The role of carboxypeptidase B1 in modulating DENV replication and release from *Aedes aegypti* midgut cells

By

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THESIS

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STATEMENT OF ORIGINALITY

The experimental work described in this thesis represents original work performed by myself in the Virus-host Interaction Research Group, Infectious Disease Laboratory, Jeffrey Cheah School of Medicine and Health Sciences, Monash University Malaysia, during the years 2011 to 2014 under the supervision of the main supervisor Associate Professor Dr. Sharifah Syed Hassan and co-supervisors Professor Dr. Iekhsan Othman and Dr. Hamdan Ahmad. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, unless otherwise acknowledge, and contains no material which has been accepted for the award of any other degree of diploma in any university.

PART A: General Declaration

Monash University

Declaration for thesis based or partially based on conjointly published or unpublished work

General Declaration

In accordance with Monash University Doctorate Regulation 17.2 Doctor of Philosophy and Research Master's regulations the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes 1 manuscript (accepted for publication, *JIDC*) and 1 article (published, *Viruses*). The core theme of the thesis is protein-protein interactions between dengue virus and *Aedes aegypti*, and protein functional elucidations during viral infection. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Jeffrey Cheah School of Medicine and Health Sciences, under the supervision of Associate Professor Dr. Sharifah Syed Hassan.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of chapters 2-4, my contribution to the work involved the following:

Thesis chapter	Publication title	Publication status*	Nature and extent of candidate's contribution
2	Protein-protein interactions between *Ae. aegypti* midgut and dengue virus 2: two-hybrid screens using the midgut cDNA library	*JIDC*, accepted for publication. *Manuscript ID: JIDC-6422* *Manuscript: page 43-72*	Experimental design and conduct, samples collection and process, data collection, result acquisition, statistical analysis, manuscript preparation
3 & 4	CPB1 of *Aedes aegypti* interacts with DENV2 E protein and regulates intracellular viral accumulation and release from midgut cells	Published in *Viruses* *Article: page 101-121*	Experimental design and conduct, samples collection and process, data collection, result acquisition, statistical analysis, manuscript preparation

I have renumbered sections of accepted or published papers in order to generate a consistent presentation within the thesis.

Signed: ……

Date: ……

ABBREVIATIONS

ADE	Antibody-dependent enhancement
AP	Alkaline phosphatase
AP-MS	Affinity purification-mass spectrometry
C	Capsid
Co-IP	Co-immunoprecipitation
CP	Carboxypeptidase
CPB1	Carboxypeptidase B1
Cryo-EM	Cryo-electron microscopy
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule 3 grabbing non-integrin
DENV	Dengue virus
DF	Dengue fever
DHF	Dengue haemorrhagic fever
dsRNA	Double-stranded RNA
DSS	Dengue shock syndrome
E	Envelope
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
GM	Genetically engineered
HCV	Hepatitic C virus
I2H	*In silico* two-hybrid
IFA	Immunofluorescent assay
IFN	Interferon
JE	Japanese encephalitis
LC	Langerhan's cells
M2H	Mammalian two-hybrid
MTase	Methyltransferase
NS	Non-structural
NTPase	Nucloside triphosphatase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PPI	Protein-protein interaction
prM	Pre-membrane
RdRp	RNA-dependent RNA-polymerase
RNAi	RNA interference
RT-PCR	Reverse transcription PCR
RT-qPCR	Reverse transcription quantification PCR
SIT	Sterile insect technique
SLE	St. Louis encephalitis
TAP-MS	Tandem affinity purified-mass spectrometry
TGN	Trans-golgi network
TLR3	Toll-like receptor 3
VLP	Virus-like particle
Y2H	Yeast two-hybrid
YF	Yellow fever

Abstract

ABSTRACT

The female *Aedes aegypti* mosquito, the principal vector for the transmission of Dengue virus (DENV), is able to support DENV propagation within its tissues, especially the midgut and salivary gland. Understanding host-virus relationships may lead to the discovery of new vector control strategies for better virus controls. In this study, preliminary biological interaction screens between DENV2 and female *Ae. aegypti*, were conducted using the yeast two-hybrid (Y2H) assays. A cDNA library of the female adult *Ae. aegypti* was constructed and used in several Y2H screens with DENV2 E, prM, M and NS1 proteins. Among the many host interacting proteins identified, carboxypeptidase B1 (CPB1) shown to bind to the E protein was selected for further studies. The interaction was also demonstrated using *in silico* docking analyses. Further analyses to verify the CPB1-E protein-protein interactions were conducted using mammalian two-hybrid, co-immunoprecipitation and double immunofluorescent co-localisation assays. It was shown that CPB1 was present in the endoplasmic reticulum (ER) of *Ae. aegypti* primary midgut cells and binds to DENV2 in the ER. The function of CPB1 with respect to the binding of virus in the ER was further investigated by the up- and down-regulation of CPB1 expression. CPB1 overexpression in mosquito C6/36 cells resulted in intracellular accumulation of DENV2 genomic RNA or virus particles, with only small amounts of virus released from the cells. In contrast, induced expression of CPB1 in Vero cells exhibited a different outcome. Therefore, it was suggested that CPB1 functions by regulating DENV2 replication exclusively in mosquito midgut cells. Based on the DENV replication cycle, we postulated that, in *Ae. aegypti* midgut cells, CPB1 binds to the E protein of the virus and regulates DENV2 genome encapsulation into the ER lumen, or inhibits immature virus particle transportation to the Golgi complexes, which subsequently reduces the amount of mature DENV2 released from the midgut cells to other tissues. The small amount of virus released from the midgut cells could be sufficient to infect the salivary gland cells for further replication and transmission to humans, but not enough to fully infect other tissues that cause adverse effects on the health of the mosquito.

Chapter 1

Literature review, research gaps, and objectives

1.1 Global situation of dengue

Dengue is recognised as a re-emerging infectious disease. Dengue fever (DF) and dengue haemorrhagic fever (DHF)/dengue shock syndrome (DSS) have become important public health problems as the global incidence of DF and DHF has steadily increased. Dengue viruses (DENV) have a worldwide distribution across the tropics and subtropics. The World Health Organisation (WHO) estimates that 2.5 billion people are at risk of dengue worldwide. It is estimated that close to 50–100 million cases of DF and 30,000 fatal cases of DHF/DSS occur annually in tropical and subtropical regions (1).

1.2 DENV infection in human

The manifestations of a DENV-infected human range from mild DF to potentially lethal DHS/DSS (2). DF is frequently uncomplicated and therefore often undiagnosed, whereas severe cases of DHF/DSS may be lethal if treatment is either poor or offered too late into the disease progression. One of the major risk factors for DHF/DSS is the patient's previous exposure to one of the DENV serotypes. Primary DENV infections usually result in non-complicated DF which facilitates the development of long-term adaptive immunity against the same serotype. However, during a second DENV infection by a different serotype, the primary anti-dengue immune response may not confer cross-protection to other serotypes. In such situations, the pre-existing antibodies may cross react with the second infecting virus generating antigen/antibody complexes and in some cases this leads to Fc receptor mediated attachment (and infection) to cells involved in the development of innate immunity. Such infections may result in

the development of DHF/DSS by the process known as antibody-dependent enhancement (ADE) (2-4).

DENV is transmitted to human host after a blood meal by an infective mosquito. Following DENV inoculation in the dermis, the primary targets for infection are skin-resident cells such as dendritic cells (DCs) and Langerhan's cells (LCs) (5). DCs, as a type of antigen-presenting cell, sense pathogens by various pathways, including Toll-like receptors (TLR), RIG-like receptors (RLR), and NOD-like receptors (NLR) (6). The specific receptor for DENV binding in DCs has been reported to be dendritic cell-specific intercellular adhesion molecule 3 grabbing non-integrin (DC-SIGN) (7). The susceptibility of DCs to DENV has provided a platform for DENV proliferation after viral inoculation by an infected mosquito (8). DENV will then enter human bloodstreams and is carried to its main target organ – liver and subsequently causes liver dysfunction (9-11). Although it is generally believed that liver as the main DENV target organ, several reports have shown the identification of DENV in various human tissues/cell lines, such as endothelial cells (12, 13), lung (14), kidney (15), spleen (16), and immune cells like macrophages and B lymphocytes (17-19).

1.3 Molecular interactions between DENV and human

Most viral replications occur in host cells, mainly in cytoplasm. A successful viral infection to its host cell requires the establishment of multiple interactions between cellular and viral molecules. This is critical in mediating viral binding, entry, replication, assembly, and release to fulfil a complete viral life cycle. The initial interaction between DENV to its susceptible host cell occurs at the point of surface attachment/binding, which can be further classified into non-specific and

specific molecular interactions. Non-specific interactions have been reported to involve in viral attachment but, instead of driving viral internalisation, it serves to concentrate the virus at the cull surface with glycosaminoglycan-like heparan sulfate (20, 21). Conversely, specific interactions include those associate with viral binding to a specific receptor proteins, which subsequently lead to viral entry. To date, despite numerous studies pertain to DENV receptor discovery, a consensus receptor protein for DENV in *Aedes* mosquito and humans is still not available (17, 19, 22-28).

1.4 Dengue virus

1.4.1 Morphology

DENV, a positive single-stranded RNA virus, is approximately 50 nm in diameter with about 11 kb RNA genome. It is a mosquito-borne member of the *Flaviviridae* family (genus: *Flavivirus*) and is related to yellow fever virus and the encephalitis-causing viruses like Japanese encephalitis (JE) virus, St. Louis encephalitis virus, tick-borne encephalitis virus, and West Nile virus. Today, DENV exists as 4 serotypes, namely DENV1, DENV2, DENV3, and DENV4.

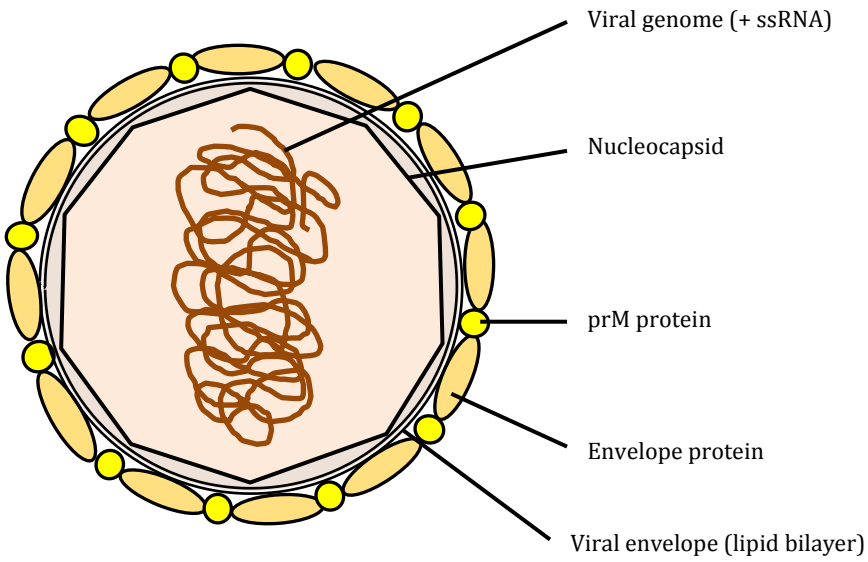


Figure 1 Schematic diagram showing the basic structure of *Flavivirus*.

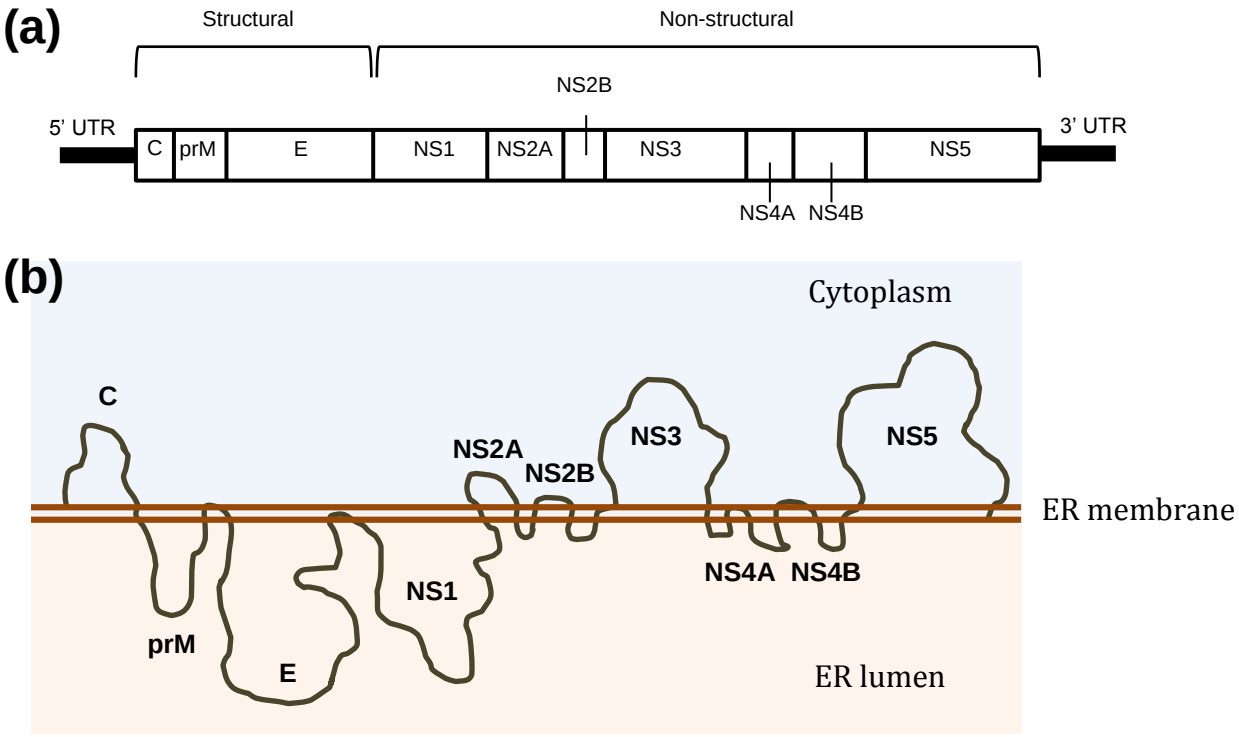


Figure 2 Schematic diagram showing DENV genome. (a) Structural and non-structural polyproteins encoded from DENV genome; **(b)** Polyprotein translated and its alignment across endoplasmic reticulum (ER) membrane.

1.4.2 DENV capsid (C) protein

Dengue virus C protein (about 13 kDa) is a structural protein responsible for assembly of viral RNA into a nucleocapsid the forms the core of a mature virus particle. Despite a general perception on DENV replication (including nucleocapsid encapsulation) occurs in cytoplasm, yet a large portion of capsid protein was found localised to the nucleus during infection (29). DENV C protein forms a stable homodimer in solution, which was reported to be a histone mimic, forming heterodimers with core histones, binding DNA and disrupting nucleosome formation (30). The same study also revealed the up-regulation of host-cell core histone level after DENV infection, which was explained to be a cellular response to C binding away the histone proteins (30).

Despite a general knowledge on the role of DENV C protein during DENV encapsulation, which requires a fine-tuned molecular orchestration between capsid-lipid (31, 32) and capsid-RNA interactions (33), the complete structural organisation of the DENV, specifically regarding the nucleocapsid structure, is still elusive.

1.4.3 DENV pre-membrane (prM) and membrane (M) protein

DENV prM protein (about 21 kDa) is synthesised in the lumen of endoplasmic reticulum (ER). It forms heterodimers with E protein, which then interact with virus nucleocapsid to form immature viral particles. Numerous studies on prM have reported the roles of this protein during DENV infection, including virus entry mediation (34), promoting ADE in human host (4), viral secretion (35), structural assembly, and as a protective protein to immature virus prior to viral release (34, 36, 37). The cleavage of prM into a short peptide “pr” and “M” marks

the maturation of DENV. This occurs when DENV transits from ER to trans-golgi networks (TGN), before viral secretion to the extracellular environment. The acidic environment in the lumen of TGN promotes structural changes of prM, which finally exposes "pr" to the host protease – furin for proteolytic cleavage (36, 38). To prevent the "mature" virus from unspecific membrane fusion, "pr" peptide was reported to remain associated with E protein until the virus is secreted into extracellular environment (37).

In nature, infectious DENV is found partially cleaved and carrying a combination of M and prM proteins. This occurrence is mirrored in the substantial levels of prM-specific antibodies that are produced the serum following dengue infection (4, 39). This has further complicated the current understandings on DENV, or, to a further extend, the *Flaviviridae* family members during viral replication and its immunological aspects during infections. Some researchers believe this is a subversive strategy employed by *Flavivirus* to evade the host immune defence system (4, 39, 40).

1.4.4 DENV envelope (E) protein

In common with all other flaviviruses, DENV E protein (about 60 kDa) is composed of 3 distinct domains, namely domain I-III (EI-EIII, respectively). It is the most exposed protein on DENV surface, which is generally believed to be involved in viral attachment to the host cells. DI is flanked by EII and EIII at both ends. EII, also known as the dimerisation domain, is located at EI's distal end. On the other hand, EIII, as the main receptor binding domain (23), is protrudes from viral surface (41), hence EIII is described as the most efficient antigenically-potential domain (42). In addition, the presence of an immunoglobulin-like fold

(FG loop) on DIII is alleged by some to hold a cell surface receptor recognition site, which is a critical structure for successful infection in mammalian cells and *Aedes aegypti* mosquitoes (43).

On a mature DENV, E proteins lie in sets of 3 of head-to-tail homodimers orientation. Each E protein in each set is nearly parallel to each other on viral surface forming a smooth "herringbone" configuration (38). During virus entry, low pH in endosome triggers conformational changes from homodimer "herringbone" structure into "fusogenic" structure, which exposes a fusion peptide buried between DI and DIII, followed by endosomal membrane fusion, nucleocapsid uncoating, and release of DENV RNA genome into host cytoplasm (38).

1.4.5 DENV non-structural 1 (NS1) protein

DENV NS1 protein (about 48 kDa) is not physically present on virus particle, only expressed to aid viral replication after infection. NS1 protein has been found on host cell surface, secreted into the extracellular environment, and binds to the uninfected cells through the interaction with heparan sulfate or chondroitin sulfate (44). In a DENV-infected cell, NS1 protein is synthesised as monomer and dimerises after post-translational modification. It is then secreted to the extracellular environment as hexamers (45-47). Scientists have not yet achieved consensus in the roles of DENV NS1 protein, but it is generally believed to involve in viral RNA replication and dengue pathogenicity (48, 49). Further studies have reported the importance of DENV NS1 protein in eliciting the development of host cross-reaction antibody against platelets (50) and endothelial cells (51). Furthermore, a few contrary findings reported the role of

NS1 protein in West Nile Virus (WNV, also a member of *Flavivirus*) in inhibiting Toll-like receptor 3 (TLR3) (52, 53).

DENV NS1 protein can potentially be a biomarker as an early indicator of DHF/DSS among patients. This hypothesis is made on the basis of high-circulating levels of NS1 protein in patients' sera during DENV infection, especially dengue illness correlating with the development of DHF/DSS (44, 54, 55). Currently, there are two commercialised dengue diagnostic Enzyme-linked Immunosorbent Assay (ELISA) diagnostic kits for the detection of NS1 antigens, which have been delivering promising and reliable results (56).

1.4.6 DENV non-structural 3 (NS3) protein

DENV NS3 protein (about 69 kda) is a multifunctional protein. It has been well-studied in several members of the *Flaviviridae*, including hepatitis C virus (HCV) (57), Dengue virus (58), West Nile virus (59), yellow fever virus (60), and Japanese encephalitis virus (61). NS3 protein is comprised of a serine protease domain (N-terminal, 1-180 amino acids) (62, 63) and a C-terminal domain involves in viral RNA replication (181-618 amino acids) (58, 64). Furthermore, NS3 protein has been reported as a RNA nucleoside triphosphatase (NTPase), also named helicase. NTPases/helicases represent a large protein family that are ubiquitously found over a wide range of organisms. The enzymes involve in cell development and differentiation, and play essential role in the replication of viral single-stranded RNA genomes (65).

1.4.7 DENV non-structural 5 (NS5) protein

DENV NS5 protein (about 105 kDa) is the largest and most well conserved *Flavivirus* protein. This protein, after infection in human host, has been shown to

perturb the host innate immune response by inhibiting type I interferon (IFN) signalling by interfering with STAT1 activation (66, 67). Specific interaction between DENV-STAT2 in human was also reported (68). NS5 protein contains two domains – the N-terminal methyltransferase (MTase) domain and the C-terminal domain. The N-terminal domain consists of two important enzymatic active sites, namely N-7, 2'-*O*-methyltransferase and guanylyltransferase, which are all required for RNA capping (69-71). On the other hand, the C-terminal domain contains RNA-dependent RNA-polymerase (RdRp). RdRp plays a role and is essential for replication of the RNA genome (72). Furthermore, this protein is frequently found localised in nuclear of infected cells (72, 73), leading to hypothesis that NS5 protein regulates host genomic regulation after infection. Hence, NS5 protein is always one of the main targets for vaccine and drug development in medical research field.

1.4.8 Other NS proteins

The mammalian interferon signalling pathway is a primary component in innate immune response to counteract viral infection. As such, viruses have devised several mechanisms to antagonise these responses. DENV encodes several proteins, including NS2A, NS4A, and NS4B that have been shown to inhibit interferon production (74). In addition, individual expression of NS4A has been shown to protect host cells against death, while NS4B and NS2A failed to protect cells against several stressors (75). Also, intraviral protein-protein interactions were also observed, mostly served as a co-factor for its interacting partner (e.g. NS4B interacts with NS3 to undergo RNA replication) (76).

1.4.9 Dengue virus replication

The establishment of multiple interactions between cell surface molecules and viruses is critical in the cell infection process that mediates viral binding, entry, replication, and multiplication of the virus. The initial interaction between DENV and its susceptible host cell occurs at the point of surface attachment/binding. Evidence suggests that the interaction is mediated by both specific and non-specific interactions between DENV and host cell surface proteins. Non-specific interactions serve to concentrate the virus at the cell surface with glycosaminoglycans like heparin sulfate (20, 21). On the other hand, specific interactions include those associate with virus binding to specific receptor proteins, which leads to viral entry. To date, several proteins have been implicated as DENV receptors, but a consensus/main receptor protein is not available (17, 19, 22-28).

After host cell entry, usually through receptor-mediated endocytosis, DENV undergoes nucleocapsid uncoating due to the slight acidic endosomal environment (34). DENV RNA genome is released into cytoplasm and translated into a single polyprotein across the endoplasmic reticulum (ER) membrane (77). The polyprotein is then processed co- and post-translationally into 3 structural proteins and 7 non-structural proteins, by both viral and host proteases (Fig. 2, page 16) (34). The structural proteins (capsid (C), pre-membrane (prM), envelope (E)) form the structure of the viral particle, while non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) are expressed only upon successful infection into the host cells. Non-structural proteins possess different functions which have been intensively investigated and they are mainly involved

in viral replication. The newly synthesised viral RNA genome is encapsulated by multiple copies of C proteins to form nucleocapsid, which is surrounded by an ER-derived lipid bilayer anchoring 180 prM/E heterodimers. They project vertically outward from the viral surface as 60 trimeric spikes (78, 79). After exposure to the acidic environment (~pH 5.9) in trans-Golgi network (TGN), the 60 prM/E trimeric spikes are altered into 90 prM/E homodimers, which renders the virus with a "smoother" surface. A host protein, furin, is then involved to cleaved prM into "pr" and "M", with "pr" capping the fusion peptide of E protein, before released into extracellular environment (41). Although it was generally believed that "pr" is released from a mature DENV, some researchers reported partial maturation as an immune-evasion strategy of DENV (39).

1.5 DENV vaccine development

Today, neither anti-dengue vaccine nor drug is available. Although dengue vaccines have been under development since the 1940s, its potential market values and appreciation of global dengue disease burden have been languished. In recent years, the development of dengue vaccines has accelerated dramatically, mainly due to its market values where 2.4 to 3.5 billion dengue vaccine doses would be needed in the first five years after vaccine introduction in public sector, including disease endemic and non-endemic countries (80). Despite a recent live-attenuated, tetravalent, dengue-yellow fever 17D chimeric virus chimeric virus vaccines given to school children in Thailand showed major but incomplete efficacy against at least two of the four DENV serotypes (81), Sanofi Pasteur (a vaccine division of Sanofi S.A.) has completed phase 3 clinical trial in Asia (July 2014) (82) and Latin America (September 2014) (83), with the

first commercialised dengue virus vaccine to be expected in mid 2015 (84). Until these vaccines are proven to be effective, protective, highly accessible, and cost effective, vector control will still remain as one of the main strategies in the fight against dengue.

1.6 DENV cycles in mosquitoes

In nature, DENV is transmitted in two independent cycles: (I) the urban cycle (85, 86), and (II) the sylvatic/zoonotic cycle. The urban cycle involves human-mosquito-human DENV transmission by the main mosquito vector *Ae. aegypti*, with other *Aedes* mosquito such as *Ae. albopictus* serving as the secondary vectors (87). In contrary to urban cycle, sylvatic/zoonotic cycle plays a role in DENV transmission between non-human primates and arboreal *Aedes* mosquitoes. These mosquitoes are usually found in sylvatic habitats of tropical and sub-tropical areas (88, 89). Extensive studies have been conducted on the relationship between the two cycles, with accumulated information revealing that, although some researchers believe DENV from sylvatic cycle carried less pathogenicity compared to DENV in urban cycle (90), sylvatic DENV share the pathogenic potential of urban DENV (91). This hypothesis is supported by a few cases of sylvatic DENV-infected patients who presented serious dengue manifestations (88, 89).

