGK1 (Phosphoglycerate kinase 1) (Khadka *et al.*, 2011). KRT8 was not followed up upon, while Ube2i and PGK1 were further validated using siRNA knock-down, and Ube2i was found to be important for DENV replication. Other than the host proteins mentioned above, there are currently not much data on

NS4B – host factor interactions available in the literature. Such paucity can be circumvented with genome-wide approaches to identify new host factors - NS4B interactions, especially in the context of viral RC, where NS4B plays an important role.

Suppression of host immune defences – NS4B was reported to suppress IFN α and IFN β signalling cascade through upstream interference by targeting Signal transducer and activator of transcription 1 (STAT 1) (Munoz-Jordan et al., 2005). This antagonism is mediated by pTMD 1 and -2, and requires proper cleavage of NS4A and NS4B by NS2B-NS3 protease. NS4B is also responsible for suppression of RNAi activity of host cells, and all four serotypes were found to be potent RNAi inhibitors (Kakumani et al., 2013). DENV infection led to down-regulation of gene expression for host proteins involved in RNAi, and silencing of these host RNAi proteins could enhance DENV replication in infected cells. Using deletion mutation studies, TMD-3 and TMD-5 were identified as the region in NS4B responsible for mediating the observed RNAi suppression. Site directed mutagenesis identified F112 for RNAi, as F112A mutation abolished RNAi activity of NS4B. Finally, using northern blot, the authors demonstrated that NS4B inhibited the ability for Dicer to process dsRNA into siRNA that is required for activating the RISC complex in the RNAi pathway (Kakumani et al., 2013).

In recent years, four DENV NS4B specific inhibitors have been published (Figure 1.12). NITD-618 and Compound 14a were identified by luciferase replicon assay during a HTS campaign using a 1.8 million compound library in NITD. NITD-618 exhibited pan-serotypic activity at half maximal effective concentration (EC₅₀) between 1 to 4 μ M, and resistance mutations were mapped to P104L and A119T amino

acid substitutions on NS4B (Xie et al., 2011). Compound 14a exhibited potent anti-DENV-2 and -3 activities, but no anti-DENV-1 and -4 activities. Resistance mutation was mapped to V63 L/M/T substitutions on NS4B (Q. Y. Wang et al., 2015). A third compound, SDM25N reported by van Cleef *et al* has only anti-DENV-2 activity, with an EC_{50} of 1.9 μ M. Resistance mutations were mapped to P104L and F164L substitution on NS4B (van Cleef et al., 2013). Using viral-like particle (VLP)-based infection assay, Zou *et al.* reported that a natural product compound – lycorine - could inhibit WNV and DENV-1 RNA replication. This inhibitory effect was also tested and observed in YFV and DENV-2. Resistance mutation was mapped to V9M substitution (G. Zou et al., 2009). Two compounds - Dasatinib and AZD0530 – exhibited anti-DENV activity, and was found to target host Fyn kinase. Interestingly, the resistance mutation was mapped to T108L amino acid substitution in NS4B instead of mutations within the targeted Fyn kinase (de Wispelaere, LaCroix, & Yang, 2013). In the related YFV, two compounds - CCG-4088 and CCG-3394 – were reported to have anti-YFV activity, and the resistance mutation was mapped to K128R substitution in NS4B (Patkar, Larsen, Owston, Smith, & Kuhn, 2009). Although resistance mutations of the inhibitors mentioned above have been mapped to NS4B, there is still no evidence that these inhibitors bind directly to NS4B, and these inhibitors might be binding to host factors involved in interacting with NS4B during viral replication instead; this awaits investigation.

Figure 1.12. Anti-viral activity and location of resistance mutations of reported compounds specifically targeting DENV NS4B.

Since no anti-dengue compound targeting NS3, NS5 and host factors has been successful in clinical trials, the above published compounds provide strong evidence that NS4B is a viable target for development of anti-dengue or anti-flaviviral therapy. However, the intrinsic hydrophobicity of the protein has made studying its biological functions very challenging. On top of this, NS4B is non-enzymatic, which makes assay development during drug discovery non-trivial. One approach to assist drug discovery and gain better understanding of the biological functions of NS4B would be to obtain a 3D structure of NS4B through X-ray crystallography or NMR.

1.6 Aims of this thesis

Dengue poses a serious healthcare burden around the world, and an estimated 40% of the world's population is at risk of dengue. Currently, only a partially efficacious

vaccine (60% efficacy) is available, while an effective anti-dengue drug is not available and is urgently needed to better manage the global spread of dengue. Therefore, this project aims to gain a better biological understanding of NS4B and contribute to anti-NS4B drug discovery efforts in three areas 1) to assist the drug discovery efforts in NS4B by obtaining the 3D structure of NS4B; 2) to characterise the interaction between NS4B and NS3hel; and 3) to identify new host proteins interacting with NS4B during DENV replication.

1.6.1 Crystallisation of NS4B

NS4B is an essential membrane-bound viral protein, and in recent years has emerged as a potential target for anti-dengue drug discovery. However, due to its hydrophobic and non-enzymatic nature, studying its functions and development of an assay for drug discovery proved to be challenging. Therefore, one aim was to solve the crystal structure of NS4B to enable structure-based drug discovery, and provide new insights into its biological functions.

1.6.2 Characterisation of NS4B – NS3 interactions

NS4B – NS3 has been reported to be important for RNA replication. However, the exact domains or residues that participate in the interaction remain unknown. We aim to characterise and map out this interaction, as this would facilitate the design of smaller proteins constructs for co-crystallisation and structural determination. This will in turn provide structural insights to design compounds that can disrupt or stabilise the PPI interface.

1.6.3 Identification of host factor involved in the viral RC

Many host factors are recruited during viral replication, and can potentially be targeted for anti-dengue therapy. This is exemplified by the discovery that FASN is important for DENV replication, and existing FASN inhibitors, C75 and Cerulenin, can inhibit virus replication. Therefore, we aim to identify new host factors involved in the interaction of NS4B within the viral RC using the SILAC proteomics approach. This will provide further insights into the NS4B – host protein interactions within the viral RC, and could also potentially identify new host targets for anti-dengue drug development.

Chapter 2. Purification and crystallisation of NS4B

2.1 Targeting DENV NS4B membrane protein for anti-dengue therapy

As described in section 1.5, Dengue NS4B is a 28 kDa transmembrane protein that is hydrophobic in nature, and forms 5 transmembrane helices on the ER membrane. In recent years, resistance mutations to several anti-dengue compounds have been mapped to NS4B, making NS4B an attractive target for anti-dengue drug development. Due to its hydrophobic and non-enzymatic nature, no assay has been developed for drug discovery purposes. Therefore, to facilitate drug discovery of this attractive target, we aim to solve the structure of NS4B to enable structural based drug discovery. However, working with membrane proteins is difficult and crystallisation is very challenging as many obstacles need to be overcome.

2.2 Challenges involved in purification and crystallisation of membrane proteins

Membrane proteins are proteins that are embedded on the lipid bilayer of cells, or on lipid membranes of organelles or vesicles. Membrane proteins have important biological roles by modulating a wide variety of cellular activities, such as receptors for signal transduction, channels for transportation of molecules or ions, and adhesion proteins for cell-cell contact. These proteins make up almost 30% of the genes in most organisms, and are targets of more than half of all approved drugs in the market (Krogh, Larsson, von Heijne, & Sonnhammer, 2001; Overington, Al-Lazikani, & Hopkins, 2006). Despite their importance, only a few structures of these molecules have been solved by X-ray crystallography or NMR. To date only 672 unique protein structures have been solved, and the total membrane protein structures represent less than 1% of

the structures deposited in the Protein Data Bank (PDB) (White, 2017). Membrane proteins are difficult to study due to several reasons.

First is the need to use detergents. Membrane protein surfaces are mostly hydrophobic and need to be embedded within the lipid bilayer. Therefore, detergents, in the form of detergent micelles, are required for membrane protein extraction, and also for keeping the proteins water soluble after extraction (Prive, 2007; Seddon, Curnow, & Booth, 2004). Due to their amphiphilic nature – consisting of a hydrophilic head and hydrophobic tails - detergents often interfere with many biochemical and biophysical assays producing high noise to signal ratio during protein characterisation experiments (detergent effect) (Alexandrov, Mileni, Chien, Hanson, & Stevens, 2008). More importantly, presence of detergents micelles could lead to protein denaturation, because the micelles do not surround membrane proteins like native lipids. This leads to loss of or incorrect lateral pressures acting on the membrane helices and may also expose embedded hydrophobic residues to the aqueous environment, eventually causing the extracted membrane protein to unfold and aggregate (Prive, 2007; Seddon et al., 2004). Detergents also adversely affect protein crystallisation in several ways. Detergent micelles usually surround membrane proteins in a non-spherical and non-uniform conformation and there is a constant exchange of detergent monomer within the aqueous solution and the detergent micelles, thus this leads to population heterogeneity (Garavito & Ferguson-Miller, 2001). Also, most membrane proteins contain limited hydrophilic surfaces that are required for forming crystal contacts during crystallogenesis, and micelles may further occlude the already limited hydrophilic surfaces (Prive, 2007). Furthermore, crystals formed usually have high

solvent contents due to presence of detergent micelles wrapping around every membrane protein unit (Carpenter, Beis, Cameron, & Iwata, 2008).

Second, due to their biological roles, membrane proteins that involve interaction with many different molecules or ions, often have flexible domains, and this is detrimental for crystallisation (Carpenter et al., 2008).

Third, data collection is also challenging due to high solvent content mentioned, and consequently membrane protein crystals are fragile and susceptible to radiation damage (Carpenter et al., 2008).

Lastly, phasing is difficult because most membrane proteins do not have a homologous model for molecular replacement, and obtaining good quality diffraction data for single-wavelength anomalous diffraction (SAD) or multi-wavelength anomalous diffraction (MAD) phasing is difficult due to radiation damage issue mentioned above (Bill et al., 2011; Carpenter et al., 2008). Therefore, many groups have developed specialised techniques for successful crystallisation of membrane proteins.

2.2.1 Techniques to overcome membrane protein crystallisation challenges

2.2.1.1 Techniques to increase hydrophilic surfaces

As mentioned above, most membrane proteins possess limited hydrophilic surfaces required for the formation of crystal-crystal contacts, and this is one of the major hurdles for membrane protein crystallisation. Several groups have overcome this problem by performing antibody-assisted crystallisation or soluble protein fusions to increase the overall hydrophilic surfaces.

Antibody-assisted crystallisation takes advantage of the large hydrophilic surfaces that is available on antibody fragments for making crystal-crystal contacts (Griffin &

Lawson, 2011; Hunte & Michel, 2002). Apart from donating hydrophilic surface for crystallisation, complexing membrane protein with antibody fragments would also increase the purity of the membrane proteins obtained which can aid crystallisation (Griffin & Lawson, 2011; Hunte & Michel, 2002). Lastly, binding of antibodies could reduce flexibility of the membrane protein by binding to and stabilising the flexible epitope (Griffin & Lawson, 2011; Hunte & Michel, 2002). Due to their bivalent binding mode and flexible linker connecting the constant and variable regions, whole antibody molecules are not suitable for crystallisation purposes. For this approach only antibody binding fragment (Fab) and single chain variable fragment (scFv) are used instead (Hunte & Michel, 2002). Fab and scFv have been successfully used for the crystallisation of several membrane proteins, such as bacterial Cytochrome C Oxidase (PDB: 1AR1), yeast Cytochrome B₁ (PDB: 1KB9), HIV-1 GP120 core (PDB: 1GC1), bacterial potassium channel KcsA (PDB: 1BL8) and WNV NS1 (PDB:4OII).

Another technique utilised to increase the hydrophilic surfaces of membrane proteins is the insertion of soluble fusion proteins. This technique was pioneered by Professors Raymond Stevens and Brian Kobilka's groups for the crystallisation of β 2-adrenergic receptor (PDB: 2RH1) through insertion of a soluble T4 lysozyme (T4L) domain. The inserted T4L domain contributed to three of the four crystal contacts found in the crystal lattice, and phasing was performed using T4L structure as a model for molecular replacement (Cherezov et al., 2007). Subsequently, more fusion partners were published for successful crystallisation of GPCR proteins (Chun et al., 2012).

2.2.1.2 Techniques to remove the detrimental detergents

Although detergents are essential for extraction and maintaining solubility of membrane protein in solution, the presence of detergent micelles could be detrimental to protein stability and protein crystallogenesis as mentioned above. Therefore, new detergent-free approaches have been devised to overcome this problem.

LCP crystallisation was invented by Landau and Rosenbusch for obtaining the structure of Bacteriorhodopsin in 1996 (PDB: 1QHJ). This technique involves formation of the lipidic meso-phase by reconstituting monoacylglycerols (MAGs) with water at a specific 3:2 ratio (Caffrey & Porter, 2010). The meso-phase is a three dimensional structure that resembles the cell lipid bilayer, consisting of curved continuous lipid bilayer network separated by two continuous networks of water channels (Figure 2.1) (Caffrey, 2015; D. Li, Lee, & Caffrey, 2011). When membrane protein samples are used as an aqueous solution for MAG reconstitution, the membrane proteins are incorporated into the meso-phase lipid bilayer, and the bound detergent molecules around the protein are removed (Caffrey, 2015; D. Li & Caffrey, 2014). The meso-phase closely mimics the membrane lipid environment, and therefore leads to better stabilisation of membrane proteins as compared to detergent micelles (Caffrey, 2015). When the correct precipitant is added, the embedded protein will start to nucleate, leading to crystallogenesis (Figure 2.1) (Caffrey, 2015; Zhao & Wu, 2012). Crystals grown in LCP have type I packing with protein molecules making contacts not only through hydrophilic but also hydrophobic surfaces resulting in lower solvent content and better crystal order (Caffrey, 2015). The development of LCP has fueled a surge in the number of membrane protein structures deposited into the PDB: more than 200 structures have been deposited into PDB since the first Bacteriorhodopsin structure;

it has now become a routine technique for membrane protein crystallisation, especially in the GPCR field (Caffrey, 2015).

Figure 2.1. Schematic representation of incorporation and crystallisation of β 2-adrenergic receptor in LCP. β 2-adrenergic receptor is incorporated into the lipid layer of the meso-phase that closely mimics the cell lipid layer (bottom left). Upon addition of a correct precipitant, temporary phase transition of the mesophase occurs leading to protein nucleation (top right). (Adapted from (Cherezov & Caffrey, 2007))

Another approach to avoid the detrimental effects of detergent was recently reported. This approach involves the use of a polymer - styrene maleic acid (SMA) – for extraction of membrane proteins in the absence of detergents (Knowles et al., 2009). SMA is an industrial polymer synthesised by the co-polymerisation of styrene and maleic anhydride monomers. Similar to detergent monomers, SMA is amphiphilic, consisting of alternating hydrophobic (styrene) and hydrophilic (maleic acid) moieties, and like detergents, is capable of disrupting the lipid bilayer (Dorr et al., 2016; Lee & Pollock, 2016). Unlike, detergent micelles that wrap around extracted proteins, SMA polymers wrap around native lipids instead, resulting in the extraction of small discs of lipid bilayer containing membrane proteins (Figure 2.2A) (Dorr et al., 2016). The

extracted lipid bilayer and membrane protein is termed SMA lipid particles (SMALP). With the retention of native lipids, the conformation and functions of the extracted membrane proteins are preserved facilitating functional and structural studies, and also render the SMALPs water-soluble (Dorr et al., 2016). After extraction with SMA, tagged recombinant membrane proteins can be purified using routine Immobilized-metal affinity chromatography (IMAC) methods (Figure 2.2B). One major advantage of this method is that SMALPs are stable once formed, and unlike detergents, supplementing buffers with SMA during purification is not required. This is a major cost advantage (Dorr et al., 2016). Lastly, because only a small amount of SMA is introduced into the protein solution, SMA produce minimal signal interference with characterisation experiments compared to detergent micelles (Dorr et al., 2016).

A

B

Figure 2.2 Schematic representations of SMALP, and SMALP purification process. (A) Comparison of detergent and SMA extracted membrane protein. Detergents micelles (red) wrap around extracted proteins in a non-uniform fashion, in the absence of lipids. SMA extracted proteins are embedded within an extracted native

lipid (green) disc that is held together by SMA polymers. **(B)** Purification of SMALPs. A heterogeneous population of SMALPs containing different membrane proteins are obtained upon SMA addition. HIS-tagged target membrane proteins can be purified using IMAC.

2.2.2 cryo-EM as alternative to x-ray crystallography

Due to the development of direct electron detection cameras and better algorithms for 3D reconstruction, cryo-EM is emerging as a technique of choice for challenging structural projects that have not been successful with traditional crystallography or nuclear magnetic resonance (NMR) spectroscopy approaches (Y. Cheng, 2015; Rawson, Davies, Lippiat, & Muench, 2016). This has led to an explosion in the number of membrane protein structures being deposited into the Electron Microscopy Data Bank (EMDB), noticeably the number of structures with resolutions better than 4Å has dramatically increased (Rawson et al., 2016). Cryo-EM has several advantages over x-ray crystallography, 1) only a small amount of starting material is required, which can overcome the low protein yield problem; 2) shorter experiment time, which eliminates the problem of protein aggregation overtime due to protein instability; and 3) a higher degree of sample heterogeneity is tolerated in cryo-EM, while heterogeneity is detrimental for crystallisation (Y. Cheng, 2015; Rawson et al., 2016; Vinothkumar, 2015). The major disadvantage and rate limiting step for cryo-EM is sample preparation, as selecting the right detergent to use and cryo-grid preparation is challenging. Another disadvantage at the moment is cryo-EM can only be utilised for high molecular weight membrane proteins or complexes (Vinothkumar, 2015).

2.3 Aims

NS4B has emerged as a good target for anti-dengue drug development. However, working with membrane proteins is very challenging, and this has hampered progress in gaining a better understanding of the biological functions of NS4B, and also hindered drug discovery efforts. In this part of the project, we aim to apply the membrane protein structural determination techniques mentioned above to obtain the atomic structure of NS4B. This would bring a better understanding of its biological functions, and also facilitate structure-based drug discovery.

2.4. Materials and Methods

Cloning of the full-length NS4B protein. The cDNA encoding the full-length NS4B of DENV-2 strain TSV01 (GenBank accession No. AY037116) with an N-terminal decahistidine tag was amplified by fusion polymerase chain reaction (PCR), digested with *NcoI* and *NotI*, and inserted into pET28a vector to generate a pET28a-NS4B full-length plasmid. The construct was confirmed by cDNA sequencing, and transformed into BL21 (DE3) *E. coli* cells (Stratagene).

Cloning of truncated NS4B protein. Cloning of all truncated constructs was performed by the Protein Production Platform (PPP) using Ligation Independent Cloning (LIC) (Savitsky et al., 2010). cDNA of all constructs were inserted into pNIC28-Bsa4 plasmid (GenBank accession EF198106), containing N-terminal hexahistidine tag and TEV cleavage site, by LIC using a RecA *E. coli* strain. The resultant plasmids were transformed into a T1-phage resistant BL21-DE3 Rosetta strain.

Expression and purification of all recombinant NS4B protein. Cells were grown in a Difco™ 2 × YT (BD) medium supplemented with 50 µg ml⁻¹ kanamycin (Sigma) to an optical density at 600 nm (OD₆₀₀) of 0.8 – 1.0. Expression of the recombinant proteins was induced by the addition of 0.2 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) and incubation at 16 °C for 16 hours (h). Cells were pelleted down by centrifugation at 7,000 × g for 7 min at 15°C, resuspended in lysis buffer (20 mM Tris(hydroxymethyl)aminomethane (Tris)-HCl, 300 mM NaCl, 10% volume per volume (v/v) glycerol, pH 8.0), and disrupted by sonication using a Digital Sonifier 450 (Branson) at 40% amplitude for 10 min. The cell lysates were clarified by

centrifugation at $10,000 \times$ gravitational force (g) for 10 min at 4°C to remove the cell debris and unlysed cells. The supernatants were centrifuged at 35,000 revolutions per minute (rpm) for 1 h at 4°C using a Beckman Ti70 rotor to pellet membranes and their associated proteins. At this stage, two approaches for membrane preparation were used, 1) without membrane washing; and 2) with membrane washing.

Without membrane washing - The membrane pellets were resuspended thoroughly in lysis buffer supplemented with 1% *n*-dodecyl- β -D-maltopyranoside (DDM) (Anatrace) and solubilized with stirring at 4°C for 2 h.

With membrane washing - The membrane pellets were subjects to three membrane washing steps. Each step involves resuspension of the membrane pellet in wash buffer by dounce homogenization, followed by ultracentrifugation at 35,000 rpm for 30 min at 4°C . A low salt buffer (20 mM Tris-HCl, 50 mM NaCl, 10% glycerol, pH 8.0) is used for the first wash, followed by a wash in high salt buffer (20 mM Tris-HCl, 750 mM NaCl, 10% glycerol, pH 8.0), and a final wash with lysis buffer (20 mM Tris-HCl, 300 mM NaCl, 10% glycerol, pH 8.0). 45 mL of wash buffer should be used for every 100 mL of culture (personal communication with NIBR scientists). The membrane pellets after the third wash were resuspended thoroughly in lysis buffer supplemented with 1% DDM (Anatrace) and solubilized with stirring at 4°C for 2 h.

After solubilisation, the post-solubilized lysates were clarified by ultracentrifugation at 35,000 rpm for 1 h at 4°C to get rid of the insolubilized fractions. Afterwards, the supernatants were purified by IMAC using HisTrap Fast Flow column (GE Healthcare) equilibrated with buffer A (20 mM Tris-HCl, 300 mM NaCl, 20 mM imidazole, 2 mM Tris-(2-carboxyethyl)-phosphine (TCEP), 0.05% DDM, pH 8.0). A 10 column volume (CV) wash was performed after sample loading. Next, the bound

proteins were eluted using a linear gradient of imidazole from 5 mM to 300 mM. The fractions containing NS4B proteins were pooled, concentrated and injected for size exclusion chromatography (SEC) using a HiLoad Superdex 200 16/60 column or HiLoad Superdex 200 10/300 column (GE Healthcare) in 20 mM Tris-HCl, 300 mM NaCl, 10% glycerol, 2 mM TCEP, 0.05% DDM, pH 8.0. Fractions containing NS4B proteins were pooled and concentrated to approximately 5 mg ml⁻¹ for crystallisation or storage. Protein concentration was estimated by Nanodrop using the respective extinction coefficient of each NS4B construct. For the bRIL constructs, protein concentration were estimated using Pierce 660 nm Protein Assay, according to manufacturer's instructions.

Crystallisation and data collection. 96 well screens using commercial kits were conducted after purification using a Phoenix liquid handling robot (Art Robbins) by mixing 200 nL of protein with 200 nL of precipitant in SD2 plates (Hampton Research). Plates were incubated at 12°C or 18°C. Optimisation of crystal hits were conducted by the hanging drop method using 24 well VDX plates (Hampton Research), where 1 µL of protein was mixed with 1 µL of precipitant on a microscope slide and vacuum sealed onto VDX plate wells using vacuum grease. For crystal harvesting, crystals were soaked in cryoprotectants for 1 min before being mounted and flash frozen in liquid nitrogen. The main cryoprotectant used was the mother liquor condition with the additional of 20% glycerol. Other cryoprotectants trialed were 25% PEG 400 or 10% increase in concentration of high molecular weight PEG present in the crystallisation condition, for example if the crystallisation condition contains 25%

PEG 3350, 35% PEG 3350 was used for cryoprotection. Data were collected on the PXIII or PXI beamline at the Swiss Light Source.

The commercial 96 well screens used were, Qiagen – Polyethylene glycol (PEG) I suite, PEG II suite, MB Class I suite, MB Class II suite, Ammonium Sulphate Suite, JCSG+; Molecular Dimensions - MemGold, MemPlus, MemSys, MemMeso.