1.6.1 Vectorial compatibility

Vectorial compatibility plays a key role in DENV dissemination. Viral tropism, infection pathways, and viral protein characteristics in mosquito each contribute to mosquito susceptibility to DENV infection. In addition, the presence of a number of genetic/non-genetic factors (92, 93), nutritional status, and larval

competition (94) in mosquitoes are generally believed to mediate the overall vectorial competence of *Ae. aegypti*. These factors explain the presence of African sylvatic *Ae. aegypti formosus* which is relatively refractory to DENV infection (93).

1.6.2 DENV tropisms in mosquitoes

Compared to vertebrate hosts, much lesser focus is applied on DENV tropisms and trafficking in *Ae. aegypti*. During a bloodmeal, the presence of DENV at the correct titer is critical for virus transmission. After ingestion of viremic bloodmeal by a mosquito, DENV is believed to attach to the midgut epithelial cells, followed by virus entry and replication (93, 95), before passing through midgut escape barrier and basal lamina. After that, DENV enters and disseminate in haemocele, followed by establishing the second infection in the next target organ – salivary gland (95). As the main DENV vector, salivary gland of *Ae. aegypti* allows the dissemination and releasing of DENV into the lumen of the salivary gland (93). To date, the knowledge on DENV tropism from the midgut to salivary gland remains largely elusive, including other organs of tissues that DENV might target before disseminate in the salivary gland. Although some researchers reported the presence of DENV in mosquito adipose tissues (96), further studies are required to complete the puzzle of DENV infection in mosquitoes. Once infected by DENV, the mosquito remains infectious for its whole life span. Some research groups believed that DENV can be transmitted vertically from DENV-infected mosquito to its eggs (97-99).

1.7 Current vector control strategies

Insecticides have historically been used to control and manage the disease spread. However, over the past 15 years, much of the medical research

community has been focused on, not only vaccine and drug developments, but also the establishment of novel vector control strategies ranging from biocontrol methods through to genetic modification of insect populations. Together with some traditional approaches, 4 major classes of interventions exist and most of them have been demonstrated success, with appropriate combination of approaches.

Environmental management is the first class of intervention, which has been practised by many nations with promising outcomes. Environmental management includes both alteration of the natural habitats of mosquitoes (to reduce population number) and modification of human habitats (to reduce biting frequencies). In Africa, extensive studies have been done on the effectiveness of mosquito nets (either insecticide-treated or non-treated) (100). Despite the success in reducing dengue cases, studies have reported the changes in mosquito biting rhythm (101). These findings presented the mosquito's ability to circumvent unfavourable environmental factors. Some of the interventions are better suited to particular vector species than to others (100). For example, community clean-up campaigns effectively reduce *Ae. aegypti* habitats surrounding households, but not for mosquito vectors breed in water-filled tree holes.

The second class of intervention, biological control, includes the use of natural predators or microorganisms against vector mosquitoes. Recently, using copepods as mosquito natural predators, mosquito population was reduced in a village in Vietnam (102). In addition, larvivorous fishes such as guppy has also been demonstrated to be a non-insecticidal biological control weapon against

mosquitoes (103). However, its drawbacks include: (I) The identification of breeding sites can be time- or labour-consuming; (II) Mosquitoes have been found selectively avoid oviposition in water containing their natural predators (104). In addition, researchers of the Eliminate Dengue Program (based in Melbourne, Australia) use *Wolbachia*-infected *Ae. aegypti* for the control of dengue virus dissemination. *Wolbachia*, a naturally exist bacteria in 60% of insects, is not found in *Ae. aegypti*. The researchers released a controlled number of *Ae. aegypti* carrying *Wolbachia* to breed with wild-type mosquitoes, resulting in a few generations of *Ae. aegypti* harbouring *Wolbachia*, which was proven to disrupt dengue transmission between humans (105). The research outcomes are being closely monitored in several countries across continents including Australia, Brazil, Vietnam, and Indonesia.

Chemical treatment, as the third class of intervention, represents the most highly used approach to date. Since World War II, DDT (dichlorodiphenyl-trichloroethane) has been used to control insects' populations (106). Indoor residual surface spraying of DDT in houses has been effective in controlling malaria transmission (107). However, there have been concerns on human health issues and non-target effect on extensive DDT usage. In addition, indoor residual surface spraying in only effective against endophilic vector species, but lack of efficacy against exophilic species such as *Ae. aegypti*. Furthermore, due to increasing concerns on insecticide-resistant mosquito species, legislative decisions between 2005 and 2009 have deregistered/limited the production of 169 insecticides, with only 9 new insecticides were registered. Disease vector

control insecticides represent only less than 1% of the total pesticide products (108).

Genetic modification is grouped as the final class of intervention strategy for mosquito vector control. This technique was first introduced in India in the mid-1970. But, the project was halted before successful completion of field trials, mainly owing to political concerns raised by the nations (109). Another potentially larger issue is that sterile insect technique (SIT) could reduce mating competitiveness in the released males in the field (110), requiring the release of large numbers to compensate. Furthermore, some level of female leakiness or low-level fertility in males may pass on some of their mutations into wild populations (111). A company based in Oxford, UK - Oxitec Ltd. has progressively released a controlled amount of genetically engineered (GM) male *Ae. aegypti* carrying dominant lethal gene (RIDL®), which cause unviability to their offsprings (112, 113). In several trials undertaken in Cayman Islands (UK), Bentong (Malaysia), Panama, and Brazil, successive releases of Oxitec mosquitoes have been shown to reduce by over 90% the wild population of dengue mosquitoes in some the treated area (114).

1.8 Problem statement

Various vector control strategies have been practised for the last few decades in the dengue endemic regions of the world, however, it seemed to be a losing battle for humans. DENV still maintains its viability regardless of the efforts taken by the authorities and governments. Despite major progressions on dengue vaccines by the company, Sanofi Pasteur (82, 83), vector controls remain the most important approach in dengue disease management in the absence of

licenced drug and vaccine. The development of more innovative vector control strategies to replace the traditional or conventional approaches should be continuously pursued. Understanding of DENV-mosquito protein-protein interactions leading to the elucidation and revelation of the mechanisms of dengue virus replication and dissemination might hold the key for the development of novel mosquito vector control strategies.

1.9 Rationale

Currently, very limited or no studies have been conducted to determine direct "biological interactions" between DENV and mosquitoes cellular proteins. Despite many studies that have been conducted to determine DENV proteins interacting with mosquitoes, most of the studies uses traditional methods like the virus overlay protein binding assay (VOPBA) and computational predictions of dengue-host protein interactions (24, 115, 116), based on structural similarity between dengue proteins and hosts proteins. Guo *et al.,* 2010 identified 4214 mosquito proteins with 10209 interactions with DENV, using computational approach. This indicates massive interactions (both direct and indirect) between DENV and mosquito, however, most of the interactions remained inconclusive and need to be further validated (116). New or more robust methods that can demonstrate and validate direct biological interactions between mosquito and DENV proteins are required to elucidate the functions and mechanisms of these interacting proteins. These approaches may lead the path of discovering novel strategies for vector control.

1.10 Overall objective

To identify *Aedes aegypti* midgut cellular proteins that interact with DENV2 viral proteins and to elucidate the functions and mechanism of one selected midgut protein, in this study carboxypeptidase B1, in viral replication and pathogenesis.

1.10.1 Specific objectives:

Objective 1

To construct and establish a robust and highly complex cDNA library of the midgut derived from female adult *Ae. aegypti*.

Objective 2

To identify midgut proteins that interact with DENV2 viral proteins through the interactions of the cDNA library established with DENV E, prM, M, NS1 proteins using an improved yeast two-hybrid system.

Objective 3

To select and validate the protein-protein interaction of one of the selected midgut protein with the respective DENV2 protein (in this study, carboxypeptidase B1 and DENV2 E protein, i.e. CPB1-E).

Objective 4

To elucidate the role of *Ae. aegypti* carboxypeptidase B1 (CPB1) during DENV2 infection in mosquito.

The activities involved to achieve all the objectives are described and simplified in a flowchart as shown in Fig. 3.

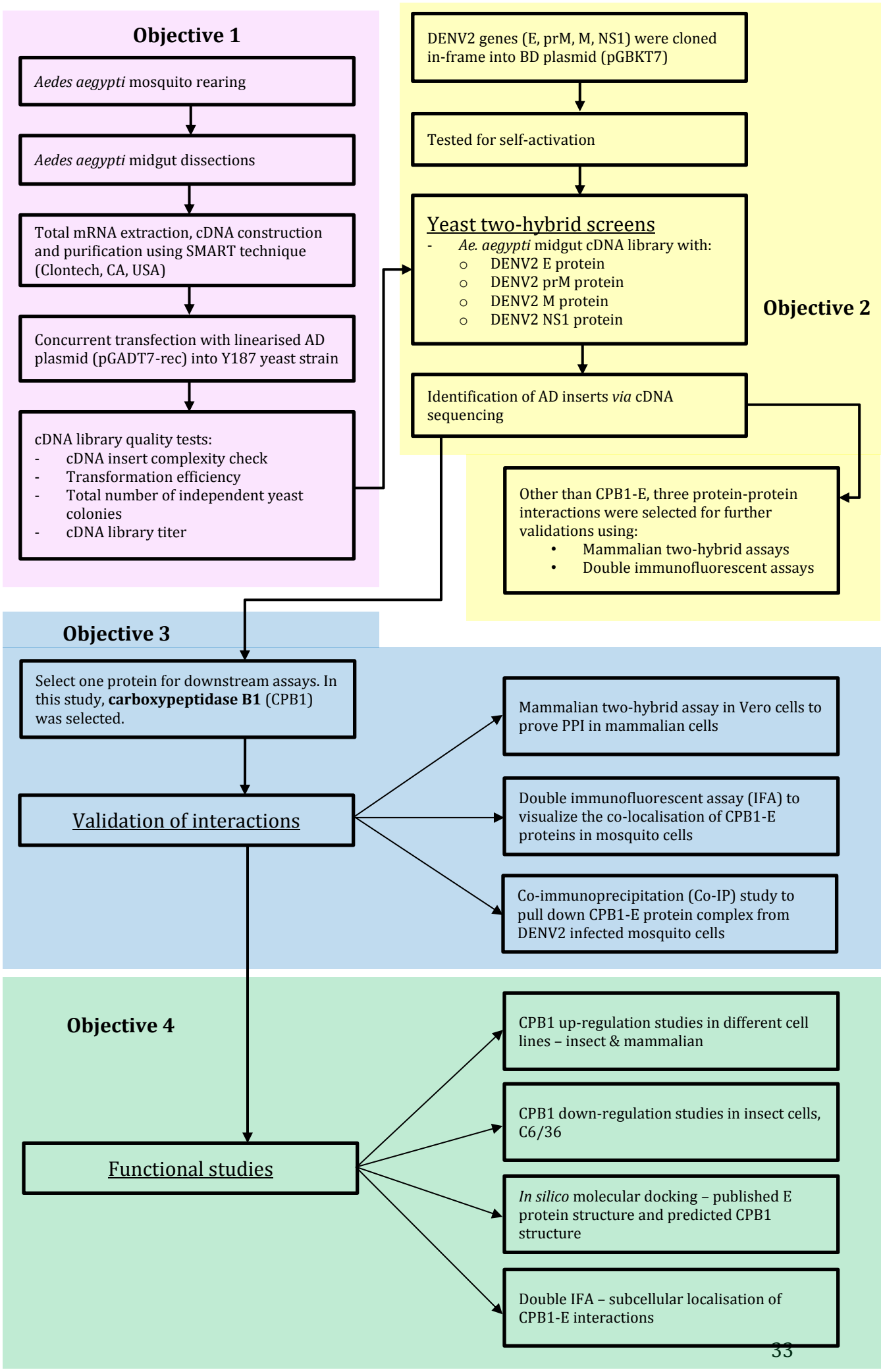


Figure 3 Flow chart of the complete study.

Chapter 2

Discovery of novel protein-protein interactions between *Aedes aegypti* mosquito midgut and DENV2 viral proteins (E, prM, M and NS1) using yeast two-hybrid system

PART B: Suggested Declaration for Thesis Chapter

Monash University

Declaration for Thesis Chapter 2

Declaration by candidate

In the case of **Chapter 2**, the nature and extent of my contribution to the work was the following:

Nature of contribution	**Extent of contribution (%)**
Experimental design and conduct, samples collection and process, data collection, result acquisition, statistical analysis, manuscript preparation	70

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	**Nature of contribution**	**Extent of contribution (%) for student co-authors only**
Vinod RMT Balasubramaniam	Participated in experiments, data collection, statistical analysis	10
Miaw-Fang Chew	Participated in experiments, data collection, statistical analysis	10
Hamdan Ahmad	Sample preparation, collection, statistical analyses	N/A (not a student registered under Monash University)
Sharifah Syed Hassan	Sample preparation, experimental design, result discussion, manuscript preparation	N/A (not a student registered under Monash University)

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature		**Date**
Main Supervisor's Signature		**Date**

2.1 General introduction

2.1.1 The importance of protein-protein interactions (PPI)

Formation of protein-protein complexes is critical for many biological functions. Disease-causing viruses are heavily dependent on PPI to achieve their viral cycles, which include virus attachment, accumulation, internalisation, replication, assembly, transportation, and release. A continuing effort to define the interactions between virus and host proteins is essential to achieve a comprehensive understanding of the processes of infection and pathogenesis. Various techniques have been utilised in interactomic studies to discover novel protein-protein interactions that carry important biological roles between viruses and hosts. Some of these techniques include *in vivo* (yeast two-hybrid, Y2H), *in vitro* (tandem affinity purification-mass spectroscopy, TAP-MS; protein microarrays; co-immunoprecipitation; phage display; X-ray crystallography), and *in silico* (*In silico* two-hybrid; phylogenetic profile) methods. Among those, the most frequently used methods are Y2H screens and TAP-MS co-complex methodologies (117).

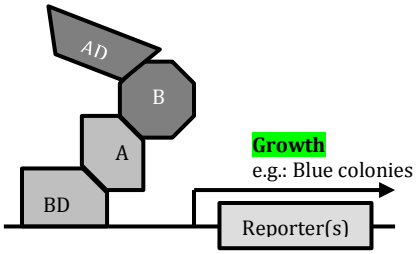
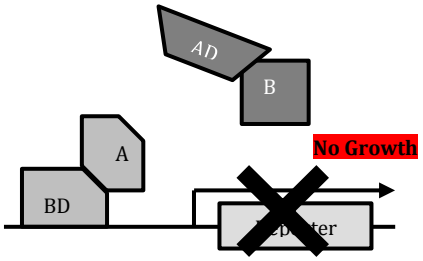
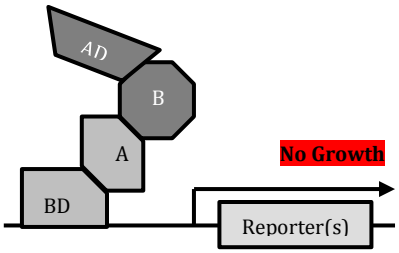
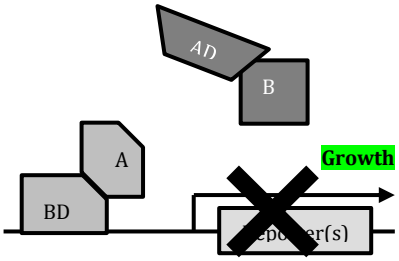
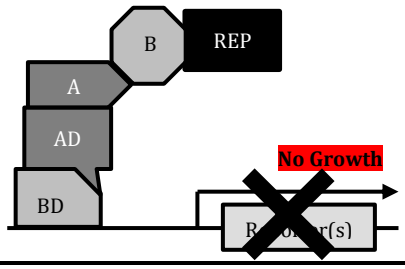
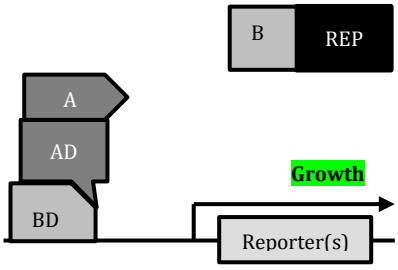
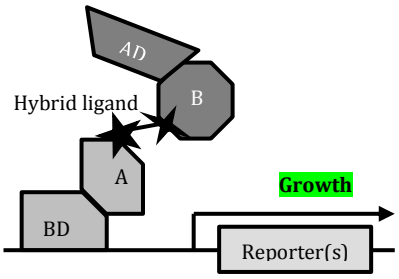
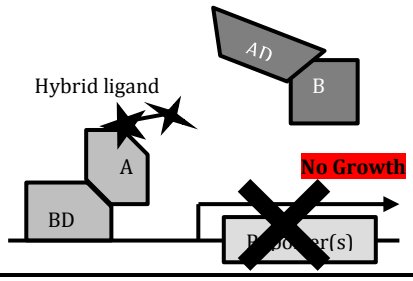
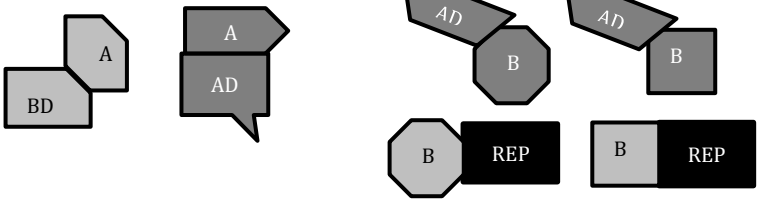
2.1.2 Y2H and TAP-MS

TAP-MS is a modified technique derived from affinity purification-mass spectrometry (AP-MS). Despite its robustness in generating biologically reliable data, TAP-MS is less accessible than Y2H due to the expensive large equipment required (e.g. mass-spectrometry machine) and highly skilled personnel. Thus, large amount of the PPI data so far have been derived from Y2H screenings. For example, approximately half of the available PPI data available on databases (e.g. IntAct and MINT) are derived from Y2H screenings (118, 119). Furthermore,

Y2H has established more than 5700 protein interaction networks in yeast (120) and about 6500 protein interactions in human (121). However, the interaction data generated by Y2H and TAP-MS are complementary to each other. TAP-MS may determine all the components of a larger protein complex, which not necessarily all interact directly with each other, although the rate of false positive is higher than that of Y2H screenings. On the other hand, Y2H studies identify defined binary protein interactions. In addition, Y2H has its limitations of detecting true positive data for membrane proteins, self-activating proteins, and proteins which require extensive and precise post-translational modifications, although articles on applying Y2H technique on these proteins are available (122, 123). Given the strengths of both methods, considerable effort is invested to overcome the remaining drawbacks, including the introduction of *LacZ* reporter gene, advancement of Y2H techniques to feed different needs, for example: reverse assay, repressed transactivator assay, and yeast 3-hybrid (Y3H) assay.

To achieve full interactome coverage, and to close the gaps in the existing PPI networks, the development and application of compartment-specific Y2H systems is increasingly important. Table 1 (page 36) summarises various existing Y2H techniques with schematic diagrams showing the respective mechanisms.

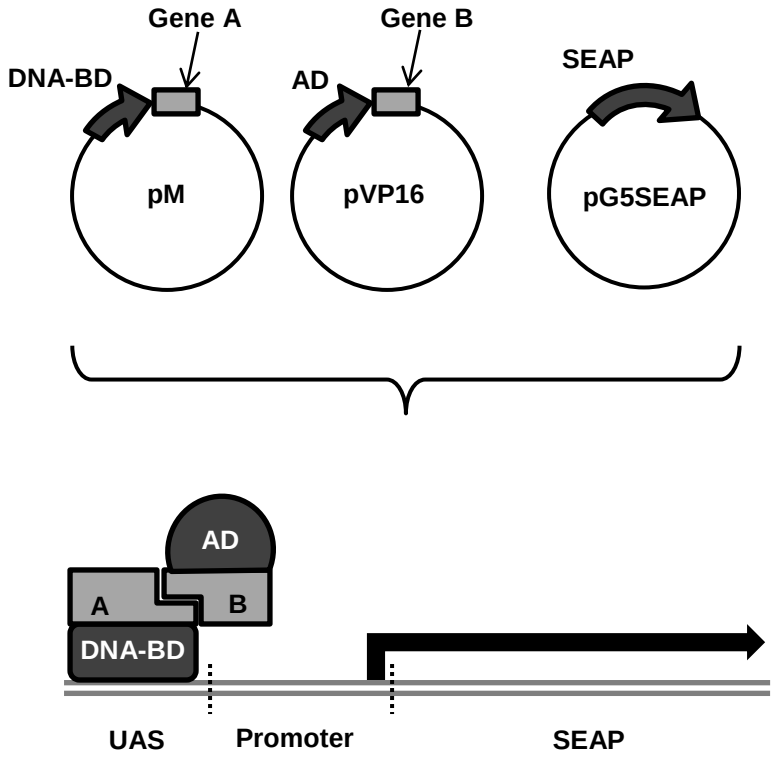
Table 1 Summary of various Y2H methodologies and schematic diagrams describe its respective mechnisms.

Y2H Methodologies	Interacting proteins	Non-interacting proteins
Forward assay		
Reverse assay		
Repressed transactivator assay		
Y3H assay		
Keys: Bait proteins Prey proteins BD: DNA binding domain AD: Activation domain A: Protein A B: Protein B REP: Repressor domain		

2.1.3 Mammalian two-hybrid (M2H) system

The M2H system features the same principle as the Y2H system – two fusion exogenous recombinant proteins are expressed *in vivo* to contain either a DNA-binding domain (DNA-BD) or an activation domain (AD). If the two proteins interact, it brings DNA-BD and AD in close proximity hence the transcription of reporter gene(s) is activated. Because these two-hybrid assays are performed in mammalian cells, the post-translational modifications of exogenous proteins are more likely to represent their native conformations, hence better mimic actual *in vivo* interactions. Therefore, the experimental results obtained are more likely to represent biologically significant interactions.

As a complementary approach to the Y2H system (124, 125), M2H system has been adopted in the research of various viruses, including highly pathogenic avian influenza virus H5N1 (126), HIV (127), SARS virus (128), and DENV (129). In this study, Matchmaker® Mammalian Assay Kit 2 (Clontech, CA, USA) was employed to validate several PPI identified through Y2H assays. The kit was equipped with Secreted Alkaline Phosphatase (SEAP) reporter gene which can be activated from the pG5SEAP plasmid upon the interaction of "bait" and "prey" proteins.



The cDNA of possible interacting protein A and B are cloned in-frame into pM and pVP16 plasmid, respectively.

pG5SEAP is the reporter plasmid.

Co-transfect mammalian cells

SEAP expression and released into the media for detection. SEAP activity reflects the binding affinity of protein A and B.

Figure 4 The procedure and mechanisms of the Matchmaker® Mammalian Two Hybrid System 2 (Clontech, CA, USA).

2.1.4 Viral interacting proteins in mosquitoes

Availability of genome sequences of the mosquito vector, *Ae. aegypti* (130), provides researchers and scientists with novel opportunities to discover multifunctional and mechanisms of genes and proteins, and the identification of host factors that are essential for dengue virus and other arbovirus infections. Research focus has been placed on arboviruses' tropisms and molecular networks/pathways that are critical for mosquito vector competency, which render infective insects to spread the respective viruses to human communities. Using computational methods, researchers have identified a large number of mosquito-DENV interacting proteins (116, 131). These interactions were mainly predicted based on protein structural similarity between pathogen and host proteins using an established method for comparing 3D structures. This was aided by known intra-species interaction of these "similar" proteins, and using data from previous screenings like RNAi or microarray screenings (131). These reports, however, are mainly designed based on *in silico* data, which lack of stringent validations and *in vivo* testing for their interaction authenticity and biological importance. On the other hand, to study protein-protein interactions between virus and mosquito cells, TAP-MS identified 18 candidates interacting with DENV and West Nile virus capsid, envelope, NS2A or NS2B proteins, these include myosin heavy or light chain, enolase, histones, and actin. These proteins are mostly involved in cellular motility, energy release, and DNA packaging, respectively (132). It was also demonstrated that down-regulation of these interacting proteins resulted in the reduction of viral replication. However, it should be noted that cell lines do not always accurately replicate or behave identically like primary cells or biologically-active organs found *in vivo*, as cell

line may lack of some of the immune privilege properties. For example, in vectorial studies, linkage to mosquito midgut epithelial barrier, midgut escape barrier, and salivary gland escape barrier are crucial. The reasons being, in a competent mosquito vector, these barriers/tissues provide the vital dynamic environment for virus penetration, replication, and release.

In this study, the midguts of female adult *Ae. aegypti* were used to construct the cDNA library. Adults mosquitoes were used as all well-differentiated tissues are found in well-developed adults, and *Ae. aegypti* has been recognised as one of the main vectors responsible for disseminating dengue viruses (96, 99, 133, 134), while the other being *Ae. albopictus*. The *Ae. aegypti* colony used in this study was of the Linnaeus strain, which has been maintained in the Institute of Medical Research, Malaysia for almost 30 years, and have been widely used in nearly all of the vector-based and transmission research studies of DENV in the country (135-138).

2.1.5 Objectives

The objectives of this chapter are:

(i) To construct and establish a robust and highly complex midgut cDNA library of derived from female adult *Ae. aegypti*.

(ii) To identify midgut proteins that interact with DENV2 viral proteins through the interactions of the cDNA library established with DENV2 E, prM, M, NS1 proteins using an improved yeast two-hybrid system from Clontech Laboratory (Matchmaker Gold Yeast Two-Hybrid System).

2.1.6 Materials and methods & Results

Both **materials and methods** and **results** have been described in the manuscript entitled:

"Protein-protein interactions between *Ae. aegypti* midgut and dengue virus 2: two-hybrid screens using the midgut cDNA library". Accepted for publication in *Journal of Infections in Developing Countries (JIDC)*. (Manuscript number: 6422)

For **materials and methods**, the manuscript includes:

1. DENV2 and cloning of viral genes
2. Mosquito rearing
3. cDNA library construction
4. DNA-Binding domain (DNA-BD) yeast preparation
5. Yeast two-hybrid screenings
6. Mammalian two-hybrid analyses
7. Double immunofluorescent assays

For **results**, the manuscript includes:

1. The quality of the cDNA library
2. Mapping DENV2 interactomes in *Aedes aegypti*
3. Identified interactomes were validated in mammalian systems
4. Double-immunofluorescence cellular co-localisations of mosquito's and DENV2 proteins

2.2 Summary of strategy and key findings

- Due to the absence of commercialised *Ae. aegypti* midgut cDNA library, the cDNA library was constructed in our laboratory using Make Your Own "Mate & Plate" cDNA Library kit (Clontech, CA, USA).
- The construction of *Ae. aegypti* midgut cDNA library required a relatively large amount of dissected midguts (>100) for total RNA extraction. This was expected as RNA was naturally unstable although RNase inhibitor cocktails were used in this study.
- The construction of midgut cDNA library derived from female adult *Ae. aegypti* allows direct "biological interactions" identification using Y2H assays.
- The Y2H system used in this study – Matchmaker® Gold Yeast Two-hybrid System, was equipped with 4 selecting markers and 2 differentiating reporter genes. This has significantly reduced the false-positive rate. However, high-stringency agars (SD/-Leu/-Trp/-His/-Ade) significantly reduced the growth rates of yeast colonies.
- False-positive yeast colonies on high stringency agars can be excluded by prolonged incubation time. False-positive colonies tend to lose its viability after a prolonged incubation period (>7 days in 30 ˚C).
- A number of putative protein interacting partners were identified from the surviving blue colonies. The list was stated in the manuscript. These protein candidates serve as fundamental knowledge for the development of new vector control strategies.

Protein-protein interactions between *Ae. aegypti* midgut and dengue virus 2: two-hybrid screens using the midgut cDNA library

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Running title

Protein interactions between mosquito and DENV

Key words

Dengue virus, *Aedes aegypti*, yeast two-hybrid, cDNA library, protein-protein interaction

Abstract

Introduction: Dengue virus (DENV), a virus causes a spectrum of disease manifestations from mild to haemorrhagic fever, is mainly transmitted by *Aedes aegypti* mosquitoes. In the absence of licensed vaccine and drug against dengue, mosquito population control remains the key strategy for disease management. One way of venturing into new vector control strategies is through understanding of the mosquito-virus relationship, especially the first mosquito organ exposed to DENV infection, the midgut.

Methodology: We constructed a midgut cDNA library derived from female adult *Ae. aegypti* mosquitoes. The cDNA library was using yeast *Saccharomyces cerevisiae* with high cDNA complexity. Gal4-based yeast two-hybrid (Y2H) assays were performed against DENV2 proteins (E, prM, M, and NS1). Validation assays such as mammalian two-hybrid (M2H) and double immunofluorescence assays (IFA) were conducted to prove the authenticity of a few selected interactions.

Results: Putative interacting proteins between *Ae. aegypti* midgut cells and DENV2 were listed. These proteins, including several hypothetical proteins, were identified from VectorBase database involved in several important biological activities, including protein transcription, translation, ATP production, immunity, DNA replication, protein digestion, and cellular ionic regulation. In addition, validation assays (M2H and double IFA assays) resulted in positive outcomes.

Conclusions: This report has not only discussed the construction of *Ae. aegypti* midgut cDNA library for Y2H screenings, but also identified and validated several putative *Ae. aegypti* mosquito midgut cell proteins interacting with DENV2. It has

paved an avenue towards a more comprehensive understanding of DENV replication *Ae. aegypti*.

Introduction

Insect-borne diseases, particularly those transmitted by mosquitoes, are among the leading causes of mortality and morbidity in humans. Dengue fever and dengue haemorrhagic fever – a globally emerging insect-borne disease threatening a third of human populations, is transmitted to humans by the mosquitoes *Aedes aegypti* and *Aedes albopictus*. During a bloodmeal of a dengue-infected person by *Aedes* mosquito, dengue virus (DENV) is ingested into the mosquito midgut. It is generally believed that mosquito midgut carries important cellular membrane receptors which facilitate viral entry through receptor-mediated endocytosis, enabling replication, exocytosis, followed by DENV disseminations to salivary glands for transmission to human.

Dengue virus, a member of the genus *Flavivirus* and family *Flaviviridae*, is an enveloped positive single-stranded RNA virus approximately 50 nm in diameter. Its 11 kb RNA genome encodes a polyprotein which, after translation, is cleaved into 10 individual proteins during maturation by host proteases (example: furin) and viral protease complex. The 10 proteins are designated as capsid (C), pre-membrane (prM), envelope (E) and 7 non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). DENV exists as 4 serotypes (DENV1-4) and is related to the viruses that cause yellow fever, Japanese, St. Louis, tick-borne, and West Nile encephalitis.

Most previous studies on DENV replication mechanisms have been conducted on mammalians or mammalian cell lines [1-5]. These studies emphasised on proteins or host cellular factors that may confer DENV susceptibility, with less research focus on DENV infection in mosquito *Ae. aegypti* and *Ae. albopictus*. Although

tubulin/tubulin-like protein and prohibitin were described as putative DENV receptors in mosquitoes [6,7], these studies utilised virus overlay protein binding assay (VOPBA), a technique that was not designed for direct *in vivo* "biological interaction". Furthermore, the capability of the VOPBA method is very limited as most of the identified proteins can only be reported as molecular weights [8-12].

To date, very limited study is conducted to determine the interactome between DENV proteins and mosquito cellular proteins using sensitive and reliable protein interaction assays. Hence, this study has been driven to identifying adult mosquito *Ae. aegypti* midgut proteins interacting with DENV2 viral proteins, using an improved yeast two-hybrid (Y2H) screening system, a high-throughput screening assay which allows identification of genuine protein interacting partners *in vivo*. This study revealed a list of putative protein interacting partners in mosquito during DENV infection. Some of these interactions were further validated by additional assays such as mammalian two-hybrid (M2H) and double immunofluorescent assay (IFA). This study allows new insights into possible virus-vector interactions.

Materials and Methods

DENV2 and the cloning of viral genes

DENV2 (MY89-88549, AJ556804) was kindly provided by Professor Sazaly AB, University of Malaya. DENV2 was propagated in C6/36 (ATCC® CRL-1660™) and Vero cells (ATCC® CCL-81™) until the development of cytopathic effect. The virus titer was determined at 10^6 $TCID_{50}$/ml. The nucleotide sequences of DENV2 genes (E, prM, M, and NS1) were obtained from the NCBI (National Centre for Biotechnology Information, http://www.ncbi.nlm.nih.gov/). These genes were amplified using *Pfu* DNA polymerase and cloned in-frame into the Y2H bait vector pGBKT7 (Clontech,

CA, USA), namely pGBKT7-E, pGBKT7-prM, pGBKT7-M, and pGBKT7-NS1, respectively. All plasmid constructs were sequenced with no mutation found.

Mosquito rearing

Mosquito (*Ae. aegypti*) colonies (Linnaeus) [13,14] were established and maintained at 28±1 ℃ under 70-75 % relative humidity, with a light/dark cycle of 12 h/12 h. Newly hatched larvae were reared in trays, each contained 750 ml of mineral water with about 2.5 cm water level. Each tray was provided with 5 cat food pellets (Friskies Senior). Adult mosquitoes were given *ad libitum* access to 10 % sucrose solution.

cDNA library construction

Adult mosquitoes were harvested and stored at -80 ^{o}C. Midguts of 50 female adult *Ae. aegypti* mosquitoes were dissected as previously described [15], and were subject to total RNA extraction by a combination method of both Trizol® reagent (Life Technologies, CA, USA) and Dynabeads® mRNA purification kit (Life Technologies, CA, USA) [16]. Purified mRNA served as the template for the first- and second-strand cDNA synthesis, using Make Your Own "Mate & Plate™" Library System (Clontech, CA, USA). Double-stranded cDNA was purified and nucleic acid less than 400 bp was discarded using Chroma Spin™ TE-400 Columns (Clontech, CA, USA). These were unwanted oligonucleotide products of incomplete first- and second-strand cDNA synthesised. To allow *in vivo* recombinational cloning, purified double-stranded cDNA, in conjunction with 3 µg of linearised pGADT7-rec vector, and 200 µg of denatured Yeastmaker Carrier DNA (Clontech, CA, USA) were co-transformed into competent yeast Y187 cells using lithium acetate (LiAc) method as previously described [17]. Transformed yeast cells were mixed with Yeast-Peptone-Dextrose

(YPD) Plus medium (Clontech, CA, USA) for better transformation efficiency. Then, pelleted cells were re-suspended in 15 ml of 0.9% (w/v) NaCl solution (primary cDNA library), prior to spreading on leucine-depleting agar plates (SD/-Leu). After 4-day incubation (30 ℃), survived colonies were pooled and kept in 50-ml or 1-ml aliquots for storage in -80 ℃ until use (amplified cDNA library). The library titer of primary and amplified cDNA library were checked by spreading 100 µl of 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}-diluted yeast culture on SD/-Leu agar plates (diluted in 0.9% NaCl solution), incubated for 4 days at 30 ℃. Then, yeast colony forming unit/ml (cfu/ml) was calculated. On the other hand, the library complexity test was conducted by randomly selecting 20 independent colonies (out of 75 colonies on the SD/-Leu agar plate of 10^{-3} dilution). The colonies were screened for cDNA inserts using Matchmaker 5' and 3' AD LD-Insert Screening Amplimers (Clontech, CA, USA). The PCR cycling parameters were as follows: 98 ℃ for 2 min; 40 cycles of 98 ℃ for 10 sec, 60 ℃ for 15 sec, 68 ℃ for 2.5 min; 68 ℃ for 5 min; hold at 4 ℃.

DNA-Binding domain (DNA-BD) yeast preparation

Yeast strains Y2HGold was transfected with respective bait plasmids, and maintained on synthetic-dropout (SD) medium lacking tryptophan (SD/-Trp). All DNA-BD yeast clones were tested for autoactivation in the absence of prey protein. No blue colonies were observed on SD/-Trp and SD/-Trp/X-α-gal agar plates, and no surviving colony was found on SD/-Trp/X-α-gal/Aureobasidin A (Aba, 125 ng/ml) plates. This indicated the lack of ability of our DNA-BD yeast clones to autoactivate the reporter genes in the absence of interacting prey protein.

Yeast two-hybrid screenings

Y2H was conducted using Matchmaker® Gold Yeast Two-Hybrid System (Clontech, CA, USA), according to manufacturer's instructions. In brief, an overnight 5-ml Y2HGold culture and a 1-ml library aliquot were mixed and mating was allowed for 24 hours in 50 ml of 2x YPDA broth (30 ℃, 45 rpm). Yeast cells were plated and incubated at 30 ℃ for 2 days on low-stringency agar plates (SD/-Trp/-Leu, DDO) in the presence of Aba and X-α-gal, followed by high-stringency agar plates (SD/-Leu/-Trp/-Ade/-His, QDO) supplemented with Aba and X-α-gal. Agar plates were incubated in 30 ℃ for 4-6 days. Blue colonies were marked and subject to plasmid extractions and DNA sequencing for cDNA identification.

Mammalian two-hybrid analyses

Three protein interactors (AAEL003582, AAEL005508, and AAEL007818) were selected for M2H analyses. All 3 genes were cloned in-frame into plasmid vector pVP16 (DNA-AD) using primers specific to ribosomal protein S15p/S13e (forward: GGGAATTCGGTCGTATGCACGCTCCCGGTAAG, reverse: ACGGATCCGGCAACCAGGGCCGAGGCGGTG), NADH-ubiquinone oxidoreductase (forward: GGGAATTCCTGACAAACTCCTTTAAAATAATTC, reverse: ACGGATCCAAAGCCTGTTTGCATTCCGAATC), and trypsin (forward: GGGAATTCAACCAATTTCTCTTTGTCAG, reverse: ACGGATCCAACCTCGGAAACCTCTCGGATC). Restriction sites were underlined. According to Fig. 4, their respective viral protein interacting partners were cloned in-frame into pM (GAL4 DNA-BD) cloning vector (Matchmaker Mammalian Assay Kit 2, Clontech, CA, USA). Using calcium phosphate transfection technique [18], along with pG5SEAP, all plasmids were co-transfected into Vero cells (ATCC® CCL-81™), according to the scheme in Fig. 4. This assay was conducted in biological triplicates. Transfected cells were incubated at 37 ℃, 5% CO_2 for 48 hours before culture media

were harvested for SEAP activity measurements using GreatEscAPe™ SEAP Chemiluminescence Detection Kit (Clontech, CA, USA). The intensity of chemiluminescence signals were measured by VICTOR™ X5 Multilabel Plate Reader (PerkinElmer).

Double immunofluorescence assays

The cDNA of *Ae. aegypti* proteins (AAEL003582, AAEL005508, and AAEL007818) were amplified and cloned in-frame into pIB/V5-His vector (Invitrogen, CA, USA). These plasmid constructs were transfected into C6/36 (ATCC® CRL-1660™) of 80% confluency using Lipofectamine® 2000 (Invitrogen, CA, USA), according to manufacturer's instructions. After 24-hour incubation at 28 ℃, cells were infected with DENV2 (MOI: 1.0), followed by an additional 48-hour incubation under the same conditions. Then, cells were fixed with 4% paraformaldehyde (15 min, room temperature). Background blocking was performed with 1% BSA in 1x SSC buffer (20 min, room temperature). Proteins of interest were captured by anti-V5 antibody (Invitrogen, CA, USA) and each respective anti-DENV2 antibodies (GTX103346, GTX128093, GTX103345), followed by fluorescent staining using AlexaFluor® 488 and 594 (Invitrogen, CA, USA). Cells were observed using an Olympus IX81 fluorescent microscope.

Results

The quality of the cDNA library

Detection of the transformation efficiency, library titer, and library quantity of the cDNA library

The transformation efficiency, library titer, and library quantity were calculated according to the formulas described previously [19]. Yeast colonies appeared on the SD/-Leu agar plates (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) were counted. The transformation efficiency of the primary cDNA library was calculated at 1.125×10^6 transformants (> 1×10^6 transformants), and the library titer was 7.5×10^4 cfu/ml (> 6.7×10^4 cfu/ml). After 4-day incubation at 30 ℃, the cell density of the liquid mixtures collected from the plates was measured using a haemocytometer. Cell density was adjusted to 8.23×10^8 cells/ml (> 2×10^7 cells/ml) by reducing the volume of the suspension by centrifugation. Then, the amplified cDNA library titer and library quantity were determined. The amplified cDNA library titer was 2.13×10^7 cfu/ml, and the amplified cDNA library quantity was 1.491×10^9 cfu (Table 1).

Identification of the length of the inserts

In order to identify the length of cDNA inserts in the cDNA library, 20 individual yeast colonies were randomly selected from SD/-Leu plates of 10^{-3} dilution, and subjected to PCR screening using Matchmaker 5' and 3' AD LD-Insert Screening Amplimers (Clontech, CA, USA). In Fig. 2, most of the fragments were more than 250 bp, with sample 11, 14, 15, and 20 which carried fragments of more than 3 kb. Since the cDNA inserts with various lengths were detected in 20/75 colonies at 10^{-3} dilution, therefore, this reflects the high complexity of cDNA inserts of various genes present in the undiluted library.

Identification of the recombination rates of the cDNA library

The insert fragments length of the screened cDNA library ranged from 153 bp to 3243 bp, with an average length of 1403 bp (Table 1). Six samples were between 0.3 and 0.5 kb (14.29%), 10 samples were between 0.5 and 1.0 kb (23.81%), 8 samples were

between 1.0 and 1.5 kb (19.05%), 7 samples were between 1.5 and 2.0 kb (17.07 %), 7 samples were between 2.0 and 2.5 kb (17.07 %), and 4 samples were more than 2.5 kb (9.52%) (Table 2). These data were obtained from the identified putative protein-protein interactions as listed in Table 3.

Mapping DENV2 interactomes in *Aedes aegypti*

We identified 42 putative protein-protein interactions between the midgut cells of *Ae. aegypti* and DENV2 proteins (E, prM, M, NS1). A biological network showing information of protein-protein interaction was constructed and visualised using Cytoscape software (Fig. 3). There were 40 nodes and 42 edges in the network. Protein IDs were in accord with VectorBase Bioinformatics Resource Centre (https://www.vectorbase.org/). Table 3 tabulated the details of each interactions including Gene IDs, coding sequence (CDS) lengths, deduced protein sizes (kDa), and protein identities.

The cDNA identification analysis showed a number of putative *Ae. aegypti* midgut protein interaction partners of their respective DENV2 proteins. Our results suggested 16 proteins interacting with DENV2 NS1 protein, 10 proteins with DENV2 prM protein, 7 proteins with DENV2 E protein, and 9 proteins with DENV2 M protein (Table 3). Although the genome of *Ae. aegypti* has been published [20], several hypothetical proteins with minimal match with any gene of other species were listed (AAEL004869, AAEL010974, AAEL007974).

Identified interactomes were validated in mammalian systems

As a complementary study to Y2H, mammalian two-hybrid (M2H) assays utilises mammalian cells which undergo more comprehensive post-translational modifications

of proteins, hence may better mimic *in vivo* interactions [21]. We conducted M2H assays using Vero cells to validate the three selected *Ae. aegypti* putative protein candidates found interacting with their respective DENV2 proteins. These proteins were ribosomal protein S15p/S13e [VectorBase: AAEL003582], NADH-ubiquinone oxidoreductase [VectorBase: AAEL005508], and trypsin [VectorBase: AAEL007818]. The reporter plasmid, pG5SEAP, when activated by the physical interaction of pM- and pVP16-conjugated proteins, encodes alkaline phosphatase which is secreted to the extracellular environment. Hence, the activity of alkaline phosphatase is a quantitative reflection of the interaction between pM- and pVP16-conjugated proteins. In Fig. 4, the alkaline phosphatase activities measured from each conjugated protein partners (Fig. 4A) were significantly higher than the background controls (Fig. 4B, C, and E) and negative controls (Fig. 4F), but lower than positive controls (Fig. 4D). Cells in positive controls were transfected with pM3-VP16, which expressed fusion proteins consisted of Gal4 DNA-BD and VP16 AD.

Double-immunofluorescence cellular co-localisations of mosquito's and DENV2 proteins

Double IFA enables the visualisation of intracellular proteins. Fig. 5 illustrated the cellular distribution and co-localisation of DENV2 proteins (NS1, prM, and E) and *Ae. aegypti* midgut proteins (ribosomal protein, NADH-ubiquinone oxidoreductase, and trypsin) after transfection of V5-tagged plasmid constructs into C6/36 cells, followed by infection with DENV2. White arrows in the merged images showed the regions where the protein-protein co-localisations may occur. Other than the interaction between NS1 and ribosomal protein, which distribution spreads across the whole cell, the rest of the protein candidates exhibited a sub-cellular co-localisation (Fig. 5B &

5C). Line profiles demonstrated the intensities of red and green fluorescence across the red lines shown in the insets (Fig. 5).

Discussion

During a bloodmeal, the midgut of *Ae. aegypti* is the first organ exposes to DENV infection, thus serves as the main target to confer infectivity to the vector. In attempts to reveal molecular interactions between the mosquito midgut and DENV proteins, a feasible approach was to construct a cDNA library of the midgut of female adult *Ae. aegypti* and applied to Y2H screens against DENV2 proteins (E, prM, M, and NS1).

Although Y2H technology is generally known to generate a high false positive rate, the Matchmaker® Gold Yeast Two-Hybrid System (Clontech, CA, USA) used in this study has been optimised and improved by the manufacturer for a remarkable reduction of false positivity. Three distinct Gal4-responsive promoters were employed to control four integrated reporter genes (*AUR1-C*, *HIS3*, *ADE2*, *MEL1*). The three promoters are unrelated except for the short protein binding sites in the UAS region (a regulatory region specifically bound by Gal4 DNA-BD). This feature automatically screens out the library proteins that interact with unrelated sequences flanking the UAS region (i.e. false positives). On the other hand, the four reporter genes (*AUR1-C*, *HIS3*, *ADE2*, *MEL1*) have distinct functions in encoding inositol phosphoryl ceramide synthase (an enzyme that confers strong resistance to the highly toxic Aureobasidin A), biosynthesis of histidine or adenine to facilitate yeast cell growth on a histidine- or adenine-depleted medium, and encodes α-galactosidase (generates blue pigments in the presence of X-α-gal) for colorimetric selection, respectively. With these features, a number of studies have revealed novel protein-protein interaction using the Matchmaker® Y2H system [22-24]. However, in order to validate the quality of the

cDNA library constructed, and to ensure the high performance of the Y2H mechanisms employed, we performed additional assays including M2H and double IFA on 3 selected mosquito midgut proteins, i.e. ribosomal protein S15p/S13e [VectorBase: AAEL003582], NADH-ubiquinone oxidoreductase [VectorBase: AAEL005508], and trypsin [VectorBase : AAEL007818] that interacts with NS1, prM and E proteins, respectively.

Previously, the first draft of the mosquito protein interaction network using a computational approach was presented by Guo *et al*. The research group reported 714 *Ae. aegypti* proteins with closely related functions in the replication/transcription/translation, immunity, transport, and metabolic pathways [25]. In another study, Mairiang *et al*. identified several mosquito protein interacting partners of DENV2 C, prM, NS3, NS4A, NS4B, and NS5 proteins [26]. Interestingly, the study identified similar proteins which were also identified in our study. These include human ribosomal protein and mosquito carboxypeptidase, which were suggested to interact with DENV C protein. However, although most of the earlier studies have identified putative interacting partners of DENV proteins, very limited information is available on mosquito protein interactors of DENV E and NS1 proteins. In our study, beside prM and M, we also identified mosquito midgut proteins interacting with DENV2 E and NS1 proteins using Y2H screenings, which have not been performed elsewhere.

This study revealed putative *Ae. aegypti* midgut proteins (identified and hypothetical) interacting with their respective DENV2 proteins. According to protein databases (VectorBase and Uniprot), these proteins were deduced to be involved in different biological processes including translation and transcriptional regulations, DNA and cell cycle regulations, mitochondria energy productions, protein digestions, immunity,

neurotransmitter regulations and cellular ionic balance regulations. Among the proteins identified, three proteins were selected for validation of the interactions (i.e. ribosomal protein S15p/S13e, NADH-ubiquinone oxidoreductase, trypsin).

Ae. aegypti ribosomal protein S15p/S13e, a candidate protein interactor of DENV2 NS1, has been shown to be present in the nucleus and cytoplasm of C6/36 cells. Despite its crucial role in protein translation, ribosomal protein's involvement in RNA virus replication and dissemination has been well studied [27,28]. Two recently published reports demonstrated the versatility of ribosomal proteins in either facilitating or inhibiting viral growth during infection [29,30]. In addition, although ribosome has traditionally been thought to function as the catalytic machinery for translational elongation, Lee *et al.* showed that ribosomal subunit protein rpL40 also acts as requisite for vesicular stomatitis virus (VSV) cap-dependent translational regulation [31]. Another ribosomal protein, p40 [VectorBase: AAEL008083/AAEL013694], is a precursor of the high affinity laminin receptor, a putative receptor for DENV entry into host cells [32]. In addition, p40 has been shown to shuttle between the cytoplasm and the nucleus [33]. Like p40, the ribosomal protein S15p/S13e was also suggested to be present throughout the C6/36 cells (in nucleus and cytoplasm).

As for proteins involved in transcription, we also identified *Ae. aegypti* TOLL pathway signaling NF-kappaB Relish-like transcription factor [VectorBase: AAEL007696], GATA transcription factor (GATAb) [VectorBase: AAEL006447], and HR3 protein [VectorBase: AAEL009588] interacting with DENV2 prM or M proteins. This finding can be linked to the ability of RNA viruses (including DENV) to regulate its host cell gene expression profiles [34]. Moreover, we also identified several mosquito proteins predicted to be involved in DNA replication or repair and

cell cycle regulation, namely DNA topoisomerase [VectorBase: AAEL007683] and cell cycle checkpoint protein rad17 [VectorBase: AAEL007649]. These proteins were heavily involved in cell replication, with processes that can lead to apoptosis, if not regulated accurately [35].

Another DENV2 prM protein interacting partner, i.e. *Ae. aegypti* NADH-ubiquinone oxidoreductase, is a 24 kDa subunit [VectorBase: AAEL005508] protein with provisional function similar to NADH-ubiquinone oxidoreductase found in mammalians. NADH-ubiquinone oxidoreductase catalyses NADH to NAD^+, reduces ubiquinone, and transports protons across the inner membrane of mitochondria. Meanwhile, this enzyme also reduces O_2 to superoxide, leading to cellular oxidative stress [36]. Previous studies reported the generation of superoxide in mosquito cells during DENV infection [37], and the overexpression of quinone oxidoreductase being recognised as the major contributor to reactive oxygen species formation [38]. Since the accumulation of this enzyme in the midgut of DENV-infected mosquito was observed [39], the biological interaction between DENV2 prM protein and *Ae. aegypti* NADH-ubiquinone oxidoreductase may be the major contributor in the regulation of the oxido-reduction mechanism to manipulate DENV infection in mosquitoes. In addition, other proteins like acyl-coa dehydrogenase [VectorBase: AAEL005732], ATP synthase beta subunits [VectorBase: AAEL002827 & AAEL003393] and voltage–dependent anion-selective channel [VectorBase: AAEL001872] predicted to be located in mitochondria, were shown to interact with DENV2 NS1 protein.