Cloning of thioredoxin tagged 44-4-7 scFv. Total RNA was extracted from 44-4-7 anti-NS4B mAb hybridoma cells (X. Xie et al., 2014) using TRIzol® (Invitrogen). cDNA of scFv was amplified using Mouse scFv Module/Recombinant Phage Antibody kit (Amersham) according to manufacturer instructions. cDNA of the scFv and the (Gly₄Ser)₃ linker were ligated into a pEXP-LiB sequencing vector, transformed into T10 competent cells (Invitrogen), and inserted into a modified pET32b vector containing a N-terminal thioredoxin – His tag and Tobacco etch virus (TEV) cleavage site after sequencing confirmation. Resultant plasmid was transformed into Rosetta Gami II cells (Invitrogen) for protein expression.

Cloning of 44-4-7 scFv containing STII signal peptide. *NcoI* and *XhoI* restriction sites were inserted into both the cDNA of STII peptide (provided by Dr Asial Ignacio) and scFv. The two cDNAs were ligated after digestion with *NcoI* and *XhoI* (NEB) into pET32b vector (Novagen), transformed into T10 competent cells (Invitrogen). After sequencing confirmation, the plasmid was transformed into Rosetta Gami II cells (Invitrogen) for protein expression.

Refolding of thioredoxin tagged 44-4-7 scFv. Cells were grown in a Difco™ 2 × YT (BD) medium supplemented with 50 µg ml⁻¹ kanamycin (Sigma) to an OD₆₀₀ of 1.0. Expression of the recombinant proteins was induced by the addition of 0.5 mM IPTG and incubation at 37 °C for 5 h. Cells were pelleted down by centrifugation at 7,000 × g for 7 min at 15°C, resuspended in lysis buffer (20 mM Tris-HCl, 300 mM NaCl, 10% glycerol, pH 7.5), and disrupted by sonication using a Digital Sonifier 450 (Branson) at 40% amplitude for 10 min. Cell lysate was centrifuged at 10,000 × g for 10 min at 4°C to remove cell debris. The supernatant was centrifuged again at 20,000 × g for 30 min at 4°C to obtain the inclusion bodies. The inclusion bodies were washed rigorously with lysis buffer thrice and were added into the solubilisation buffer (0.1 M Tris-HCl, 50 mM Glycine, 8 M Urea, 300 mM NaCl, pH 8.0) and stirred at 4°C overnight. Insoluble aggregates were separated from solubilised proteins by centrifugation at 20 000 x g for 30 min at 4°C. Supernatant was placed into a 5000 molecular weight cut-off (MWCO) dialysis tubing and dialysed overnight at 4°C with refolding buffer (0.1 M Tris, 300 mM NaCl, 0.4 M arginine, 0.25 mM oxidised glutathione, 2.5 mM reduced glutathione, pH 8.0). Nickel - nitrilotriacetic acid (Ni-NTA) beads were added into the dialysed samples to pull-down the refolded proteins. Three washes with 10 mM, 20 mM, and 30 mM imidazole were performed before the refolded proteins were eluted with 200 mM imidazole. The eluate was concentrated for gel filtration using a HiLoad Superdex 75 16/60 column (GE Healthcare) in 20 mM Tris-HCl, 300 mM NaCl, 10% glycerol, pH 8.0.

Periplasmic extraction of 44-4-7 scFv. Cells were grown in a Difco™ LB (BD) medium supplemented with 50 µg ml⁻¹ kanamycin (Sigma) to an OD₆₀₀ of 1.0.

Expression of the recombinant proteins was induced by the addition of 1 μ M IPTG and incubation at 15 °C for 12 h. Cells were pelleted down by centrifugation at $7,000 \times g$ for 7 min at 15°C, resuspended in lysis buffer (20 mM Tris-HCl, 300 mM NaCl, 10% glycerol, pH 7.5). Cell pellet was resuspended in 1 pellet volume of ice-cold 1X TES buffer (0.2 M Tris-HCl, 0.5 mM Ethylenediaminetetraacetic acid (EDTA), 0.5 M sucrose, pH 8.0). 2 pellet volumes of ice-cold milliQ water were added to the resuspended pellet and incubated by gentle stirring for 60mins at 4°C. Unwanted spheroplasts (*E. coli* cells without outer membrane) were pelleted by centrifugation at $15,000 \times g$ for 15min at 4°C. Ni-NTA agarose beads (Qiagen) were added into the supernatant for pull-down of the soluble scFv proteins. Two washes with 20 mM, and 30 mM imidazole were performed before the scFv proteins were eluted with 200 mM imidazole. The eluate was concentrated for gel filtration using a HiLoad Superdex 75 16/60 column (GE Healthcare) in 20 mM Tris-HCl, 300 mM NaCl, 10% glycerol, pH 8.0.

Western blotting (WB) analysis. Proteins were separated on 12% or 15% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gels, and transferred onto a PVDF membrane using the Trans-Blot® Turbo™ Transfer System (Bio-Rad). The blots were blocked in TBST buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) supplemented with 5% skim milk for 1 h at room temperature, followed by probing with primary antibodies for 1 h. After two washes with TBST buffer, the blots were incubated with a goat anti-mouse Immunoglobulin gamma (IgG) conjugated to horse radish peroxidase (HRP) (1:10,000 dilution) in TBST buffer with 5% milk for 1 h, followed by three washes with TBST buffer. The

antibody-protein complexes were detected using the Amersham Enhanced chemiluminescence (ECL) Prime Western Blotting detection reagent (GE Healthcare). Anti-His tag WB was conducted using mouse monoclonal penta-His antibody (1:1000) from Qiagen. Anti-NS4B WB was conducted using in-house mouse monoclonal 44-4-7 antibody (1:1000) (X. Xie et al., 2014)

Surface plasmon resonance (SPR). SPR biosensor measurements were performed using a Biacore T200 instrument (GE Healthcare) in 20 mM Tris-HCl pH 8.0, 150 mM NaCl, 10% glycerol, 1 mM TCEP, 0.03% weight per volume (w/v) DDM, 5 mM CaCl_2 at 25°C. Purified recombinant biotinylated NS4B protein was directly immobilized onto Streptavidin sensor chips (GE Healthcare) Different concentrations of NITD-618 or compound 14a were diluted into running buffer to concentrations ranging from 39 nM to 2.5 μM as 2-fold serial dilutions and injected across immobilized chip surface. Raw sensorgrams were double referenced and responses were corrected using a reference flow cell and a blank buffer injection. For each corrected sensorgram, the zero for x- and y-axis was taken at the point of sample injection. Responses for the varied concentrations were overlaid and fit to a 1:1 simple binding model to obtain binding affinities using Biacore Evaluation software.

Circular dichroism (CD). CD analyses were conducted using a Jasco J-810 CD spectropolarimeter. Protein was dialysed into buffer containing 10 mM Tris-HCl, 50 mM NaCl, and 0.01% DDM, pH 8.0; NS4B protein was diluted 0.5 mg/mL using the same buffer. Measurement collected using a 0.1 mm quartz cuvette. Spectra in far ultraviolet region between 190 to 260 nm in triplicates at room temperature. Solvent

backgrounds were substrated using the Pro-Data Chirascan software, version 4. Ellipticity measured in millidegrees was converted to mean residue ellipticity ($\text{deg.cm}^2.\text{dmol}^{-1}$), using the formula:

$$\theta \text{ MRE} = \theta .M/10.c.l.(n - 1)$$

where θ is the measured ellipticity of the protein, M is the molecular weight of protein expressed in Da, c is the concentration of protein expressed in mg/mL, l is the pathlength of the cuvette expressed in mm and n-1 is the number of peptide bonds in the protein.

Data deconvolution was performed using SELCON 3 program available on the Dichroweb server (Whitmore & Wallace, 2008).

LCP crystallisation. LCP was performed according to instructions from (Caffrey & Porter, 2010). Briefly, 10 – 20 mg/mL of purified NS4B or NS4B-Fab complex were mixed with molten monoolein at a lipid : protein ratio of 3:2. Resultant monoolein-protein mixture is dispersed onto a LCP glass sandwich plate and immediately covered with crystallisation solution. The sandwich plate is covered with a glass cover slip and incubated at 20°C. Harvesting of crystals were performed according to (D. Li, Boland, Aragao, Walsh, & Caffrey, 2012).

SMA protein extraction. Steps for purification of membrane protein SMA are the same as purification of NS4B using detergent as described above. However, during the membrane solubilisation step, a final concentration of 2.5% (w/v) SMA is added instead of detergents, and solubilisation is performed at RT for 2 h instead of 4°C.

Negative-stain Electron Microscopy (EM). The grid preparations and data collection for the EM studies were done with assistance from Professor Shashi Bhushan's laboratory at NTU.

bRIL-NS4B-mAb complex or bRIL-NS4B SMALP prepared in our laboratory was diluted to 10 µg/ml with 20 mM Tris-HCl, pH 7.5, 150 mM NaCl. 4 µl of protein sample was applied to a glow-discharged carbon-coated transmission EM grid and stained with 2% (v/v) uranyl acetate. The grid was air-dried for 10 minutes. EM was performed using FEI 120 kV EM equipped with 4K Eagle camera. Negative stained grids were imaged at nominal magnification of 49,000× (calibrated 66,350×) with -1.0 to -2.0 µm defocus applied resulting in a 2.11 Å pixel size at the objective scale. About 1,000 particles of box size 72 x 72 pixels were selected manually by Prof Shashi Bhushan and applied to 2D class averaging using EMAN2 image processing package (Tang et al., 2007).

2.5 Results

2.5.1 Optimisation of expression and membrane solubilisation conditions using full-length NS4B construct

Initial work for this project was focused on optimisation of expression and purification of the pET28a deca-histidine tagged (HIS₁₀-tag) full-length NS4B construct from Dengue 2 (DENV-2) TSV01 strain. This work led to the discovery of *in vitro* dimerisation of NS4B, which supported findings by Dr Jing Zou *et al.* that NS4B dimerises in cell culture (discussed in Section 2.5.3). Results of this preliminary work, including optimisation of expression conditions and detergent screen are summarised on Figures 2.3 - 2.5. This construct was not pursued further after new truncated DENV-2 TSV01 constructs with better soluble expression were produced by the PPP.

A

Figure 2.3. Optimisation of expression conditions in *E. coli* BL21 (DE3) strain.

Optimisation was performed by growing cell at different IPTG concentrations, temperatures or inductions at different O.Ds. WB performed after solubilisation with DDM using 44-4-7 anti-NS4B mAb shows that all these conditions gave similar yields, based on the size of their bands. Culturing cells at 0.2 mM IPTG, 16°C, and 0.8 O.D was chosen to be default culture conditions, as these conditions would keep the rate of protein expression low and allow more time for proper protein folding (Freigassner, Pichler, & Glieder, 2009).

A **B**

Figure 2.4 Screen for high yielding *E. coli* expression strains. Strains tested were specialised membrane expression cell lines – C41, C41P, C43, C43P, and a tunable expression strain pLemo (Miroux & Walker, 1996; Wagner et al., 2008). BL21 (DE3) was used as a control for comparisons Expression was induced with 0.2 mM IPTG at 1.0 O.D, at 16°C overnight. Expression levels were tested by anti-NS4B WB. **(A)** Expression of NS4B using tunable pLemo expression strain. Protein expression can be controlled through the addition of rhamnose to control the expression of lysozyme (Wagner et al., 2008). Lysozyme inhibits T7 polymerase expression. Therefore, by regulating lysozyme through rhamnose, the level of expression of NS4B can be regulated, and prevent toxicity due to overexpression. Unexpectedly, WB shows that BL21 (DE3) has better NS4B expression compared to the pLemo strains grown with different levels of rhamnose. **(B)** The C41, C41P, C43 and C43P strains were not able to express NS4B at all. Therefore, BL21 (DE3) was chosen as the expression strain.

Figure 2.5. Detergent screen performed to determine suitable detergent(s) for membrane solubilisation. A panel of detergents routinely used for membrane protein extraction and crystallisation - DDM, 6-Cyclohexyl-1-hexyl- β -D-maltoside (CYMAL 6), 5-Cyclohexyl-1-hexyl- β -D-maltoside (CYMAL 5), Lauryldimethylamine-oxide (LDAO), *n*-octyl- β -D-glucopyranoside (OG) and *n*-Decyl- β -D-maltopyranoside (DM) – were used to solubilise 10 mL of overnight cultures, followed by assessment of the protein yield by anti-NS4B WB. WB shows that most of the detergents, except LDAO and OG, are suitable for membrane solubilisation. DDM has a slightly better yield compared to the rest, and therefore was chosen as the detergent of choice for membrane solubilisation.

2.5.2 Expression, purification and characterization of NS4B using multiple constructs approach

A total of 18 different constructs were made using the multiple-construct strategy to identify optimal expressing construct(s). The truncated constructs - vc008, vc009, vc010, vc011, vc013 (Figure 2.6 and Table 2.1) of NS4B were designed based on NMR findings that the first 20 residues at the N-terminus of NS4B are flexible (Y. Li et al., 2015; Y. Li, Wong, et al., 2016). Each construct contains a hexa-histidine tag and TEV cleavage site at the N-terminus. Detergent screening was performed by PPP. DDM and CYMAL 6 were found to be suitable detergents for membrane solubilisation (performed by Dr Agnes Jong, Figure 2.7). For this report, data from vc008 will be used as representative result, because it produced the best diffracting crystals, and were used for majority of the experiments presented in this thesis.

Figure 2.6. Schematic representation of NS4B constructs made by PPP using the multiple constructs approach. These constructs were made to remove a flexible region in the N-terminus observed in NMR studies. Reduced flexibility will increase the chances crystallisation. Small scale expressions were carried out by PPP, only constructs with good expression (green) were picked for scaled up expression and purification. vc012 was not pursued because the size of the protein was much larger than expected. (Adapted from PPP, www.proteins.sg)

Table 2.1. Properties of NS4B constructs used for multiple construct strategy.

Construct	Length (amino acids)	Molecular weight (kDa)	Isoelectric Point
pNIC-vc008	15-248	27.8	9.07
pNIC-vc009	21-248	27.3	9.07
pNIC-vc010	25-248	26.8	9.27
pNIC-vc011	27-248	26.6	9.27
pNIC-vc012	31-248	26.2	9.05
pNIC-vc013	24-230	25	7.68

Figure 2.7. Detergent screen on vc008 truncated NS4B construct. CYMAL 6 and DDM produced the desired peak in SEC. As expected, LDAO and FC12 solubilised more NS4B proteins, but these proteins are not soluble as reflected by the SEC profiles. [Performed by Dr Agnes Jong (NTU, PPP)]

A two-step purification strategy using IMAC with HisTrap fast flow nickel column, followed by size exclusion chromatography (SEC) was employed for purification after membrane solubilisation using DDM. The UV absorbance was almost negligible for all the constructs on the chromatogram, and the presence of NS4B protein could only be visualised using SDS-PAGE (Figure 2.8). NS4B protein could be observed in almost all IMAC eluate fractions. This is an indication that a hexa-histidine tag (HIS₆-tag) may not be sufficient for strong IMAC column binding, as the detergent micelles that are wrapping around NS4B might have partially masked the His tags, leading to weak binding to the His trap columns. Therefore, a vc008 construct containing octa-histidine tag (HIS₈-tag) was subsequently designed.

Figure 2.8. Chromatogram and SDS-PAGE visualisation fractions from vc008 IMAC. NS4B is observed in all fractions indicating weak binding with the Nickel resin.

After SEC, two different pools of proteins were collected and concentrated based on the SEC profile (Figure 2.9A). On average, the total yield for vc008 from 8 litres of *E. coli* culture was approximately 0.8 mg/L. However, SDS-PAGE indicated that the protein is not homogenous, as several other bands were present. Subsequently, a different purification strategy involving vigorous washing of the membrane pellet after membrane preparation with high and low salt buffers was employed (personal communications with NIBR membrane protein scientists). When used in conjunction with a HIS₈-tag, a pure and homogenous NS4B population was obtained, but the yield decreased from 0.8 mg/L to approximately 0.4 -0.5 mg/L (Figure 2.9B).

A **B**

Figure 2.9. Representative result for purification and characterisation of vc008 construct. (A) SEC chromatogram and SDS-PAGE gel of initial vc008 purification performed without membrane washing step. Two major peaks were obtained and were pooled separately (fractions under the peaks 48.59 mL and 73.09 mL) for crystallisation screens. SDS-PAGE gel lanes were not pure indicating the presence of NS4B oligomers or contaminants. (B) SEC chromatogram and SDS-PAGE gel of vc008 purification performed with membrane washing step. Only a single peak was obtained, fractions under the peak were pooled for subsequent characterisation or crystallisation. Fractions appeared clean on the SDS-PAGE gel.

Concentration of the SEC fractions was initially monitored using Dynamic light scattering (DLS). However, due to detergent effect, the size distributions of the concentrated proteins were adversely affected (Figure 2.10A). Therefore, the concentration process was monitored using SEC instead. The elution peak started broadening for protein concentration higher than 5 mg/mL, indicating formation of oligomers or soluble aggregates (Figure 2.10B). For this reason, the optimal protein concentration was set at 5 mg/mL for subsequent purifications.

Figure 2.10. Monitoring oligomerisation of vc008 during protein concentration.

(A) Monitoring oligomerisation with DLS. A large size distribution is observed, indicating polydispersed protein population. This is most likely due to the presence of DDM detergent micelles. (B) Monitoring oligomerisation with SEC. Merged SEC chromatogram of vc008 at various concentrated concentrations. The peak of purified vc008 gradually shifted to left (forming oligomers) as the concentration process proceeded. At 10 mg/mL, two peaks were obtained at elution volumes (V_e) of 12.62 mL and 13.96 mL, indicating oligomerisation. Therefore, 5 mg/mL was determined to be optimally concentrated concentration for crystallisation.

The thermostability of vc008 to vc011 constructs in SEC buffer was tested by Thermofluor using Sypro Orange to identify the most stable construct(s) for

crystallisation. Again, due to detergent effect, the results were adversely affected (Figure 2.11).

Figure 2.11 Thermofluor analysis of purified NS4B constructs. 5 μ l each protein at 2.5 mg/ml was mixed with 7.5 μ l of 300 $\times$ Sypro Orange, and water was added to top up the volume to 25 μ l according to (Ericsson, 2006). The samples were incubated at temperatures from 20 to 90 $^{\circ}$ C in increments of 0.2 $^{\circ}$ C using CFX96 real time PCR machine. Fluorescence changes in the wells of the plate at excitation and emission were 490 and 575 nm respectively were monitored using the built-in CCD camera. **(A)** Control thermofluor experiment using buffer, soluble full-length NS3 and NS2B-NS3pro proteins in the presence and absence of 0.03% DDM. In the absence of DDM, the buffer did not produce any signal, while NS3 and NS2B-NS3pro produced a typical melting curve. However, in the presence of DDM, the signals for these 3 conditions were adversely affected. The signals for the NS4B constructs in **(B)** and **(C)** were also adversely affected by the presence of DDM.

The purified protein was subjected to Matrix-assisted laser desorption/ionization – time of flight (MALDI-TOF) MS (Figure 2.12) and CD to further ensure homogeneity and proper folding (Figure 2.13).

A

Figure 2.12. MALDI-TOF MS spectrum of purified vc008. Results indicate that majority of the protein population is monomeric NS4B (28.0 kDa), and a small population NS4B dimers exists (55.62 kDa), which might have formed during the concentration process. The high background was caused by the presence of DDM in the sample buffer.

Result	Helix1	Helix2	Strand1	Strand2	Turns	Unordered	Total
Final	0.680	0.254	-0.003	-0.001	0.036	0.161	1.128

Figure 2.13. Plot of experimental CD spectrum of vc008 (green) and theoretical CD data (cyan) using SELCON 3 analysis program (Whitmore & Wallace, 2008). The experimental data of the purified sample exhibited an α -helical structure, indicating that the sample is correctly folded. Further data deconvolution using SELCON 3 analysis predicted that the purified protein contains approximately 93.4% α -helix, 3.6% β -turns, and 16.1% unordered regions.

2.5.3 Crystallisation assays of purified NS4B

The first round of 96-well screens using commercial screens was conducted immediately after purification, but no hits were observed. A second round of screening was conducted after DDM was exchanged for CYMAL 6 by dialysis; no hits were observed either. There is high concentration of calcium ions within the rER, we speculated that addition of calcium chloride (CaCl_2) might help in crystallisation since NS4B resides on the ER membrane. Therefore, CaCl_2 was added directly to the purified NS4B before crystallisation, which finally produced crystals hits. The majority of the hits were from PEG I suite and MemGold screening kits. However,

only vc008 and vc011 constructs gave crystals. Small crystals started appearing after 3 weeks, and a total of 15 hits were obtained after 6 weeks. The hits were confirmed with UV microscopy, as shown in Figure 2.14.

A **B** **C** **D**

Figure 2.14. Representative light and UV microscope pictures of crystals hits in 96-well screens. (A) vc008 (NS4B 15-248) in PEG I suite A12 – 0.1 M 2-(N-morpholino)ethanesulfonic acid (MES) pH 6.5, 25% (w/v) PEG 2000 monomethyl ether (MME). (B) vc011 (NS4B 27-248) in PEG I suite H3 – 0.2 M Sodium phosphate, 20% (w/v) PEG 3350. (C) vc008 NS4B 15-248) in PEG I suite B2 – 0.1 M Na – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 7.5, 30% (v/v) PEG 300. (D) vc011 (NS4B 27-248) in PEG I suite F11 - 0.2 M Ammonium formate, 20% (w/v) PEG 3350. (Scale bar is 90 μ m)

Next, 24-well grid screens around the initial hit conditions were conducted for optimisation. In total only 5 conditions were reproducible (Figure 2.16A–E, hit conditions are summarised in Appendix I). Three crystal forms were obtained: cubic; rod; pyramidal (Figure 2.15A-B). Crystals from an optimisation screen were harvested and washed thoroughly with fresh crystallisation buffer for SDS-PAGE analysis. However, no protein band was observed (Figure 2.15C). Since the crystals were very small (between 20 – 40 μ m), a more sensitive WB analysis was used to show that the crystals were indeed NS4B crystals. From WB, it was observed that the crystals could be formed by a truncated form of NS4B with a size of approximately 22.5 kDa (Figure 2.15D). An anti-HIS WB of the washed crystals indicated that the protein forming the crystals did not have HIS tags (Figure 2.15E).

Figure 2.15. Representative photos of grid screen optimisation of crystal hits. (A) vc008 crystals obtained from optimising PEG I D7 conditions - 0.1 M Tris-HCl pH 8.5, 25% (w/v) PEG 3000. **(B)** vc008 crystals obtained from optimising PEG I B2 conditions – 0.1 M Sodium HEPES pH 7.5, 30% PEG 300. **(C)** SDS-PAGE gel of 20 picked and washed crystals. No protein band was observed. **(D)** WB analysis of 20 picked and washed crystals using 44-4-7 anti-NS4B mAb. A protein band was observed around 22.5 kDa. **(E)** Anti-HIS WB analysis of washed crystals. The vc008 crystals did not have a HIS tag.

Optimised crystals were harvested and tested at the Swiss Light Source using the PX I or PX III beamline (Figure 2.16A-E). The resolutions obtained ranged from 10 Å – 18 Å. With kind assistance from Professor Lars Redecke from University of Lübeck, the maximum resolution of vc008 crystals was pushed to approximately 7 Å using the DESY micro-focused beamline (Figure 2.17). This was the best resolution obtained so far, but was not sufficient to obtain the unit cell dimensions.

A

B

C

D

E

Figure 2.16. Summary of diffraction data of five weakly diffracting crystals. These crystals were obtained from 24 well optimisation of original hit conditions.

Figure 2.17. Best diffraction of approximately $7\text{\AA}$ obtained from vc008 crystal so far. Data was collected with micro-focused beamline in DESY.

Several other optimisation approaches such as the use of additive screens, macro and micro-seedings, compound (NITD proprietary compounds) soakings or co-crystallisations, and dehydrations were attempted, but the crystals either did not grow or dissolved into the precipitants. Crystallisation using 200 μL of Al's oil (50% (v/v) paraffin oil : 50% (v/v) silicon oil) was also attempted. This was used to grow bigger crystals by slowing down vapour diffusion rate. There is a chance that the bigger crystals might have better crystal packing, and thus better resolution. This approach was successful in obtaining bigger crystals, but there were no improvements in diffraction resolution of the crystals (Figure 2.18). On the contrary, the resolution actually decreased.

Figure 2.18. Optimisation of vc008 using Al's oil in conditions from PEG D7. The crystal grew significantly larger, but the diffract quality was adversely affected, as compared to original condition.

After repeating various optimisation trials without any progress, we came to a conclusion that the existing constructs were not optimum for obtaining high resolution structures. Most probably because there are highly flexible regions within NS4B, for example the cytoplasmic loop, pTMD-1 and -2, and these regions are destabilising the

crystal contacts within the crystal. Therefore, to mitigate this problem, we moved on with the antibody assisted crystallisation strategy in an attempt to stabilise NS4B for crystallisation (Section 2.5.6).

2.5.4 Dimerisation of NS4B

During purification of the pET28a deca-histidine full-length NS4B construct, apart from the expected 28 kDa NS4B band, a distinct band at approximately 56 kDa could be seen on SDS-PAGE gels from both IMAC and SEC, as well on the SEC profile (Figure 2.19A - C). An anti-NS4B WB confirmed that the 56 kDa band was also NS4B (Figure 2.19D). This result indicates that NS4B is able to form stable dimers *in vitro*. The observation was further confirmed using size exclusion chromatography - multi-angle static light scattering (SEC-MALS) by Dr Jing Zou and Ms Aline Reynaud, where the molecular mass of WNV NS4B was calculated to be 60.02 kDa, indicating that NS4B exists as a dimer in the assay solution (Figure 2.19E). Efforts to reduce the dimers to monomer using high concentrations of DTT did not break up the dimers. Further experiments performed by Dr Jing Zou demonstrated that NS4B forms dimers in transient transfected cells and in virus infected cells. The results were published in (J. Zou et al., 2014).

Figure 2.19. Dimerization of recombinant DENV-2 NS4B protein [Adapted from (J. Zou et al., 2014)]. **(A)** SDS-PAGE analysis of NS4B after IMAC. NS4B dimer and monomer are indicated by an asterisk and arrow respectively. **(B)** SEC chromatogram of NS4B of pooled fractions 8 -10 from (A). A dimer peak elution (asterisk) is observed between fractions 8 - 12, while a monomer peak (arrow) is observed between fractions 14 - 18. **(C)** SDS-PAGE analysis of SEC eluates. A clear dimer band is observed from Lane 9 -13, Lane I was loaded with pooled NS4B after concentration. **(D)** WB analysis of pooled NS4B. **(E)** SEC-MALS analysis of WNV NS4B dimerization. Purified WNV NS4B was loaded into a S200 5/150 GL column using a buffer containing 20 mM Tris-HCl (pH 8.0), 300 mM NaCl, 2 mM TCEP, 0.03% DDM, and 0.006% cholesterol (CHS) for SEC-MALS analysis. The calculated molecular mass of the protein - detergent conjugate is 455.0 kDa (red), NS4B – 60.02 kDa (blue), and DDM-CHS micelles – 395.0 kDa (green).

2.5.5 Effects of calcium ions (Ca^{2+}) on NS4B

A concentration of up to 10 mM CaCl_2 was necessary for successful NS4B crystallisation. Therefore, the effects of calcium ions (Ca^{2+}) on NS4B were investigated. First, 10 mM CaCl_2 was added during NS4B purification steps. A broadening of NS4B elution peak was observed (V_e from 40 – 90 mL) as compared to previous purifications without CaCl_2 , when only a single peak elution at 75.19 mL was observed (Figure 2.20). This result indicates that Ca^{2+} may promote NS4B oligomerisation.

Figure 2.20. Effects of Ca^{2+} on NS4B during SEC. Addition of 10 mM Ca^{2+} led to broadening of NS4B elution peak during SEC.

Next, the effect of Ca^{2+} on the stability of NS4B was investigated by incubating purified NS4B with 10 mM CaCl_2 at room temperature over 7 days. Comparisons with SDS-PAGE analysis of NS4B incubated in absence of CaCl_2 show that NS4B aggregation was accelerated by the presence of Ca^{2+} (Figure 2.21A and B). Most of the

NS4B protein aggregated over 7 days, while in the absence of Ca^{2+} only approximately 50% of NS4B aggregated.

Figure 2.21. Effects of Ca^{2+} on NS4B stability. (A) SDS-PAGE of purified vc008 in the absence CaCl_2 incubated over 7 days. NS4B slow aggregates over time. (B) SDS-PAGE of purified vc008 in the presence of CaCl_2 incubated over 7 days. Presence of CaCl_2 accelerates the rate of aggregation of NS4B.

Using SPR spectroscopy, it was observed that Ca^{2+} is required for proper binding of NITD-618 compound to chip immobilised NS4B (Figure 2.22), leading binding saturation, while no saturation was observed in the control experiment without Ca^{2+} (experiment performed by Dr Christian Noble, NITD).

Figure 2.22. Effects of Ca²⁺ on NS4B binding to NITD-618 and Compound 14a using SPR. SPR was performed by adding 2-fold serially diluted NITD-618 compound or Compound 14a (39 nM to 2.5 μ M) to chip immobilized NS4B in absence or presence of 5 mM Ca²⁺. No binding was observed for Compound 14a in absence or presence of Ca²⁺. A concentration dependent binding of NITD-618 was observed without Ca²⁺ in the running buffer, while binding saturation was observed at high NITD-618 concentrations when Ca²⁺ was added to the running buffer. (SPR data were obtained from Dr Christian Noble)

2.5.6 Antibody assisted crystallisation

An antibody assisted crystallisation approach was attempted for three reasons, 1) to increase purity of NS4B during purification of the protein – antibody complex; 2) to reduce flexibility of NS4B during crystallisation, and increase hydrophilic surfaces for crystallisation; 3) to help structure determination by molecular replacement for phasing. Three mouse anti-NS4B monoclonal antibodies are available for use for this project,

44-4-7, 3-7, 10-3, were produced by immunising mice with purified recombinant full-length NS4B (Figure 2.23) (X. Xie et al., 2014).

Figure 2.23. Epitope mapping of the three anti-NS4B mAbs used in this project. The epitopes recognised by each antibody are indicated by arrows [Modified from (X. Xie et al., 2014)].

2.5.6.1 Co-crystallisation using scFv

The first attempt in this strategy was to generate scFv fragments for co-crystallisation. scFv was chosen because it has less flexibility compared to Fab fragments, and is therefore more favourable for obtaining high resolution structure. 44-4-7 antibody was chosen first because it binds to the flexible cytoplasmic loop, and we believe that stabilisation of this loop will greatly increase the chance of obtaining more stable crystal packing, and thus better diffracting crystals. 44-4-7 scFv fragments were generated from the hybridoma RNA using Mouse scFv Module/Recombinant Phage Antibody kit, consisting of a variable light and heavy chain that are linked together by a flexible (Gly₄Ser)₃ linker (performed by Ms Yee Hwa Wong). Initial expression was performed using a modified pET32b vector containing a thioredoxin – hexa-histidine tag in *E. coli* Origami expression strain (Refer to Appendix II for the scFv protein

sequence). However, no soluble protein was obtained, and refolding was performed instead. The yield from a 10 L culture after refolding was only 0.05 mg/L (Figure 2.24).

A.

B.

C.

Figure 2.24. Refolding of pET32b thioredoxin tagged 44-4-7 scFv. (A) Schematic representation of pET32b thioredoxin tagged 44-4-7 scFv. The molecular weight of the full-length construct is 44 kDa, and 27 kDa when the thioredoxin – His tag is cleaved off by TEV protease. (B) SDS-PAGE of pull-down experiment after refolding using Ni-NTA agarose beads. Lane 1: Refolded scFv mixture; Lane 2: Supernatant after pull down; Lanes 3 – 5: Wash (10mM, 20mM, 30mM imidazole respectively); Lanes 6 - 7: Elutions (200mM Imidazole each). The two elutions on lanes 6 and 7 were pooled for TEV protease cleavage and SEC. (C) SDS-PAGE of SEC fractions after TEV protease cleavage. The cleaved 27 kDa scFv fragments (lanes 9-11) were pooled and concentrated.

Next, to increase yield and reduce time required for purification (the scFv refolding process is long), a periplasmic expression approach was attempted. A STII signal peptide was inserted to the N-terminus of the construct, which directs the translated protein into the periplasmic space where chaperones are available to assist the formation of proper di-sulphide bonds that are required for correct scFv folding. The

folded scFv can then be extracted from the periplasmic space as a soluble protein, eliminating the need to go through a lengthy refolding process. Unfortunately, after optimisation of expression conditions, a 10L culture could only yield approximately 0.1 mg/L of soluble 44-4-7 which was still too low for co-crystallisation (Figure 2.25).

A

B.

Figure 2.25. Purification of STII - 44-4-7 scFv by periplasmic extraction. (A) Schematic representation of pET32b STII 44-4-7 scFv. The molecular weight of the whole construct is 30 kDa and 28 kDa when the STII peptide is cleaved off. **(B)** SEC profile and SDS-PAGE of scFv after batch IMAC. Proteins from 90 mL to 112.5 mL (Lanes 6 – 13) were pooled and concentrated.

Next, a direct refolding from inclusion bodies approach was performed, followed by IMAC and SEC. A construct without thioredoxin or STII peptide was made for this approach (Figure 2.26A). The refolded scFv produced two peaks after SEC. The first

peak (peak elution at 48.01 mL) precipitated rapidly after SEC, while the second peak (peak elution at 106.06 mL) remained soluble even after concentration (Figure 2.26B).

A.

B.

Figure 2.26. Representative purification and characterisation of refolded 44-4-7 scFv from inclusion bodies. (A) Schematic representation of scFv construct used for refolding. (B) SEC chromatogram of 44-4-7 scFv after refolding. Fractions from the first peak precipitated during collection. The fractions from the second peak were collected for SDS-PAGE and subsequent experiments.

The concentrated scFv were mixed in excess with purified vc008 at a molar ratio of 2:1 for 2 hrs, and subsequently subjected to SEC. A shift in both the SEC V_e of vc008 and scFv was observed. SDS-PAGE analysis shows that both vc008 and scFv co-eluted out at 82.14 mL, indicating the two proteins might have formed a complex together (Figure 2.27). Yield from the co-elution was much less than expected, and most of the

scFv remained unbound and eluted out at 92.35 mL (a change in V_e could be due to the presence of DDM in the SEC buffer).

Figure 2.27. Complexing of refolded scFv with purified vc008. SEC chromatogram of refolded scFv in complex with vc008. Co-elution was observed at V_e 82.14 mL on SDS-PAGE of the fractions containing the complex peak.

This hinted that most of the refolded scFv might not be active or refolded incorrectly due to formation of the wrong intra – molecular disulphide bonds. A single cycle kinetics SPR experiment was conducted to study the binding of refolded scFv to immobilised vc008 (Figure 2.28). A fast on and off rate was observed from the SPR run, with an estimated equilibrium dissociation constant (K_D) of 120 nM; much lower affinity than the expected 10 – 20 nM. This confirmed that wrongly refolded scFv populations were present. Nevertheless, the co-eluted fractions were setup for crystallisation trials. However, no hits were obtained. The approach was stopped, and we moved on to co-crystallisation using antibody binding fragments (Fab).

Figure 2.28. SPR sensogram of single cycle kinetic run of refolded scFv with immobilised NS4B. A fast on and off rate of was observed. The K_D was estimated to be 120 nM.

2.5.5.2 Fab co-crystallisation

Full 44-4-7 mAb were purified in large scale using hybridoma cells, and subsequently purified by dialysis and Protein A affinity chromatography. First, the hybridoma culture supernatant containing secreted mAb was filtered using a 100 kilo-daltons (kDa) MWCO filtration cassette to remove the major components present in fetal bovine serum (FBS) including bovine serum albumin. Next, the filtered mixture was subjected to Protein A chromatography to obtained highly pure mAb. The purified mAb was then digested with activated papain (10 μ g of papain per mg of mAb) to obtain the desired Fab fragments. Optimisation of papain digestion was performed, and an optimum digestion time of 10 hrs at 37°C was determined (Figure 2.29A). Cleaved fragment crystallisable (Fc) region was subsequently removed with Protein A beads to obtain pure Fab fragments (Figure 2.29B).

Figure 2.29. Fragmentation 44-4-7 mAb and complex formation of 44-4-7 Fab with NS4B. (A) SDS-PAGE analysis of time point digestion of 44-4-7 mAb with papain. 10 hr was the optimum timing for complete digestion, without over digesting the mAb. (B) SDS-PAGE analysis of purification of papain digested Fab by Protein A removal of cleaved Fc regions.

Purified Fab and vc008 were mixed together at a molar ratio of 2:1 for 2 hrs then SEC was performed. SPR analysis indicates that the Fab binds to vc008 with high affinity (Figure 2.30A). Co-elution of Fab and NS4B was observed at V_e 11.3 mL, as compared to a V_e of 12.45 mL and 14.50 mL for NS4B only and Fab fragments only run respectively (Figure 2.30B and C). The co-eluted fractions were collected and concentrated for crystallisation trials.

Figure 2.30. Complex formation of 44-4-7 Fab with NS4B. (A) SPR sensogram of Fab binding to immobilised vc008. Tight binding was observed. (B) Merged chromatogram of NS4B only and 44-4-7 Fab only SEC run before complexing. (C) SEC chromatogram of NS4B and 44-4-7 Fab complex. A shift in the elution of NS4B from V_e 12.45 mL to 11.30 mL was observed, and the NS4B – Fab complex was visualised on SDS-PAGE (box). The protein marker for Fab and NS4B are inserted on the right.

Crystallisation screens were performed using membrane protein and antibody specific screening kits. Needle like crystals were formed after 2 weeks in three conditions (conditions are summarised in Appendix I). However, needle-like crystals were also observed in the corresponding Fab only control well, implying that crystals formed were from Fab fragments only (Figure 2.31). This is an indication that either NS4B degraded due to detergent intolerance or the antibody – protein interaction was destabilised by detergent during the crystallisation process. Further optimisations of the hit conditions were unsuccessful.

Figure 2.31. UV micrograph of NS4B-Fab complex crystallisation. (A) Needle-like crystals were observed from both NS4B-Fab and Fab only control wells (0.1 M Tris.HCl, pH 8.5, 1.5 M Ammonium sulphate, 15 % (w/v) Glycerol), indicating that the crystals formed were most likely Fab only crystals. (Scale bar is 70 μ m)

2.5.7 Fusion protein strategy

The successful crystallisation and structure determination of the β 2-adrenergic receptor fused with a T4L domain was a proof-of-concept that the fusion protein strategy is a powerful technique for membrane protein crystallisation (Cherezov et al., 2007). Similar to the antibody-assisted crystallisation approach, insertion of fusion partners will increase the hydrophilic surface area for crystal contacts, and the fusion

partner can also be used for phasing during data processing. For this approach, we chose T4L and cytochrome b₅₆₂RIL (bRIL) for fusion with NS4B, because they were identified to be the most productive for crystallisation (Figure 2.32) (Chun et al., 2012). Following the same strategy, a NS4B-NS5 MTase construct was made. Another construct, NS4A-NS4B, was made as an exploratory attempt.

Construct		Yield	Legend - No expression / precipitated + 0.1 - 1.0 mg/L ++ 1.0 - 2.5 mg/L +++ > 2.5 mg/L
N—	NS4A—NS4B—C	+	
N—	T4L—NS4B—C	-	
N—	BRIL—NS4B—C	+++	
N—	NS4B—NS5meth—C	-	
N—	NS4B—T4L—C	-	
N—	NS4B—BRIL—C	+	

Figure 2.32. Representation of fusion constructs made for the fusion protein strategy. The yield of each construct is indicated on the right.

2.5.7.1 Purification of fusion NS4B proteins

Expression of these constructs were tested using 4 litre cultures; only bRIL-NS4B construct produced higher yield compared to the non-fusion constructs (Figure 2.33A), while the other constructs did not produced any soluble yield. MALDI-TOF analysis confirms that the protein is of the correct size (Figure 2.33B). The purification results are summarised on Table 2.2. Since the yield of bRIL-NS4B was much better; it was chosen to replace vc008 as the main focus for all subsequent crystallisation efforts. However, no hits were obtained from 96-well screens, or 24-well screens with the 5 conditions that produced hits previously.

A. **B.**

Figure 2.33. Purification of bRIL-NS4B fusion protein. (A) SEC profile of bRIL-NS4B construct. The protein eluted out as a single peak, with peak elution at 76.37 mL. SDS-PAGE gel analysis showed that the eluted fractions were homogenous. (B) MALDI-TOF analysis of purified bRIL-NS4B.

Table 2.2. Properties and expression yield of fusion NS4B constructs

Construct	Molecular weight ^a (Uncleaved /Cleaved) (kDa)	Isoelectric Point ^a (Uncleaved /Cleaved)	Yield per litre (mg)
pET28a - NS4A - NS4B	45.9 / 43.7	7.22 / 7.62	0.4 – 0.8
pET28a -T4L - NS4B	45.6 / 43.1	8.85 / 9.15	Precipitated
pET28a - BRIL - NS4B	44.1 / 41.7	6.32 / 6.34	2.0 - 5.0
pET28a - NS4B – NS5meth	62.2 / 59.7	8.99 / 9.14	Precipitated
pET28a - NS4B – T4L	45.4 / 42.9	8.32 / 8.86	Precipitated
pET28a - NS4B – BRIL	43.8 / 41.2	6.01 / 5.76	0.7 - 1.0

^a Predicted by ExPASy Protparam program

2.5.7.2 Exploring cryo-EM structure determination using mAb – bRIL-NS4B complex

With cryo-EM emerging as a viable technique for high resolution structural determination, and since we have access to state-of-the art EM at the SBS microscopy facility, we decided to explore the possibility of solving the structure of NS4B by cryo-EM. Due to the detection limit of cryo-EM, we first had to direct bind full-length 44-4-7 or 10-3-7 mAb to bRIL-NS4B, to increase the total protein-mAb complex size to approximately 190 kDa. Complexing both mAbs with NS4B was also attempted. mAb and NS4B were mixed at a molar ratio of 0.8 : 1 for at least 2 hrs, as recommended by Professor Shashi Bhushan. A portion of the resultant mAb – NS4B complex was directly used for negative staining, while the remaining samples were subjected to SEC before negative staining was performed. Unfortunately, even after repeating sample preparation several times, none of the samples were sufficiently homogenous (Figure 2.34).

Figure 2.34. Representative negative-stain EM micrographs. Samples were not homogenous. (Scale bar: 100 nm)

2.5.8 Baculo-virus expression system (BEVS)

A BEVS approach was attempted after failing to obtain crystals from the new fusion constructs for 2 reasons, 1) allows for proper folding of eukaryotic proteins (DENV is replicating in mammalian host cells), thus could increase protein yield; 2) allows for formation of any post-translation modifications that are required for proper protein function. With help from Ms Vivian Lim, three constructs – full-length NS4B, vc008, and bRIL-NS4B, were made using the Bac-to-Bac system. Protein expression was tested using 500 mL Sf9 cell cultures. Yield from both vc008 and full-length NS4B were low, and were not worth pursuing. Yield from bRIL-NS4B was estimated to be approximately 5 mg/L, making this the most productive construct (Figure 2.35). The SEC produced a 2 peak elution profile similar to that of vc008 construct observed on Figure 2.9A. Increasing the number of membrane washes during purification did not reduce the formation of the larger peak.

Figure 2.35. SEC profile of bRIL-NS4B fusion protein expressed using BEVS. The protein eluted out as 2 peaks – Peak 1 at 55.60 mL and Peak 2 at 72.01 mL. 2 bands are present on the SDS-PAGE of the eluted fractions.

To investigate if the two populations are in dynamic equilibrium, which will hinder crystallogenesis due to conformation heterogeneity, the two peaks were collected separately, and re-injected for a second SEC run. No new peaks were formed during the second run, indicating that the peaks do not exist in dynamic equilibrium, but as separate populations (Figure 2.36).

Figure 2.36. Reinjection of SEC peaks to assess the oligomerisation state of peaks 1 and 2 from bRIL-NS4B purification. Merged SEC profile of Peak 1 and 2 re-injected for SEC. No new peaks were formed during the elution of the two re-injected peaks.

Two proteins bands were observed on the SDS-PAGE after SEC, a larger band at approximately 42 kDa, which corresponds to the correct size of bRIL-NS4B, and an unknown smaller band at approximately 30 kDa (Figure 2.35). MALDI-TOF analysis confirmed the presence of bRIL-NS4B at 42479 Da, but did not identify any major peak corresponding to the smaller band (Figure 2.37). Further protein identification using MS confirmed that the smaller band is produced by bRIL-NS4B (Appendix III). The lower molecular weight band could be an artefact during SDS-PAGE due to incomplete SDS denaturation. Both peaks were pooled for crystallisation screening. However, no crystal hit has been obtained yet.

Figure 2.37. Characterisation of bRIL-NS4B Peak 2 by MALDI-TOF. A single major peak is observed at 42.479 kDa. The high background is due to the presence of DDM.

2.5.9 Crystallisation of NS4B using LCP

LCP screens were performed on all existing constructs, with special focus on bRIL-NS4B – Fab complex, as instructed by Professor Martin Caffrey (Trinity College, Dublin) (Caffrey & Porter, 2010). The LCP matrix is very sensitive to destabilisation by crystallisation screen components such as high molecular weight PEGs, high salt concentration, and presence of sulphate or citrate anions, making most commercial crystallisation screens unsuitable for LCP screening (Cherezov, Fersi, & Caffrey, 2001). Only MemMeso (Hampton Research) that is specifically formulated for LCP is commercially available. Therefore, to increase the amount of chemical space for screening, a home-made 96-well screen was produced by searching through the Protein Data Bank (PDB) for published LCP crystal structures and their crystallisation conditions; a total of 91 different conditions were selected. The remaining 5 wells were

conditions that produced hits for our crystallisation trials, but with high molecular weight PEG substituted with PEG 400 (Appendix IV).

Two potential protein crystal hits were obtained, but due to technical difficulties, none of the conditions could be reproduced. Harvesting crystal hits was very challenging, and many crystals were adsorbed to the spacer tape lining the crystallisation well during the process of removing the glass coverslips using a glass cutter. So far, none of the LCP samples sent for data collection produced diffractions. One reason is because the LCP crystals are transparent under bright-field microscope, which make harvesting of the crystals very challenging, and at times only the LCP matrix was harvested.

2.5.10 Detergent-free membrane protein purification

We are interested in detergent-free membrane protein purification mainly for 2 reasons, 1) to facilitate characterisation and compound screening with NS4B by removing detergent effect; 2) to better mimic the lipid bilayer for preserving structural integrity for cryo-EM studies using mAb–NS4B complexes. Two techniques – nanodiscs and SMALP, were chosen because both had been successfully used for screening and cryo-EM structure determination of membrane proteins (Denisov & Sligar, 2016; Postis et al., 2015).

Attempts to incorporate bRIL-NS4B into nanodisc failed (Figure 2.38).

A **B**

Figure 2.38. Incorporation of bRIL-NS4B into nanodisc using MSP1D1 and POPC. (A) SEC profile of empty nanodiscs formed using MSP1D1 (24.7 kDa) and POPC in the absence of NS4B. **(B)** SEC profile of nanodiscs formed using MSP1D1, POPC and bRIL-NS4B (44 kDa). A shift in V_e was observed, with a new peak forming at V_e 8.80 mL. However, no bRIL-NS4B was observed on SDS-PAGE gel, indicating that bRIL-NS4B was not incorporated into the nanodiscs.