Cold-induced protein (BnC24A) [VectorBase: AAEL005097] was suggested to interact with DENV2 prM protein. This protein is provisional and a homologue to the cold-induced protein BnC24 found in rapeseed (*Brassica napus*). Although Saez-

Vasquez J. *et al*. suggested the possible role of rapeseed BnC24A protein in facilitating mRNA accumulation in response to cold treatment [40], the function of BnC24A in *Ae. aegypti* midgut cells upon DENV infection has not been reported. Another protein, the class B scavenger receptor CD36 (SRB), which is a cell surface glycoprotein, is present on a variety of cell types, including *Ae. aegypti* hemocytes [41]. The possibility of *Ae. aegypti* SRB [VectorBase: AAEL005981] interacting with DENV2 prM/M proteins opened a new avenue towards a more comprehensive understanding of mosquito antiviral mechanisms during DENV infection. Previously, SRB in ixodid ticks (*Haemaphysalis longicornis*) was found to play a key role in granulocyte-mediated phagocytosis to invading *Escherichia coli* and contributed to the first-line host defense against various pathogens [42]. Also, other studies on SRB in insects revealed the critical roles of this protein in cellular lipid regulations [43] and dietary carotenoids uptake [44].

We also identified a few digesting enzymes that were found interacting with prM, E, or M, namely zinc carboxypeptidase [VectorBase: AAEL008599, AAEL001863, AAEL012781] and trypsin [VectorBase: AAEL007818]. Carboxypeptidase, a well-known hydrolytic enzyme involved in C-terminal peptide cleavage, was also found to be highly regulated after bloodmeal [45]. In mosquito, carboxypeptidase has not only been found to be involved in sexual development of malarial protozoan parasites in the midgut [46], but also in the interaction between carboxypeptidase and DENV capsid protein in the salivary gland of *Ae. aegypti* [26]. In addition, carboxypeptidase D has been continuously proven to be a receptor for duck hepatitis B virus [47]. These studies show the diverse roles of carboxypeptidases in pathogen invasions, including viruses.

The interaction between trypsin and E protein was further validated using M2H assays and double IFA. It was shown that trypsin-E interaction was located in the subcellular region of C6/36 cells, however, the cellular organelle at which the interaction may occur needs to be identified. This interaction is supported by the presence of trypsin activity in mosquito midgut which peaked at 3 hours after blood feeding, and that tryptic digestion of viral surface proteins enhances the infectivity of DENV2 in mosquito midgut cells, but was unable to support viral replication [48].

Our results also suggested that *Ae. aegypti* sensory neuron membrane protein-1 [VectorBase: AAEL005374] and acetylcholinesterase [VectorBase: AAEL000511] interact with DENV2 E or M proteins. These interactions may be associated with behavioural changes of mosquito after DENV infection. Behavioural changes, especially in the avidity to feed in *Ae. aegypti* after infection with DENV has been well studied [49]. Therefore, these interacting proteins may be involved in the pathogenesis of behavioural changes of its vector mosquitos.

Some immune-related proteins, namely suppressors of cytokine signalling [VectorBase: AAEL000393] and clip-domain serine protease family C [VectorBase: AAEL012713], were also putative interacting proteins for DENV2 NS1 protein. These proteins were reported to be related to innate immunity in insects, such as malaria parasites and bacteria [50,51]. In a recent study, the possible role of NS1 in regulating host immune system has also been reported [52]. Lastly, several hypothetical proteins were identified in this study [VectorBase: AAEL012947, AAEL004869, AAEL010974, AAEL007974]. These proteins were documented in VectorBase without predicted functions and identifications.

In conclusion, in this study, we reported the construction of a new cDNA library of female adult *Ae. aegypti* midgut, with tests to justify its complexity, robustness, and quality for use in Y2H screening studies against DENV2 proteins (E, prM, M, NS1). A number of putative mosquito midgut proteins that interact with DENV proteins were identified. Additional validation assays (M2H and double IFA) conducted for some selected mosquito midgut proteins supported the quality of the cDNA library and the robustness of the Y2H mechanisms. Although investigation into the biological relevance of the reported interactions is necessary, this preliminary study has paved a pathway and direction towards the investigations of the candidate proteins on their importance for the virus replication cycle inside insect cells. The employment of several decisive methodologies, such as RNA interference (RNAi), could aid in functional validations. Besides DENVs, the cDNA library can also be applied for the discovery of novel interacting proteins of other mosquito-borne viruses such as Yellow Fever and Chikungunya virus. This study will guide future research into dissecting and targeting these proteins in vector control or dengue prevention.

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Conflict of Interest

The authors declare no conflict of interest

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Figures

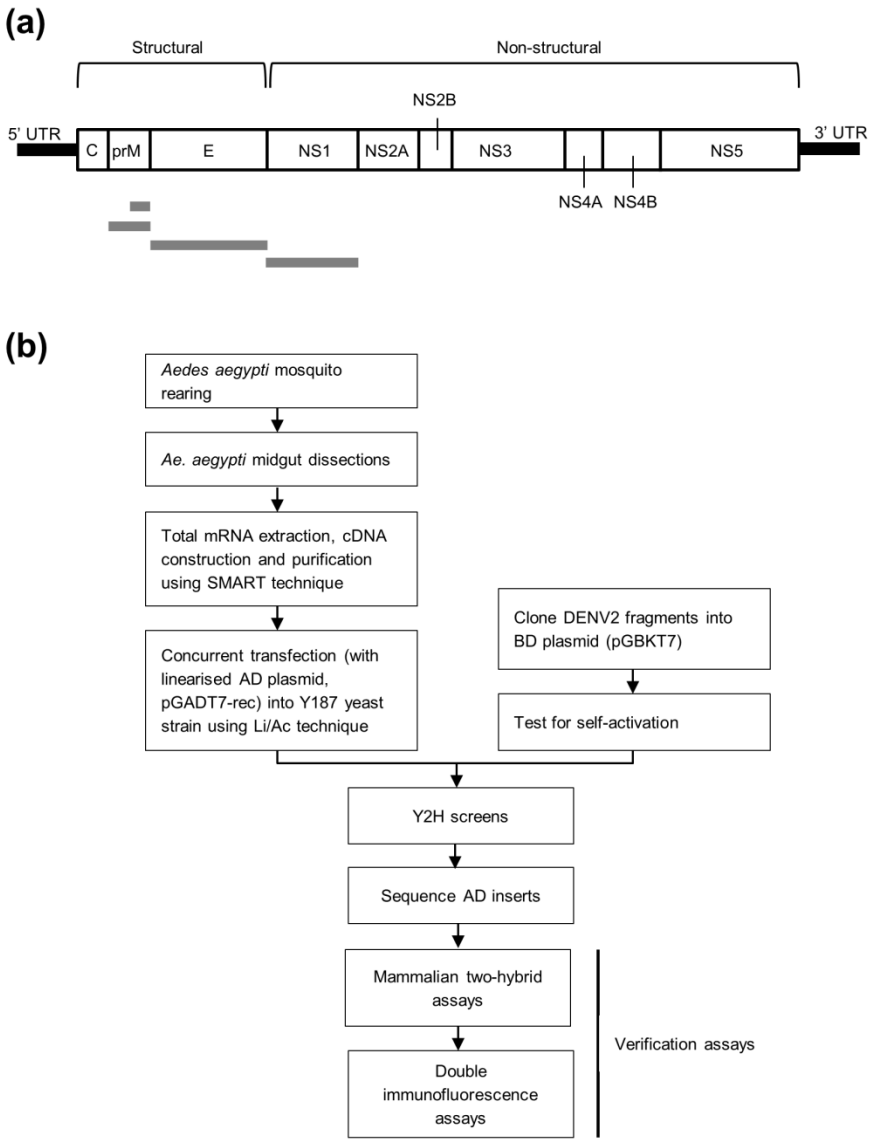

Figure 1. Overview of yeast two-hybrid (Y2H) screens to identify putative *Ae. aegypti* midgut cellular proteins interacting with DENV2 proteins. (**a**) The organisation of DENV2 genome and fragments (in grey) used in Y2H screens with *Ae. aegypti* cDNA library. (**b**) Flowchart of the Y2H approach to screen for *Ae. aegypti* proteins targeted by DENV2.

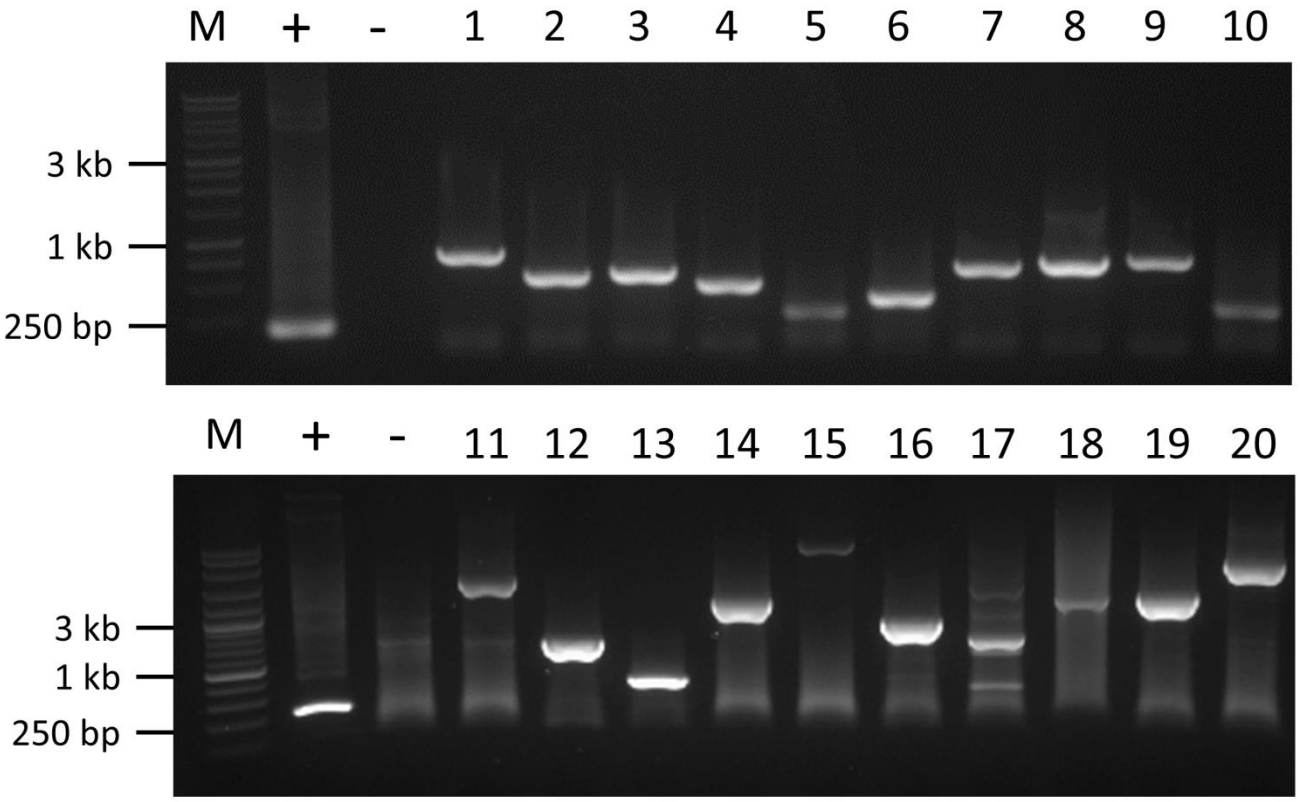

Figure 2. Library complexity check via colony PCR amplification from 20 randomly-selected yeast colonies. (+) Positive controls, amplification of the empty vector pGADT7 using Matchmaker 5' and 3' AD LD-Insert Screening Amplimers. (-) Negative controls, template was substituted by nuclease-free water. (**M**) DNA marker. (**1-20**) 20 randomly selected yeast colonies from 10^{-3}-dilution SD/-Leu agar plate yielded individual cDNA fragments ranged between 300 bp to 4500 bp. This indirectly reflects the high cDNA complexity present in the undiluted library.

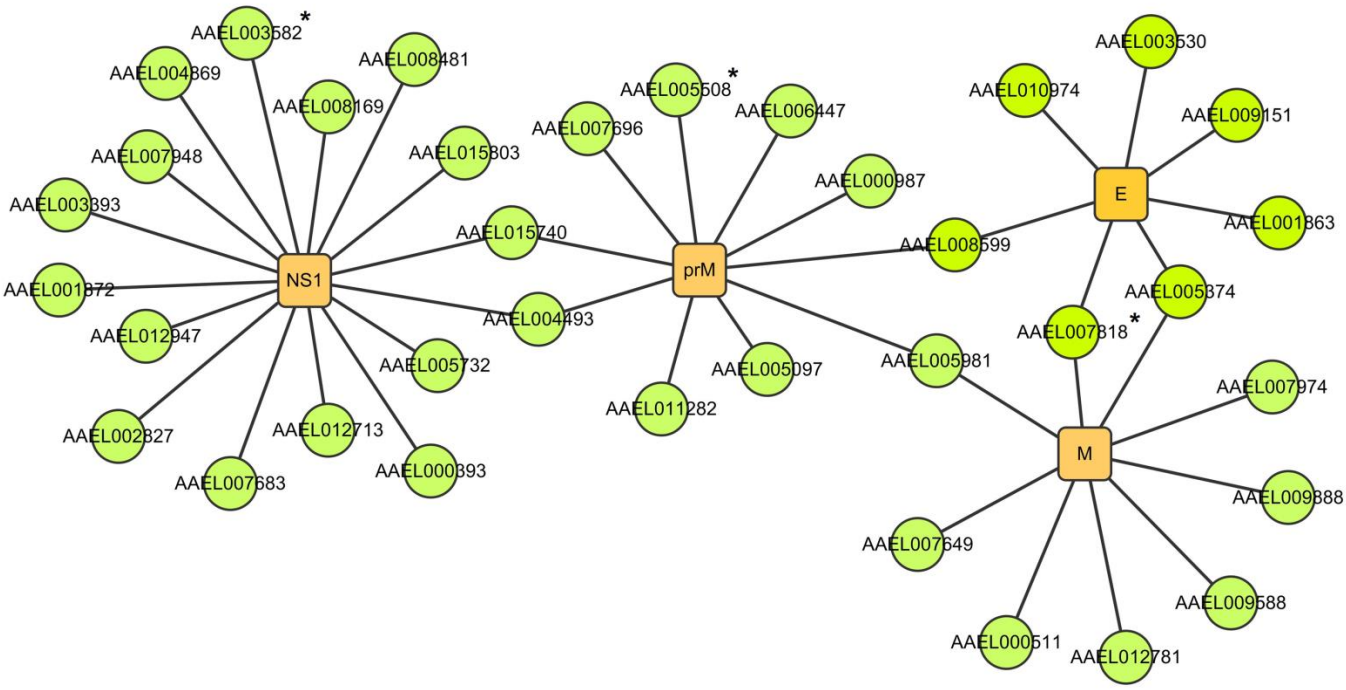

Figure 3. The putative *Aedes aegypti* midgut cellular protein candidates which were suggested to interact with their respective DENV2 proteins. Square nodes represent DENV2 proteins while round nodes represent proteins identified from female adult *Ae. aegypti* midguts. Nodes are connected to indicate the PPIs found in our yeast two-hybrid screenings in this study. Proteins marked asterisks (*) were subjected to mammalian two-hybrid and double immunofluorescent assays. Protein IDs are in accord with VectorBase Bioinformatics Resource Centre.

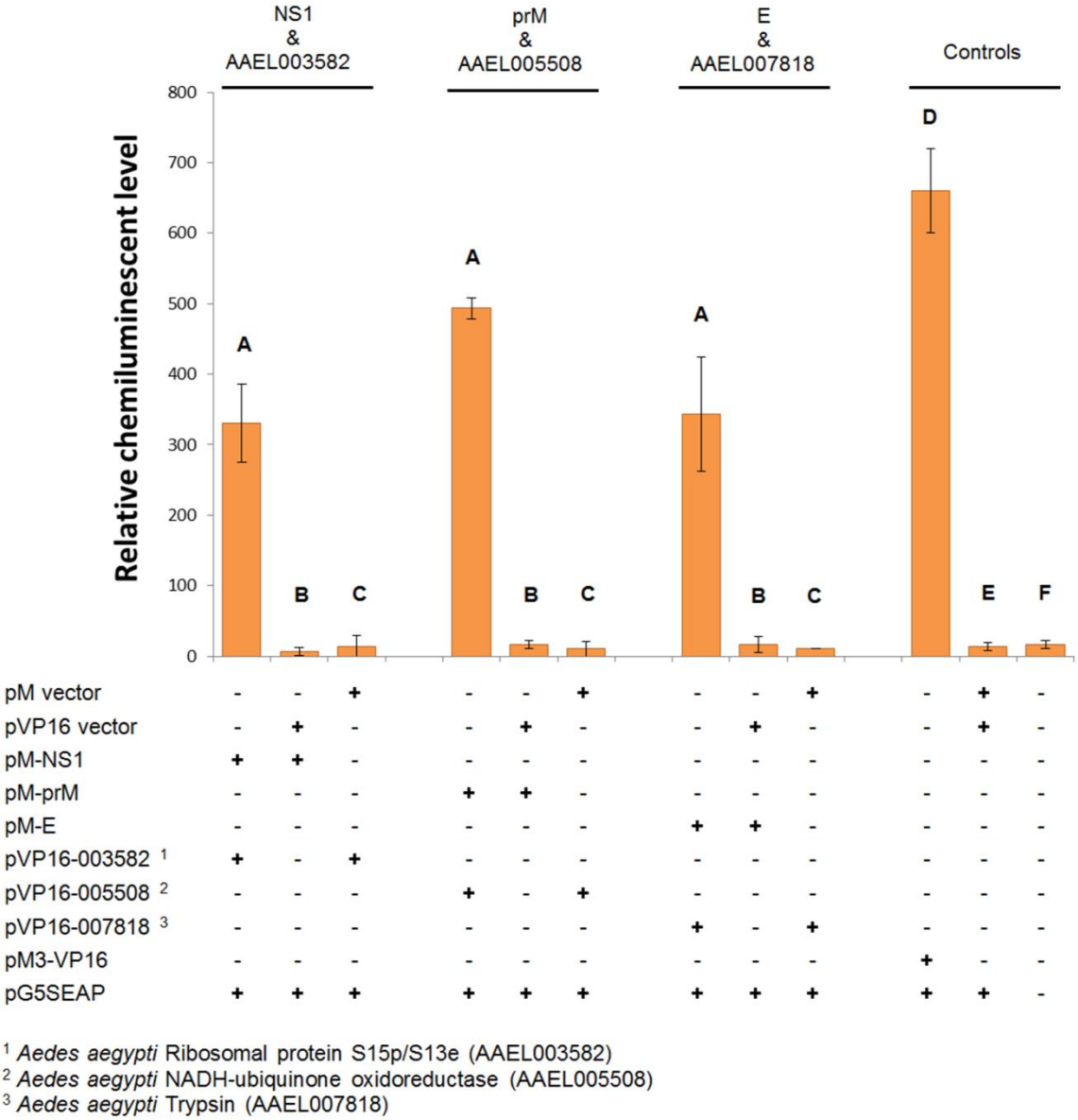

Figure 4. Mammalian two-hybrid (M2H) assays to validate the interaction between *Aedes aegypti* midgut proteins and DENV2 proteins in Vero cells. Columns show mean values, error bars standard deviation of 3 samples (n=3). Each column represents a treatment and is labelled "+" for the plasmid transfected and "-" for the plasmid that is absent. Each independent experiment was constituted of 3 assays, labelled A, B, and C. Cells were transfected with: (**A**) pM & pVP16 vectors with respective cDNA inserts; (**B**) pM with cDNA inserts & empty pVP16 vectors; (**C**) empty pM vectors & pVP16 with cDNA inserts. For controls, cells were transfected with: (**D**) positive control plasmid pM3-VP16; (**E**) empty pM & pVP16 plasmids. (**F**) Non-treated controls.

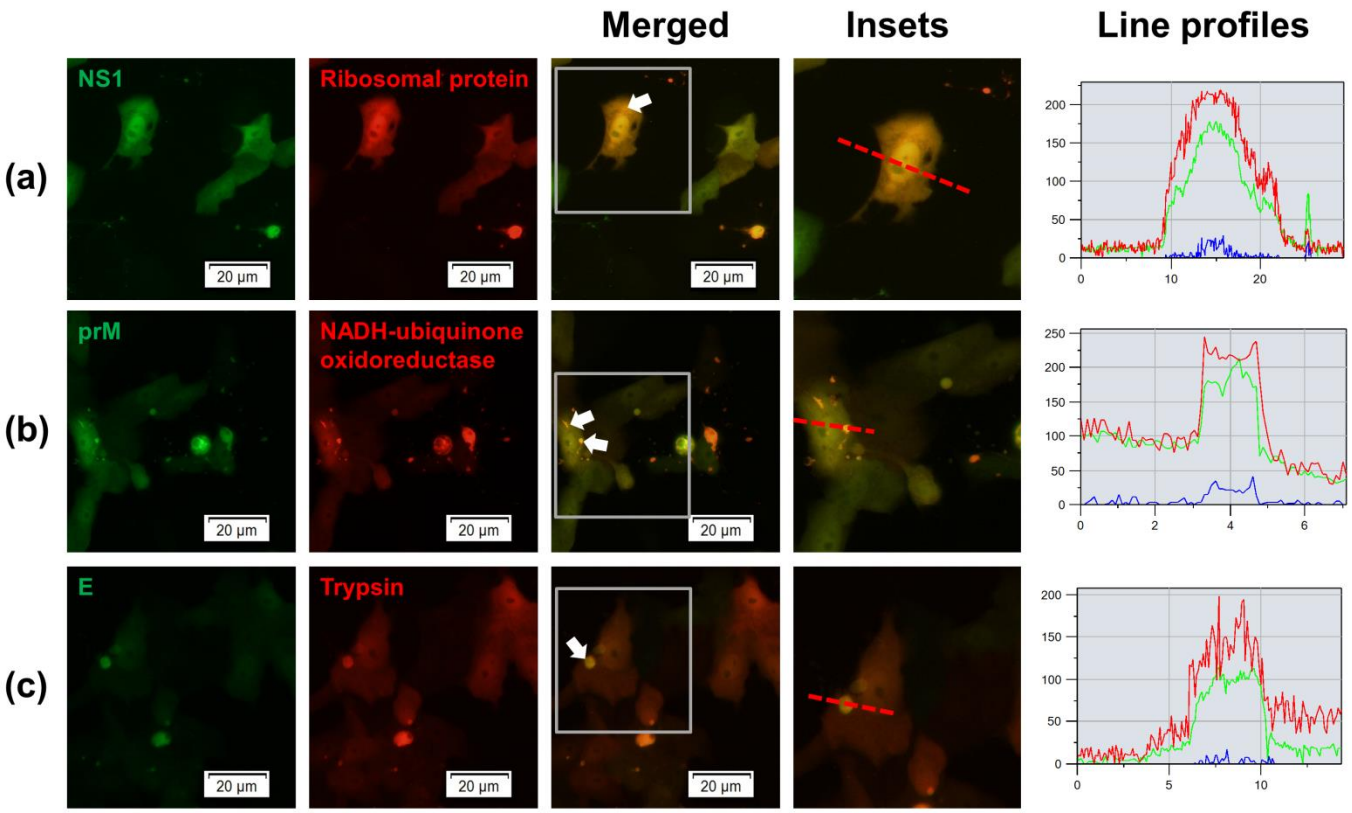

Figure 5. Cellular co-localization of *Aedes aegypti* midgut proteins and DENV2 proteins in DENV2-infected C6/36 cells. The plasmid constructs (pIB/V5-His) carrying the cDNA of midgut proteins were transfected into C6/36 cells, which were subsequently infected with DENV2. Midgut proteins (ribosomal protein, NADH-ubiquinone oxidoreductase, trypsin) were captured using anti-V5 antibody, while DENV2 proteins were captured using antibody against NS1, prM, and E, respectively. Co-localisation of: (**a**) Ribosomal protein S15p/S13e [VectorBase: AAEL003582] and DENV2 NS1 protein; (**b**) NADH-ubiquinone oxidoreductase [VectorBase: AAEL005508] and DENV2 prM protein; (**c**) Trypsin [VectorBase: AAEL007818] and DENV2 E protein. Co-localisations are indicated by white arrows. Line profiles were shown to demonstrate the intensity of red, green, and blue fluorescence across the cross-sectional regions indicated by the red lines in the insets.

Tables

Table 1. The *Aedes aegypti* midgut cDNA library transformation efficiency, insert sizes, and the quality of the library.

	cDNA library	Expected
Transformation efficiency	1.125×10^6 transformants/3 µg pGADT7-Rec	$\geq 1 \times 10^6$ transformants/3 µg pGADT7-Rec
Total number of independent yeast colonies	1.125×10^6	$\geq 1 \times 10^6$
Insert size (kb)		
Minimum screened	0.15 kb	
Maximum screened	3.24 kb	
Average	1.40 kb	
Cell density of frozen library (cells/ml)	8.23×10^8 cells/ml	$> 2 \times 10^7$ cells/ml
cDNA library titer (cfu/ml)		
Primary cDNA library	7.5×10^4 cfu/ml	6.7×10^4 cfu/ml
Amplified cDNA library	2.13×10^7 cfu/ml	$> 1 \times 10^7$ cfu/ml
cDNA library quantity		
Primary cDNA library	1.125×10^6 cfu	
Amplified cDNA library	1.491×10^9 cfu	

Table 2. Insert fragments length by sequencing

Insert size (kb)	Number of fragments	Rate (%)
0.3 – 0.5	6	14.29
0.5 – 1.0	10	23.81
1.0 – 1.5	8	19.05
1.5 – 2.0	7	17.07
2.0 – 2.5	7	17.07
> 2.5	4	9.52

Table 3. Identification of putative *Aedes aegypti* midgut proteins interacting with their respective DENV2 proteins through Y2H screens.