In contrast, incorporation of bRIL-NS4B into SMA polymer was successful and a single peak was observed on the SEC profile after protein extraction and purification (Figure 2.39A). The purified bRIL-NS4B SMALPs were submitted to Prof Shashi Bhushan for negative-stain EM. For the first time, a relatively homogenous population was obtained, with good contrast, and individual particles could be picked for 2D reconstruction (Figure 2.40). However, further experiments need to be performed for optimization of sample preparation, and eventually production of larger amounts of bRIL-NS4B and 44-4-7 mAb for cryo-EM data collection. This work is ongoing.

A.

Figure 2.39. Purification of bRIL-NS4B SMALP. SEC profile of bRIL-NS4B purified using SMALP. A single sharp peak is obtained, and SDS-PAGE analysis shows that the eluates are pure.

Figure 2.40. Negative-stain EM of bRIL-NS4B SMALP. A relatively homogenous population of bRIL-NS4B SMALP could be observed with good contrast on the EM micrograph. Representative of selected particles for class averaging are highlighted in white boxes. Selected class average images are shown on the right panel (scale bar: 5 nm). This work was performed by Prof Shashi Bhushan (NTU).

2.6 Discussion

DENV NS4B is a small hydrophobic integral membrane protein that has been identified as a good target for development of anti-Dengue therapy. However, its inherent hydrophobicity and lack of enzymatic activity has hampered such efforts. In this chapter, we aimed to determine the structure of DENV2 TSV01 NS4B in an attempt to gain better understanding of the biological functions of NS4B and provide structural insights for structure-based drug discovery. We have explored several different strategies for this purpose. Evaluation of the results has identified several possible factors and new approaches that could contribute to successful structural determination in the near future.

2.6.1 Crystals obtained from multiple construct approach might be *E. coli* contaminant - AcrB

It has been reported that contamination with host membrane protein – acriflavine resistance protein B (AcrB) - is a common issue when using *E. coli* as the heterologous expression host (Veesler, Blangy, Cambillau, & Sciara, 2008; Wiseman et al., 2014). AcrB is a 110 kDa multidrug efflux pump sitting on the outer *E. coli* membrane, and its expression is upregulated during the protein overexpression process as a stress response mechanism (Glover et al., 2011). AcrB has a histidine rich C-terminus that readily binds to immobilised nickel ions during IMAC purification of His-tagged membrane proteins. Other histidine rich contaminants such as succinate dehydrogenase and OmpF have also been reported (Wiseman et al., 2014). However, these contaminants can be removed easily by either extensive imidazole washes or reduction of detergent concentration used during extraction respectively, but it is very

difficult to remove AcrB contamination completely (Wiseman et al., 2014). AcrB has reported to readily crystallise even at picogram concentration in many different conditions, which is a major concern when crystallising membrane proteins (NS4B in this case) expressed in *E. coli* (Psakis, Polaczek, & Essen, 2009).

There is definitely a possibility that initial crystal hits obtained from the multiple construct approach were AcrB crystals, because initial purification was performed without membrane washes, and the SDS-PAGE gel lanes obtained were not pure with the presence of several unexpected bands, including a band near 100 kDa in Pool 2 (Figure 2.9A). This could explain why subsequent crystallization screens of the new fusion constructs and mAb-NS4B complex using the five identified crystal hit conditions did not produce any crystals. Furthermore, SDS-PAGE analysis of washed crystals did not produce a protein band, which is not consistent with protein crystals because crystals are formed from densely packed proteins.

A counter argument to this contaminating AcrB notion would be hit conditions obtained were different from the AcrB crystallisation conditions published in PDB. Furthermore, hits were only obtained from vc008 and vc011, while no hits were obtained from vc009, vc010, vc013. Logically all these constructs should have AcrB contamination and would have produced AcrB crystals as well. More importantly, if the crystals were indeed AcrB crystals, crystal hits should be obtained during the initial screens performed without calcium chloride. The fact that hits were only obtained after calcium chloride addition, and that calcium ions has an effect on the oligomerisation of NS4B may indicate that the crystals obtain were indeed NS4B crystals.

In hindsight, samples should have been sent for purity check by MS or western blot using anti-AcrB antibody before setting up crystallisation screens. Samples

contaminated with AcrB should be disposed or re-purified. To prevent future AcrB contamination, 1) a AcrB deficient strain could be used; 2) use an alternative affinity tags such as Strep-Tag, GST-Tag, MBP-Tag; 3) use insect cell or mammalian cell for protein expression. Therefore, BEVS expression of NS4B should be the main focus moving forward, since it also led to better yield with high purity.

2.6.2 Characterization and optimization of the stability of NS4B constructs in detergent and storage buffer is required

Successful protein crystallisation is a multi-factorial process, where optimization of each factor will increase the probability of crystallogenesis. These factors include high yield in mg quantities, high purity, monodispersity, proper folding, intrinsic protein and buffer stability (Bill et al., 2011; Carpenter et al., 2008; Drew, Lerch, Kunji, Slotboom, & de Gier, 2006). Thus far, only yield (milligram quantity), purity (clean bands on SDS-PAGE gel), correct folding (CD), monodispersity (single peak on SEC) has been assessed. However, the stability of NS4B constructs in detergents and storage buffers could not be tested.

2.6.2.1 Artificial evolution to improve thermostability of membrane proteins in detergents

Two results obtained suggest that the extracted NS4B protein is not stable in DDM after purification, 1) the amount of soluble NS4B was decreasing when placed at RT for 7 days (Figure 2.21B); 2) crystallisation using Fab-NS4B complex was unsuccessful, and only Fab crystals were obtained, indicating that NS4B aggregated during the crystallisation process. To overcome this issue and improve the likelihood

of crystallogenesis, artificial evolution to improve the stability of NS4B could be adopted. This technique had been successfully implemented by different membrane protein groups to promote thermostabilisation of membrane proteins in detergents (Ghosh, Kumari, Jaiman, & Shukla, 2015; Heydenreich, Vuckovic, Matkovic, & Veprintsev, 2015; Scott, Kummer, Tremmel, & Pluckthun, 2013).

As mentioned in section 2.2, detergent micelles are poor substitutes for the native lipid bilayers, leading to the loss of or incorrect lateral pressure on the membrane helices, and eventually causing the extracted membrane protein to unfold and aggregate (Prive, 2007). To make matters worse, it has been shown that successful crystallisation requires incorporation of membrane proteins into shorter chain detergents that form small micelles, such as OG, *n*-Nonyl- β -D-Maltopyranoside (NM), or LDAO. These detergents form smaller micelles compared to DDM, and expose more hydrophilic surfaces on the proteins for forming crystal contacts. Consequently, short chain detergents could potentially produce better diffracting crystals with stronger lattices due to formation of more crystal contacts (Bill et al., 2011; Hardy, Bill, Jawhari, & Rothnie, 2016; Prive, 2007). However, these short chain detergents are usually much harsher than DDM and would accelerate the process of protein aggregation. Purification of vc008 using OG lead to aggregation during IMAC (results not shown). Therefore, thermostabilisation of target proteins in detergent by artificial evolution becomes essential.

Alanine scanning mutagenesis and directed evolution are the two most commonly utilised artificial evolution approaches for thermostabilisation of membrane proteins. The two techniques have been successfully applied to improve the detergent stability and structural determination of proteins such as the adenosine A_{2a} receptor, β 1-

adrenergic receptor, and rat neurotensin receptor-1 (P. Egloff et al., 2014; Lebon, Bennett, Jazayeri, & Tate, 2011; Serrano-Vega, Magnani, Shibata, & Tate, 2008) Unfortunately, these techniques rely on ligand binding assays for assessment. Since NS4B is non-enzymatic, and assay with known ligands (NITD-618, and NS3hel) has not been developed yet, these approaches are not suitable at the moment. A ligand-free approach using directed evolution has been described by Asial *et al.* The technique is known as ‘Hot CoFi’, where mutant plasmids generated from directed evolution are directly cloned into *E. coli* (Asial et al., 2013). The resultant colonies are heated at various temperatures before the cells are lysed and passed through a Durapore membrane filter onto a nitro-cellulose membrane. Mutant proteins that are soluble at a specific heating temperature will be able to pass through the Durapore membrane, while aggregated mutants do not. Qualitative WB analysis is performed to identify the mutants with higher thermostability compared to wild-type. An iterative selection process of better mutants is performed for subsequent screenings to improve thermostability by additional mutations. This led to, apart from other membrane protein targets, 26.6 °C thermostability improvement of NXR1 after iterative screenings, and led to improvement of diffraction quality from 2.1Å and 1.5Å. However, the feasibility of this approach on membrane proteins remains to be tested. Another potential disadvantage of this approach is that it does not provide information on how the stabilizing mutations affect the function and conformation of the proteins. Since artificial evolution is not possible due to the lack of a ligand binding reporter assay, ‘hot-cofi’ is a promising approach to follow up to generate more thermostable NS4B mutants in detergents such as OG or LDAO for crystallisation.

2.6.2.2 Using green fluorescence protein (GFP) -fusion protein for identification of stabilising buffer conditions

Another factor that requires optimization is the identification of stabilising buffers used for storage of NS4B after purification. It was first demonstrated by Ericsson *et al* that fluorescence-based thermal stability assay (Thermofluor) is an efficient method for identifying stabilising buffer conditions of soluble proteins that improves crystallisation rates in a high throughput manner (Ericsson, Hallberg, Detitta, Dekker, & Nordlund, 2006). Crystallisation screenings of proteins stored in the optimised buffers led to the doubling of crystal hits as compared to control plates in non-optimal buffers. Thermofluor assay relies on a hydrophobic fluorophore, Sypro Orange, to bind to unfolded hydrophobic protein regions as the protein is gradually heated up with increasing temperature. Unfortunately, the hydrophobic regions of detergents would directly bind to Sypro Orange causing significant interference to the final result, making this technique not suitable for optimization of membrane protein buffers. Therefore, our attempts to determine the stability of NS4B constructs in purification buffer constructs using Thermofluor failed. An alternative dye, N-[4-(7-diethylamino-4-methyl-3-coumarinyl)phenyl]maleimide (CPM), was found by Alexandrov *et al.* to be suitable replacement for Sypro Orange for membrane protein buffer optimization using Thermofluor (Alexandrov et al., 2008). CPM is a thiol-reactive dye that readily binds to cysteine residues exposed during the thermos-unfolding process to produce a fluorescent readout. However, the excitation and emission wavelength of CPM is 387 nm and 463 nm respectively, and we do not have a suitable filter on our RT-PCR machine to analyze at these wavelengths. Fortunately, an alternative but low throughput method using GFP fused NS4B is available for the goal of buffer optimisation.

Hattori et al. demonstrated that it is possible to screen for stabilising additives using GFP-based thermostability assay (Hattori, Hibbs, & Gouaux, 2012). In this method, the group incubated purified GFP fused zebrafish P2X purinoceptor 4 receptor (P2X4) and *C. elegans* glutamate-gated chloride channel (GluCl) at temperatures from 4 – 80°C in buffer containing various additives, followed by centrifugation, and subjecting each condition to SEC analysis (Figure 2.41). In theory, only folded protein after heat treatment would produce GFP fluorescence, which could be detected by a fluorometer that is attached to the FPLC machine. By comparing the GFP and UV elution profiles of proteins in control buffer without additives and sample profiles with additives, the group was able to identify ligands, ions and lipids that provided stabilising effects to the two membrane proteins. This led to the successful crystallisation of GluCl in the presence of POPC lipid additives (Althoff, Hibbs, Banerjee, & Gouaux, 2014). Therefore, it will be useful to adopt this approach for identifying buffers, ions, and additives that would stabilise NS4B in solution before crystallisation screens are performed. It would be even more powerful if this approach is performed on the thermostabilised mutants generated by artificial mutagenesis describe above.

Figure 2.41 Schematic flow of GFP-based thermostability assay. [Adapted from (Hattori et al., 2012)]. Purified GFP-membrane protein samples are heated on a thermocycler and injected into a SEC column for separation. Proteins that are still stable after heat treatment would retain GFP fluorescence which would be detected by the fluorometer attached, while unstable proteins would aggregate and lose GFP fluorescence.

2.6.3 Effect of calcium ion on NS4B oligomerisation state needs to be investigated

Three effects of calcium ions on NS4B were observed, 1) calcium ions are required for crystallogensis of NS4B; 2) purification of NS4B in the presence of calcium ions led to oligomerisation NS4B; and 3) addition of calcium ions to purified NS4B led to accelerated aggregation of NS4B. Calcium is present in millimolar quantities within the ER, since NS4B resides on the ER membrane; it is not surprising that calcium ions might have an effect on the function or oligomerisation state of NS4B. However, the

significance of these effects was not followed up during the course of crystallising NS4B.

One interpretation of the observed results is calcium ions promote the oligomerisation of NS4B that might act as scaffolding during the formation of IV and CM or the oligomers are required from tethering of the RC to the ER membrane (J. Zou et al., 2014). Serendipitously, oligomerisation assisted in the nucleation process for the formation of NS4B crystals. However, the oligomers might be thermodynamically unstable, and might require other viral proteins such NS1, NS4A or host proteins for stabilisation (Youn et al., 2012; J. Zou, Xie, et al., 2015). Therefore, in the absence of a stabilising protein, the oligomers aggregated rapidly. This is highly speculative and would require further investigations. A quick method to test the effect of calcium ions on the stability of NS4B would be to perform the GFP thermostability assay described in Section 2.6.2.2 in the absence and presence of NS1, NS4A or host cell lysates.

2.6.4 Antibody engineering

Antibody assisted crystallisation was a major focus for this project, because this technique was instrumental for structural determination of many membrane proteins. The 44-4-7 mAb used in this project might not be optimal for crystallisation purposes. First, it could be used for immuno-staining of virus infected cells and WB; indicating that 44-4-7 is recognizing a linear epitope on the cytoplasmic loop that is present in both folded and unfolded NS4B. For crystallisation purposes, an antibody that recognizes a discontinuous (conformational) epitope is preferred, because the antibody would only bind to properly folded proteins (Carpenter et al., 2008). Second, the interaction of NS4B with 44-4-7 could be broken up by the presence of detergents.

Therefore in collaboration with Dr Wang Chang-I (SIgN, A*STAR), an attempt to generate detergent stable and conformational anti-NS4B Fab was undertaken using phage display technology. However, the attempt failed to produce any clones during the first round of panning, most probably due to occlusion of NS4B epitopes by DDM micelles. To overcome this issue, NS4B prepared in nanodisc or SMALP could be used for panning instead. It was demonstrated by Dominik *et al.* that the phage display hits rate for generating conformational antibody fragments is greatly improved when using membrane proteins purified in nanodiscs, as these proteins are presented in a more native like environment (Dominik et al., 2016). Incorporation of NS4B into nanodiscs did not work in our hands. Incorporation of NS4B into SMALP, on the other hand, worked very well, and represents an attractive alternative to nanodiscs for the generation of new Fab fragments targeting NS4B by phage display.

With advancements in antibody engineering, it might be possible to produce a bispecific antibody that targets both N4B and its known interacting partner, for example NS3 helicase, for structural determination by co-crystallisation or by cryo-EM. However, this is an ambitious speculation.

2.6.5 Crystallisation via LCP is challenging, but is should be explored further

Crystallisation using LCP was attempted because it allowed membrane proteins to be reconstituted into a native-like environment for better protein stabilisation compared to detergent micelles, and the detergents that could adversely affect crystallisation are removed after incorporation of membrane protein into the LCP matrix (Caffrey, 2015; Caffrey & Porter, 2010). However, the protein reconstitution, dispensing, and crystal

harvesting steps are all technically challenging and time consuming to perform (Caffrey & Porter, 2010).

The reconstitution step involves loading of purified protein and molten monolein in separate Hamilton syringes. The step must be performed quickly to prevent monolein from solidifying, while avoiding air bubble formation. Mixing is done using a special syringe coupler which must be tightly secured to avoid leakage, but over tightening will lead to shearing and overheating of the membrane protein. Due to the small volumes involved, the loading and mixing frequently led to sample loss in our hands due to of lack of experience. Dispensing of the protein-LCP mixture (bolus) is manual and therefore is slow, and setting up one 96-well plate usually requires an hour to complete. This frequently led to dehydration of samples, and complicates reproduction of crystal hits. Harvesting of crystal hits is the most challenging step for LCP, because it involves manual cutting of the glass coverslip on top of the crystallisation bolus using a glass cutter. During the process of cutting, the LCP bolus can be easily sucked into the spacer at the side of each well. This is the most frequent step where we lose our crystals. After cutting open the well, the crystals are usually transparent on the microscope, and are only visible under polarized light. Therefore, harvesting the crystals will require shifting between bright-field and polarized field, which is very challenging as the bolus shifts during harvesting. This had led to harvesting of only the LCP matrix for data collection many times.

Despite all of the challenges mentioned above, LCP is still a promising technique for crystallisation of NS4B, as demonstrated by the successful determination of over 250 membrane protein structures since 1997. In order to achieve this, better training on this technique with an experienced collaborator is essential.

2.6.6 Non-crystallographic structural determination using SMALP

Cryo-EM has been utilised to determine many membrane proteins or complexes in recent years. Most of these structures were solved in the presence of detergents, which may lead to some structural deviation from the natural conformation (Hardy et al., 2016). SMALP, however, is a true detergent free method, as membrane protein could be directly extracted out of the lipid bilayer without using detergents in any purification steps (Dorr et al., 2016). This makes SMALP advantageous for single particle cryo-EM structural determination. On top of that, the presence of detergents will adversely affect the contrast of the sample prepared for cryo-EM, leading to sub-optimal data quality (Postis et al., 2015).

As discussed above, our crystallisation efforts were unsuccessful, and much more efforts are required before structural determination is possible. Currently, SMALP is the most promising technique in our hands for structural determination of NS4B using cryo-EM to examine the structure of NS4B-SMALP alone or in complex with mAb. We have demonstrated that a relatively homogeneous population of bRIL-NS4B SMALP could be observed using negative stain EM (Figure 2.40). The challenge now is to optimise sample preparation for cryo-EM. Addition of 44-4-7 mAb should be performed as well to increase the size of the NS4B-SMALP complex. At the same time, the mAb can provide better orientation and density during particle reconstruction.

2.6.7 ‘Divide and conquer’ constructs for structural determination and compound screening

A divide and conquer approach was conceptualised to crystallise small truncations of NS4B. The region flanking the cytoplasmic loop, TMD-3 and TMD-4 (Figure 2.42),

was chosen for this approach because the cytoplasmic loop is the most biologically interesting region, and because 44-4-7 mAb could be used for co-crystallisation.

Figure 2.42. Truncated NS4B construct designed for structural determination and possibly compound screening.

This construct could also be used for compound screening either by NMR or SPR. Preliminary data indicate that the truncated protein could be purified at reasonably high yield, and interestingly is stable and did not aggregate in the absence of detergent during SEC (experiment performed by Dr Abbas El Sahili, Figure 2.43). Further experiments to test the stability of this construct are underway. If this approach is successful, other truncated constructs from the N-terminus and C-terminus regions could be made for the same purpose.

Figure 2.43. SEC profile of truncated NS4B (TMD-3 and -4) in the presence and absence of DDM. The truncated NS4B proteins did not form aggregates in the absence of DDM.

Chapter 3. Mapping the interaction between DENV NS4B and NS3

3.1. Introduction

RNA replication of DENV and other related flavivirus is a tightly regulated and well-orchestrated process. This process occurs within a virus-induced IV ultrastructure on the ER membrane (Paul & Bartenschlager, 2013). Within the IV RNA replication is mediated by the RC, consisting of both viral and host proteins. All of the NS viral proteins have been reported to co-localise with dsRNA, a marker for RNA replication, within the RC, indicating the involvement of these proteins during RNA replication (Klema et al., 2015). Apart from the role of individual proteins, a myriad of different PPIs are believed to take place during RNA replication (Klema et al., 2015; Krishnan & Garcia-Blanco, 2014). For example, the interactions of NS4A-NS4B and NS1-NS4B have been reported to be important for successful RNA replication (Youn et al., 2012; J. Zou, Xie, et al., 2015). However, current knowledge of the protein-protein interactome within the RC and how these interactions affect RNA replication still remains limited. A better understanding of the PPIs involved during RNA replication could potentially assist in designing inhibitors or stabilisers of these PPI as anti-dengue therapy to disrupt the well-orchestrated RNA replication process.

One such PPI is the NS4B-NS3 interaction. It was reported that interaction with NS4B increases the dsRNA-unwinding activity of NS3 by dissociating NS3 from the ssRNA template, and allows the displaced NS3 to unwind the dsRNA duplex, therefore increasing the overall processivity of the viral replicase complex (Umareddy et al., 2006). However, the exact domains or residues that participate in this important interaction remains to be characterized.

3.2 Aims

In this project, we aim to utilise biochemical and biophysical techniques to map regions within NS4B and NS3 that are mediating the interaction. Using these information, we aim to design new protein constructs for co-crystallisation and structural determination of the NS4B-NS3 complex. The structure would provide valuable insights for designing compounds that can disrupt or stabilise the PPI interface to stop viral replication.

3.3. Materials and Methods

Cloning, expression and purification of NS4B. As described in Section 2.4.

Enzyme-linked immunosorbent assay (ELISA). ELISA was performed according to a previous report (38) with minor modifications. In brief, purified NS4B protein was diluted in coating buffer (50 mM carbonate/bicarbonate buffer, pH 9.6) and incubated in 96-well ELISA plates (Nunc) (150 ng/well) at 4°C overnight. Next day, the plates were incubated at room temperature for 2 h in a blocking buffer (10 mM Tris-HCl, pH 8.0, and 150 mM NaCl [TBS buffer] supplemented with 2% FBS). After three washes with TBS buffer plus 0.05% Triton X-100, blocked wells were incubated with serial dilutions of full-length NS3 protein in blocking buffer at room temperature for 2 h. After another three washes with TBS buffer plus 0.05% Triton X-100, the plates were incubated with a rabbit anti-NS3 pAb (GeneTex), followed by incubation with a secondary antibody (goat anti-rabbit antibody conjugated to HRP). After extensive washing, the plates were incubated with the substrate 3,3',5,5'-tetramethylbenzidine (TMB) (Thermo Scientific) at room temperature for less than 3 min. The reactions were stopped by addition of equal volumes of 1 M sulfuric acid. The absorbance at 450 nm was measured using a Safire II plate reader (Tecan). Two sets of controls were used for background subtraction: (i) wells coated with NS4B were not sequentially incubated with NS3; (ii) NS4B-free wells were sequentially incubated with NS3. The concentration of NS3 at which half of NS4B proteins were bound by NS3 was defined as the K_d (dissociation constant), which was calculated using the one-site specificity model in Prism, version 6.

Surface plasmon resonance (SPR). SPR biosensor measurements were performed using a Biacore 3000 instrument (GE Healthcare) in 20 mM Tris-HCl pH 8.0, 150 mM NaCl, 10% glycerol, 5 mM DTT at 25°C. The purified recombinant NS4B protein was directly immobilized onto CM5 sensorchips (GE Healthcare) using standard amine-coupling chemistry at a flow rate of 10 mL/min. Carboxyl derivatives on CM5 dextran surface were activated with a 10 min injection of 1:1 mixture of 0.2 M N-ethyl-N'-[3-(diethylamino)propyl]carbodiimide (EDC) and 50 mM N-hydroxysuccinimide (NHS). Recombinant NS4B was diluted into 10 mM sodium acetate, pH 4.5 (concentration) and injected across the activated dextran surface until an immobilization level of about 11,000 RU was reached. Unreacted activated carboxyl derivatives were blocked with 0.5 M ethanolamine – HCl at pH 8.5 for 10 min. Different concentrations of binding partners (either the entire NS3 helicase domain or its subdomains 1, 2 or 2+3, see Fig 2) were diluted into running buffer with concentrations ranging from 3.125 nM to 1 μ M as 2-fold serial dilutions and injected across immobilized surface in replicates. Raw sensorgrams were double referenced and responses were corrected using a reference flow cell and a blank buffer injection. For each corrected sensorgram, the zero for x- and y-axis was taken at the point of sample injection. Responses for the varied concentrations were overlaid and fit to a 1:1 simple binding model to obtain binding affinities using Biacore Evaluation software.

Cells, viruses, and antibodies. BHK-21 cells were purchased from the American Type Culture Collection (ATCC), and maintained in a high glucose Dulbecco modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (HyClone Laboratories, Logan, UT) and 1% penicillin/streptomycin

(Invitrogen). 293T cells were grown in low-glucose DMEM (Invitrogen) containing 10% FBS and 1% penicillin/streptomycin. DENV2 (strain NGC; GenBank number AF038403) was generated from its corresponding infectious cDNA clone (pACYC-NGC FL). The following antibodies were used in this study: mouse monoclonal antibody (mAb) 44-4-7 against DENV2 NS4B (26), rabbit polyclonal antibody (pAb) against DENV2 NS3 (Genetex), goat anti-rabbit antibody conjugated to horseradish peroxidase (HRP) (Sigma), mouse IgG2b negative control antibody and protein A conjugated to HRP (Sigma), mouse mAb 4G2 against DENV E protein (ATCC), Alexa Fluor 488 goat anti-mouse IgG (life technologies).

Plasmid construction. Standard molecular biology procedures were performed for all plasmid constructions. The fragments encoding the NS2B-NS3, C-terminally HA-tagged 2K-NS4B were amplified from pACYC-DENV-2 TSV01 full-length by PCR using corresponding primer pairs. The PCR products were digested by restriction enzymes and cloned into the pXJ expression vector. All constructs were validated by DNA sequencing. Primers sequences are available upon request.

Co-IP. 293T cells in 10-cm dish transfected with various constructs using XtremeGENE 9 DNA transfection reagent (Roche). At 48 h post transfection (p.t), cells were lysed in 1 mL IP buffer (20 mM Tris HCl, pH 7.5, 100 mM NaCl, 0.5% DDM, and EDTA-free protease inhibitor cocktail [Roche]) by rotating at 4°C for 1 h. Lysates were clarified by centrifugation at $20,000 \times g$, 4°C for 30 min and subjected to co-IP using protein A conjugated magnetic beads according to the manufacturer's instructions (Millipore). Briefly, immune-complexes were formed at 4°C overnight by

mixing 200 μ l of cell lysates with mouse mAb anti-NS4B (4 μ g) in a 500- μ l reaction system containing 250 mM sodium chloride. Subsequently, the complexes were precipitated with protein A-conjugated magnetic beads at 4°C for 1 h with rotation, followed by washing extensively with IP buffer (250 mM sodium chloride). Finally, proteins were eluted with 4 $\times$ lithium dodecyl sulfate (LDS) sample buffer (Life technologies) supplemented with 100 mM DTT, heated at 70°C for 10 min, and analyzed by WB. Specifically, NS4B was detected by mAb 44-4-7 against NS4B (generated in house) and protein A conjugated to HRP (1:2000 dilution) as the primary and secondary antibodies.

BHK-21 cells in a T-175 flask were infected with DENV-2 NGC (MOI=1). At 48 h post infection (p.i), cells were lysed in 1 ml IP buffer. Clarified supernatant was subjected to co-IP using protein A Sepharose (GE). Immune-complexes were formed at 4°C overnight by mixing 100 μ l of cell lysates with mouse mAb anti-NS4B (4 μ g) or mouse IgG2b negative control antibody (4 μ g) in a 500- μ l reaction system containing 250 mM sodium chloride. Subsequently, complexes were precipitated with protein A Sepharose at 4°C for 1 h with rotation. The following steps were performed as described as above.

***In situ* proximity of ligation assay (PLA).** PLA was performed according to the manufacturer's instruction (Olink Bioscience). Briefly, BHK-21 cells were seeded in an 8-well chamber (Nunc). The next day cells were infected with DENV-2 NGC (MOI=1) or transiently transfected with pXJ-NS2B-NS3 and pXJ-2K-NS4B-HA. At 48 h p.i or p.t., cells were fixed by 4% paraformaldehyde at room temperature for 30 min, and then permeabilized by PBS supplemented with 0.1% Triton X-100 at room

temperature for 10 min. Subsequently, cells were incubated with PBS containing 1% FBS and 0.05% Tween-20 (PBST) at room temperature for 1 h, and then incubated with primary antibodies anti-NS4B mouse mAb 44-4-7 (26) and anti-NS3 rabbit polyclonal antibody (pAb). The secondary antibodies supplied by the kit (PLA probe anti-mouse PLUS and anti-rabbit MINUS) were used at a 1:5 dilution and incubated at 37°C for 1 h. Afterwards, ligation-ligase solution was added and incubated at 37°C for 30 min. Signal amplification was performed by adding amplification-polymerase solution and incubating at 37°C for 100 min. Finally, chamber slides were mounted by Duolink in situ mounting medium containing 4', 6-diamidino-2-phenylindole (DAPI). Signals were observed on a Leica DM4000 B system at 598 nm. Images were merged using the Adobe Photoshop CS3 software.

RNA transcription, electroporation, Immunofluorescence assay and virus production. DENV2 NS4B mutations were engineered using a QuickChange II XL site-directed mutagenesis kit (Stratagene). Wild type (WT) and mutant DENV2 genome-length RNAs were transcribed *in vitro* using a T7 mMessage mMachine kit (Ambion) from cDNA plasmids pre-linearized by XbaI. RNA transcripts (10 µg) were electroporated into BHK-21 cells following a protocol described previously (X. Xie et al., 2014).

BHK-21 cells electroporated with viral RNA were seeded in an 8-well Lab-Tek chamber slide (Thermo Fisher Scientific). At indicated time points, the cells were fixed in 100% ethanol at -20°C for 30 min. After 1 h of blocking in PBS supplemented with 1% FBS at room temperature, the cells were treated with mouse mAb 4G2 for 1h, followed by three washes with PBS. Cells were then incubated with Alexa Fluor 488

goat anti-mouse IgG for 1 h. After three washes, the cells were mounted in a mounting medium with DAPI (Vector Laboratories, Inc.). Fluorescence images were taken and processed as described above.

BHK-21 cells transfected with genome-length RNA were seeded in one T-75 flask. After incubation at 37°C for one day, the medium was replaced with fresh DMEM supplemented with 2% FBS and cells were cultured at 30°C for another four days. On day 5 p.t, culture fluids were collected and virus titer was quantified by plaque assay as described previously (J. Zou et al., 2014).

Protein expression and purification. To express and purify the NS4B cytoplasmic loop region, the cDNA encoding residues I121 to V171 of NS4B from DENV2 was cloned into the PNIC28-Bsa4 vector. The resulting plasmid was transformed into *E. coli* BL21 (DE3) competent cells. The recombinant protein was soluble and was first purified using metal affinity chromatography. The N-terminal hexahistidine tag was then removed using the TEV protease. The cytoplasmic loop was further purified using gel filtration chromatography using a Superdex-200 column (GE healthcare). The expression and purification of the complete helicase region of NS3 from the DENV2 and DENV4 serotypes (residues 168-618) was conducted as previously reported (24, 25). Expression and purification of the full-length NS3 protease-helicase from DENV4 (fused to 40 hydrophilic residues from the NS2B cofactor) was done as described (Luo, Xu, Hunke, et al., 2008). Expression of the individual subdomains of the NS3 helicase gave poorly soluble proteins using the DENV2 serotype but soluble proteins for the DENV4 serotype. Protein fragments encompassing subdomains 1 (residues 168-322), subdomain 2 (residues 323- 484) and

subdomains 2+3 (residues 323-618) from DENV4 were expressed and purified using metal affinity followed by SEC.

NMR measurements. Prior to the titration experiments, uniformly ^{15}N and ^{13}C -labeled NS4B cytoplasmic loop as expressed above, was purified in an NMR buffer containing 20 mM sodium phosphate, pH 6.5, 150 mM NaCl and 1 mM DTT. All NMR spectra were acquired at 298 K on a Bruker Avance II 600 MHz or 700 MHz spectrometer equipped with a triple-resonance cryoprobe. Spectra were processed with Topspin 2.1 (Bruker) and NMRPipe (Delaglio et al., 1995) and visualized with NMRView (Johnson, 2004) or Sparky (<http://www.cgl.ucsf.edu/home/sparky/>). Uniformly $^{13}\text{C}/^{15}\text{N}$ -labeled NS4B cytoplasmic loop (0.5 mM) in the NMR buffer was used for data collection. The backbone ^1HN , ^{15}N , $^{13}\text{C}\alpha$ and $^{13}\text{C}'$ resonances were assigned using two dimensional (2D) and three dimensional (3D) experiments that included ^1H - ^{15}N -HSQC, 3D-HNCO, HNCACB, CBCA(CO)NH and HNCA (Q. Li, Raida, & Kang, 2010). All pulse programs were from the Bruker standard library (Topspin 2.1). The protein secondary structure was analyzed using the $\text{C}\alpha$ chemical shift (Andersen, Cao, & Chen, 1992). Steady-state $[^1\text{H}]$ - ^{15}N NOEs were obtained using two datasets that were collected with and without initial proton saturation for a period of 3 s (Kay, Torchia, & Bax, 1989). For the titration experiment, 2D- ^1H - ^{15}N -HSQC spectra were collected for the ^{15}N ^{13}C -labeled NS4B in the absence and presence of different amounts of unlabeled helicase.

3.4 Results

The NS4B-NS3 interaction reported by Umareddy *et al.* was first re-investigated using direct ELISA. The ELISA plate was coated with full-length NS4B, blocked with 2% FBS, and incubated with two-fold serial diluted N2B-NS3. The captured NS2B-NS3 was detected using primary rabbit anti-NS3 mAb and secondary goat anti-rabbit IgG conjugated to HRP. As shown in Figure 3.1, NS2B-NS3 bound to NS4B in a dose-dependent fashion, with an estimated dissociation constant (K_D) of 197.7 nM. The ELISA result confirms that NS3 interacts with NS4B.

Figure 3.1. Re-investigation of the reported NS4B – NS3 interaction with ELISA.

Purified full-length NS4B (100 ng) was coated onto ELISA plate wells, and 75 nM – 1.5 μ M of purified NS2B-NS3 in 2-fold serial dilutions were added. The bound NS2B-NS3 proteins were probed with primary rabbit anti-NS3 mAb, followed by secondary goat anti-rabbit pAb conjugated to HRP. TMB was added and resultant signal at 450 nm was measured. The experiment was performed in triplicates. The K_D was estimated to be 197.7 ± 26.25 nM, using a one-site specificity model on Prism 6 software. A negative control experiment using NS2B-NS3 S135A protein was performed separately.

3.4.1 NS4B-NS3 binding is mediated by NS3hel subdomains 2-3

After validating the reported NS4B-NS3 interaction, we investigated this interaction *in vivo* and *in vitro*. Two sets of *in vivo* experiments were conducted in cell culture by Dr Jing Zou to investigate the interaction between NS4B and NS3 in the context of viral infection in BHK-21 cells, as well as in the absence of viral infection using transient transfection in 293T cells. Using co-IP and proximity-ligation assay (PLA), Dr Jing Zou was able to demonstrate that NS4B and NS3 can associate during viral infection and in absence of infection during transient transfection (Figure 3.2).

Figure 3.2. Analysis of the interaction between NS4B and NS3 by co-IP and PLA.

(A and B) BHK-21 cells were infected with DENV-2 at an MOI of 1. At 48 h p.i., the infected cells were subjected to co-IP (A) and PLA (B). (C) 293T cells were co-transfected with expression vectors pXJ-NS2B-NS3 and/or pXJ-2K-NS4B. At 48 h p.t.,

the transfected cells were subjected to co-IP. **(D)** BHK-21 cells were co-transfected with expression vectors pXJ-NS2B-NS3 and/or pXJ-2K-NS4B. At 48 h p.t., the transfected cells were analyzed by PLA. BHK-21 cells, rather than 293T cells, were chosen for the PLA because PLA requires many washing steps, and 293T cells are easily washed off during these steps. In the PLA, red fluorescent signals indicate NS3-NS4B proximity. Nuclei were stained in blue by DAPI. (Experiment performed by Dr Jing Zou, NITD)

Next, we performed SPR binding experiments to further investigate if NS4B is directly interacting with NS3, and not mediated by a host or viral protein. Recombinant NS2B-NS3, NS3hel domain, and NS4B from DENV2 were expressed and purified; the purity of the proteins was assessed by SDS-PAGE gel (Figure 3.3A). CD spectroscopy was performed to ensure that NS4B is correctly folded; the result indicated a predominantly α -helical structure which is consistent with the NS4B membrane topology model reported by Miller *et al* (Figure 3.3B). SPR was performed by attaching NS4B onto a CM5 sensor chip, and NS2B-NS3 or NS3hel proteins were injected at two-fold serially diluted concentrations ranging from 53 nM to 3.4 μ M. The SPR results showed that NS2B-NS3 interacted with NS4B with an K_D of 163 nM (using 1:1 interaction fitting) (Figure 3.3C). Next, we performed the same binding experiment using individual NS3 protease (NS3pro – with NS2B cofactor) and NS3hel domains to determine which domain of NS3 is responsible for binding with NS4B. The SPR results showed that the NS3hel domain could bind to NS4B at a K_D of 222 nM, while no binding was observed for the NS3pro domain (Figure 3.3 C and D). These results demonstrate that NS4B binds to the helicase domain of NS3.

Figure 3.3. Identification the NS3 domains responsible for interacting with NS4B by SPR. (A) SDS-PAGE analysis for purity assessment of the purified proteins used in this experiment. The gel indicates that all three proteins used are pure. (B) CD analysis of purified NS4B folding. The CD spectrum shows that NS4B is folded, and is predominantly an α -helical structure. (C) SPR sensograms of injected full-length NS3 (53 nM to 3.4 μ M) and immobilised NS4B. A K_D of 163 nM was determined using 1:1 simple binding model on the Biacore evaluation software 4.1. (D) SPR sensograms of injected NS3hel domain (53 nM to 3.4 μ M) and immobilised NS4B. A K_D of 222 nM was observed, using 1:1 simple binding model (orange curves). (E) SPR sensograms of injected NS2B-NS3 protease domain and immobilised NS4B. No binding was observed. All SPR experiments were performed in triplicates, and only one replicate is shown for clarity.

The NS3hel domain comprises of three subdomains - subdomains 1 (residues 168 to 322) and 2 (residues 323 to 484) and subdomain 3 (residues 485 to 618) (Figure 3.4A). To further pinpoint the region within NS3hel that is interacting with NS4B, we expressed and purified individual subdomains 1, 2 and subdomains 2-3 from DENV4 (Figure 3.4B). DENV4 was used instead of DENV2 because the individual DENV2 NS3hel subdomains 1 and 2 have poor solubility, while subdomain 3 alone has poor solubility in both DENV2 and DENV4. Fortunately a construct with subdomains 2-3 from DENV4 was soluble and was used in place of subdomain 3. CD analyses were performed on these subdomains after purification to ensure proper folding (Figure 3.4C). Using the same SPR approach, binding the subdomains to immobilised NS4B were tested. No binding was observed for subdomain 1, while subdomain bound with a K_D of 4.84 μ M. The subdomains 2-3 construct interacted with NS4B at a K_D of 340 nM, and is comparable with the full-length NS3hel which bound to NS4B at a K_D of 540 nM (Figure 3.4D). These results suggest that NS4B binds to subdomains 2 and 3 of the NS3 helicase.

Figure 3.4. Mapping the region within NS3hel mediating interaction with NS4B.

(A) Schematic diagram of the three NS3hel subdomains used for SPR. Subdomain 1 is shown in red, Subdomain 2 in green, and subdomain 3 in yellow. The subdomain boundaries were defined previously by Dr Ting Xu from our group. (T. Xu et al., 2005). (B) Purity analysis using SDS-PAGE of the purified proteins – full-length NS3hel, subdomain 1, subdomain 2, subdomains 2-3 - used in this experiment. (C) Folding analysis of the proteins used by CD. The CD spectra indicated that all the proteins are properly folded. (D) SPR sensograms showing interaction of the NS3hel subdomains (7.8 nM to 1 μM) with immobilised NS4B. The K_D obtained for full-length NS3hel, subdomain 1, subdomain 2, subdomains 2-3 are 530 nM; negligible response; 4.8 μM ; and 340 nM respectively. A simple 1:1 binding model was used to fit all the experimental data (orange curves).

3.4.2 The NS4B cytoplasmic loop is responsible for mediating interactions with NS3hel subdomains 2-3

Next, we moved on to map the region within NS4B involved in interacting with NS3hel. Using the proposed NS4B topology as a reference (Figure 1.7A), we rationalised that the most likely region for interacting with NS3hel would be the cytoplasmic loop, because it is the only segment of NS4B that is exposed to the cytoplasm and with adequate length to mediate PPIs; residues 245-248 are also exposed in the cytoplasm, but are most likely too short. With this in mind, three biotinylated peptides - representing the N-terminal half of the cytoplasmic loop (residues 130 to 148), C-terminal half (residues 149 to 167), and a full-length cytoplasmic loop (residues 130 to 167) – were synthesised (Figure 3.5A). The peptides were immobilised onto a CM5 chip that was pre-immobilised with neutravidin, and two-fold serial diluted full-length NS3hel were injected for SPR analysis. The result showed all the peptides were able to bind to NS3hel. The full-length cytoplasmic loop has an affinity of 1.3 μM , while the N- and C-terminal halves have an affinity of 2.8 μM and 1.6 μM for the NS3 helicase respectively; a negative control protein – Tyrosine Kinase Non Receptor 2 (TNK2) – did not display any binding (Figure 3.5B and C). These results suggest that binding of NS4B to NS3hel is mediated by the cytoplasmic loop.

Competitive ELISA experiments were performed to determine if the synthesised peptides could compete with full-length NS4b for NS3hel binding, but this effort was not reproducible.

A

Full-length loop (130 – 167) – Biotin - TREAQKRAAAGIMKNPTVDGITVIDLDPIPYDPKFEKQ

N-terminal half (130 – 148) – Biotin - TREAQKRAAAGIMKNPTVD

C-terminal half (149 – 167) – Biotin - GITVIDLDPIPYDPKFEKQ

Figure 3.5. Mapping the region of NS4B cytoplasmic loop that mediates interaction with NS3hel. (A) Amino acid sequences of the biotinylated synthesized for SPR analysis. (B) SPR sensorgrams of the interaction between immobilised cytoplasmic loop peptide and NS3hel. The peptides were captured onto CM5 sensor chips that were pre-immobilised with Neutravidin., and two-fold serial diluted NS3hel (1.6 to 200 μ M) was injected. The K_D for the full-length cytoplasmic loop was estimated to be 1.3 μ M, while both the N- and C-terminal halves bound to NS3hel with an affinity of 2.8 μ M and 1.6 μ M respectively. The negative control, TNK2, did not produce any detectable signals for all three peptide (lower panel).

3.4.3 Identification of NS4B cytoplasmic loop residues important for NS4B-NS3 interactions

Next, Dr Congbao Kang performed solution NMR to identify the cytoplasmic loop residues that are interacting with NS3hel. From the 2D ^1H - ^{15}N -HSQC experiments, chemical shift perturbations upon addition of NS3hel were assigned to 15 residues on the cytoplasmic loop - G124, K128, T130, Q134, G140, N144, I150 to D156, I158,

at a molar ratio of 1:2 is shown. The affected residues are shown with residue name and number. **(C)** Schematic diagram of the topology of NS4B from DENV2. Residues identified from the chemical shift perturbation experiment are indicated in red. **(D)** Sequence alignment of the NS4B fragment (residues 121 to 171) among different flaviviruses. The numbers in parentheses are the numbers of NS4B sequences that were used for the alignment. Sequences were downloaded from the National Center for Biotechnology Information (NCBI) protein database. The alignment was performed using CLC Main Workbench software (CLC bio). The amino acid positions of NS4B are numbered according to the DENV2 NGC strain (GenBank number AF038403). The flavivirus-conserved residues are shaded in gray. The amino acids identified from the chemical shift perturbation analysis are shown in red in the DENV2 sequence, among which four residues (indicated by arrowheads), belonging to the cytoplasmic loop [defined as residues 129 to 165, according to (Miller et al., 2006)], are strictly conserved across flaviviruses. (Experiment performed by Dr Congbao Kang, ETC, A*STAR)

Dr Jing Zou followed up on the NMR experiment, and performed alanine substitution mutagenesis analysis on G124, Q134, G140, N144, and D154 using a full-length DENV2 infectious clone to study the importance of these residues during viral replication (Figure 3.7). These amino acids were chosen because they are conserved in all DENV serotypes and various related flavivirus, indicating biological importance. Using IFA and plaque assay to monitor viral replication, Q134 and G140 were found to be essential, and mutagenesis abolished virus replication, although G140A mutation rapidly reverted back to WT. On the other hand, N144A led to 1000-fold reduction in virus production, while G124A and D154A did not affect viral replication. These results suggest that Q134, G140 and N144 of NS4B are essential for viral replication, and might be due to their role in mediating NS4B – NS3 interactions.

Figure 3.7. Analysis of NS4B mutations in DENV2 genome-length RNA. (A) IFA. BHK-21 cells were electroporated with equal amounts (10 μ g) of WT or mutant genome-length RNAs (containing the indicated amino acid substitutions in NS4B). The expression of viral E protein (indicated in green) was monitored on day 1 and day 4 p.t. The nuclei were stained with DAPI. (B) Plaque morphologies of mutant and WT viruses. Culture fluids derived from the transfected cells in the experiment shown in panel A were analyzed for plaque morphology and virus yields on day 5 p.t. Virus titers were quantified by plaque assay. (C) Sequencing analysis of the recovered viruses. The NS4B region of the viruses recovered on day 5 p.t. from the experiment shown in panel A was sequenced. Mutant G140A virus reverted to the wild-type sequence, whereas mutant P124A, N144A, and D154A viruses retained the engineered substitutions. (Experiment performed by Dr Jing Zou, NITD)

3.4.4 Co-crystallisation assays of NS4B cytoplasmic loop with NS3hel

Previously, co-crystallisation with full-length NS3 or NS3hel with full-length NS4B was performed to no avail. This is most likely due to the presence of detergents that are required to stabilise NS4B in solution. With confirmation that the cytoplasmic loop

peptides could interact with NS3hel, we attempted to obtain the co-crystal structure of NS4B and NS3hel again; this time with the synthesised cytoplasmic loop peptides to avoid detergent interference. A hit was obtained from PEG I suite condition B1. After optimization using 24-well grid screens, large plate crystals were obtained from the condition 0.1 M MES pH 6.7, 15% PEG 3350 after macro-seeding (Figure 3.8). The crystals were harvested and data collection was performed using the School of Biological Sciences in-house X-ray machine equipped with a copper rotating anode and a MarCCD detector. The structure was solved by molecular replacement using the published DENV 2 NS3hel structure (PDB: 2JLQ). Unfortunately, only the NS3hel protein was observed in the structure, and the full-length cytoplasmic loop peptide was not present. The electron density map, data collection and refinement statistics after first refinement are presented in Appendix V.