DENV2 proteins	*Ae. aegypti* Gene ID (VectorBase)	Gene names (*Aedes aegypti*)	Size of CDS (bp)
NS1	AAEL003582[a]	Ribosomal protein S15p/S13e	456
	AAEL008169	40S ribosomal protein S12	333
	AAEL008481	60S ribosomal protein L18	570
	AAEL015803	5.8S ribosomal RNA	153
	AAEL005732	Acyl-coa dehydrogenase	1209
	AAEL000393	Suppressors of cytokine signaling	597
	AAEL012713	Clip-Domain Serine Protease family C	1092
	AAEL007683	DNA topoisomerase	3066
	AAEL002827	ATP synthase beta subunit	1515
	AAEL012947	Hypothetical protein	2070
	AAEL001872	Voltage-dependent anion-selective channel	849
	AAEL003393	ATP synthase beta subunit	1515
	AAEL007948	Glutathione-s-transferase theta	672
	AAEL004869	Hypothetical protein	1578
	AAEL015740	Small subunit ribosomal RNA, 5' domain	592
	AAEL004493	Ribosome biogenesis protein tsr1	2430
prM	AAEL007696	TOLL pathway signalling NF-kappaB Relish-like transcription factor	1740
	AAEL005508[a]	NADH-ubiquinone oxidoreductase 24 kda subunit	723
	AAEL006447	GATA transcription factor (GATAb)	2313
	AAEL000987	60S ribosomal protein L8	786
	AAEL005097	cold induced protein (BnC24A)	432
	AAEL011282	Ribosomal RNA small subunit methyltransferase b (sun)	2439
	AAEL008599	Zinc carboxypeptidase	1182
	AAEL005981	Class B Scavenger Receptor (CD36 domain)	1494
	AAEL015740	Small subunit ribosomal RNA, 5' domain	592
	AAEL004493	Ribosome biogenesis protein tsr1	2430
E	AAEL010974	Hypothetical protein	3021
	AAEL003530	Acidic ribosomal protein P1	339
	AAEL009151	30S ribosomal protein S8	393
	AAEL001863	Zinc carboxypeptidase	1239
	AAEL005374	Sensory neuron membrane protein-1	1590
	AAEL007818[a]	Trypsin	765
	AAEL008599	Zinc carboxypeptidase	1182
M	AAEL007974	Hypothetical protein	2166
	AAEL009888	Bumetanide-sensitive Na-K-Cl cotransport protein	3192
	AAEL009588	Expressed protein (HR3)	1407
	AAEL012781	Protease m1 zinc metalloprotease	1635
	AAEL000511	Acetylcholinesterase	2109
	AAEL007649	Cell cycle checkpoint protein rad17	3243
	AAEL005981	Class B Scavenger Receptor (CD36 domain)	1494
	AAEL007818	Trypsin	765
	AAEL005374	Sensory neuron membrane protein-1	1590

[a] Protein interactions were validated by additional assays – Mammalian two-hybrid and double immunofluorescent assays.

Authors' contributions

S.S.H., H.A. and H.T. conceived and designed the experiments. H.T., V.R.M.T.B. and M.C. performed the experiments. H.T., V.R.M.T.B. and M.C. analysed the data. H.A., S.S.H. contributed reagents/materials/analysis tools. S.S.H. and H.T. wrote the paper.

2.3 Concluding remarks

To date, there has been no report on the establishment of *Ae. aegypti* midgut cDNA library. In this chapter we had shown the successful establishment of a midgut cDNA library which was screened against DENV2 proteins (E, prM, M and NS1) by Gal4-based yeast two-hybrid assays. This assay resulted in the identification of 16, 10, 7, and 9 putative cellular host proteins interacting with NS1, prM, E and M, respectively (Table 3, page 71). Some of the similar proteins have also been identified by other workers in their studies using Y2H and other protein-protein interaction screening assays, including *in silico* approaches (116, 139). Although the Y2H system is known to generate an extremely high false positive rate, as noted by us, however, according to Clontech Laboratories, the system has been improved to result in a remarkable reduction of false positivity. Three distinct Gal4-responsive promoters were employed to control four integrated reporter genes (*AUR1-C*, *HIS3*, *ADE2*, *MEL1*). The three promoters are unrelated except for the short protein binding sites in the UAS region (a regulatory region specifically bound by Gal4 DNA-BD). This feature automatically screens out the library proteins that interact with unrelated sequences flanking the UAS region (i.e. false positives). On the other hand, the four reporter genes have distinct functions:

AUR1-C : Encodes the enzyme inositol phosphoryl ceramide synthase that confer strong resistance (*AbA*^{r}) to the highly toxic Aureobasidin A (AbA), a cyclic depsipeptide antibiotic toxic to yeast at low concenrations (0.1-0.5 µg/ml). Aba-based selection greatly favours the growth and identification of genuinely

positive clones by killing sensitive yeast cells, rather than retarding yeast growth.

HIS3 : Histidine is essential for the growth of Y2HGold (the "bait" strain). *HIS3* expression is triggered by the close-proximity interaction of two proteins (bait and prey) which permits the cell to biosynthsise histidine and allows growth on His-drop-out minimal medium.

ADE2 : Y2HGold is also unable to grow on adenine-depleted minimal medium. Gal4-responsive *Ade2* expression allows the cells to grow on –Ade minimal medium.

MEL1 : Similar to *LacZ* gene commonly used in bacterial system, *MEL1* encodes α-galactosidase, an enzyme generates blue pigments to the yeast colonies in the presence of chromogenic substrate X-α-gal (not X-gal).

The putative *Ae. aegypti* midgut proteins identified (known and hypothetical) interacting with the respective DENV2 proteins were deduced to be involved in different biological processes such as translation and transcriptional regulations, DNA and cell cycle regulations, mitochondria energy productions, protein digestions, immunity, neurotransmitter regulations and cellular ionic balance regulations (VectorBase and Uniprot).

In this chapter we are merely reporting the interacting cellular proteins with DENV2 proteins. We did not design any experiments to prove the functional relevance of these interactions, although experiments such as RNAi methods in

C6/36 cells that can validate the putative functions of these proteins. Attempts to validate the interactions of each of the proteins cannot be conducted due to time limitation. However, to determine and validate whether the Y2H functions, 3 putative proteins were selected and subjected to the M2H and protein co-localisation assay by IFA. It is also understood that on their own, the M2H assay may provide false positive results and the IFA may not be a confirmative methodology for protein-protein interactions as proteins can localise without interaction. However, with positive results in all the systems tested, the possibility of false positivity in the interactions of the 3 cellular proteins with DENV2 proteins tested is highly unlikely, which is also an indication that the Y2H system was functioning precisely. In the next chapters, we showed and validated the function of one of the proteins that was identified here using Y2H assay, by M2H assay, co-localisation assay and more definitive assays such as co-immunoprecipitation, siRNA and protein up-regulation studies.

Chapter 3

Interaction of *Ae. aegypti* carboxypeptidase B1 and DENV2 E protein

PART B: Suggested Declaration for Thesis Chapter

Monash University

Declaration for Thesis Chapter 3 and 4

Declaration by candidate

In the case of **Chapter 3 and 4**, the nature and extent of my contribution to the work was the following:

Nature of contribution	**Extent of contribution (%)**
Experimental design and conduct, samples collection and process, data collection, result acquisition, statistical analysis, manuscript preparation	70

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	**Nature of contribution**	**Extent of contribution (%) for student co-authors only**
Vinod RMT Balasubramaniam	Participated in experiments, data collection, statistical analysis	10
Bimo Ario Tejo	Participated in experiments, data collection, statistical analysis	N/A (not a student registered under Monash University)
Hamdan Ahmad	Sample preparation, collection, statistical analyses	N/A (not a student registered under Monash University)
Sharifah Syed Hassan	Sample preparation, experimental design, result discussion, manuscript preparation	N/A (not a student registered under Monash University)

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature		**Date**
Main Supervisor's Signature		**Date**

3.1 General introduction

Since the initial CPB1-E PPI was derived from Y2H data, validation assays are necessary. The results obtained from different PPI validation assays should complement each other from different perspectives. The examples of these methods include mammalian two-hybrid (M2H) assay (direct biological interaction *in vivo*), co-immunoprecipitation (co-IP) assay (protein complex formation *in vivo* and precipitated *in vitro*), and double immunofluorescent assay (double IFA) (*in-situ* biological assay).

3.1.1 CPB1 in *Aedes aegypti*

Carboxypeptidases (CPs) are hydrolases that cleave one amino acid from the carboxyl-terminus of proteins and peptides at a time. In mammalians, CPs perform various functions in the body, mainly involved in the digestion of food (carboxypeptidases A1, A2, and B). There are other members in CPs family that thought to play a role in processing of intercellular peptide messengers, such as carboxypeptidase D, E, M, and N. In *Ae. aegyiti*, CPs can be categorised as either digestive or regulatory CPs. Digestive CPs are mainly involved in protein digestion and degradation in the digestive tract. Researchers have analysed and identified the expression patterns of digestive carboxypeptidase A (CPA) and carboxypeptidase B (CPB) in *Ae. aegypti* (140). In the study, several CPAs and CPBs expression, including CPB1, CPB3, and CPB4, were 40-fold up-regulated ($P<0.01$) 24 hours after a blood meal. Although CPB1 can be involved in nutrient digestion, the possibility of the abundance CPB1 aiding in DENV replication should not be neglected. Vendrell *et. al.* reported that CPA efficiently cleave the carboxyl-terminus of protein/peptide containing aspartic acid, glutamic acid,

arginine, lysine, or proline (141). On the other hand, CPB enzymes prefer hydrolysis of arginine and lysine residues. Since these digestive CPs require the presence of zinc as co-factor for effective enzymatic activity, they are termed "metallocarboxypeptidases". Study on regulatory CPs in insect is very limited. There were reported evidences demonstrating the presence of vitellogenic carboxypeptidase which degrades/regulates yolk proteins in mosquito fat body (142). In addition, angiotension-converting enzyme (ACE), a dipeptidyl carboxypeptidase, which plays a role in the metabolism of peptide hormones involved in regulating reproduction, was found to regulate egg-laying activity in mosquitoes by adding two selective inhibitors of ACE, captopril and lisinopril, to the blood meal (143).

It has been generally known that CPB1 is mainly expressed in mosquito's midgut cells, involved in protein digestion after blood meal (140, 144). The discovery of CPB1 binding to DENV2 E protein in our study will pave an avenue towards a new understanding of the versatility of CPB1 and DENV infection mechanisms in *Ae. aegypti*.

3.1.2 Significance of PPI

In cellular biology, proteins interact with each other in a highly specific manner, determining the outcome of most cellular processes. The distortion of PPI may lead to the development of many diseases. On the other hand, a successful pathogenic infection also requires the involvement of PPI for signal transduction inter- or intracellularly. For example, DENV morphogenesis involves host proteins like ribosomes and furin for virus maturation. At the meantime, viral proteins such as capsid protein was found binding to core histones and inhibits

nucleosome formation in human liver cells (30). In addition, the presence of NS1 in host serum facilitates viral growth either *via* interaction with human complements (145) or heparan sulfate and chondroitin sulfate E (146).

3.1.3 PPI databases

PPI databases have been well developed in recent years, due to the advancement of PPI discovery and verification techniques, and the improvement in screening size of high-throughput PPI screening techniques. The experimental/computational techniques and databases were discussed in detail by Shoemaker & Panchenko (147, 148) and Rao *et al*. (117). In light of summarising most of the major PPI databases available, Shoemaker & Panchenko and Rao *et al.* had included various experimental techniques contributed to PPI data, including Y2H as one of the most major methods used in PPI studies. However, interaction data between human pathogens (i.e. DENV) and insect vectors is largely incomplete. Very limited data can be obtained from major databases such as IntAct (http://www.ebi.ac.uk/intact/), DIP (http://dip.doe-mbi.ucla.edu/dip/Main.cgi), IMEx Consortium (http://www.imexconsortium.org/), or Reactome (http://www.reactome.org/). Nevertheless, with the establishment of VectorBase (a Bioinformatics Resource Center focused on invertebrate vectors of human disease, https://www.vectorbase.org/), interaction data between *Ae. aegypti* and DENV is believed to expand in a rapid pace. This is also supported by the increasing interest on virus-vector interaction research, where various methods from different categories were used to explore novel PPI data between viruses and their vectors, including *in silico* computational approach (116, 131), *in vivo* Y2H

or M2H approach (139), *in vitro* techniques such as affinity chromatography approach (149), and other experimental systems like 2D-difference gel electrophoresis (2D-DIGE) (96).

3.1.4 Verification of CPB1-E interaction

Technological and methodological advances have allowed high-throughput discovery of protein-protein interactions in many organisms. PPI detection methods can be categorised into three types, namely *in vitro* (TAP-MS, Co-IP), *in vivo* (Y2H, M2H), and *in silico* (I2H, phylogenetic profile, structure-based approaches). However, most of these methods have experienced high rates of noise or false positives. In light of this issue, several research groups have reported different methods to assess the reliability and accuracy of experimental PPI data. In terms of computational assessment, Deane *et al.* presented Expression Profile Reliability (EPR) index and Paralogous Verificaion Method (PVM) (http://dip.doe-mbi.ucla.edu/dip/Services.cgi) to evaluates the quality of data sets of a large-scale PPI or PPI probable if the putatively interacting pair has paralogs that also interact (150). In addition, Saito *et al*. reported a "interaction generality" computational measurement, which is helpful in effectively constructing reliable protein-protein interaction networks (151). Some researchers utilised the cellular localisation and cellular-role properties to provide a sound quantitative estimate of PPI data (152). For example, for true interactions, the interacting proteins should be localised to the same cellular compartment. Also, the proteins should share a common cellular role. In light of this, several groups have conducted PPI verification assays such as M2H, co-IP, and double IFA to support their Y2H data (126, 153-155).

In order to verify CPB1-E interaction in mosquito midgut cells, this study employed several PPI verification assays, which have been mainly conducted in most of the PPI studies. These assays include mammalian two-hybrid assays (*in vivo*), double immunofluorescent assays (*in vivo*), co-immunoprecipitation assays (*in vitro*), and molecular docking analyses (*in silico*, chapter 4).

3.1.5 Objective

The objective of the study in this chapter is therefore:

(i) To select and validate the protein-protein interactions of one of the selected midgut protein with the respective DENV2 protein (i.e. carboxypeptidase B1 and DENV2 E protein, CPB1-E).

3.1.6 Materials and methods & Results

Unless stated otherwise, both **materials and methods** and **results** have been published in the article entitled:

Tham H-W, Balasubramaniam V, Tejo B, Ahmad H, & Hassan S (2014) CPB1 of *Aedes aegypti* Interacts with DENV2 E Protein and Regulates Intracellular Viral Accumulation and Release from Midgut Cells. *Viruses* 6(12):5028-5046.

For **materials and methods**, unless stated otherwise, the article includes:

1. Virus and cell cultures
2. Plasmid construction and yeast transfection
3. *Ae. aegypti* primary midgut cell preparations
4. Verification assays
    a. Mammalian two-hybrid analysis
        i. M2H assay using Vero cells

ii. M2H assay using MDCK cells *(not included in the article)*

b. Double immunofluorescence assay

i. Using mosquito midgut cells without endoplasmic reticulum (ER) stain *(not included in the article)*

ii. Using mosquito midgut cells with ER stain *(discussed in chapter 4)*

c. Co-immunoprecipitation analysis

For **results**, unless stated otherwise, the article includes:

1. CPB1 protein interacts with the DENV2 E protein
    a. In Vero cells
    b. In MDCK cells *(not included in the article)*
2. CPB1 co-localises with DENV2 E protein through IFA
    a. Using mosquito midgut cells without ER stain *(not included in the article)*
    b. Using mosquito midgut cells with ER stain *(discussed in chapter 4)*
3. CPB1-E protein complexes co-immunoprecipitate from DENV2-infected *Ae. aegypti* primary midgut cells

The two assays – "M2H assay using MDCK cells" and "Double IFA using mosquito midgut cells without ER stain" that were conducted but not stated in the article are described and discussed below:

3.2 Additional assays

3.2.1 M2H assay using MDCK cells

Materials and methods. M2H assay was initially performed on MDCK cells using the same protocol described in the paper (page 105, section 2.7), with MDCK cells instead of Vero cells.

Results. M2H assays were initially conducted in MDCK cells to validate CPB1-E interaction. The direct reflection of the binding affinity of the two recombinant proteins was measured by relative alkaline phosphatase (AP) activity. The binding affinity of CPB1-E in MDCK cells was not significant when compared to negative and positive controls (Fig 5, page 85). Although minimal activity was detected from mock-treated control (Fig. 5B, page 85), relatively strong AP activities were measured from the rest of the control samples (Fig. 5C, D, and E, page 85). About 50% cell death was observed 48 hours after plasmid transfection.

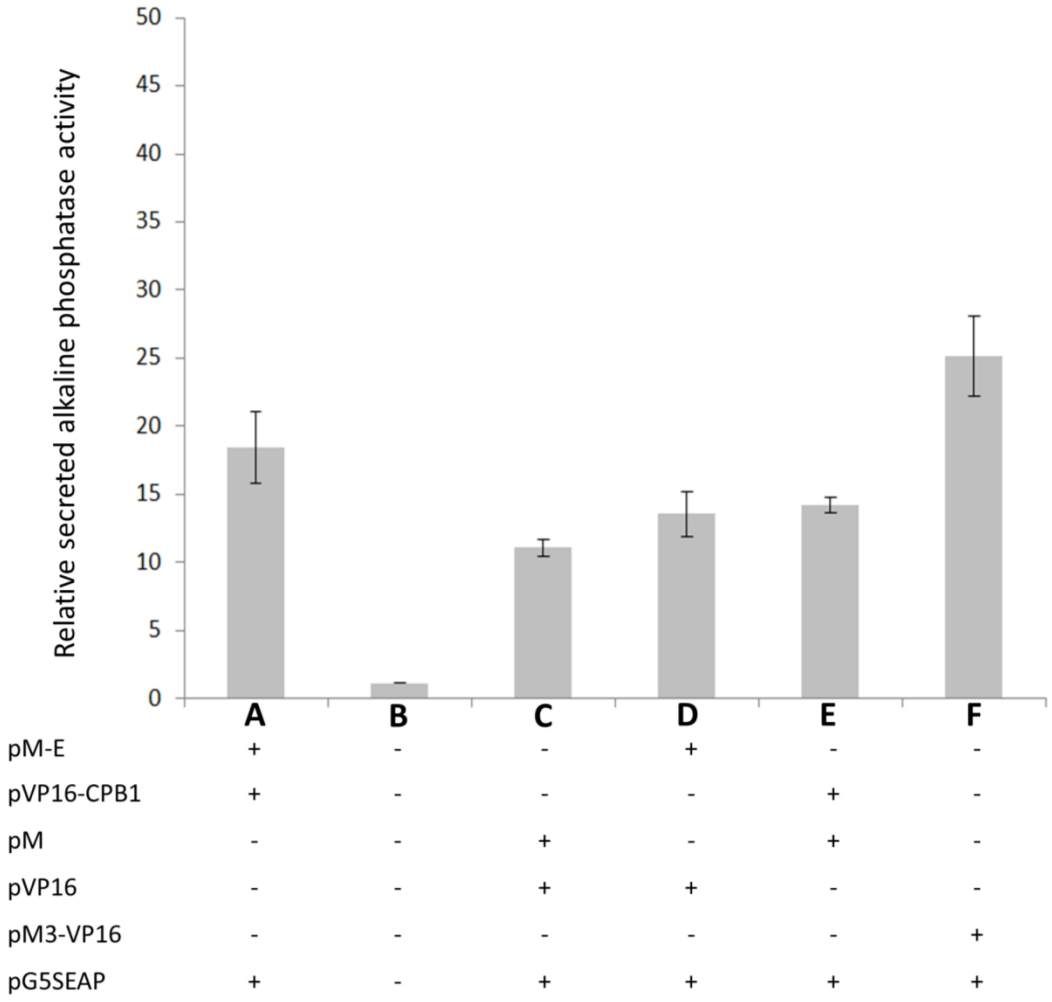

Figure 5 Mammalian two-hybrid assays performed to confirm the physical interaction of CPB1 and E protein in MDCK cells. Each column represents a treatment and is labelled (+) for the plasmid transfected and (-) for the plasmid that is absent. **(A)** Interaction between CPB1 and E proteins. The secreted alkaline phosphatase (SEAP) activity is not significant when compared to the negative controls. **(B-E)** Various negative controls with the presence/absence of different combinations of plasmid construct. **(F)** Positive controls harbouring the relatively highest SEAP activity because M3 and VP16 are known protein interacting partners, which are known to induce SEAP gene expression under normal circumstances.

Discussion. Mammalian two-hybrid assays require the use to any mammalian cells for the biosynthesis and biological interactions of two recombinant proteins. However, upon using the MDCK cells, it was observed that there was a low cell-viability rate after plasmid transfection. This may be explained by the intolerance of MDCK cells to the extrinsic plasmids carrying insect's and viral genes, and also the inability of the cell type to withstand an excess of plasmids (>10 µg) in a

single transfection, which could cause toxicity to the cells. In addition, MDCK may not be suitable as it was reported to undergo autophagy during *Flavivirus* infection (75), which can partly contribute to the cell death observed in this experiment. The high AP activities in the controls C, D, and E were deduced as due to the high self-activation rate of the pG5SEAP reporter gene or false-positive results retrieved from MDCK cells. The autophagy events occurred in the presence of DENV2 proteins may have affected the outcomes of this assay. Therefore, this assay was repeated using another mammalian cell line – Vero cells. The results were reported in the manuscript attached.

3.2.2 Double IFA using mosquito midgut cells without ER stain

Materials and methods. Anti-E (GeneTex, GTX43296) and anti-CPB1 antibody (Abnova, H00001360-D01) were obtained commercially. *Ae. aegypti* midgut dissection and primary cell line maintenance were described in the manuscript (page 104, section 2.4). This assay was conducted as described previously (126). In brief, primary midgut cells were infected with DENV2 at a multiplicity of infection (MOI) of 10, and viral proliferation was allowed for 24 hours. Cells were fixed (4% paraformaldehyde, 1x PBS, 10 min), permeabilised (0.25% Triton X-100, 1x PBS, 10 min), and blocked (5% BSA, 1x PBS, 1 hour) before treating with primary (GeneTex, GTX43296, 1:2000 dilution, 1 hour) and secondary antibodies (AlexaFluor® 488, AlexaFluor® 594, 1:2000 dilution, 1 hour). The final nuclear staining was performed with ProLong® Gold Antifade Reagent with DAPI (Invitrogen, CA, USA). Slides were viewed under IX80 confocal microscope (Olympus). This assay was conducted at room temperature unless stated otherwise.

Results. Double IFA study enables visualisation of intracellular protein localisation. In figure 6 (page 87), co-localisations of CPB1 and E proteins in the cytoplasm of DENV2-infected *Ae. aegypti* midgut cells were observed (white arrowed). In addition, the cell was scanned from the X-axis and Y-axis (Z-stack scanning, 0.3 µm intervals, Fig. 6A, page 87), which presented a clear intracellular co-localisation of CPB1 and E proteins. Co-localisation was mainly found in close proximity to the nucleus.

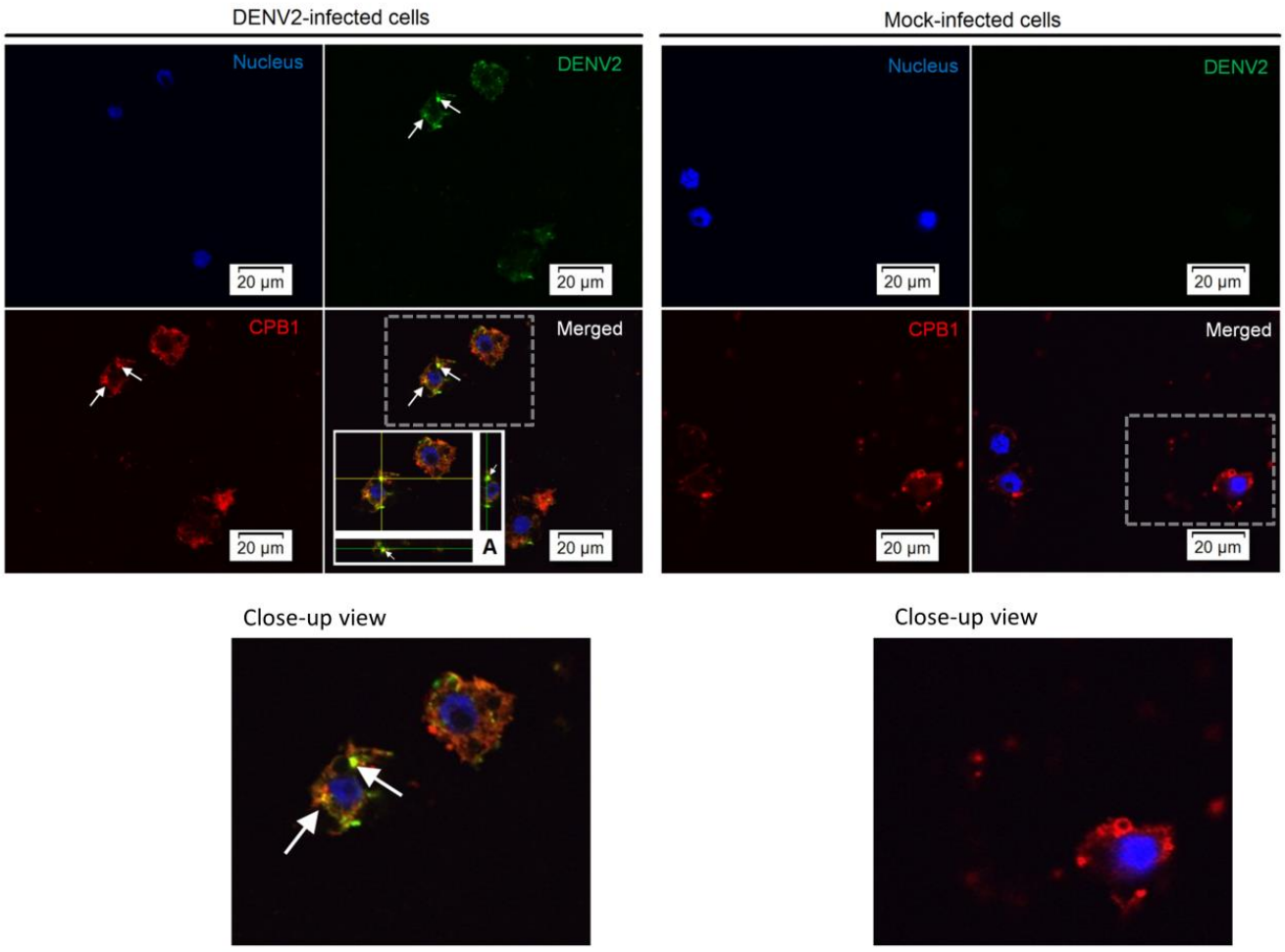

Figure 6 Cellular localisation of CPB1 and DENV2 in *Aedes aegypti* midgut cells. The midgut cells were isolated from *Ae. aegypti* and maintained in MEM media supplemented with 10% FBS. Cells were fixed at 48 hpi and stained for nuclear DNA (DAPI, blue), DENV2 (green), and CPB1 (red), viewed under a confocal microscope at 60x with immersion oil. The DAPI, DENV2, and CPB1 images are overlaid into a merged image (Merged). CPB1 is mainly observed in cell cytoplasm, where DENV2 was also mainly present. The co-localisation of CPB1 and DENV2 is arrowed. (Inset, A) The arrowed cells were subjected to Z-stack scanning (0.3 µm) which gave rise to the horizontal (y-axis) and perpendicular (x-axis) images. White arrows in "A" show the co-localisation of CPB1 and DENV2 at x- and y-axis. On the other hand, no DENV2 was detected in mock-infected cells. Grey dotted boxes indicate the regions with close-up views. Bars = 20 µm.