Figure 3.8. Crystal hit from initial 96-well screen and optimisation of NS3hel and full-length cytoplasmic loop. Initial hit was obtained from PEG I suite condition B1 - 0.1 M MES pH 6.5, 25% PEG 3000. The optimised condition was 0.1 M MES pH 6.7, 15% PEG 3350

3.5 Discussion

NS4B-NS3 interaction was first reported through Y2H screening, and was validated by further biochemical pull-down and IP experiments (Umareddy et al., 2006). This interaction is important for RNA replication, where NS4B enhances NS3 helicase unwinding activity by helping NS3 dissociate from ssRNA and freeing NS3 to unwind the another dsRNA, thus improving processivity of the DENV replicase complex. In this study, we aimed to map the sites of interaction between these two proteins using biochemical and biophysical method, and tried to obtain a co-crystal structure of this complex for drug discovery purposes.

We first demonstrated the interaction *in vivo* during viral infection, and also in the absence of other viral factors by transient protein expression (Figure 3.2). Next, we demonstrated using SPR that the interaction occurs *in vitro* in the absence of other viral or host factors (Figure 3.4). Further SPR studies, mapped the interaction sites to subdomains 2-3 of NS3hel and the cytoplasmic loop NS4B (Figure 3.5). Using NMR, 15 residues on the cytoplasmic loop exhibited chemical shifts upon addition of NS3hel (Figure 3.6). The importance of four of the 15 residues (Q134, G140, N144, and D154) for viral replication was investigated by alanine substitution mutagenesis; these residues are conserved across several flavivirus residues. The mutagenesis studies showed that Q134A mutation was lethal; G140A mutation almost abolished viral replication but reverted back to WT after four days, N144A mutation led to a 1,000-fold reduction in virus titer, while D154A mutation did not affect virus replication (Figure 3.7). Lastly, co-crystallisation trials were performed using synthesised NS4B peptides and full-length NS3hel, but only NS3hel crystals were obtained.

3.5.1 Further experiments are needed to confirm and identify residues on NS4B involved in direct interaction with NS3

Although we observed and identified 15 residues exhibiting chemical shifts upon addition of NS3hel using NMR. The observed chemical shifts could be due to indirectly conformational changes to the cytoplasmic loop induced by NS3hel binding. However, we did not perform any experiment to demonstrate that these residues directly participate in interacting with NS3. We also demonstrated through alanine substitutions that Q134, G140 and N144 are important for viral replication. Again, these results do not imply that these mutated residues affected NS4B-NS3 binding, as the mutations could affect other important biological roles played by NS4B. Therefore, further experiments to demonstrate direct binding is needed.

In an article published shortly after our publication, Professor Ralf Bartenschlager's group confirmed that Q134 on the cytoplasmic loop is important for interacting with NS3 (Chatel-Chaix et al., 2015). Using NS2B-NS3 expressing Huh-7 stable cell line, it was demonstrated that the transfected NS4B Q134A lethal mutant abolished NS4B-NS3 interaction during co-IP experiments, while this mutant did not affect NS4B-NS4A interactions (Chatel-Chaix et al., 2015). This is the first evidence that Q134 is directly involved in interacting with NS3, and this interaction is essential as abrogating this interaction is lethal for virus replication.

Although our previous co-crystallisation efforts using full-length proteins or synthesised peptides did not work, obtaining the crystal structure of the interaction complex remains the best method to visualise residues involved. This might be more feasible with our new truncated NS4B construct (Figure 2.40) consisting of only TMD3 – cytoplasmic loop – TMD4. As mentioned in section 2.6.7, the yield of this

construct is good and more importantly this construct is stable in absence of detergent. Therefore, co-crystallisation of NS3hel with this construct should be tested.

3.5.2 Possible model of RC architecture

Figure 3.9. Possible architectural model of viral RC determined based on results from this study and previously published data. [Adapted from (J. Zou, Lee le, et al., 2015)]

Based on our mapping data and published literatures, we proposed a model of the possible organisation of viral proteins within the viral RC (Figure 3.9). In this model, NS2B is anchored to the IV membrane through its 4 transmembrane helices. The NS3pro domain is responsible for tethering the replicase complex to the IV membrane through interactions with its exposed NS2B cofactor (residues 49 to 96), and also through an interaction with the IV membrane mediated by an exposed hydrophobic loop within the NS3pro (G29-L30-F31-G32). Subdomain 3 of NS3hel forms contact

with NS5 through residue K330 of NS5, while subdomains 2 and 3 of NS3hel interacts with the NS4B cytoplasmic loop.

NS1, NS2A and NS4A have been reported to be important for the formation of viral RC, and how these proteins are oriented in this model remains to be determined. On top of the viral proteins, how host factors contribute to the RC need to be investigated as well. As RNA replication is spatially and temporally regulated, the RC will most likely require different proteins and take up different architectures at various stages of RNA replication. A better understanding of the proteins involved would greatly advance knowledge of the biology and pathogenesis of DENV that is currently lacking.

Chapter 4. Identification of host factors interacting with NS4B during DENV viral replication

4.1 Introduction

Dengue viral replication is a highly complex and tightly regulated multi-stage process (Figure 1.3), and 10 viral proteins are not sufficient to accomplish this demanding task (Fernandez-Garcia et al., 2009). Therefore, DENV and other related +RNA viruses have evolved ways to recruit host factors or hijack host biological pathways to facilitate viral replication and for evasion of the host immune defences (Nagy & Pogany, 2011; Paul & Bartenschlager, 2015). During the RNA replication stage, all seven NS proteins have been reported to be involved in the formation the RC, which is the multi-protein complex required for nascent RNA synthesis (Klema et al., 2015; Paul & Bartenschlager, 2015). On top of the viral proteins, many studies have reported the involvement of host factors essential for RNA replication, and some host factors might be directly involved in forming part of the RC (den Boon, Diaz, & Ahlquist, 2010; Klema et al., 2015). Due to their importance, these host factors can potentially be targeted for anti-dengue therapy, and possibly pan-flaviviral therapy as the flaviviruses share many evolutionarily conserved replication mechanisms (Acosta & Bartenschlager, 2016). One more advantage is that the drug discovery process will be significantly shortened if an approved drug targeting the host factor is already available, and could be repurposed for anti-dengue treatment (for example Celgosivir). There will also be a higher barrier for emergence of resistance by targeting host factors (Acosta & Bartenschlager, 2016; Krishnan & Garcia-Blanco, 2014). An excellent example is the host factor, FASN, which is important for DENV replication. Existing FASN

inhibitors, C75 and Cerulenin, can inhibit virus replication in not only DENV but also for the related WNV and YFV (Heaton et al., 2010).

In recent years, the identification of host factors involved in flavivirus replication has been accelerated with the advent of various global profiling techniques such as Y2H assay, RNAi, quantitative proteomics [For more details, see review by (Krishnan & Garcia-Blanco, 2014)]. In this study, we utilised an IP-MS strategy to couple IP using NS4B mAb with SILAC-based quantitative proteomics to identify host factors interacting with DENV NS4B during viral replication.

4.2 Aims

We aim to identify and validate new host factors involved in the interaction of NS4B within the viral RC using IP coupled SILAC-based quantitative proteomics. This will provide further insights into the biological relevance of NS4B – host protein interactions within the viral RC and could also potentially identify targets for anti-dengue drug development.

4.2 Materials and methods

SILAC labeling, DENV infection and IP for SILAC. Huh-7 cells were passaged at least 6 times in light or heavy label media containing Arg- and Lys-free DMEM FLEX MEDIA (Life Technologies) supplemented with 4 mM L-glutamine, 1 mM sodium pyruvate, 10% (v/v) dialyzed FBS, 1000 mg/mL glucose solution, 1% (v/v) Penicillin-Streptomycin (10,000 U/mL), 84 mg/L L-Arginine hydrochloride or L-Arginine-¹³C₆ hydrochloride (Sigma), 146 mg/L L-Lysine hydrochloride or L-Lysine-¹³C₆ hydrochloride (Sigma). Infected 4×10^7 Huh-7 cells grown in heavy or light media with DENV-2 (strain NGC) at an MOI of 2, at 48 hpi the culture supernatant was removed, and cell monolayer was washed three times with ice cold PBS. Cells were lysed with 2 mL of lysis buffer containing 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.5% (w/v) DDM, and 1× EDTA-free protease inhibitor cocktail (Roche) and incubated for 1 h at 4°C with rotating, and followed by harvesting the supernatants by pelleting the cell debris at $20,000 \times g$ for 15-30 min at 4°C. The total protein concentration was determined by Pierce 660 nM protein assay kit, and equal amounts of total cell lysates were used for immune-complex formation at 4°C overnight with rotation, using 5 µg of anti-NS4B mAb 44-4-7 (Xuping Xie et al., 2014) for heavy-labeled cell lysates, and 5 µg of control mIgG2b (Millipore) for light-labeled cell lysates, respectively. The salt concentration was adjusted to 300 mM using NaCl prior to immune-complex formation. The heavy and light immune-complexes were pooled together and IP was performed by adding 50 µL of protein G conjugated magnetic beads (Millipore), rotating at 4°C for 1 h. The beads were washed five times with PBS containing 0.1% (v/v) Tween-20, and the bound protein was eluted from the beads in 60 µL of $2 \times$ LDS sample buffer (Life technologies) containing 100 mM DTT by

heating at 70°C for 15 min with a shaking speed of 1200 rpm on a 164 ThermoMixer (Eppendorf). The eluted protein was then separated by one-dimensional SDS-PAGE and stained using Commassie blue. Each of the lanes was excised and cut into ten fractions; then in-gel digested with trypsin and subjected to nano LC-MS/MS analysis.

Nano LC-MS/MS analysis. The nano-flow RPLC separation of peptides was performed on a Picochip C18 column (75 μm ID $\times$ 15 μm tip packed w/105 mm, New Objective, Woburn, MA) which was coupled online to a Q-Exactive MS instrument (Thermo Scientific, Hudson, NH). Mobile phases Buffer A (0.1% (v/v) formic acid in water) and Buffer B (0.1% (v/v) formic acid in CH_3CN) were delivered by an Easy-nLC1000 system (Thermo Scientific, Hudson, NH). The chromatographic gradient was 0-3% Buffer B from 0 to 2 min, 3-35% Buffer B from 2 to 69 min, 35-100% Buffer B from 69 to 82 min, and maintaining 100% Buffer B for 8 mins. After LC separation, the peptides were directly analyzed online by MS using the MS/MS data-dependent acquisition mode. All parameters were as follows: positive ion mode; mass range: 300-3500 m/z; NCE: 27; resolution: 70,000 for MS and 17,500 for tandem MS; data-dependent acquisition: top 10; charge exclusion: 1, >8; dynamic exclusion: 20.0 s.

Database searching and data processing. The database search was performed with the SEQUEST algorithm using Proteome Discoverer software (version 1.4.1.14, Thermo Fisher Scientific, San Jose, CA). The database was created by adding all ten DENV mature protein sequences into the human Uniprot database. Search criteria were set up as follows: signal to noise (S/N): 1.5; enzyme: trypsin; miss-cleavage sites: 2; dynamic modification: carboxymethylation on cysteine, oxidation on methionine, and

label: $^{13}\text{C}_6$ of lysine and arginine; precursor ion accuracy: 10 ppm; fragment ion accuracy: 0.1 Da; $\Delta\text{Cn} \leq 0.05$; FDR (q value, strict ≤ 0.01 , medium ≤ 0.05).

Scaffold (version 4.4.1, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identification. Peptide identifications were accepted if they could be established at a greater than 92.0% probability to achieve an FDR less than 1.0 % by the Scaffold local FDR algorithm. Protein identifications were accepted if they could be established at greater than 95.0% probability to achieve an FDR less than 2.0 % and containing at least two unique peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii, Keller, Kolker, & Aebersold, 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide overlap were grouped into clusters.

Selection of 106 human target candidates from 1023 identified proteins.

Candidate selection for follow up studies was based on the following three parameter combinations:

- P-value ≤ 0.01 AND mean (enrichment across valid measurements) ≥ 1.2 AND minimum (enrichment across valid measurements) ≥ 0.5 AND number of valid measurements ≥ 3
- P-value ≤ 0.02 AND mean (enrichment across valid measurements) ≥ 1.1 AND minimum (enrichment across valid measurements) ≥ 0.8 AND number of valid measurements ≥ 3

- $P\text{-value} \leq 0.05$ AND $\text{mean (enrichment across valid measurements)} \geq 1.5$ AND $\text{minimum (enrichment across valid measurements)} \geq 1.0$ AND $\text{number of valid measurements} \geq 3$

Functional analysis of the Proteomics-based target candidates. Uniprot protein identifier mapping to gene symbols and further annotation was performed via the Uniprot “Retrieve/ID mapping” functionality (<http://www.uniprot.org/uploadlists/>), selecting the options “From UniProtKB AC/ID To UniProtKB” and selecting the following columns for output: Entry, Entry name, Status, Protein names, Organism, Length, Gene names, Gene names (primary), Gene names (synonym), Domain, Protein families, Tissue specificity, Subunit structure, Interacts with, Pathway, Function, Gene ontology (biological process), Gene ontology (molecular function), Gene ontology (cellular component), Subcellular location.

Gene set enrichment analysis was performed with a proprietary web tool GLAAT [unpublished] that assesses statistical over-representation (enrichment) using a one-sided hypergeometric test (Hackenberg & Matthiesen, 2008). Using this tool, the 106 target candidates were tested for enrichment against the following gene set collections: GeneOntology, InterPRO, NCBI BioSystems and MetaBase; among these, GeneOntology and MetaBase are organized into thematic sub-collections. For each (sub)collection, a maximum of the ten most highly enriched gene sets were reported up to a P-value of 0.05. Negative log-transformed P-values were reported as “enrichment scores”.

siRNA Knockdown. ON-TARGETplus™ siRNA was purchased from Dharmacon (GE Healthcare) in a 96-well format. Each well was resuspended into a 5 μ M stock with nuclease-free water according to manufacturer's instructions, and stored at -20°C as 10 μ L aliquots. See supplementary information for the full list.

One day prior to siRNA transfection, 100 μ L of Huh-7 cells were seeded into 96-well plates using a BioTek dispensing robot (BioTek) to a final concentration of 14,000 cells per well. Each well was transfected with a final concentration of 25 nM siRNA using Lipofectamine 3000 (Invitrogen). Briefly, for each siRNA, 0.2 μ L of Lipofectamine 3000 (Invitrogen) was first diluted with 4.8 μ L of Opti-MEM reduced serum media (Invitrogen), while 0.5 μ L of siRNA stock was mixed with 4.5 μ L of Opti-MEM. Both diluted mixture were then added together and incubated at RT for 30 mins. 10 μ L of mixture was then added into the cell culture plate and incubated for 48 h. All dilutions and transfer steps were performed using a Mosquito liquid-handling robot (TTP Labtech). Cells were infected with DENV-2 NGC reporter virus 48 h.p.t at an MOI of 0.4. Virus infection was quantified using ViviRen™ Live Cell Substrate (Promega) 24 h.p.i and cell viability was quantified afterwards using CellTiter-Glo® Luminescent Cell Viability Assay (Promega). For both assays, luciferase activities were measured using a Clarity luminescence microplate reader (BioTek). Experiments were performed in triplicates.

For data analysis, raw siRNA knockdown data from 12 plates (4 batches with 3 plates each) were uploaded into a proprietary system (HELIOS [unpublished]) for managing and analyzing plate-based screening data. Since inspection of the raw data revealed systematic plate geometry (edge) effects on the data, both data normalization and correction procedures as implemented within HELIOS were applied. For data

normalization, two approaches were used: 1) for each plate, well-specific read-outs were divided by the well value of the 'SCRAMBLED' control, 2) for each plate; well-specific read-outs were divided by the plate median.

For data correction, systematic error pattern modeling, using local polynomial regression was applied on a plate by plate basis. Analysis of normalized, corrected data did not reveal a discernible batch effect, so plate data was treated as 12 independent replicates. Genes were then scored by the rank averages across all 12 plates, with the two normalization approaches leading to similar rank orders.

RT-PCR. One day prior to siRNA transfection, 2 mL of Huh-7 cells were seeded into a 6-well plate to a final concentration of 250,000 cells per well. Each well was transfected with a final concentration of 25 nM siRNA using Lipofectamine 3000 (Invitrogen) as described above. After 48 h.p.t, total RNA was extracted from the cells in each well using Trizol (Invitrogen), following manufacturer's instructions. RT-qPCR was performed using 100 ng of total RNA extract using iTaq™ Universal One-Step RT-qPCR kit), following the manufacturer's instructions, with a primer pair targeting exon 3 - 5 of Sec61 α (Integrated DNA Technologies) -

Primer 1: 5' – CTCCATCAATGTGCCTCTGT – 3',

Primer 2: 5' – CCGCTATCACCTCTTTATCTTC – 3',

β -actin primers were used as house-keeping gene control. Relative gene expression was normalized following instructions from Dharmacon using the $\Delta\Delta C_q$ calculation method (Dharmacon, 2015; Livak & Schmittgen, 2001).

Immuno-fluorescence microscopy. 50,000 cells were seeded into a chamber of an 8 chamber Bio-Tek II Chamber Slide (Nunc) and infected with DENV-2 NGC strain 1 day later. At 48 h.p.i, cells were washed once with warm phosphate-buffered saline (PBS), fixed with warm 4% paraformaldehyde (37°C) and incubated at room temperature for 30 min. Subsequently, cells were and permeabilized with 0.25% (v/v) Triton X-100 in PBS for 10 min. Cells were then blocked with PBS containing 3% bovine serum albumin (BSA) for 1 h, and incubated with primary antibody (anti-NS4B – 1:200, anti-Sec61 α – 1:100) for 1 h at 37°C, followed by three 5 min washes with PBST buffer (PBS containing 0.05% Tween 20 and 1% BSA). Next, the cells were incubated with Alexa Fluor 488 goat anti-mouse IgG or Alexa Fluor 594 goat anti-rabbit IgG (1:1,000) secondary antibodies for 1 h in PBST buffer, and washed three times with PBST. The stained cells were mounted in Vector shield mounting medium with 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Inc.). Fluorescence images were acquired with a Leica DM4000 B microscope system.

Confocal microscopy. Huh-7 cells were transfected with 10 μ g of pXJ-NS4B and 10 μ g of pCDNA3-Sec61 plasmid using TransIT 293 transfection reagent (Mirus) according to manufacturer's instructions. Cells were prepared for confocal microscopy as described above for IFA. Confocal images were acquired using a Zeiss LSM 510 META laser scanning confocal microscope equipped with an oil immersion 100 $\times$ objective lens (IMB Microscopy Unit, A*STAR). Images were merged using Fiji image software (Schindelin et al., 2012).

Proximity ligation assay (PLA). PLA was performed using a Duolink in situ kit (Sigma-Aldrich). Cells were grown with an MOI of 5 and prepared as described above for IFA until the addition of secondary antibodies. Steps from addition of secondary antibody onwards were performed according to the manufacturer's instructions. The secondary antibodies supplied by the kit (PLA probe anti-mouse Plus and anti-rabbit Minus) were used at a 1:5 dilution and incubated at 37°C for 1 h. Afterwards, ligation-ligase solution was added and incubated at 37°C for 30 min. Signal amplification was performed by the addition of amplification - polymerase solution and incubation at 37°C for 100 min. Finally, chamber slides were mounted by Duolink in situ mounting medium containing DAPI. Fluorescence images were acquired with a Leica DM4000 B microscope system.

Antiviral testing and cytotoxicity measurement. Antiviral testing using the CFI assay was performed as described previously (Q.Y. Wang et al., 2009). In brief, 2×10^4 A549 cells per well in 96-well plate were infected with DENV at an MOI of 0.3 in the presence of two-fold serial dilutions of compound. At 48 h post-infection (p.i.), the infected wells were washed, fixed, and immuno-blotted with primary antibody (4G2; American Type Culture Collection) and horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Sigma) to determine the yield of viral E protein. The dose-response curve was plotted using the Prism 5 software (GraphPad Software) and EC_{50} (effective concentration of compound to reduce 50% E protein production) was calculated using non-linear regression analysis.

Antiviral testing using the viral titer reduction assay was performed as described previously (Yin et al., 2009). Approximately 2×10^4 vero cells seeded per well of 96-

well plates. At 24 h post-seeding, cells were infected with POWV, WNV, YFV, WEEV, or VSV (MOI 0.1). The infected cells were immediately treated with series dilutions of compound. Cell culture fluids were collected at 42 h p.i followed by quantification for viral titers using plaque assay (Puig-Basagoiti et al., 2006).

Cell viability was measured using the Cell Counting Kit-8 (measuring cellular dehydrogenase activity; Dojindo Molecular Technologies) according to the manufacturer's protocol. HepG2 cells were seeded at 5×10^3 cells per well in a 96-well plate. After 24 h incubation at 37°C in 5% (v/v) CO₂, the cells were treated with two-fold serial dilutions of compound. At 48 h post-treatment, 4 µl of CCK-8 solution was added to each well. After another 90 min of incubation at 37°C, the absorbance was measured at 450 nm using a microplate reader (Tecan).

4.3 Results

4.3.1 Identification by SILAC MS/MS of host factors interacting with NS4B during DENV replication

To determine the optimum viral infection condition for proteomic analysis, Huh-7 cells were infected with DENV-2 New Guinea C (NGC) strain at multiplicity of infections (MOIs) of 0.5, 1, and 2. Huh-7 was chosen because it represents on the target cells during natural DENV infection, and has been shown to be most efficient for viral infection studies in our hand. Optimum viral infection condition was determined by monitoring NS3 expression using immunofluorescence analysis (IFA) at 24, 40, 48, 64 and 72 h.p.i. We found that at 48 hpi, approximately 80% of the Huh-7 cells infected at an MOI of 2 were IFA-positive, while no obvious differences in the morphology of the infected cells compared to uninfected control cells, indicating that there was little cell death (Figure 4.1A). Therefore, 48 h infection with an MOI of 2 was chosen as the condition for subsequent proteomics studies.

Our proteomics strategy employs SILAC labeling, followed by immuno- enrichment using NS4B 44-4-7 mAb, then subjecting the enriched samples for nano LC-MS/MS, and finally bioinformatics data analysis to identify unique NS4B binding host proteins (Figure 4.1B). Huh-7 cells were metabolically labelled for at least six cell doublings in either light (containing ^{12}C labelled arginine and lysine) or heavy (containing ^{13}C labelled arginine and lysine) culture media before DENV infection using the optimised condition identified previously. Cells were harvested and lysed 48 hpi, followed by addition of 44-4-7 anti-NS4B mAb or control mouse IgG2b antibody to the heavy-labelled or light-labelled cells lysates respectively. co-IP was then performed using Protein G conjugated magnetic beads, followed by 1D SDS-PAGE, alkylation and in-

gel tryptic digestion. The digested peptides were sent to Drs Yuan Gao and Yanyan Yu (CNIBR) for nano LC-MS/MS analysis. This procedure was repeated four times to ensure reproducibility and accuracy of the results.

A

B

Figure 4.1. (A) IFA of NS3 expression in DENV-2 NGC virus infected Huh-7 cells. Huh-7 cells were infected with DENV-2 at an MOI of either 0.5, 1, or 2, and IFA analysis was performed 48 hpi by monitoring the expression of NS3 protein. (B) Schematic diagram illustrating our SILAC strategy for identifying host proteins involved in DENV replication. In this strategy, Huh-7 cells were labelled with either light ($^{12}\text{C}_6$) or heavy ($^{13}\text{C}_6$) arginine and lysine isotopes for 6 passages to ensure 100% isotope incorporation. Next, the cells were infected with DENV-2 for 48 hours, before the cells were lysed. IP was performed on the heavy labelled lysate to enrich for NS4B associated proteins and complexes using 44-4-7 anti-NS4B mAb, while a mouse IgG2b mAb was added to the light labelled lysate as a control. Both the light and heavy IP samples were pooled MS sample preparation and MS analysis. Using the SILAC labeling strategy, specific NS4B binders will only appear as isotopically heavy, while non-specific binders will appear isotopically light and heavy with a 1:1 intensity ratio.

4.3.2 Bioinformatics data analysis

MS data acquisition and processing were performed by Drs Yuan Gao and Yanyan Yu. Functional and statistical analysis of the MS data was performed by Dr Sebastian Hoersch (NIBR). During the preliminary analysis, 713, 882, 801, and 770 host proteins were quantified from the four experiments respectively. In total, 1023 non-redundant proteins, including all 10 viral proteins were identified. Further data analysis identified 106 human protein hits that were significantly enriched within the affinity-purified NS4B complex (Figure 4.2A and Appendix VI). In addition, eight DENV viral proteins (E, PrM, NS1, NS2A, NS2B, NS3, NS4A, and NS4B) were also significantly enriched, whereas the C and NS5 proteins were not. Gene set enrichment analysis (GSEA) and pathway analysis was performed on the 106 host proteins to reveal their major functional roles (Figure 4.2B).

Figure 4.2. Bioinformatics analysis of SILAC MS data. (A) Bioinformatics workflow for the analysis of candidates for viral NS4B association resulting from quantitative proteomics. Filtering criteria are described in Materials and methods. (B) Gene Set Enrichment Analysis (GSEA) results for 106 genes (X-axis) corresponding to 106 host proteins determined to be NS4B-associated by our analysis. Gene sets from

different gene set collections with significant enrichment were hierarchically clustered based on gene membership (Y-axis). Major functional themes in common to select subclusters are indicated.

Overall, these proteins are found to be involved in ER protein processing including UPR, lipid metabolism, glycosylation, and protein folding. In summary, as expected, the spectrum of gene set enrichment observed, overlaps with and recapitulates known dengue infection biology, underscoring the involvement of host factors at multiple functional levels (Marceau et al., 2016; Savidis et al., 2016).

4.3.3 Validation of SILAC MS/MS results using siRNA knock-down

A total of 92 of the 106 were selected for further validation using siRNA mediated knock-down with ON-TARGETplus SMARTpool siRNAs (Appendix VII). Huh-7 cells were first grown 96-well culture plates and transfected with siRNA. At 48 h.p.t, the transfected cells were infected with DENV-2 NGC *Renilla* luciferase (Rluc) reporter virus (MOI of 0.4) for another 24 h before analysis of luciferase level and cell viability was performed (Figure 4.3A). A previously validated siRNA targeting NS3 was used as positive control for inhibition of viral replication (Ng et al., 2007), and the ON-TARGETplus non-targeting control siRNA (scrambled) as a negative control. Four batches of siRNA screens were performed each in triplicates. The Rluc readouts exhibited considerable variability and systematic edge effects, which required advanced data correction and normalisation. Therefore, data were pooled across all batches, resulting in 12 replicates for each siRNA condition, and sent to Dr Sebastian Hoersch for data normalization and analysis using local polynomial regression

(Novartis proprietary software – HELIOS, unpublished) (Result summarized in Appendix VII). From the analysis of normalised and corrected data, Sec61 α , a component of the Sec61 translocation complex, was identified to consistently exhibit the strongest RNAi phenotype, and was chosen for further biochemical validation (Figure 4.3B). Sec61 α -targeting siRNAs resulted in 75% knocked down in Sec61 α mRNA levels (Figure 4.3C), and showed the strongest inhibition of Rluc expression by 60% (Figure 4.3D), with no obvious adverse effect on cell viability. Collectively, these results suggest that Sec61 α is important for DENV replication.

Figure 4.3. Functional validation of identified NS4B interaction partners. (A) Schematic work flow of the siRNA-mediated screening strategy. (B) Boxplot showing the distribution of normalized and corrected siRNA knock down readouts from 12 replicates across 92 genes and 3 controls (media only, NS3 positive control, non-targeting negative control) in Huh-7 cells. SEC61A1 is highlighted in red. (C) Sec61 α transcript level is decreased upon siRNA-mediated knock down in Huh-7 cells. Error bars represent the standard deviation of three independent biological replicates. (D)

DENV infectivity is reduced in Huh-7 cells as indicated by a reduction in luciferase activity upon silencing of Sec61 α or NS3. Error bars represent the standard deviation of four independent biological replicates.

4.3.4 Association of NS4B with Sec61 during DENV infection

To validate if the involvement of Sec61 α during DENV replication indeed requires association with NS4B, we performed confocal microscopy and proximity ligation assay (PLA). Huh-7 cells were co-transfected with two plasmids - pXJ-NS4B and pCDNA3-Sec61, and the cells were harvested at 48 hpt for sample preparation and immuno-staining. Confocal microscopy analysis of the stained samples was performed at the Biopolis Microscopy Unit. Signals for NS4B and Sec61 α were observed to overlap with each other, giving a calculated Pearson's coefficient of 0.66 (Fig 4.4A). To further examine the association of NS4B with Sec61 α in the context of viral infection, Huh-7 cells were infected with DENV-2 NGC at an MOI of 5. Cells were harvested at 48 hpt and subjected to proximity ligation assay, which allows the detection of two proteins locating within a distance of 400 Å in the cell. As shown in Fig 4.4B, red fluorescent signals were detected only in the infected cells probed with both NS4B and Sec61 α antibodies, while no signal was observed in the negative control, as well as infected cell controls probed with either NS4B or Sec61 α antibody alone. These results indicate that NS4B and Sec61 α can colocalise in the absence of other DENV proteins, and also confirm that NS4B and Sec61 α colocalise during the course of viral infection.

Figure 4.4. Colocalization analysis of NS4B and Sec61. (A) Confocal microscopy. Huh-7 cells were transfected with 10 μ g of pXJ-NS4B and 10 μ g of pCDNA3-Sec61 plasmid. At 48 h.p.t, NS4B distribution was probed with mouse anti-NS4B primary antibody (44-4-7) and Alexa Fluor 488 goat anti-mouse IgG secondary antibody (green), while Sec61 α distribution was probed with rabbit anti- Sec61 α primary antibody and Alexa Fluor 594 goat anti-rabbit IgG secondary antibody (red). Nuclei were stained with DAPI (blue). (B) PLA. Huh-7 cells were infected with DENV-2 NGC at MOI of 5. At 48 h.p.i, cells were probed with mouse anti-NS4B primary antibody (44-4-7) and rabbit anti- Sec61 α primary antibody, followed by anti-mouse Plus and anti-rabbit Minus PLA probes. The red signals generated after the ligation and amplification steps indicate that NS4B and Sec61 are in close proximity. Nuclei were stained with DAPI (blue).

4.3.5 Inhibition of DENV replication by decatransin, a Sec61 translocon inhibitor

In an orthogonal approach, pharmaco-validation to investigate the involvement of Sec61 during DENV infection was performed by Ms Haoying Xu using decatransin, a known Sec 61 translocon inhibitor (Junne et al., 2015). Sec61 α is one of the three subunits, including Sec61 - β , and - γ , that forms the Sec61 translocation complex

(Greenfield & High, 1999). The compound was found to exhibit pan-serotypic activity, with EC₅₀ of 0.086, 0.093, 0.019, and 0.13 μ M against DENV-1, -2, -3, and -4, respectively (Table 4.1). Further experiments, showed that decatransin has broad anti-viral activities against +RNA viruses (WNV, YFV, JEV, Powassan virus (POWV), and Western equine encephalitis virus (WEEV), but not –RNA virus [Vesicular stomatitis virus (VSV)] (Table 4.1). These results suggest that Sec61 might be essential for replication of DENV and other +RNA viruses, and targeting Sec61 might be a potential pan-flaviviral therapy.

Table 4.1. Antiviral spectrum of decatransin

Virus	Strain	EC ₅₀ (μ M) ^a
DENV-1	MY97-10245	0.086
DENV-2	MY97-10340	0.093
DENV-3	MY05-34640	0.019
DENV-4	MY01-22713	0.10
JEV	Nakayama	0.015
POWV	LB	0.018
WNV	NY003356	0.051
YFV	17b	0.022
WEEV	62-7564	0.005
VSV	New Jersey	>2

^a EC₅₀ values were obtained by nonlinear regression analysis of the dose-response curves that were plotted from the viral E protein production or viral titer versus the log of the concentration of the test compound. The values were obtained from two independent experiments and reported as the mean.

4.4 Discussion

Host factors play important roles during various stages of the DENV replication, and many host factors and host pathways have been reported to be recruited to facilitate efficient viral replication. Due to their biological importance during viral replication, these host factors are attractive targets for anti-viral drug development. In this project, we aimed to contribute to the host factor drug discovery efforts by using SILAC-based quantitative proteomics to identify and validate new host factors involved in interacting with NS4B and important for virus replication.

Using this approach, we identified and quantified 1013 cellular proteins and 10 viral proteins involved in DENV-2 NGC strain replication. Among the quantified 1013 host factors, 106 were significantly enriched within the NS4B complex isolated from infected cells (Figure 4.2A). Gene enrichment and pathway analysis showed that these enriched proteins are involved in ER protein processing, UPR, lipid metabolism, glycosylation, and protein folding, which is consistent and overlaps with existing literature (Figure 4.2B)(Marceau et al., 2016; Savidis et al., 2016). Guided by validation results from four independent siRNA knockdown screens, Sec61 α emerged as a prime candidate for follow up analysis as this protein had the strongest RNAi phenotype in our screens (Figure 4.3B-D). Confocal microscopy and PLA analysis confirmed the association of Sec61 α with NS4B during virus infection and absence of viral factors respectively (Figure 4.4A and B). Finally, inhibition of Sec61 α using decatransin, a Sec61 translocon inhibitor, led to potent pan-serotype inhibition of DENV replication. Furthermore, decatransin also inhibited viral replication of other related flaviviruses and a +RNA virus (Table 4.1).

4.4.1 Advantages and limitations of coupling IP with SILAC MS/MS

The introduction of an IP step before LC-MS/MS reduces sample complexity by enriching for NS4B associated protein complexes, this allows for a focused study on the interacting proteins or complexes specific for NS4B during viral replication. This is not achievable via quantitative proteomics alone. Enrichment also facilitates the detection of low abundance proteins by increasing their concentrations within the sample, which would be masked in the typical non-enriched quantitative proteomics studies. Unlike Y2H (used in several genome-wide DENV PPI studies) which is an exogenous system, our IP-MS strategy is performed in near physiological conditions. Thus, this allows for the detection of PPIs that are more reflective of the nature course of DENV infections *in vivo*. Also, Y2H studies can only detect direct PPIs, while IP-MS allows for detection of both direct and indirect PPIs. Although powerful, weak or transient interactions may be disrupted during the washing steps after IP. Li *et al.* circumvented this problem by performing an additional round of IP-MS with the incorporation of formaldehyde cross-linking (Y. Li, Collins, et al., 2016). Other potential disadvantage of our IP-MS strategy is the use of a mouse IgG2b (same isotype as anti-NS4B mAb) as a control antibody for to rule out antibody cross-reactivity. This approach may not achieve the intended purpose because different antibodies, even of the same isotype, will have differences in cross-reactivity. Thus, this could lead to false positive results.

4.4.2 Further experiments are needed to characterise Sec61 α and NS4B association

We have demonstrated through PLA analysis that Sec61 α and NS4B associate during DENV replication. However, we do not know whether the interaction between Sec61 α and NS4B is direct, or if it is mediated by other host or viral factors. If the interaction is direct, the domains of each protein involved should be further characterised to elucidate the molecular determinants and biological relevance of this interaction. A strategy similar to the one applied in chapter 3 could be adopted to achieve this.

4.4.3 Sec61 might be an important host factor for pan-flaviviral replication and a potential target for pan-flaviviral therapy

The Sec61 translocon is a heterodimeric complex made up of three subunits Sec61 α , - β , and - γ (Greenfield & High, 1999). The subunits are universally conserved, and the Sec61 complex is important for translocation of proteins across the ER membrane, and also for the integration of proteins into the ER membrane (Greenfield & High, 1999; Park & Rapoport, 2012). Our pharmacological data showed that inhibition of the Sec61 translocon with decatransin resulted in the potent inhibition of viral replication for all of the flaviviruses tested, while not affecting -RNA virus replication. Therefore, we speculate that DENV and other flaviruses might exploit this host machinery for polyprotein translocation and integration into the ER membrane. This is also consistent with recent findings from genome-wide CRISPR screens that identified SEC61A1 and SEC61B as candidate host factors required in flavivirus infection (Marceau et al., 2016). With a calculated therapeutic index of at least 20-fold in cell culture, targeting the Sec61 translocon with decatransin might be a potential candidate for pan-flaviviral

therapy. The immediate next step would be to perform *in vitro* toxicity profiling to determine the 50% cytotoxicity concentration (CC₅₀) for calculating the actual therapeutic index of decatransin and establish the safety margins. Using these new information, *in vivo* toxicity should be evaluated in mice, followed by pharmacokinetics (PK) studies to understand the Absorption, Distribution, Metabolism, and Elimination (ADME) of decatransin *in vivo* to assess the developability of decatransin.

4.4.4 Other host factors that might be important for DENV replication

We chose to validate Sec61A, because we had decatransin against SEC61 translocon within Novartis and this allowed us to perform the pharmaco-validation experiments. However, SEC61A was ranked third in our RNAi validation experiments, and Eukaryotic Translation Initiation Factor 4A3 (EIF4A3) and Valosin Containing Protein (VCP) were ranked higher than SEC61A1, implying that these two proteins might have even higher importance during DENV replication and their roles should be explored. However, there was no time to these two proteins validated in this study due to the abrupt closure of my institute.

EIF4A3 is a putative RNA helicase that contain a conserved Asp-Glu-Ala-Asp (DEAD) motif. This protein is involved in recognizing and altering RNA secondary structures (Genecard, 2017). The mechanism of RNA recognition by DENV NS3hel is very similar to eIF4A3 (Luo, Xu, Watson, et al., 2008). Currently, there is no evidence in the literature that eIF4A3 is involved in DENV replication. However, several other host DEAD box proteins have been implicated in flavivirus replication. For example DEAD-box RNA helicase DDX5 acts as a positive regulator of JEV replication by

binding to viral 3' UTR (C. Li et al., 2013). DDX56 is relocated from the nucleolus to the site of WNV replication and plays a role in the correct formation of infectious WNV particles (Z. Xu & Hobman, 2012).

VCP has been implicated together with SEC61A to facilitate Sindbis virus (Alphavirus) entry into human cells, and could function similarly during DENV infections (Panda et al., 2013). An ATPase orthologue of VCP found in mosquitoes was reported to interact with DENV NS3 during a Y2H screen by Mairiang *et al* (Mairiang et al., 2013). The ATPase activity of VCP was found to be essential for poliovirus (Enterovirus) replication, where VCP mutants without ATPase activity inhibited viral replication (Arita, Wakita, & Shimizu, 2012). However, no studies have been conducted to examine the role of VCP in DENV replication.

The manuscript for this work is in preparation, and further data analysis on gene set enrichment and siRNA knock-down is still ongoing. The results presented are the latest data at the time of writing this thesis.

5. Conclusions and future directions

NS4B from DENV has been identified as an important player during viral replication. It is involved in the formation of IV on the ER membrane that accommodates the viral RC, as well as participating in the formation of the RC itself. NS4B also important for the modulation of host immune responses to facilitate viral replication. However, its natural hydrophobicity and non-enzymatic nature has impeded our understanding of this essential protein. In this project, we aimed to gain a better understanding of the biological roles of NS4B and also to assist in drug development of anti-dengue compound through inhibition of NS4B activities. This goal was executed using three approaches, 1) to express and attempt to crystallise NS4B for structural determination; 2) to map the reported NS4B-NS3hel interaction; and 3) to identify new host factors interacting with NS4B during viral replication.

1. Structural determination of NS4B

For this approach, various recombinant NS4B constructs were successfully expressed and purified to monodispersity and sufficient yield was obtained for crystallisation. Putative NS4B crystals were successfully obtained using vapour diffusion, antibody-assisted crystallisation, and LCP methods. However, only weak diffraction was obtained from vapour diffusion crystals, while Fab only crystals were obtained using antibody-assisted crystallisation and lastly crystals from LCP approach were not reproducible. Crystals obtained were from constructs purified with DDM, which forms large micelles, and might occlude hydrophilic surfaces on NS4B required for forming stable crystal lattices. The failure to obtain good quality crystals could be due to the poor protein stability of the NS4B constructs in detergents and storage buffers.

However, thermostabilisation of these constructs to remedy this problem was not explored. Purification of NS4B using SMA polymer was performed. This technique is a detergent-free method and eliminated the use of detergents that might have destabilising effects on NS4B. NS4B-SMALPs purified were homogenous under negative-stain EM and individual particles could be picked for 2D reconstruction. The next step is to optimise sample preparations for cryo-EM. This is the most promising technique in our hands to obtain a much awaited atomic resolution structure of NS4B.

Future directions

- Thermostabilisation using ‘Hot-CoFi’ approach to generate new detergent stable mutants and using GFP fused NS4B to determine stabilising buffer conditions would be critical for obtaining better diffracting NS4B crystals.
- LCP is the most productive technique for membrane protein crystallisation but is technically challenging for researchers from non-specialised laboratories. Therefore, it will be important to receive proper training from specialised membrane protein laboratories to effectively execute the LCP procedures.
- With convenient access to the NTU EM facility, structural determination of NS4B SMALP using cryo-EM must be explored. This is the most promising approach so far.

2. Mapping NS4B-NS3hel interaction

In the second part of this project, in collaboration with Dr Jing Zou and Dr Congbao Kang, the reported NS4B-NS3 interaction was mapped using a combination of biochemical, biophysical and genetic approaches. The interaction was confirmed using

co-IP and proximity ligation assay (PLA) in the presence or absence of other viral and host proteins. Using SPR, we mapped the interaction to domains 2 and 3 of NS3 helicase (NS3hel) and the cytoplasmic loop of NS4B. Nuclear magnetic resonance (NMR) analysis identified 12 residues on the NS4B cytoplasmic loop that might be involved in mediating the interaction. Further genetic analysis of 4 of the 12 residues (Q134, G140, N144, and D154) using alanine substitution, demonstrated that Q134, G140 and N144 are important for DENV replication, possibly due to their involvement in interacting with NS3hel. Co-crystallisation of full-length NS3hel with synthesised NS4B cytoplasmic loops was explored, but only apo-NS3hel crystals were obtained.

Future directions

- Crystallisation of full-length NS3hel with the new TM3-TM4 NS4B construct (Figure 2.21) should be attempted. This construct is stable in the absence of detergent, which will eliminate the detergent interference problem and therefore might be advantageous for co-crystallisation with NS3hel. Structural determination would be crucial for the development of PPI stabilisers or inhibitors to disrupt NS4B-NS3hel interaction.

3. Identification of new host factors interacting with NS4B during viral replication

In the last part of this project, in collaborations with Drs Qing Yin Wang, Yuan Gao, Yanyan Yu and Sebastian Hoersch, we identified 106 host proteins that interacts with NS4B during DENV replication using a novel approach of coupling SILAC MS/MS with co-IP. These proteins were involved in cellular processes that were previously

reported to be important for flaviviral replication. siRNA validation identified Sec61 α as an important host factor for DENV replication. Using PLA and confocal microscopy, we found that Sec61 α associates with NS4B during viral replication and in absence of other viral proteins during transient transfection respectively. Finally, we demonstrated that inhibition of the Sec61 complex (Sec61 α is part of this complex) led to potent of inhibition not only DENV, but also other flavivirus - JEV, WNV, YFV and POWV. Therefore, targeting Sec61 translocon might be a potential pan-flaviviral therapy.

Future directions

- The direct interaction of NS4B with Sec61 α was not established in our studies. A similar approach using to map the NS4B-NS3hel interaction could be employed to identify the possible domains or residues participating in the interaction. This will advance our understandings on the biological roles played by both proteins.
- Validation of decatransin as a potential pan-flaviviral compound should be performed *in vivo*. Special attention should be placed on assessing the toxicity of this compound, as it targets an essential protein complex. A standard test would be a 2 week rat toxicity study to evaluate the developability of decatransin.

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Appendix

Appendix I - Crystallisation conditions of crystal hits

Protein	Detergent used	Method	Screen	Condition
vc008 [#]	DDM	Vapour diffusion	PEG I suite	0.1 M Tris, pH 8.5, 25%(w/v) PEG 4000
vc008 [#]	DDM	Vapour diffusion	PEG I suite	0.1 M Tris pH 8.5, 25%(w/v) PEG 3000
vc011 [#]	DDM	Vapour diffusion	PEG I suite	0.2 M Zinc acetate, 20%(w/v) PEG 3350
vc011 [#]	DDM	Vapour diffusion	PEG I suite	0.2 M Tri-lithium citrate, 20%(w/v) PEG 3350
vc011 [#]	DDM	Vapour diffusion	PEG I suite	0.2 M Sodium chloride, 0.1M Hepes, pH7.0, 22%(w/v) PEG550 MME
vc008 - 44-4-7 Fab*	DDM	Vapour diffusion	Ammonium Sulphate suite	0.1 M Tris.HCl, pH 8.5, 1.5 M Ammonium sulfate, 15 %(w/v) Glycerol
vc008 - 44-4-7 Fab*	DDM	Vapour diffusion	PEG I suite	0.2 M Ammonium chloride, 20 %(w/v) PEG 3350
vc008 - 44-4-7 Fab*	DDM	Vapour diffusion	JCSG+	0.17 M Ammonium sulfate, 25.5 %(w/v) PEG 4000, 15 %(v/v) Glycerol

*Crystals contain only Fab fragment

[#]10 mM calcium chloride added 2 h before crystallisation screen

Appendix II – Protein sequence of pet32a 44-4-7 scFv

Colour legend

Thioredoxin tag – His tag - Thrombin cleavage site – S tag- TEV protease cleavage site - NcoI site-Light Chain - G4S linker - Heavy Chain - stop codon

Protein sequence

MSDKIIHLTDDSFDTDVLKADGAILVDFWAEWCGPCKMIAPILDEIADEYQGKLT
 AKLNIDQNPGTAPKYGIRGIPTLLLFKNGEVAATKVGALSKGQLKEFLDANLAGSG
 SGHMHHHHHSSGLVPRGSGMKETAALKFERQHMDSPDLGTENLYFQSPWDIVL
 TQSPASLAVSLGQRAAISCRASESVDSYGNSFMYWYQQKPGQPPKLLIYRASNLESG
 IPARFSGSGSRTDFTLTINPVEADDVATYYCQQSYEDPPTFGAGTKLEIKRGGGSG
 GGGSGGGGSQVKLQESGGGLVQPGGSLKLSCAASGFDERRYWMSWVRQAPGKG
 LEWIGEINPDSSTINYTPSLKDKFIISRDNAKNTLYLQMTKVRSEDTALYYCANYN
 DGGFAYWGQGTITVTVSS-

Appendix III – Result of protein ID by MALDI TOF/TOF

Rank	Protein Name	Species	Accession No.	Protein MW	Protein Score	Protein Score C.I.%	Total Ion Score	Total Ion C.I.%
1	Soluble cytochrome b562	Escherichia coli O157:H7	sp P0ABE8 C562_ECO57	14054.3	458	100	394	100
2	Soluble cytochrome b562	Escherichia coli	sp P0ABE7 C562_ECOLX	14052.3	451	100	392	100
3	Soluble cytochrome b562	Escherichia coli O6:H1 (strain CFT073 / ATCC 700928 / UPEC)	sp Q8CVG7 C562_ECOL6	14008.3	437	100	394	100
4	Genome polyprotein	Dengue virus type 1 (strain Singapore/S275/1990)	sp P33478 POLG_DEN1S	382740.8	212	100	205	100
5	Genome polyprotein	Dengue virus type 3 (strain China/80-2/1980)	sp Q99D35 POLG_DEN3C	381280	207	100	207	100
6	Genome polyprotein	Dengue virus type 3 (strain Singapore/8120/1995)	sp Q5UB51 POLG_DEN3I	381090.9	207	100	207	100
7	Genome polyprotein	Dengue virus type 3 (strain Martinique/1243/1999)	sp Q6YMS3 POLG_DEN3M	381019.6	206	100	206	100
8	Genome polyprotein	Dengue virus type 3 (strain Sri Lanka/1266/2000)	sp Q6YMS4 POLG_DEN3S	380987.6	206	100	206	100

Rank	Protein Name	Species	Accession No.	Protein MW	Protein Score	Protein Score C.I.%	Total Ion Score	Total Ion C.I.%
9	Genome polyprotein	Dengue virus type 1 (strain Brazil/97-11/1997)	sp P27909 POLG_DEN1B	382025.6	205	100	205	100
10	Genome polyprotein	Dengue virus type 1 (strain Nauru/West Pac/1974)	sp P17763 POLG_DEN1W	381822.5	205	100	205	100
11	Genome polyprotein	Dengue virus type 2 (strain 16681-PDK53)	sp P29991 POLG_DEN27	382890	93	99.975	93	100
12	Genome polyprotein	Dengue virus type 2 (isolate Thailand/0168/1979)	sp P14337 POLG_DEN28	382400.8	93	99.975	93	100
13	Genome polyprotein	Dengue virus type 2 (strain Jamaica/1409/1983)	sp P07564 POLG_DEN2J	382256.7	93	99.975	93	100
14	Genome polyprotein	Dengue virus type 2 (strain Thailand/NGS-C/1944)	sp P14340 POLG_DEN2N	382506.7	93	99.975	93	100
15	Genome polyprotein	Dengue virus type 2 (strain Puerto Rico/PR159-S1/1969)	sp P12823 POLG_DEN2P	382225.4	93	99.975	93	100
16	Genome polyprotein	Dengue virus type 2 (strain Peru/IQT2913/1996)	sp Q9WDA6 POLG_DEN2Q	382792.8	93	99.975	93	100
17	Genome polyprotein	Dengue virus type 3 (strain Philippines/H87/1956)	sp P27915 POLG_DEN3P	381127	93	99.975	93	100

Rank	Protein Name	Species	Accession No.	Protein MW	Protein Score	Protein Score C.I.%	Total Ion Score	Total Ion C.I.%
18	Genome polyprotein	Dengue virus type 2 (strain Thailand/16681/1984)	sp P29990 POLG_DEN26	382552.6	92	99.967	92	100
19	Uncharacterized protein APE_2601.1	Aeropyrum (strain ATCC 700893) pernix	sp Q9Y8N2 Y2601_AERPE	29668.2	48	0	37	46.086
20	Sulfite reductase, dissimilatory-type subunit beta	Desulfovibrio (strain Hildenborough) vulgaris	sp P45575 DSVB_DESVH	43232.4	45	0	-	-

Appendix IV – Home-made LCP recipe based on published LCP protein structures.