Discussion. The presence of CPB1 in the cytoplasm as illustrated in figure 6 above has suggested the possibility that CPB1 is not a receptor of DENV2. Since co-localisation of CPB1 and DENV2 E protein was observed close to the nucleus, it was an indication that the CPB1-E co-localisation might have occurred in the endoplasmic reticulum (ER). In the organisation of organelles in a eukaryotic cell, it was shown that the ER is arranged in close proximity to the nucleus, compared to other organelles. In addition, ER facilitates DENV RNA replication, viral protein translation and DENV budding to form immature viral particles (156). Therefore, this observation has sparked our interest in investigating the role of CPB1 in the ER of *Ae. aegypti* during DENV2 infection. Further studies in determining the co-localisation of CPB1-E in the ER was undertaken and the results were discussed in the manuscript.

3.3 Summary of strategies and key findings

The overall materials and methods, results, and discussion are part of the published article entitled:

Tham H-W, Balasubramaniam V, Tejo B, Ahmad H, & Hassan S (2014) CPB1 of *Aedes aegypti* Interacts with DENV2 E Protein and Regulates Intracellular Viral Accumulation and Release from Midgut Cells. *Viruses* 6(12):5028-5046.

- Y2H assays identified CPB1-E interaction, the three additional validation assays (M2H, Co-IP, double IFA) further confirmed the interaction.
- M2H assays conducted in Vero cells presented with the most significant results. Perhaps Vero cell is monkey origin with the closest lineage to humans.

- M2H assays conducted in MDCK cells had suboptimal outcomes (Fig. 5, page 85). This assay was not included in the article.
- CalPhos™ Mammalian Transfection Kit gave a much higher cell viability compared to Lipofectamine™ 2000. This was mainly due to the high ratio of Lipofectamine transfection reagent : plasmid mass required when 15 µg of plasmid cocktail was transfected. On the other hand, CalPhos™ Mammalian Transfection reagent forms nanoparticles as plasmid carriers, which had minimal adverse effect on mammalian cells.
- *Aedes aegypti* primary midgut cells were successfully isolated and maintained in MEM media supplemented with 10% FBS with 1x PSK antibody. However, since the primary cell line was composed of various cell types, about 40-50% of the cells isolated were suspension cells and minimal centrifugation was required during passaging.
- Co-IP assay detected the presence of CPB1-E protein complexes in DENV2 infected *Ae. aegypti* primary midgut cells. However, CPB1 was prone to degradation with the observation of multiple protein bands detected in Western blot assay (section 3.4, page 110).
- CPB1-E interaction was found in proximity to cell nucleus. Double IFA assay was not included in the paper but presented in a separated section in this thesis (section 3.2.2, page 86).

Article is attached in page 101-121

3.4 Concluding remarks

For the interaction study, CPB1 was selected out of the many putative proteins identified in the Y2H studies, as the potential candidate for exploration into the functional relationship in response to DENV infections in the midgut cells of *Ae. aegypti* mosquito. The reasons for selecting CPB1 was, besides its proteolytic activities, CPB1 and its other counterparts seemed to play crucial diverse roles in various pathogenic infections such as duck hepatitis B virus (157-159), DENV capsid protein binding in the salivary gland of *Ae. aegypti* (139), and as a target for transmission-blocking vaccine development (160).

In attempts to validate the interactions between CPB1 and DENV2 E proteins, we have successfully demonstrated the interactions through the M2H, co-IP assays, and double IFA in *Ae. aegypti* primary midgut cells, where cells were originally sourced from adult mosquitoes. The study was therefore conducted on primary midgut cells of the mosquitoes. One reason for this is to capture the cell heterogeneity of the midgut of the mosquitoes (161, 162). The midgut is composed of an epithelial layer of large endoreplicating cells with various functions like absorption of nutrients (with brush borders of microvilli), carbohydrate storage, secretion of digestive enzymes and mucus. Interspersed among the endoreplicating cells are diploid regenerative cells, endocrine and muscle cells. These primary cells are passaged to about 2-3 times only, as increased in the number of sub-passaged will cause in the loss of heterogeneity of the cells thus resulting in a quite homogenous cell line.

Mammalian two-hybrid system is a platform for *in vivo* protein-protein interaction, which confers biologically significant data with more precise post-

translational protein modifications in mammalian cells (128, 153, 163). Although M2H assays resulted in suboptimal results in MDCK cells (*Canis familiaris* kidney cells), promising results were obtained when the same assay was conducted using Vero cells (discussed in manuscript). Second, CPB1 and E proteins were found co-localised *in vivo* in the primary midgut cell cultures of *Ae. aegypti*. Our results clearly indicated that the *Ae. aegypti* midgut cells were susceptible to DENV2. Under confocal fluorescence microscope, co-localisation of CPB1 and E proteins was observed in cell cytoplasm, with a close proximity to nucleus (Fig. 6, page 87). This phenomenon was also presented by Brazzoli M. *et. al.* in 2007 (164), and Welsch *et. al.* in 2009 when the research team reported a 3-dimensional (3D) architecture of dengue virus replication sites (165). This result led us to discovering the possibility of CPB1-E interaction in cellular organelles in proximity to the nucleus, such as endoplasmic reticulum (ER). Third, to detect the presence of CPB1-E protein complex in infected cells, the CPB1-E protein complex was successfully precipitated by resins conjugated with either anti-E or anti-CPB1 antibody (Fig. 3, page 110). This assay validates two important components of this study: (I) the presence of CPB1-E complex in DENV2 infected mosquito cells, and (II) the authenticity of CPB1 and E protein to be readily detected by their respective antibodies during Western blotting assay. Gathering the abovementioned findings, our data strongly suggest the biological interaction between *Ae. aegypti* CPB1 and DENV2 E proteins.

However, several limitations were identified. Although Vero or MDCK cells were employed, M2H assays did not 100% reflect the actual molecular activities found in insect cells. With the absence of commercialised two-hybrid validation assays

specifically designed for insect cell lines, M2H can only partly support our hypothesis that CPB1-E interaction carries a biologically significant role during DENV2 infection. Also, the *Ae. aegypti* primary midgut cell line developed in this study consisted of various cell types, including epithelial cells, luminal cells, *etc*. Although most of the cells isolated were susceptible to DENV2 infection (Fig. 6, page 87), the identity of these cells are yet to be clarified.

In conclusion, Chapter 3 marked the completion of CPB1-E protein interaction validation assays. Five validation assays were conducted, with three assays being included in part of the published article (166). Nevertheless, the two assays with suboptimal results (section 3.2, page 84) were discussed and the results obtained had effectively directed this study to comprehend our understanding on DENV2 replication mechanism in mosquitoes. Additional assays for molecular functional studies were discussed in chapter 4.

Chapter 4

Elucidating the role of *Aedes aegypti* carboxypeptidase B1 (CPB1) during DENV2 infection in mosquito

PART B: Suggested Declaration for Thesis Chapter

Monash University

Declaration for Thesis Chapter 3 and 4

Declaration by candidate

In the case of **Chapter 3 and 4**, the nature and extent of my contribution to the work was the following:

Nature of contribution	**Extent of contribution (%)**
Experimental design and conduct, samples collection and process, data collection, result acquisition, statistical analysis, manuscript preparation	70

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	**Nature of contribution**	**Extent of contribution (%) for student co-authors only**
Vinod RMT Balasubramaniam	Participated in experiments, data collection, statistical analysis	10
Bimo Ario Tejo	Participated in experiments, data collection, statistical analysis	N/A (not a student registered under Monash University)
Hamdan Ahmad	Sample preparation, collection, statistical analyses	N/A (not a student registered under Monash University)
Sharifah Syed Hassan	Sample preparation, experimental design, result discussion, manuscript preparation	N/A (not a student registered under Monash University)

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature		**Date**
Main Supervisor's Signature		**Date**

4.1 General introduction

Once a protein-protein interaction has been identified, its cellular roles can be revealed through various functional assays. In this regard, the involvement of each protein in a particular cellular pathway or biological process can be deduced *via* systematic *in vivo* experiments.

In the previous chapters we have described the construction of a robust and complex *Ae. aegypti* midgut cDNA library, which was subjected to Y2H screenings and revealed a number of putative mosquito's proteins that interact with DENV2 proteins (chapter 2). Then, CPB1 was selected for downstream assays including a series of stringent validation assays (section 3.2, page 84 and section 2.7-2.9, page 105). The results suggested that CPB1-E interaction was authentic and possibly involved in an important biological event during DENV2 infection in mosquito's midgut cells (chapter 3).

4.1.1 Objective

Based on all the achievements described earlier, the next objective is therefore:

(i) To elucidate the role of *Ae. aegypti* carboxypeptidase B1 (CPB1) during DENV2 infection in mosquito.

4.1.2 Materials and methods & Results

Unless stated otherwise, both **materials and methods** and **results** have been published in the article entitled:

Tham H-W, Balasubramaniam V, Tejo B, Ahmad H, & Hassan S (2014) CPB1 of *Aedes aegypti* Interacts with DENV2 E Protein and Regulates Intracellular Viral Accumulation and Release from Midgut Cells. *Viruses* 6(12):5028-5046.

For **materials and methods**, unless stated otherwise, the manuscript includes:

1. *In silico* molecular docking analysis
2. CPB1 up-regulation studies in C6/36 cells
3. CPB1 down-regulation studies in C6/36 cells (*not included in the article*)
4. Double IFA with ER strain

For **results**, unless stated otherwise, the manuscript includes:

1. *In silico* docking suggests possible CPB1-E interaction
2. Over-expression of CPB1 in mosquito C6/36 cells results in intracellular accumulation of DENV2 genomic RNA and viral components
3. CPB1 down-regulation studies in C6/36 cells (*not included in the article*)
4. CPB1 co-localises with DENV2 E protein in the ER of *Ae. aegypti* primary midgut cells

Assay 3 (i.e. "CPB1 down-regulation studies in C6/36 cells") was not included in the published article as this assay required optimisations. The assay is described and discussed below:

4.2 Additional assay

4.2.1 CPB1 down-regulation assay

Materials and methods. The siRNA targeting mosquito CPB1 mRNA is not commercially available. Due to this, custom SMARTpool ON-TARGET*plus* siRNA was designed through Dharmacon (Thermo Scientific, USA) webtool – siDESIGN Center (http://www.thermoscientificbio.com/design-center/) based on *Ae. aegypti* carboxypeptidase B1 gene sequence (NCBI accession number AY590494.1). The siRNA was resuspended in siRNA buffer (60 mM KCl, 6 mM

HEPES-pH 7.5, 0.2 mM $MgCl_2$) and aliquot into 100 µM stocks, which were kept in -20 ^oC until use.

C6/36 was seeded on 24-well plates at $1x10^4$ cells/well. Cells were incubated in MEM basal media supplemented with 10% FBS and 15 mM HEPES until 70-80% confluent. SMARTpool ON-TARGET*plus* siRNA was transfected into C6/36 using Lipofectamine RNAiMAX (Invitrogen, CA, USA) at a mass increment (20 pmol, 50 pmol, 100 pmol, 150 pmol) according to the manufacturer's protocol. After 48 hours, cellular total RNA was extracted for CPB1 down-regulation efficiency test. The remaining replicates were infected with DENV2 and incubated for an additional 48 hours. Then, cells were harvested for total RNA extraction (Trizol, Invitrogen, CA, USA) followed by mRNA purification (PolyATract, Promega, WI, USA). Reverse transcription (RevertAid cDNA Synthesis Kit, Fermentas, Leicester, U.K.) was performed, followed by real-time qPCR assays for DENV2 genomic RNA quantification.

Results. The DENV2 genomic RNA quantification from C6/36 cells transfected with different mass of siRNA did not show any significant difference (Fig. 3A, page 98). There was no DENV2 detected in the mock-infected control. CPB1 mRNA extracted from all samples was also quantified. DNA band intensities were observed to be similar across different samples including the mock-infected control (Fig. 3B, page 98).

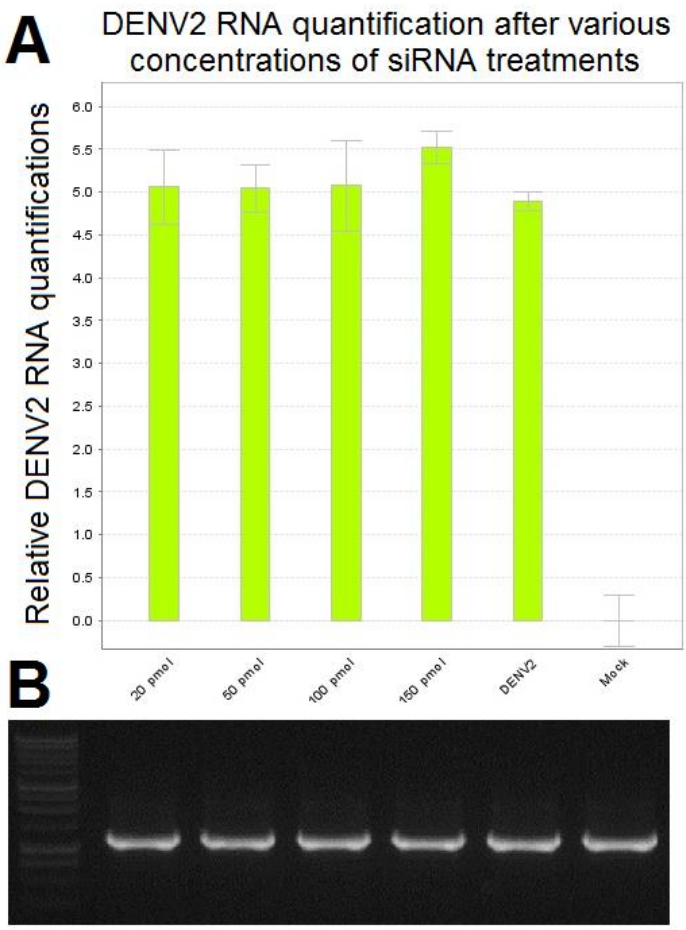

Figure 7 DENV2 genomic RNA quantification from C6/36 cellular lysates transfected with various mass (pmol) of siRNA targeting CPB1 gene. (A) Real-time quantification of DENV2 genomic RNA extracted and purified from C6/36 cellular lysates. Each sample was transfected with an ascending amount of siRNA before DENV2 infection. Columns show mean values, error bars SD of three samples (n=3). **(B)** End-point PCR analyses show equal amounts of CPB1 mRNA present in each respective samples.

Discussion. Further optimisations were required for the suppression of CPB1 expression in C6/36 using siRNA (SMARTpool ON-TARGET*plus*, Dharmacon). Since the SMARTpool ON-TARGET*plus* siRNA was optimised for mammalian cells, very limited or no information was available for protein down-regulation in C6/36 using the product. The efficiency of antiviral RNA interference (RNAi) mechanism in mosquito cells remained uncertain (167). To our knowledge, using double-stranded RNA (dsRNA), protein down-regulation was successfully

demonstrated in adult mosquitoes (168). These methods could be modified and applied into this study as an alternative to ON-TARGET*plus* siRNA.

4.3 Summary of strategies and key findings

- CPB1 up-regulation assay revealed that the excessive amount of CPB1 in mosquito cells regulated DENV2 growth. On the other hand, this phenomenon was not observed in mammalian cells like Vero cells, which secreted more viruses to the extracellular environment in the presence of excessive CPB1 (Fig. 5, page 113).
- Our *in silico* molecular modelling showed that the residues at E protein where CPB1 binds are Thr66, Asn67, Thr68, Lys122, and Val251. According to an atomic-level functional model study of DENV, these amino acids were not in the list of amino acid residues critical for the fusion-loop, hinge or E-M protein "latch" (156). This has drawn our attention to the possible new mechanisms performed by CPB1 to regulate DENV2 growth.
- It was hypothesised that CPB1 in mosquito cells retained DENV2 replication by inhibiting viral budding or viral transport from ER to trans-Golgi network. This hypothesis was made based on the double IFA results reported in the paper (Fig. 2, page 119) where the DENV2-infected *Ae. aegypti* primary midgut cells were stained with ER stain (ER-Tracker™ Blue-White DPX, Invitrogen, CA, USA) showed co-localisation of CPB1-E protein within the ER regions.
- CPB1 down-regulation assay was not successful and required more optimisations. It was concluded that ON-TARGET*plus* siRNA was designed

with molecular modifications optimised for mammalian cellular mechanisms.

- A research group reported the absence of functional siRNA response in C6/36 cell lines (167). Due to time constrain, in future, a different mosquito cell line (i.e. Aeg2 or CCL-125) can be used. In addition, dsRNA successfully down regulated mosquito protein in some previous studies (168). Similar technique can be applied in this study.

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Article

CPB1 of *Aedes aegypti* Interacts with DENV2 E Protein and Regulates Intracellular Viral Accumulation and Release from Midgut Cells

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Abstract: *Aedes aegypti* is a principal vector responsible for the transmission of dengue viruses (DENV). To date, vector control remains the key option for dengue disease management. To develop new vector control strategies, a more comprehensive understanding of the biological interactions between DENV and *Ae. aegypti* is required. In

this study, a cDNA library derived from the midgut of female adult *Ae. aegypti* was used in yeast two-hybrid (Y2H) screenings against DENV2 envelope (E) protein. Among the many interacting proteins identified, carboxypeptidase B1 (CPB1) was selected, and its biological interaction with E protein in *Ae. aegypti* primary midgut cells was further validated. Our double immunofluorescent assay showed that CPB1-E interaction occurred in the endoplasmic reticulum (ER) of the *Ae. aegypti* primary midgut cells. Overexpression of CPB1 in mosquito cells resulted in intracellular DENV2 genomic RNA or virus particle accumulation, with a lower amount of virus release. Therefore, we postulated that in *Ae. aegypti* midgut cells, CPB1 binds to the E protein deposited on the ER intraluminal membranes and inhibits DENV2 RNA encapsulation, thus inhibiting budding from the ER, and may interfere with immature virus transportation to the trans-Golgi network.

Keywords: dengue virus; *Aedes aegypti*; carboxypeptidase; yeast two-hybrid

1. Introduction

The *Aedes aegypti* mosquito is more widely dispersed today, exposing a third of the world's population to the risk of infections with one or more of the four dengue virus serotypes (DENV1-4). DENV is a positive-sense, single-stranded RNA virus with a total genome size of approximately 11 kb. It belongs to the *Flavivirus* genus of the *Flaviviridae* family. In its host, the DENV genome is translated into three structural proteins, C, prM and E, and seven non-structural proteins, NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5, from a single open reading frame. The multiple proteins are then cleaved into individual components via proteolytic cleavage by either viral or host proteases. DENV assembly occurs in the rough endoplasmic reticulum (rER) [1]. Then, the virus is channeled into the trans-Golgi network (TGN) for maturation to occur [2,3], followed by virus release from the infected host cell.

Recently, there has been an increased interest in understanding viral tropism and the molecular activities of DENV in *Ae. aegypti* and *Ae. albopictus* [4–7]. Following a blood meal from an infected human, DENVs multiply in the midgut of the mosquito for seven to 14 days. The viruses are disseminated from the midgut and proliferate in the salivary gland, where the virus is transmitted to another host when the infective mosquito takes another blood meal. The vital role of mosquitoes in the transmission cycle of DENV makes vector control an important strategy in disease management. The use of pesticides, however, has resulted in various issues, which hinder the on-going dengue management efforts [8–14]. Other strategies for mosquito control must therefore be considered. Some of these include directing research towards determining mechanisms of viral survival, virulence and tropism in the *Ae. aegypti* mosquito.

In nature, the mosquito vectorial capacity has been linked to the mosquito midgut epithelial barrier, midgut escape barrier and salivary escape barrier [15]. These differentiated tissues are only present in adult mosquitoes. In this study, female adult *Ae. aegypti* midgut tissues were

collected for cDNA library construction and then used for yeast two-hybrid (Y2H) screening to identify interacting dengue virus proteins.

We constructed the cDNA library using a homologous recombination-mediated approach with the Gal4 activation domain-based vector, pGADT7. This library was screened against the DENV2 E protein, resulting in the identification of a number of interacting proteins, with carboxypeptidase B1 (CPB1) as one of the predominant DENV2 E protein interaction partners. To date, there have been no studies reporting the possible role of *Ae. aegypti* CPB1 protein during DENV infection. This study reports the molecular interactions between *Ae. aegypti* CPB1 and the DENV2 E protein, as well as the role of CPB1 in regulating viral replication and release from *Ae. aegypti* primary midgut cells.

2. Materials and Methods

2.1. Virus and Cell Cultures

Dengue virus type 2 (MY89-88549, AJ556804) was kindly provided by Prof. Sazaly Abu Bakar, University of Malaya. The virus was propagated in C6/36 (ATCC® CRL-1660™) and Vero cells (ATCC® CCL-81™) until cytopathic effects were observed. The titer of DENV2 grown in C6/36 and Vero cells was determined in Vero cells and was 10^4 and 10^6 median tissue culture infective dose/mL ($TCID_{50}$/mL), respectively. Vero cells were incubated at 37 °C with 5% CO_2, in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 1% PSK (working concentration of 0.5 U/mL of penicillin "G" sodium, 0.5 mg/mL of streptomycin and 1 mg/mL kanamycin sulfate) and 1% HEPES buffer (Gibco®, Life Technologies, Carlsbad, CA, USA). The C6/36 cells were incubated at 28 °C with 5% CO_2, maintained in minimal essential medium (MEM) identical to the supplements described above, with 1.5% HEPES buffer. Both cell lines were passaged once every 4–5 days.

2.2. Plasmid Construction and Yeast Transfection

The DENV2 envelope (E) gene (forward primer: AATGCGTTGTATAGGAATATC; reverse primer: AACCACTATCGGCCTGCACCAT) was cloned in-frame into the pGBKT7 plasmid (Clontech, Mountain View, CA, USA) and named pGBKT7-E. The plasmid was transfected into the yeast strain Y2HGold using the lithium/acetate method described previously [16]. Positively-transfected yeast colonies were maintained on selective agars with depleted tryptophan (SD/-Trp, synthetic dropout media without tryptophan) and cryopreserved at −80 °C until use.

2.3. Aedes aegypti Mosquitoes

Ae. aegypti mosquitoes (Linnaeus) [17,18] were maintained in the insectarium at the Universiti Sains Malaysia (USM), School of Biological Sciences, at 28 ± 1 ℃ under 70%–75% relative humidity, with a light/dark cycle of 14 h/10 h. The larvae were reared in round trays

with mineral water (approximately 3 cm height) and provided with cat food (chicken sourced). The adults were fed with sterilized 10% sucrose solution *ad libitum*.

2.4. Aedes aegypti Primary Midgut Cell Preparations

Mosquitoes were cold anaesthetized, and all dissections were performed under sterile conditions following previously described protocols [19,20] with minor modifications. In brief, the mosquitoes were surface sterilized with a 70% ethanol solution [21] and rinsed with sterile phosphate-buffered saline (PBS). *Ae. aegypti* midguts were carefully dissected [22] under a dissecting microscope (15× magnification). The midguts were immersed in sterile PBS solution supplemented with 1× penicillin-streptomycin-neomycin (PSN) antibiotic mixture (Gibco®, Life Technologies) and trypsinized with 1× Krebs-Ringer bicarbonate solution supplemented with 0.25% trypsin (37 ℃ for 25 minutes). The cells were pelleted (500× *g*, 4 ℃, 15 min), resuspended in MEM media supplemented with 10% FBS and incubated in 96-well plates (28 ℃, 5% CO_2) with one passage every 12 days.

2.5. cDNA Library Construction

A total of 50 dissected *Ae. aegypti* primary midguts [22] were subjected to total RNA extraction using TRIzol® Reagent (Invitrogen™, Life Technologies). Following the manufacturer's instructions and previously reported studies [23,24], first- and second-strand cDNA were synthesized using the Make Your Own "Mate & Plate™" Library System (Clontech). Double-stranded cDNA was purified, and nucleic acids less than 400 bp were discarded using Chroma Spin™ TE-400 Columns (Clontech). About 4.16 µg (10 µL) of purified double-stranded cDNA, in conjunction with 3 µg linearized pGADT7-rec vector and 200 µg denatured Yeastmaker Carrier DNA (Clontech) were mixed and transformed concurrently into competent yeast Y187 cells [16]. Then, the pelleted cells were re-suspended in 15 mL sterile 0.9% (w/v) NaCl solution prior to spreading on selection agar plates with depleted leucine (SD/-Leu). To detect transformation efficiency, 100 µL was spread in dilutions of 10^{-1}, 10^{-2} and 10^{-3} on 100-mm SD/-Leu agar plates, and others were spread on 150-mm plates for 150 µL each (a total of 100 plates). After incubation at 30 ℃ for 96 hours, the surviving colonies were counted to calculate the library titer and the number of independent clones [25]. Then, the plates were harvested with 5 mL YPDA (yeast extract solution with peptone, dextrose, and adenine) freezing medium each. All liquids were collected in a sterile 1-L flask and mixed well. After that, they were stored in 1-mL or 50-mL aliquots at −80 °C until use.

2.6. Yeast Two-Hybrid Screening

Y2H screening was conducted according to previous studies [26,27] and the manufacturer's instructions. Prior to mating, Y2HGold was plated on SD/-Ade/-His/-Trp agar supplemented with X-α-gal (40 µg/mL) for the auto-activation test. In the absence of the pGADT7 plasmid, no auto-activation of any reporter was detected in our Y2HGold harboring pGBKT7-E. Yeast

mating was conducted aseptically by inoculating an overnight 5-mL Y2Hgold culture (bait) with a 1-mL library aliquot (prey). The mixture was diluted to 50 mL using 2× YPDA broth. Mating was allowed for 24 hours at 30 ℃ with 45-rpm orbital shaking. Then, the yeast cells were pelleted and spread on low-stringency selection agar SD/-Leu/-Trp (DDO) in the presence of Aureobasidin A (Aba, 125 ng/mL) and X-α-gal, followed by high-stringency selection agar SD/-Leu/-Trp/-Ade/-His (QDO) supplemented with Aba and X-α-gal. After a 96-hour incubation at 30 ℃, the plasmids were extracted from the surviving blue yeast colonies and sequenced for cDNA insert identification (BLAST).

2.7. Mammalian Two-Hybrid Analysis

Mammalian two-hybrid analysis was performed as a complementary approach to the Y2H [28]. The cDNA of DENV2 E and *Ae. aegypti* CPB1 were cloned in-frame into the pM (GAL4 DNA-BD) and pVP16 (AD) cloning vectors (Matchmaker Mammalian Assay Kit 2, Clontech), respectively. Using the CalPhos™ Mammalian Transfection Kit (Clontech), Vero cells were co-transfected with different plasmid cocktails consisting of 5 μg each pM-E, pVP16-CPB1 and pG5SEAP according to the scheme in Figure 1. The transfected cells were incubated (37 ℃, 5% CO_2, 48 hours), and the culture media were harvested for SEAP (secreted alkaline phosphatase) activity analysis using the GreatEscAPe™ SEAP Chemiluminescence Detection Kit (Clontech). Chemiluminescent signals were detected using a PerkinElmer VICTOR™ X5 Multilabel Plate Reader. This assay was conducted in biological triplicates.

2.8. Double Immunofluorescence Assay

This assay was performed using a previously-described procedure [29] with minor modifications. Anti-E (GeneTex, GTX43296) and anti-CPB1 antibody (Abnova, H00001360-D01) were obtained commercially. *Ae. aegypti* primary midgut cells were infected with DENV2 at multiplicity of infection (MOI) of 10, and viral proliferation was allowed for 48 hours. Live cells were stained with ER-Tracker™ Blue-White Dapoxyl™ (1 μM/mL) (Molecular Probes®, Life Technologies™), fixed (4% paraformaldehyde, 1× Hank's Balanced Salt Solution (HBSS), 10 min), permeabilized (0.25% Triton X-100, 1× HBSS, 10 min), blocked (1% BSA, 1× HBSS, 1 hour) and incubated with primary (anti-E and anti-CPB1 antibodies, 1:2000 dilution, 1 hour) and secondary antibodies (AlexaFluor® 488, AlexaFluor® 594, 1:2000 dilution, 1 hour). The slides were mounted and viewed under an IX81 confocal microscope (Olympus). This assay was conducted at room temperature, unless otherwise stated.

2.9. Co-Immunoprecipitation Analysis

Ae. aegypti CPB1 and DENV2 E protein were co-immunoprecipitated using the Dynabeads® Co-Immunoprecipitation Kit (Invitrogen™, Life Technologies) following the manufacturer's instructions. In brief, DENV2-infected (MOI: 1) *Ae. aegypti* primary midgut cells were pelleted and lysed with lysis buffer (110 mM KOAc, 0.5% Triton X-100, 100 mM

NaCl, 2 mM $MgCl_2$, 1 mM DTT and 0.8 mM PMSF, pH 7.4). Anti-CPB1 and anti-E antibody-coupled resins were prepared according to the manufacturer's instructions. In two independent experiments, antibody-coupled resins were mixed with cell lysates on a tube rotator (4 °C, 30 min). The resin-antibody-proteins complexes were collected using magnetic stands. The complexes were washed three times (0.05% Tween-20, 100 mM NaCl, 2 mM $MgCl_2$ and 0.8 mM PMSF, pH 7.5) before the final elution of the protein complexes. The eluents were subjected to western blot assay with both primary antibodies, anti-E antibody (Abnova, MAB8902) and anti-CPB1 antibody (Abnova, H00001360-D01). Alkaline phosphatase (AP)-conjugated anti-rabbit antibody (host: goat) was used as the secondary antibody. The bands were visualized after incubation with the 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT) substrate. In parallel, 10% cellular lysate and non-antibody-coated beads were included in the western blot assays.

2.10. Molecular Docking Analysis

The three-dimensional structure of the DENV E was obtained from the Protein Data Bank (PDB: 1OAN). For *Ae. aegypti* CPB1, the protein structure was predicted using Iterative Threading ASSEmbly Refinement (I-TASSER) [30,31]. The PatchDock algorithm [32] was used to determine a rigid docking model between the CPB1 and E protein. The three-dimensional transformations of the Cartesian coordinate of the proteins were further refined using the FireDock algorithm [33,34]. The complex structure with the lowest interaction energy was used for further analyses.

2.11. CPB1 Upregulation Study

CPB1 upregulation studies were conducted in insect [35] and mammalian cell lines [36], C6/36 and Vero cells, respectively. *Ae. aegypti* CPB1 cDNA was cloned in-frame into the pIB/V5-His and pcDNA3.1(+) plasmids (Invitrogen™, Life Technologies). The cells were seeded on 24-well plates at 3×10^5 cells/well one day before transfection. A total of 2 µg of plasmid constructs were transfected into each respective well using Lipofectamine® 2000 transfection reagent (Invitrogen™, Life Technologies). CPB1 expression was allowed (28 °C, 5% CO_2, 48 hours) before DENV2 infection (MOI: 1). After 48 hours, the cells and media were harvested for RNA extraction using the RNeasy Kit (Qiagen, Valencia, CA, USA). The RNA concentration and quality were determined using a NanoPhotometer® (Implen, München, Germany). The cDNA was constructed with the RevertAid Premium First-strand cDNA Synthesis Kit (Fermentas, Leicester, U.K.). Quantitative real-time PCR (qPCR) was conducted using a StepOnePlus qPCR System (Applied Biosystem®, Life Technologies) with the Power SYBR® Green PCR Master Mix (Applied Biosystems®, Life Technologies). The temperature cycle was as follows: 95 °C, 10 min; 40 cycles of 95 °C, 15 s, 60 °C, 30 s, and 72 °C, 40 s. A melt curve setting was included. The relative quantification of DENV2 genomic RNA was assessed using the comparative C_T ($\Delta\Delta C_T$) algorithm [37]. This assay was conducted in

triplicate. Results expressed as $\Delta\Delta C_T$ were reported as the mean standard deviation and analyzed using paired Student's *t*-test. *p* values <0.05 were considered statistically significant.

3. Results

3.1. Ae. aegypti Midgut cDNA Library Construction

The library titer and the total number of independent clones were calculated according to previous reported formulas [23,25]. Based on the colony number on SD/-Leu plates, the Y187 library titer (cfu/mL) was determined at 7.5×10^4, which met the minimum requirement standard (6.7×10^4 cfu/mL) of the manufacturer (Clontech). The total number of independent clone, which was an indication of the cDNA library complexity, was 3.75×10^7.

3.2. CPB1 Protein Interacts with the DENV2 E Protein

Using the Y2H assays, a total of 265 surviving blue yeast colonies were obtained from low-stringency selection agars (SD/-Leu/-Trp). This number was reduced to 56 by eliminating false-positive interactors on high-stringency selection agars (SD/-Leu/-Trp/-His/-Ade). The final step of the assay, including the sequencing of these colonies, resulted in 11 positive candidate proteins, because of multiple copies of sequences of frequently-interacting protein candidates. CPB1 was identified in seven of 56 yeast colonies surviving on high-stringency selecting agars.

A mammalian two-hybrid (M2H) assay was also performed as a complementary experiment to the Y2H to confirm the interactions. Vero cells were co-transfected with the pM and pVP16 plasmids along with a pG5SEAP reporter vector, according to the scheme in Figure 1 (the full experimental procedure is described in the Materials and Methods section). pG5SEAP is transcriptionally activated by physical interaction between pM- and pVP16-conjugated proteins and expresses SEAP (secreted alkaline phosphatase), which can be determined maximally 48 hours following transfection. The SEAP reporter gene encodes alkaline phosphatase without the membrane-anchoring domain, which contains the protein to be secreted from the transfected cells into the culture medium. Therefore, the level of SEAP activity is a direct quantification of protein-protein interactions, because its activity is directly proportional to the intracellular amount of mRNA and proteins [38,39]. The results showed that the CPB1-E interaction was relatively strong (Figure 1A) compared to the positive (Figure 1F) and negative controls (Figure 1B–E). The positive controls were Vero cells transfected with the pM3-VP16 plasmid, which encodes a Gal4 DNA binding domain fused to the transcription activator, VP16. On the other hand, negative controls consisted of mock-transfected controls (Figure 1B), empty pM and pVP16 controls (Figure 1C), pM with insert control (Figure 1D) and pVP16 with insert control (Figure 1E). These negative-control samples showed a minimal chemiluminescence level, which denoted the genuine interaction of CPB1-E.

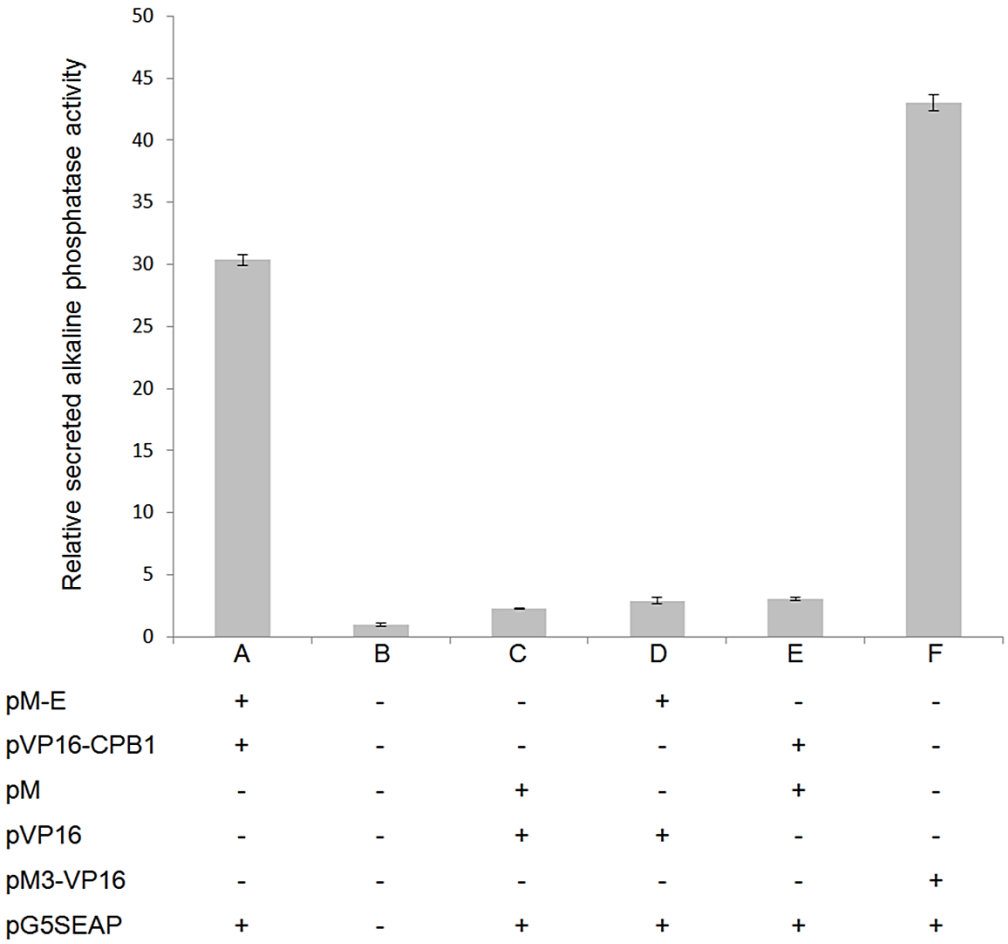

Figure 1. Mammalian two-hybrid assays confirm the physical interaction of CPB1 and E protein in Vero cells. The columns show the mean values and the error bars are the SD of three samples (n = 3). Each column represents a treatment and is labelled "+" for the plasmid transfected and "-" for the plasmid that is absent. (**A**) Interaction between CPB1 and E proteins. The secreted alkaline phosphatase (SEAP) activity is significantly higher than the negative controls. (**B**–**E**) Various negative controls in the presence/absence of different combinations of plasmid construct. (**F**) Positive controls transfected with pM3-VP16, which expresses a fusion of the GAL4 DNA-binding domain to the VP16 activation domain. Therefore, the positive controls had the highest SEAP activity. The media was harvested 48 hours post-transfection.

3.3. CPB1 Co-Localizes with Dengue Virus 2 E Protein in the Endoplasmic Reticulum (ER) of Ae. aegypti Primary Midgut Cells

The distribution of CPB1 in mosquito cells has not been previously assessed. Therefore, we investigated the cellular localization of CPB1 in *Ae. aegypti* midgut cells during DENV2 infection. Figure 2 shows the DENV2-infected (Figure 2A) and mock-infected primary midgut cells (Figure 2B). CPB1 was found throughout the cells, including the nuclei, regardless of the presence of DENV2 (Figure 2A,B, *CPB1*). Not all midgut cell types were susceptible to DENV2 infection, and the distribution of DENV2 varies between cell types (Figure 2A, *DENV2*). The CPB1 and E proteins co-localize in proximity to the nucleus, in the ER of DENV2-infected *Ae. aegypti* midgut cells (Figure 2A, white arrows in the close-up view). The ER in the cytoplasm was stained blue surrounding a "hollow" region in the majority of cells (Figure 2A,B, endoplasmic reticulum). These hollow regions are the nuclei, labelled "N". No DENV2 was detected in the mock-infected control (Figure 2B, *DENV2*).

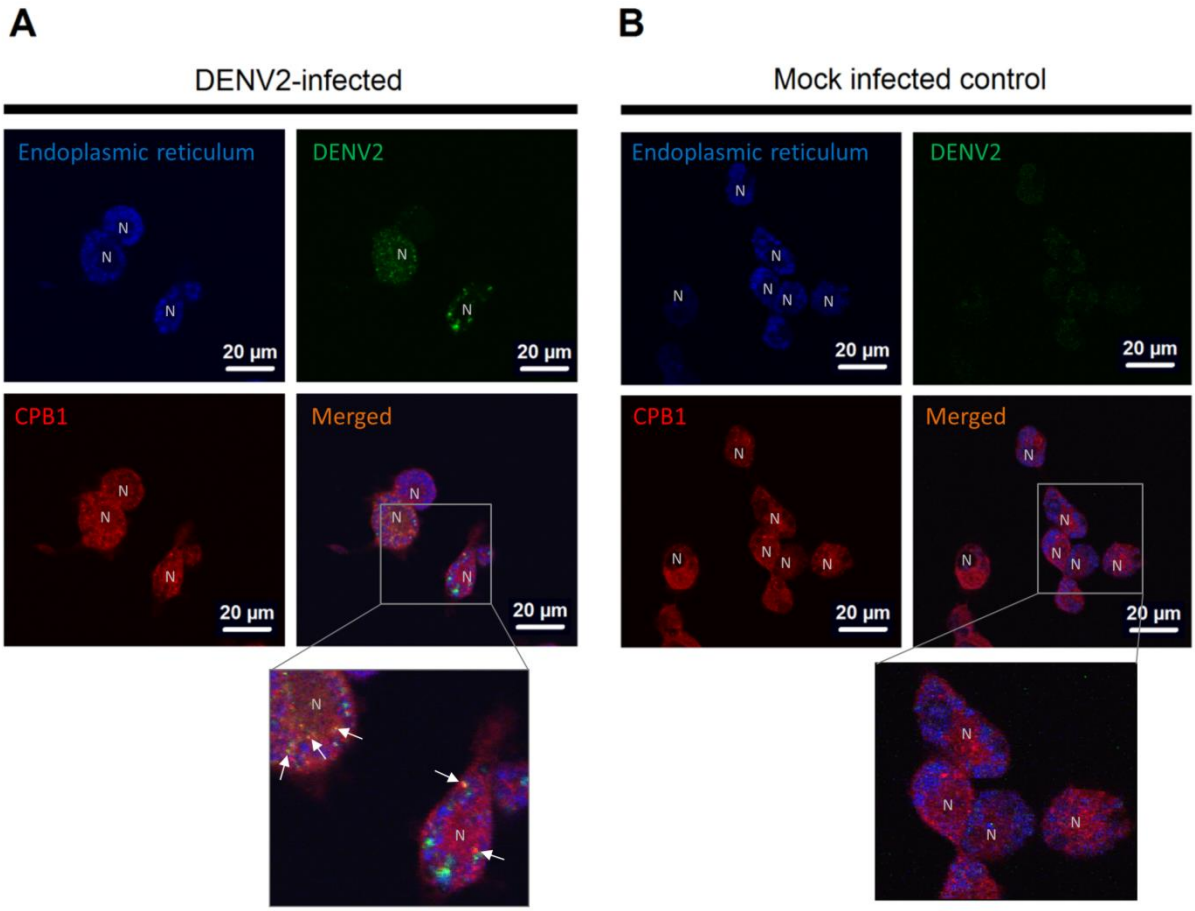

Figure 2. Cellular localization of CPB1 and DENV2 in the ER of *Aedes aegypti* primary midgut cells. (**A**) DENV2-infected mosquito primary midgut cells. (**B**) Mock-infected control. The midgut cells were isolated from female adult *Ae. aegypti* and maintained in MEM media supplemented with 10% FBS. At 48 hpi, the cells were stained with ER-Tracker™ (blue, endoplasmic reticulum), fixed and stained for DENV2 (green, DENV2) and CPB1 (red, CPB1).

The cells were mounted and viewed under a confocal microscope at 100× magnification with immersion oil (Olympus IX81). The ER, DENV2 and CPB1 images are overlaid into a merged image (merged). CPB1 is mainly observed throughout the cell cytoplasm (red), whereas DENV2 was found in DENV-infected cells (green). N, nuclei that were not stained to avoid confusion with the ER. White arrows in the close-up view indicate the co-localization of CPB1 and DENV2 within the blue ER regions. Bars = 20 μm.

3.4. CPB1-E Protein Complexes Co-Immunoprecipitate from DENV2-Infected Ae. aegypti Primary Midgut Cells

To further confirm the presence of CPB1-E protein complexes in *Ae. aegypti* during DENV2 infection, a co-immunoprecipitation assay was performed using the cell lysates of DENV2-infected *Ae. aegypti* primary midgut cells. In this assay, 10% input of total cellular lysate indicates the amount of CPB1 and E proteins in the lysate (Figure 3A). The non-coated beads served as the "resin-only" control, which showed its ultra-low background binding ability (Figure 3B). Anti-CPB1 antibody-coupled resin pulled down the 46-kDa CPB1 and the 56-kDa E protein of DENV2 (Figure 3C, infected). The same two bands were observed when anti-E antibody-coupled resin was used in an independent co-immunoprecipitation experiment (Figure 3D, infected). In the uninfected controls, only CPB1 was pulled down when the anti-CPB1 antibody-coupled resin was used (Figure 3C, control). By contrast, no visible bands were observed when the anti-E antibody-coupled resin was used to co-immunoprecipitate E or CPB1 proteins from the uninfected cells (Figure 3D, control). The multiple bands, which were consistently observed with CPB1, were degraded products of this protein. The β-actin bands indicate that equal amounts of total cellular proteins were applied to each lane.

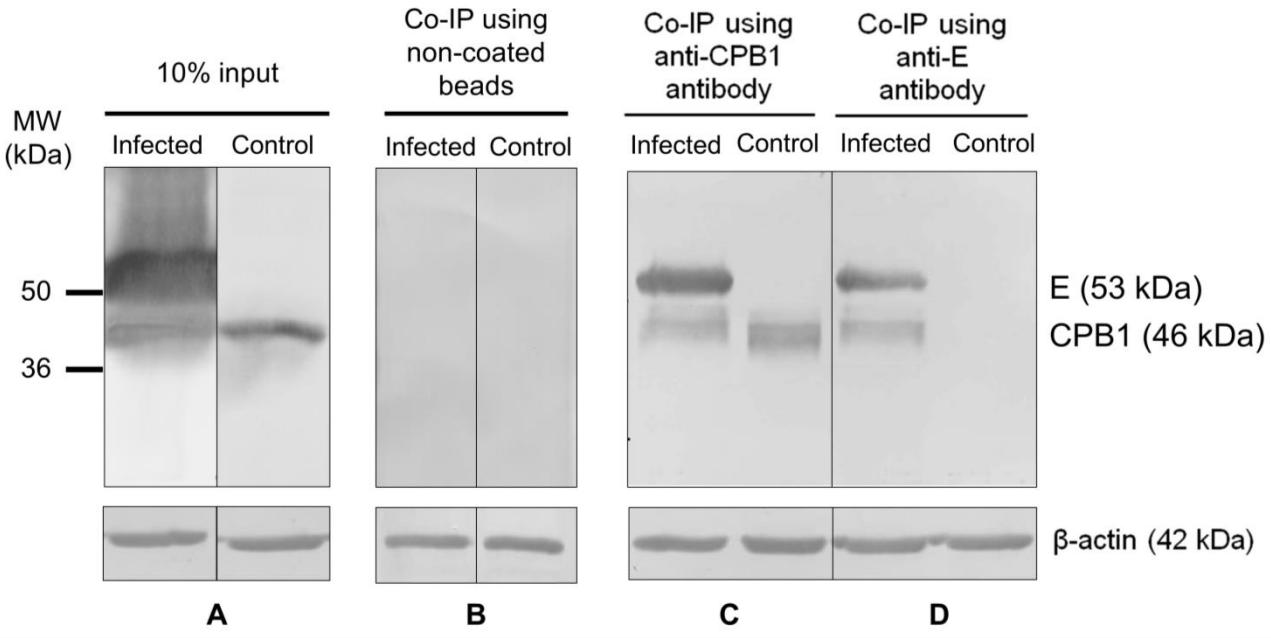

Figure 3. Co-immunoprecipitation of CPB1 and E proteins from DENV2-infected and mock-infected *Aedes aegypti* primary midgut cell lysate. SDS-PAGE and western blot were performed using Dynabeads® conjugated with anti-CPB1 or anti-E antibodies. (**A**) 10% of the total cellular lysate. (**B**) Non-antibody-coated bead controls. Cell lysate derived from DENV2-infected *Ae. aegypti* primary midgut cells (infected). Control, cell lysate derived from mock-infected *Ae. aegypti* primary midgut cells. (**C**,**D**) Anti-CPB1 and anti-E antibodies pulled down the CPB1 and E protein from DENV2-infected samples (infected). For controls, anti-CPB1 antibody pulled down the CPB1 protein ((**C**), control), whereas no band was observed in the same sample when anti-E antibody was used ((**D**), control). Western blot analysis and β-actin protein levels of each sample are shown.

3.5. In Silico Docking Suggests Possible CPB1-E Interaction

The docking algorithm supports CPB1-E interaction with a binding energy of −22.7 kcal/mol. The result also suggests that CPB1 binds to domain II of the E protein (EII), where CPB1 binds to Thr66, Asn67, Thr68, Lys122 and Val251. Figure 4 shows the predicted CPB1-E protein complex, with CPB1 illustrated in yellow, E in grey and red (grey: domain I and II; red: domain III) (Figure 4).