			PEG		Buffer		Salt 1		Salt 2 / Additive		Additive	
1	A1	26%	PEG 400	100 mM	MES pH 6.5		200 mM	Lithium sulphate	4%	DMSO	3.50%	1,4 butanediol
2	A2	25%	PEG 400	100 mM	Sodium citrate pH 5.5		300 mM	Sodium malonate	5 mM	Nickel chloride		
3	A3	25%	PEG 400	100 mM	Bis-Tris Propane pH 6.5		120 mM	Sodium potassium tartrate			3%	1,3 butanediol
4	A4	20%	PEG 400				300 mM	Sodium malonate				
5	A5			500 mM	Hepes pH7.5		700 mM	Ammonium sulphate	100 mM	Sodium citrate, pH 5.5	18%	JEFFAMINE M-600
6	A6	30%	PEG 400				185 mM	Lithium sulphate	100 mM	Sodium citrate pH 6.5		
7	A7	28%	PEG 400	100 mM	Bis-Tris propane pH 7.0		300 mM	Potassium formate				
8	A8	27%	PEG 400	100 mM	Tris pH 7.5		220 mM	Sodium formate			5%	1,4 butanediol
9	A9	25%	PEG 400	100 mM	Tris pH 7.0		300 mM	Potassium sodium tartrate				
10	A10	20%	PEG 400	100 mM	Tris pH 8.0		100 mM	Potassium formate				
11	A11	30%		100 mM	Bis-Tris propane pH 6.5		100 mM	Sodium sulphate			5%	1,4-Butanediol
12	A12			50 mM	MES pH 5.3				350 mM	Potassium chloride		
13	B1	5%	PEG 4000				100 mM	Sodium acetate pH4.6	100 mM	Potassium chloride		
14	B2	18%	PEG 400	50 mM	Hepes pH 7.0		100 mM	Potassium sulphate				
15	B3	7%	PEG 3000	100 mM	Tricine pH 7.4		50 mM	Lithium sulphate				
16	B4			100 mM	Sodium citrate pH 5.6		100 mM	Sodium chloride	100 mM	Lithium nitrate	4%	1,4-Butanediol
17	B5			100 mM	Sodium citrate pH 5.6		100 mM	Sodium chloride	100 mM	Lithium nitrate	8%	MPD
18	B6	30%					100 mM	Sodium citrate pH 5.0	100 mM	Sodium chloride	100mM	Magnesium chloride
19	B7	3%	PEG 4000	20 mM	Tris pH 7.5		66 mM	Sodium citrate pH 5.0				
20	B8	30%	PEG 400	100 mM	Hepes pH7.5		150 mM	Sodium citrate pH 5.0	350 mM	Magnesium chloride		
21	B9	30%	PEG 300	100 mM	Hepes pH7.5		100 mM	Ammonium phosphate			2%	MPD
22	B10	15%	PEG 300	100 mM	Hepes pH7.5				50 mM	EDTA	1.20%	1,2,3-heptanetriol
23	B11	30%	PEG 400	100 mM	Bis-Tris propane pH 7.5		300 mM	Ammonium acetate				
24	B12	28%	PEG 500 DME	100 mM	Hepes pH 7.0		200 mM	Ammonium sulphate				
25	C1	27%	PEG 500 DME	100 mM	MES pH 6.5		100 mM	Ammonium nitrate				
26	C2	36%	PEG 400	100 mM	MES pH 6.0		100 mM	Zinc acetate				
27	C3	25%	PEG 600	100 mM	MES pH 6.0		50 mM	Potassium thiocyanate				
28	C4	42%	PEG 200				100 mM	Sodium acetate pH 4.5	50 mM	Magnesium chloride		
29	C5	30%	PEG 400	100 mM	Hepes pH 7.0				300 mM	Lithium sulphate	7.50%	DMSO
30	C6			100 mM	MES pH 6.5		150 mM	Ammonium formate			10%	MPD
31	C7			100 mM	Tris pH 8.0				100 mM	Potassium formate		
32	C8			800 mM	Hepes pH7.5		560 mM	Ammonium sulphate			14.40%	Jeffamine M-600
33	C9						100 mM	Sodium citrate pH 5.6	100 mM	Magnesium chloride	4%	MPD
34	C10	40%	PEG 400	100 mM	Tris pH 8.0				4%	DMSO	1%	1,2,3-heptanetriol
35	C11	20%	PEG 400	100 mM	MES pH 6.5		400 mM	Potassium nitrate	1 mM	TCEP		
36	C12	28%	PEG 400				300 mM	Ammonium phosphate	10 mM	Magnesium chloride	100 mM	Sodium citrate pH 4.5
37	D1	30%	PEG 400	100 mM	Hepes pH 7.0		400 mM	Sodium malonate				
38	D2	20%	PEG 400				100 mM	Sodium citrate pH 5.5	300 mM	Sodium malonate		
39	D3	26%	PEG 400	100 mM	MES pH 6.0		300 mM	Sodium malonate	5 mM	Strontium chloride		
40	D4	30%	PEG 400	100 mM	Hepes pH 7.0		100 mM	Potassium sodium tartrate	100 mM	Sodium malonate		
41	D5			500 mM	Hepes pH 6.5		550 mM	Sodium acetate pH 4.6	100 mM	Potassium sodium phosphate		
42	D6			500 mM	Hepes pH 8.0		700 mM	Ammonium sulphate			20%	Jeffamine M-600
43	D7	30%	PEG 400				100 mM	Sodium citrate pH 6.0	400 mM	Potassium nitrate		
44	D8	30%	PEG 300	100 mM	Hepes pH7.5		100 mM	Ammonium phosphate			1%	1,2,3-heptanetriol
45	D9	30%	PEG 500 DME	100 mM	Ammonium sulphate		100mM	Sodium citrate pH 6.0	100 mM	Magnesium chloride	100 mM	Sodium chloride
46	D10			100 mM	Hepes pH 7.0		200 mM	Lithium sulphate			10 mM	ATP
47	D11	40%	PEG 400				1 M	Sodium chloride	100 mM	Sodium cacodylate pH 6.0		
48	D12	30%	PEG 400				100 mM	Sodium citrate pH 5.5	200 mM	Magnesium chloride		

			PEG		Buffer		Salt 1		Salt 2 / Additive		Additive
49	E1	25%	PEG 600	100 mM	MES pH 6.0		50 mM	Potassium thiocyanate			
50	E2	30%	PEG 400	100 mM	Hepes pH 7.0				2 mM	beta-mercaptoethanol	
51	E3	30%	PEG 600	100 mM	Tris pH 8.0		120 mM	Magnesium formate	100 mM	Sodium chloride	
52	E4	30%	PEG 300	100 mM	Hepes pH 7.5		100 mM	Manganese chloride			
53	E5	20%	PEG 400	100 mM	Hepes pH 7.5		150 mM	Calcium chloride	100 mM	Sodium thiocyanate	
54	E6	28%	PEG 500 DME	100 mM	Sodium cacodylate pH 6.0		2.5 mM	Cadmium chloride			
55	E7	24%	PEG 500 DME	100 mM	MES pH 6.0		10 mM	Copper II chloride	200 mM	Ammonium formate	
56	E8	28%	PEG 550 MME	100 mM	Tris pH 8.0		350 mM	Magnesium sulphate			
57	E9	23%	PEG 550 MME	100 mM	Tris pH 8.0		350 mM	Ammonium citrate			3% DMSO
58	E10	23%	PEG 550 MME	100 mM	Tris pH 8.0		150 mM	Ammonium sulphate			
59	E11	30%	PEG 500 DME	100 mM	Sodium citrate pH 6.0		100 mM	Magnesium chloride	100 mM	Ammonium sulphate	100 mM Sodium chloride
60	E12	31%	PEG 500 DME	100 mM	Hepes pH 7.0		200 mM	Lithium sulphate			
61	F1	28%	PEG 400	50 mM	MES pH 6.5		100 mM	Sodium thiocyanate			
62	F2	26%	PEG 550 MME	100 mM	Tris pH 8.0		100 mM	Lithium sulphate			
63	F3	30%	PEG 400	50 mM	MES pH 6.5		100 mM	Potassium thiocyanate	20 mM	Calcium chloride	
64	F4	30%	PEG 400	100 mM	MES pH 6.5		100 mM	Magnesium acetate			
65	F5	36%	PEG 400	100 mM	MES pH 6.5		160 mM	Lithium sulphate	5 mM	Strontium chloride	
66	F6	36%	PEG 400	100 mM	MES pH 6.5		300 mM	Lithium sulphate	5 mM	Strontium chloride	
67	F7	26%	PEG 400	100 mM	Tris pH 8.0		300 mM	Ammonium sulphate			
68	F8	9%	PEG 400	100 mM	MES pH 6.0		150 mM	Potassium sodium tartrate			0.50% Jeffamine M-600 pH7
69	F9	20%	PEG 3350	100 mM	Bis-Tris propane pH 6.5		270 mM	Calcium chloride			
70	F10	5%	MPD	50 mM	Sodium citrate pH 5.5		100 mM	Sodium chloride	60 mM	Magnesium acetate	
71	F11	30%	PEG 400	100 mM	Tris pH 8.8		100 mM	Sodium chloride			
72	F12	32%	PEG 400	100 mM	Hepes pH 7.0		150 mM	Sodium chloride			
73	G1		PEG 400	100 mM	Hepes pH 7.0		100 mM	Ammonium diPhosphate			
74	G2	27%	PEG 400	100 mM	Hepes pH 7.0		100 mM	Ammonium diPhosphate	2 mM	TCEP	
75	G3	30%	PEG 400	100 mM	Sodium citrate pH 5.0		450 mM	Ammonium acetate			3% 2-Propanol
76	G4	35%	PEG 400	100 mM	Sodium citrate pH 6.0		150 mM	Ammonium acetate			4% MPD
77	G5	27%	PEG 400	100 mM	Hepes pH 7.0		150 mM	Ammonium fluoride			2.50% Jeffamine M-600 pH7
78	G6	36%	PEG 400	100 mM	Hepes pH 7.0		100 mM	Ammonium chloride			
79	G7	30%	PEG 400	100 mM	MES pH 6.0		100 mM	Magnesium sulphate			
80	G8	25%	PEG 400	100 mM	MES pH 6.0		200 mM	Sodium chloride			
81	G9	29%	PEG 2000	100 mM	Sodium potassium phosphate pH 5.6						
82	G10	20%	PEG 400	100 mM	Hepes pH 7.0		100 mM	Sodium citrate pH 5.5			
83	G11	10%	PEG 2000	100 mM	Sodium citrate pH 6.5		200 mM	Ammonium diPhosphate			
84	G12	20%	PEG 400	100 mM	Sodium citrate pH 5.5		500 mM	Ammonium diPhosphate			5% DMSO
85	H1	28%	PEG 400	100 mM	Sodium citrate pH 5.5		450 mM	Ammonium diPhosphate			4% DMSO
86	H2	30%	PEG 400				50 mM	Hexa-cobalt chloride			
87	H3	28%	PEG 400	50 mM	Sodium acetate pH 5.5		50 mM	Magnesium acetate			
88	H4	25%	PEG 400	100 mM	Sodium citrate pH 5.0		400 mM	Potassium diPhosphate			6% DMSO
89	H5	26%	PEG 400	100 mM	Sodium citrate pH 5.5		100 mM	Ammonium diPhosphate			
90	H6	28%	PEG 400	100 mM	Sodium citrate pH 5.5		120 mM	Ammonium diPhosphate			
91	H7	30%	PEG 400				100 mM	Sodium citrate pH 5.5			4% MPD
92	H8	20%	PEG 400	100 mM	Tris pH 8.5						
93	H9	20%	PEG 400	100 mM	Tris pH 8.5						
94	H10	20%	PEG 400				200 mM	Zinc acetate			
95	H11	20%	PEG 400				200 mM	Tri-lithium citrate			
96	H12	22%	PEG 550 MME				200 mM	Sodium chloride			

Appendix V – Electron density map and refinement statistics of NS3hel after first refinement

Figure 1. Electron density map NS3hel structure after first refinement. The structure was solved by molecular replacement using published NS3hel structure (PDB: 2JLQ). No new densities were observed at the interface between Domain 2 and Domain 3 (white box), indicating that the synthesised NS4B loop peptide is not present in the structure.

Table 1. Data collection and refinement statistics

Parameters	Native value
Wavelength (Å)	1.54187
Resolution range (Å)	19.83 - 2.8 (2.9 - 2.8)
Space group	P 1 21 1
Unit cell	
a, b, c (Å)	49.74, 98.83, 53.02
α , β , γ (°)	90, 93.38, 90
Total reflections	24769 (2451)
Unique reflections	12614 (1251)
Parameters	Native value
Multiplicity	2.0 (2.0)
Completeness (%)	99.43 (99.76)
Mean I/ σ (I)	8.48 (0.74)
Wilson B-factor	81.34
R-merge	0.09149 (1.226)
R-meas	0.1294 (1.734)
R-pim	0.09149 (1.226)
CC1/2	0.994 (0.216)
CC*	0.998 (0.596)
Reflections used in refinement	12607 (1251)
Reflections used for R-free	603 (62)
R-work	0.2393 (0.2334)
R-free	0.3529 (0.3796)
CC(work)	0.927 (0.389)
CC(free)	0.800 (0.221)
Number of non-hydrogen atoms	3591
macromolecules	3591
Protein residues	451
RMS bonds (Å)	0.012
RMS angles (°)	1.63
Ramachandran favored (%)	78.17
Ramachandran allowed (%)	17.59
Ramachandran outliers (%)	4.23
Rotamer outliers (%)	17.14
Clashscore	21.92
Average B-factor	85.57
macromolecules	85.57

Statistics for the highest-resolution shell are shown in parentheses.

Appendix VI - Table of 106 significantly enriched host proteins identified from SILAC MS/MS

Gene name	Mean enrichment score	Enrichment P-value
ABCD3	1.10	< 0.0001
ACADSB	1.25	< 0.0001
ACSL4	1.27	0.00022
ACTG1	4.63	0.021
ACTL6A	1.70	0.009
AHSA1	1.28	0.0067
ALDH3A2	1.30	< 0.0001
APOB	7.50	< 0.0001
ARF4	1.53	0.00016
ARL1	1.73	0.0027
ASNS	1.50	0.05
ATP1A1	1.83	0.021
ATP2A2	3.30	< 0.0001
ATP5C1	1.25	< 0.0001
ATP5J2-PTCD1	1.20	0.00078
B4GAT1	1.97	0.0039
BAG2	2.75	0.00016
CANX	2.40	< 0.0001
CAV1	9.37	0.021
CLTC	1.23	< 0.0001
COPA	1.10	< 0.0001
CPS1	1.48	0.0035
CYC1	1.30	0.016
DDOST	1.30	0.00038
DDX41	1.18	0.017
DHCR24	1.40	< 0.0001
DHCR7	1.73	< 0.0001
DNAJA1	2.03	0.028
DYNC1H1	1.20	< 0.0001
EIF3L	2.33	0.0026
EIF4A3	1.23	< 0.0001
ESYT1	1.58	0.016
FADS2	1.50	0.00016
FASN	1.25	< 0.0001
FDPS	1.63	0.016

Gene name	Mean enrichment score	Enrichment P-value
FNDC3B	1.38	0.0071
GAK	1.40	0.014
GALNT2	1.57	0.0017
GFPT1	1.25	< 0.0001
GOLT1B	3.70	0.021
HACD3	1.83	< 0.0001
HIGD1A	1.93	0.021
Histone H2B type 1-C	3.25	0.01
HIST4H4 histone	3.78	0.00046
Histone H2A type 2-A	3.43	0.019
HM13	3.63	0.0017
HNRNPA0	1.15	0.0011
HNRNPDL	1.13	0.012
HSD17B11	1.25	0.00066
HSD17B4	1.13	< 0.0001
HSDL2	1.15	0.0012
HSPA5	1.40	< 0.0001
HSPD1	1.10	0.0015
IARS	1.15	0.003
KDEL3	9.43	0.021
MATR3	1.35	0.016
MTCH2	1.48	0.00035
MYH9	8.03	< 0.0001
NADK2	1.30	0.0039
NCL	1.30	0.0011
NPM1	1.20	0.006
NSDHL	1.23	< 0.0001
PCBP1	1.18	0.0039
PDIA6	1.58	< 0.0001
PHGDH	1.45	0.00023
PON2	2.10	0.009
PRKDC	1.25	< 0.0001
PRPF38A	1.50	0.047
PRPF8	1.13	< 0.0001
RAB14	1.33	0.00018
RAB2A	1.50	0.0065
RALY	1.40	0.00097
RBM4	1.37	0.009

Gene name	Mean enrichment score	Enrichment P-value
RPN1	1.23	< 0.0001
RPN2	1.60	0.00067
RPS15A	1.18	< 0.0001
RUVBL2	1.68	< 0.0001
SAP30BP	1.15	0.00084
SDHA	1.13	0.0061
SEC61A1	1.35	< 0.0001
SEC61B	1.28	0.0017
SERPINH1	1.28	< 0.0001
SFXN1	1.40	0.014
SIGMAR1	3.27	0.021
SLC25A1	1.37	< 0.0001
SLC25A11	1.50	< 0.0001
SLC25A13	1.35	0.0032
SLC25A3	1.50	< 0.0001
SLC3A2	1.67	0.0017
SNRNP200	1.18	0.003
SRM	1.97	0.009
SRPRB	1.35	0.0039
SSR1	1.80	0.009
STOM	10.45	< 0.0001
STOML2	1.95	< 0.0001
STRAP	1.23	0.012
STT3A	1.75	0.00078
SURF4	2.00	0.0039
TECR	1.13	0.0033
TMED10	1.58	0.0017
TUBB	1.25	0.0083
TUFM	1.20	< 0.0001
VCP	1.80	< 0.0001
VDAC2	2.05	< 0.0001
VDAC3	1.73	0.00078
WFS1	3.13	0.021

Appendix VII - Ranking of genes based on normalised RNAi know-down data

Gene name	Mean Rluc signal^a	Rank by mean Rluc signal
Media	1.0	Control
NS3	2.5	Control
EIF4A3	13.7	1
VCP	20.5	2
SEC61A1	21.3	3
CLTC	21.9	4
RPS15A	22.9	5
SLC25A1	24.8	6
WFS1	28.0	7
FADS2	30.3	8
SIGMAR1	31.6	9
CANX	31.9	10
PON2	31.9	10
SSR1	32.8	12
FNDC3B	34.0	13
ACTL6A	34.9	14
MATR3	35.3	15
DNAJA1	36.1	16
TMED10	36.4	17
ATP5C1	37.1	18
RPN1	37.7	19
PRKDC	37.8	20
IARS	38.3	21
CAV1	38.8	22
GOLT1B	38.8	23
TUFM	39.0	24
EIF3L	41.8	25
RPN2	42.5	26
NSDHL	42.5	26
HSPA5	43.3	28
DHCR7	43.4	29
MYH9	44.4	30
HIST2H2AA3	44.8	31
PRPF38A	45.1	32
ATP5J2-PTCD1	45.1	32
Lipofectamine	45.2	Control
TECR	45.2	34

Gene name	Mean Rluc signal^a	Rank by mean Rluc signal
STRAP	45.7	35
HNRNPA0	45.8	36
SLC25A3	46.4	37
HSPD1	46.5	38
HSD17B4	47.2	39
ACSL4	47.2	39
GAK	47.3	41
ESYT1	47.4	42
PRPF8	47.6	43
ATP1A1	47.8	44
DDOST	48.0	45
STT3A	48.6	46
COPA	49.2	47
SAP30BP	49.3	48
CYC1	49.7	49
HSDL2	50.8	50
AHSA1	50.8	51
ACTG1	51.1	52
SLC25A11	51.8	53
SNRNP200	52.8	54
ACADSB	52.8	54
ATP2A2	52.8	54
TUBB	53.3	57
ARL1	53.4	58
HIGD1A	53.8	59
PHGDH	54.3	60
DYNC1H1	54.9	61
VDAC3	55.3	62
SDHA	55.8	63
HM13	56.6	64
ASNS	56.8	65
RUVBL2	56.8	65
PCBP1	58.2	67
HNRNPDL	58.4	68
Scrambled	58.6	Control
CPS1	59.0	69
RBM4	59.3	70
GFPT1	59.3	71
KDEL3	59.5	72

Gene name	Mean Rluc signal^a	Rank by mean Rluc signal
SERPINH1	60.6	73
RAB14	61.1	74
RAB2A	62.1	75
SFXN1	62.5	76
MTCH2	63.7	77
SRM	63.8	78
HSD17B11	64.7	79
SLC3A2	65.8	80
NCL	66.7	81
NPM1	67.0	82
SURF4	68.0	83
ALDH3A2	68.3	84
DDX41	68.3	84
NADK2	68.5	86
B4GAT1	69.4	87
GALNT2	70.8	88
ABCD3	71.0	89
SEC61B	73.1	90
ARF4	75.3	91
FASN	87.1	92

^a Due to edge effect plate, plate normalisation and data correction based on plate region and mean scaling was performed using HELIO.