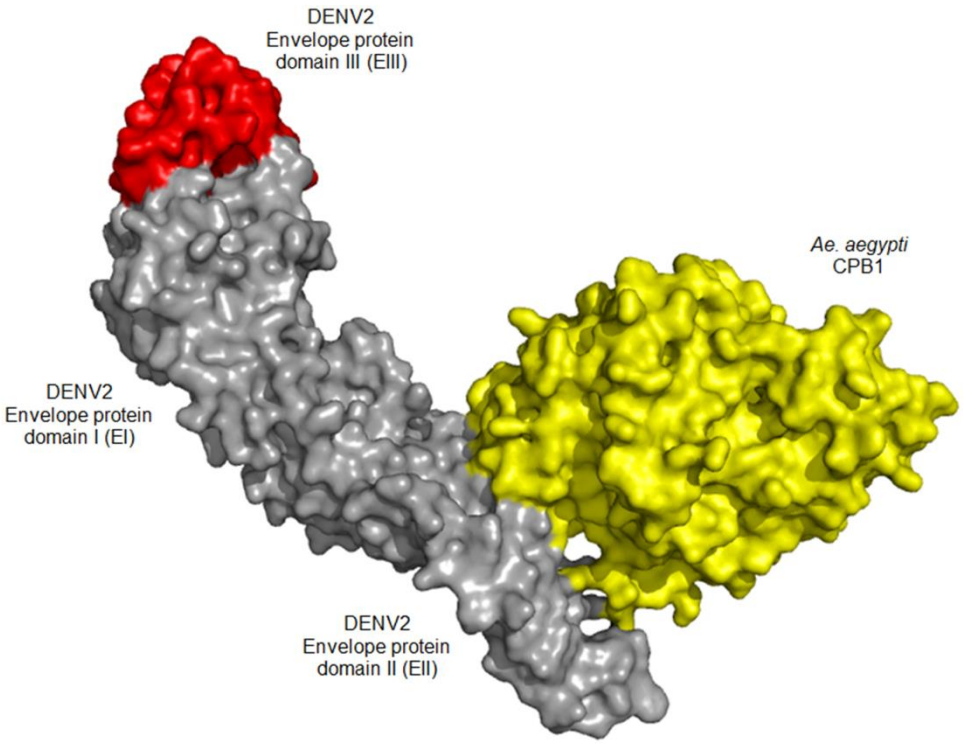

Figure 4. *In silico* molecular docking surface structure of the *Aedes aegypti* CPB1 and DENV2 E protein (PDB: 1OAN). The CPB1 protein structure was predicted using Iterative Threading ASSEmbly Refinement (I-TASSER). The CPB1-E protein rigid docking model was predicted using the PatchDock algorithm and further refined using the FireDock algorithm. The CPB1-E docking algorithm suggests the lowest binding energy of −22.7 kcal/mol. It also supports an

interaction between CPB1 and the domain II of the E protein (EII), at residues Thr66, Asn67, Thr68, Lys122 and Val251. CPB1 is shown in yellow, and E in grey and red, where grey represents domains I and II and red represents domain III.

3.6. Overexpression of CPB1 in Mosquito C6/36 Cells Results in Intracellular Accumulation of DENV2 Genomic RNA and Viral Components

The morphology of C6/36 cells was altered, with increased size and cell elongation after DENV2 infection (Figure 5A). We also performed the relative quantification of intra- and extracellular DENV2 mRNA genomes of various C6/36 and Vero treatment groups by qPCR (Figure 5B,C). In both cell lines, DENV2 was not detected in either the cellular lysate or control sample media of non-infected controls (Figure 5B,C, mock and *CPB1*). For DENV2-infected samples, a higher amount of DENV2 genomic RNA was detected in the media (extracellular) compared to the cellular lysate (intracellular) (Figure 5B,C, DENV2). However, after *Ae. aegypti* CPB1 upregulation, opposite outcomes were observed between C6/36 and Vero cell lines. For C6/36 cells, the DENV2 genomic RNA was primarily detected intracellularly and was 9.4-fold higher than the control ($p < 0.0001$), whereas significantly less DENV2 was released ($p < 0.0001$) (Figure 5B, CPB1 + DENV2). For Vero cells, overexpressed CPB1 resulted in an increase in intracellular and extracellular viral accumulation compared to Vero cells without CPB1 overexpression ($p < 0.05$) (Figure 5C, CPB1 + DENV2). Distinct outcomes were observed for DENV2-infected C6/36 and Vero cells after CPB1 overexpression (Figure 5B, C, CPB1 + DENV2). The results implicate that CPB1 may exclusively regulate DENV2 replication in mosquito cells, in order to confer infectivity to *Ae. Aegypti* while maintaining the healthiness of its vector mosquitoes.

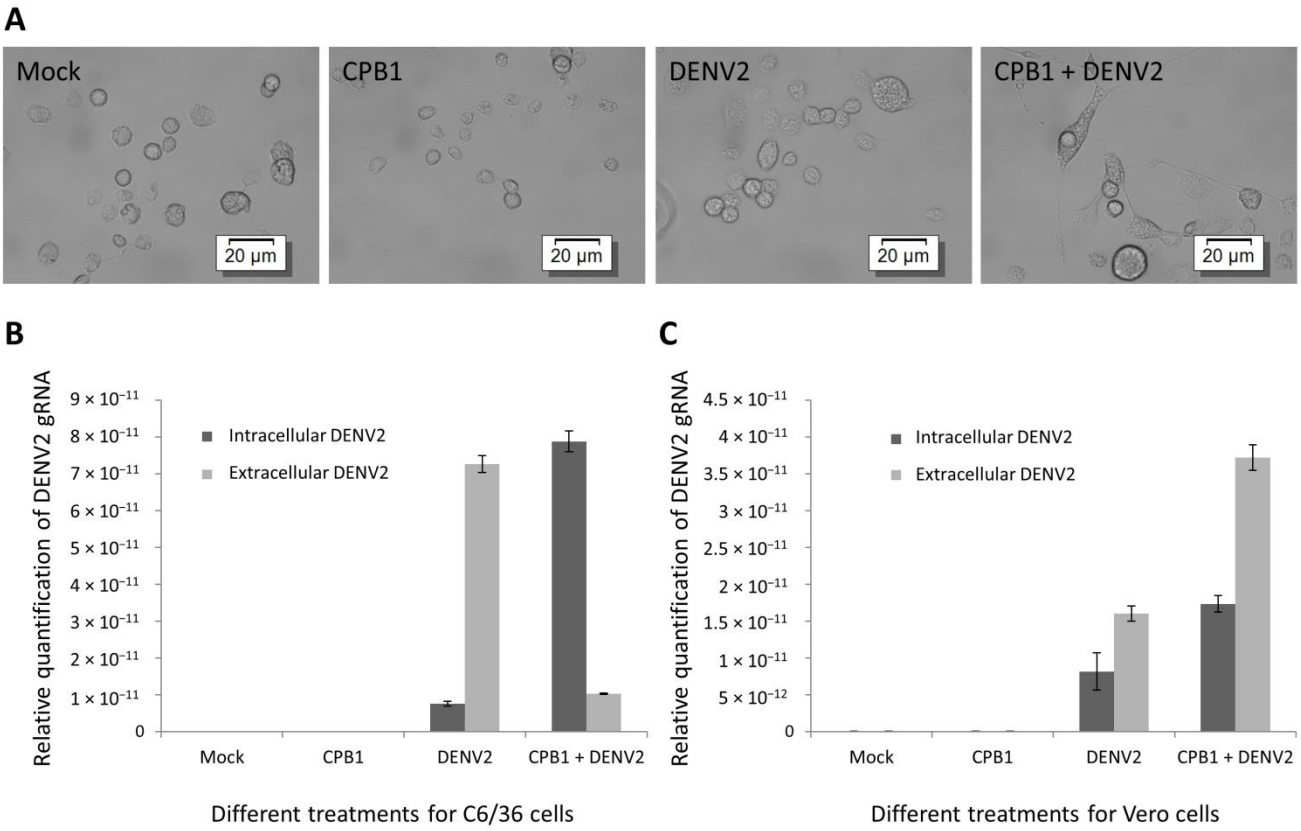

Figure 5. Intra- and extra-cellular DENV2 genomic RNA quantification of C6/36 and Vero cells during the upregulation of CPB1 expression. The cells were seeded in 24-well plates until 80% confluency, before pIB-CPB1 (for C6/36) or pcDNA-CPB1 (for Vero) was transfected. Protein expression was allowed for 48 hours, followed by DENV2 infection. Mock, mock-infected cells. CPB1, cells with upregulated CPB1 expression without DENV2 infection. DENV2, cells with DENV2 infection without CPB1 upregulation. CPB1 + DENV2, cells with DENV2 infection after CPB1 upregulation. (**A**) The cell morphology of C6/36 cells with different treatments. (**B**,**C**) The columns show the mean values and the error bars are the standard deviation (SD) of three samples (n = 3). Cellular lysates and media were harvested for RNA extractions at 48 hpi. DENV2 genomic RNA was quantified using relative RT-qPCR. Excessive CPB1 leads to the intracellular accumulation of DENV2 genomic RNA in C6/36 cells ($p < 0.0001$), with a significant decrease in virus released into the extracellular media ($p < 0.0001$). By contrast, Vero cells exhibited an opposite trend, in which despite the accumulation of DENV2 genomic RNA ($p < 0.05$), the amount of virus released was directly proportional to the intracellular genomic RNA accumulation ($p < 0.05$).

4. Discussion

In this study, we identified and localized CPB1, an *Ae. aegypti* protein that interacts with the E protein of DENV2. CPB1 binds to the DENV2 E protein in yeast two-hybrid screenings and mammalian two-hybrid assay, and the interaction was confirmed by co-immunoprecipitation and co-localization assays.

Various characteristics and functions of CPB1 have been reported in mosquito. The rapid induction of carboxypeptidase genes in the midgut of mosquito [40] and the upregulation of carboxypeptidase B1 and B3 in *Ae. aegypti* midgut have been demonstrated at 24 hours post blood meal (PBM) [41]. CPB1 is a well-known hydrolytic enzyme, which is involved in C-terminal peptide cleavage after blood ingestion [41]. Polyclonal antibodies against this mosquito-derived CPB1 antigen have also been shown to block the sexual development of malarial protozoan parasites in the midgut of *Anopheles stephensi* [42]. Carboxypeptidase D, a counterpart of CPB1, is a receptor for duck hepatitis B virus [43–45]. In addition, a recent study reported the interaction between carboxypeptidase A (CPA) and the DENV capsid (C) protein in the salivary gland of *Ae. aegypti* [6]. Based on these diverse roles of carboxypeptidases, we explored the functional relationship of *Ae. Aegypti*-derived CPB1 in response to DENV2 infections in the midgut cells of *Ae. aegypti* mosquito.

To elucidate the mechanisms involved in the interaction between CPB1 and the E protein, it was necessary to determine where this interaction occurred. Macrophages are a primary target for dengue virus infection. Harris *et al.* showed that a vitellogenic-like carboxypeptidase (CPVL) was highly localized in the endoplasmic reticulum (ER) of human THP-1 monocytic cells and macrophages [46]. Specifically, in macrophages, CPVL is glycosylated and retained in the ER. The results of our study are consistent with this finding. We showed that using *Ae. aegypti* primary midgut cells, the CPB1 also localises in the ER and is not the surface of the cells (Figure 2).

Studies have shown that CPB1 is naturally upregulated in mosquito midgut cells [41,47]. However, the natural upregulation of CPB1 during DENV infections in C6/36 and Vero cells has not been previously reported. To mimic the effect of upregulation of CPB1 in these cells, they were transfected with protein-expression plasmid containing the CPB1 cDNA insert, followed by DENV infection. Interestingly, overexpression of CPB1 protein in DENV2-infected C6/36 cells caused intracellular accumulation of viral genomes and immature virus (Figure 5B, CPB1 + DENV2). However, overexpression of CPB1 protein in Vero cells during DENV2 infection resulted in a higher amount of intracellular accumulation and extracellular virus released compared to DENV2-infected cells without CPB1 overexpression (Figure 5C).

It is hypothesized that, during DENV2 replication in *Ae. aegypti* midgut cells, CPB1 binds to the E proteins that are deposited on the ER intraluminal membrane or to the E proteins on immature virions. The interaction may reduce the encapsulation process of the newly-formed RNA into immature virus, resulting in DENV genomic RNA accumulation. Meanwhile, CPB1-E interaction on newly-assembled immature virus, including viruses that are stacked in the dilated ER cisterna, may hamper further processing into mature viruses in the trans-Golgi network. The accumulation of intracellular genomic materials or viruses causes cellular enlargement (Figure 5A, CPB1 + DENV2) and can, therefore, be explained by the accumulation of excessive DENV2 genomic RNA, DENV2 viral proteins and host cell materials [48], in addition to the cytopathic changes that occur during infection [49]. A similar effect of DENV genomic RNA accumulation during viral inhibition was also observed when autophagy was inhibited by spautin-1 (specific and potent autophagy inhibitor 1) [50]. Autophagy is required for optimal

DENV RNA replication, and cellular lipid metabolism is necessary for energy production [51]. In addition, a reduction in the amount of secreted infectious virions was also observed in a study where the kinase inhibitor, SFV785, was used to dislocate the DENV envelope protein, thus blocking virus assembly [52].

We cannot exclude the possibility that the interaction between CPB1 and E could lead to inhibition of viral morphogenesis, such as viral glycoprotein processing, as described previously [53,54]. However, our *in silico* molecular modelling not only supports CPB1-E protein interaction, but also suggests that the residues of E protein to which CPB1 binds are Thr66, Asn67, Thr68, Lys122 and Val251. These residues are found in EII, and none of these residues are critical for the fusion-loop, hinge or E-M protein "latch" functionalities [55]. Further experimental results derived from mutational or isothermal titration calorimetry (ITC) analyses may support this computational finding.

The differences in the outcomes of DENV infections between cell lines suggest that there are dissimilarities in viral propagation pathways between insect and mammalian host systems. Many studies also confirmed the differential inhibition of DENV infection in mosquito and mammalian cells by the dissimilarity of the DENV E protein, polysaccharide composition between virions derived from mosquito and mammalian cells [56,57], different antiviral susceptibility to drug inhibitors of DENV2 entry [58] and endocytic uptake in Vero and C6/36 cells [59]. In this study, the differences in the outcomes could be due to several possible reasons. Firstly, CPB1 gene/protein used in the upregulation study is derived from mosquito. Sequence alignment conducted in our lab between the mammalian CPB1 (GenBank Accession Number: AAP36803.1) and mosquito CPB1 (GenBank Accession Number: AY590494.1) showed that the amino acid sequence homology is approximately 50%, thus indicating a significant differences in the protein characteristics. Therefore, the Vero cells might react differently to an insect CPB1 protein. Secondly, as a digestive enzyme for blood digestion after blood meal, CPB1 could therefore be suggested that the genes encoding the protein might be easily regulated in mosquitoes compared to Vero cells, where CPB1 has not been reported to be involved in any functional activities during DENV infection.

In this study, we demonstrated an anti-virus role for *Ae. aegypti* CPB1. Anti-viral mechanisms in insects have been widely studied, with reports showing delicate balances between viruses and the immune systems of insect vectors. These mechanisms include RNA interference (RNAi), Toll pathways, Janus kinase (JAK)-signal transducer and activator of transcription (STAT) (JAK-STAT) pathways and autophagy [60–66]. These systems control or contain viral growth, fine tune the virus titer and confer infectivity to the insect vector without impairing the health of the insect. Our results demonstrate the existence of a novel virus-control mechanism by CPB1 in the ER of *Ae. aegypti* midgut cells during DENV2 infection.

During a blood meal from a DENV-infected human, viral infection of midgut cells occurs. It is hypothesized that CPB1 upregulation after a blood meal [41] functions to regulate the intracellular accumulation and minimal extracellular secretion of infectious DENV2. The viral infection causes cytopathic changes due to viral RNA and immature virus accumulation, and these cells most likely progress to lysis or death, followed by virus release. The released viruses

are mostly immature viruses, CPB1-bound immature viruses and a low titer of mature infectious viruses. This may favor the health of the vector mosquito while keeping the virus titer at a low level to reduce infection that may cause devastating damage to uninfected midgut and adjacent cells. Consistent with previous studies on DENV and Chikungunya virus expression profiles in different mosquito organs [67–69], mosquito salivary glands contained a lower virus titer compared to the midgut cells. As shown in this study, although the small amount of virus released from the midgut cells is not likely to cause secondary infection in other cell types in the mosquito host, the virus titer is strategically reduced for a delicate balance between viruses and the immune systems of insect vectors [60–66]. Our results demonstrate a novel virus-control mechanism using CPB1 in the ER of *Ae. aegypti* midgut cells during DENV2 infection.

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Author Contributions

S.S.H., H.A. and H.-W.T. conceived and designed the experiments. H.-W.T. and V.R.M.T.B. performed the experiments. H.-W.T., V.R.M.T.B. and B.A.T. analyzed the data. H.A., S.S.H., B.A.T. contributed reagents/materials/analysis tools. S.S.H. and H.-W.T. wrote the paper.

Conflicts of Interest

The authors declare no conflict of interest.

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4.4 Concluding remarks

Despite all the methodologies that were conducted to define the role of CPB1 during DENV infection in midgut cells of mosquitoes, one of the most convincing ways would definitely be determining the antiviral effects of CPB1 through protein down-regulation studies, such as RNAi assays. However, since the siRNA construct to knockdown insect CPB1 gene is not commercially available, a custom-made siRNA (SMARTpool ON-TARGET*plus*) from Dharmacon, Thermo Fisher was used for the siRNA work. The siRNA which was transfected into *Ae. aegypti* primary midgut cells resulted in extensive destruction/high toxicity (probably also due to the relatively unstable nature of primary midgut cells). The next option was to use a commonly available continuous cell line (i.e the C6/36) for the knockdown study. There were, however, no significant changes in DENV2 replication when CPB1 knockdown was conducted in C6/36 cells. In addition, the results of end-point PCR showed similar CPB1 band intensities before and after siRNA transfection. It was speculated that this result was aligned with Brackney *et al.* who showed C6/36 cells have a dysfunctional antiviral RNAi response (167). We used C6/36 cell lines in this study although *Ae.aegypti* cell line (e.g. CCL-125) will seemed to be appropriate as both *Ae. albopictus* and *Ae. aegypti* are two well-recognised important vectors for dissemination and transmission of DENVs. Moncayo *et al.* also demonstrated equivalent vector competence of both mosquito species to some DENV2 strain (169). Moreover, since the DENV2 used in this study was initially propagated and maintained in C6/36 cells, the cells support the growth and provide the optimal conditions for viral pathogenicity. Similarities in the proteins of both species of mosquitoes have also been shown when the same DENV-interacting mosquito

proteins were identified from *Ae. aegypti* and C6/36 cells (149). However, due to the dysfunctional antiviral RNAi response, further protein knockdown studies will need to be conducted in *Ae. aegypti* cell lines.

In this study we showed that CPB1 was found in the ER during DENV2 infection in mosquito midgut. Also, CPB1-E interaction is suggested to potentially modulate DENV2 morphogenesis through blocking the viral RNA encapsulation (Fig. 8, page 123). These, in turn, contain DENV2 growth in the midgut cells, yet allow minimal rate of virus growth to spread the virus to other organs, such as salivary glands, to confer infectivity to the mosquito vector while maintaining a well physical conditions of the vector.

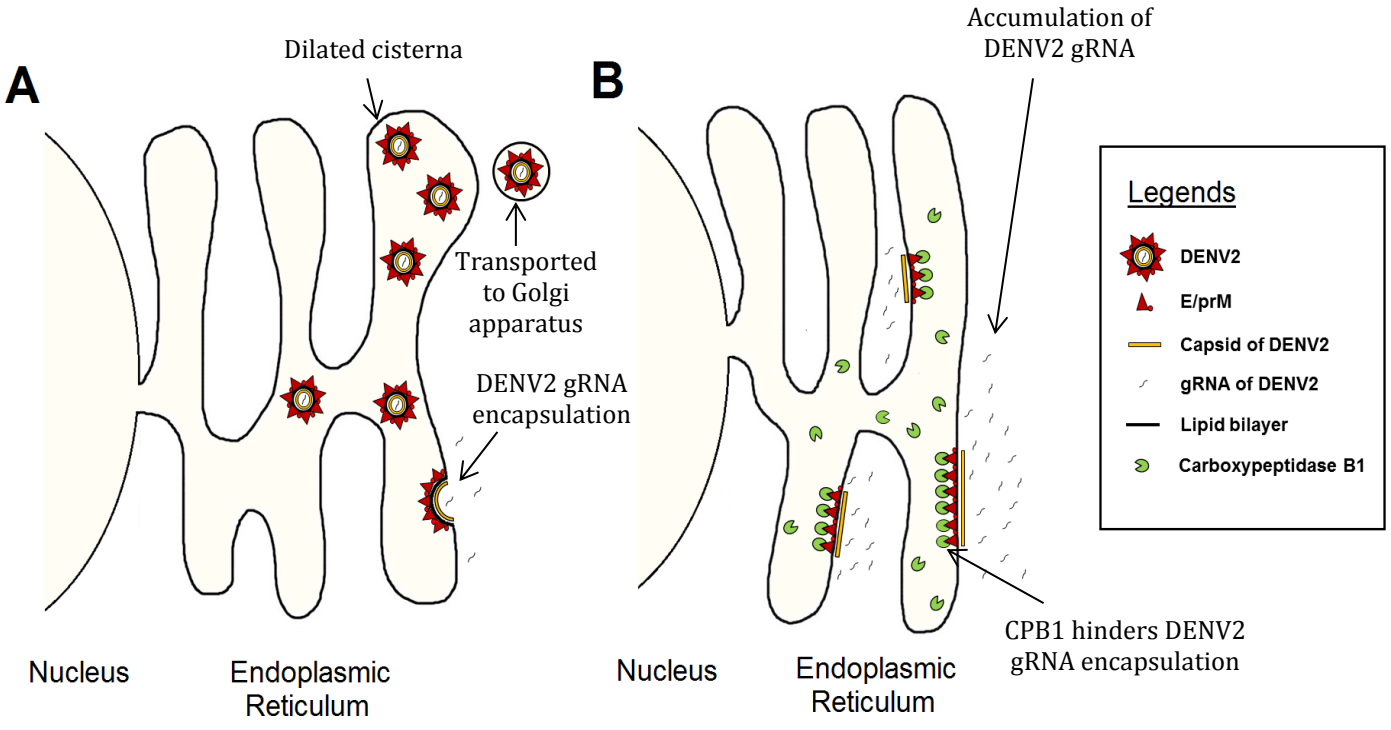

Figure 8 The proposed mechanism employed by CPB1 to regulate dengue virus growth. (**A**) Under normal conditions, encapsulation of genomic RNA happens in the endoplasmic reticulum (ER) of the host cells. This allows the formation of immature viral particles which are stacked in the dilated ER cisterna, followed by transportation to Golgi apparatus for further processing. (**B**) When CPB1 is up-regulated, excessive CPB1 proteins in the lumen of ER bind to DENV2 E proteins, which hindered the encapsulation of DENV gRNA. This has caused the accumulation of DENV gRNA in the cytoplasm, which in turn reduces the formation and release of mature virus from the host cells.

Several limitations exist for functional assays conducted in chapter 4. First, the cytotoxic effect of excessive CPB1 on mosquito cells like C6/36 was yet to be determined. This is important to maintain the cell viability during DENV2 infection. However, since cell death was not observed in our samples, we believed that CPB1 did not affect cell's healthiness, or the up-regulation CPB1 expression did not reach a cytotoxic level. Second, in order to observe virus particle accumulation, more advance technique such as electronic microscopy (EM) is required. Third, the binding energy of CPB1-E was computationally predicted. Another option to measure CPB1-E interaction energy is isothermal titration calorimetry (ITC), which is a physical technique used to determine the thermodynamic parameters of interactions of small (i.e. medical compounds) to large (i.e. proteins) in solution.

Chapter 5

General discussion, conclusion and future studies

5.1 Overall summary

Dengue viruses belong to the genus *Flavivirus* of the family *Flaviviridae*. There are nearly 70 viruses in this genus, out of which many are important human pathogens, including DENV, Japanese encephalitis (JE) virus, St. Louis encephalitis (SLE) virus, and yellow fever (YF) virus (170). *Flavivirus* cause a variety of diseases such as mild fevers (mostly undiagnosed), haemorrhagic fevers, and encephalitis. Among these viruses, DENV is one of the most important mosquito-borne viral threats to the public health. Although Sanofi's vaccine has completed phase-3 clinical trial recently (82), no vaccine or antiviral therapy is approved for dengue fever to date. Many tropical and sub-tropical countries have experienced dengue outbreaks for decades, and in some countries, like Malaysia, dengue has become an endemic disease. In Malaysia, the number of reported dengue cases nationwide from January 2014 until 8th November 2014 has increased by 185% to 88,806 cases (cumulative death toll: 169), compared with 31,170 cases (cumulative death toll: 63) recorded during the same period in 2013. These data showed the alarming situation has again highlighted the urgent needs of the development of more reliable diagnostic techniques, efficient and cost effective viral drugs and vaccines, and new strategies in vector controls.

Being the most prevalent arbovirus, DENV is disseminated and propagated in competent mosquito vectors such as *Aedes aegypti* and *Ae. albopictus*. *Ae. aegypti* is considered as the primary DENV vector and has repeatedly been incriminated as a driving force in dengue's worldwide emergence. The dramatic global expansion of both of these vectors in the last decades has significantly increased public health concern (171). In the battle against dengue, understanding of

mosquito-virus relationship is one of the vital elements that may result in the development of innovative strategies for vector and DENV control.

Realising that protein-protein interactions between viral and host proteins, especially the midgut proteins, is one of the best ways to identify and understand the interactions, we developed a cDNA library of the midgut cells in order to facilitate the molecular interaction studies with DENV proteins. During the 4-year research period, the cDNA library was successfully constructed with satisfying qualities, which was subsequently used in several Y2H screens to identify a number of mosquito-DENV2 interacting proteins (Chapter 2). Although another research group published a similar study on the construction of *Ae. aegypti* cDNA library, the insect organs subject to this purpose were not specified (139). In addition, the same group did not report any mosquito interacting protein for DENV E and NS1 proteins, while the midgut cDNA library discussed in this thesis identified several proteins interacting with, not only DENV2 E, NS1, but also prM and M proteins.

The Y2H system has been known to result in false-positive interactions, yeast intracellular environment can lead to spurious results for exogenous proteins due to differences in post-translational modifications or the presence/absence of bridging proteins (172). However, the Matchmaker® Gold Y2H system (Clontech) has been greatly improved to tackle the false positivity. Compared to the conventional Y2H systems, the Matchmaker® Gold Y2H system is equipped with at least 6 reporter genes and 3 distinct promoters to screen out false positivity. Therefore, this Y2H system is a more powerful method for identifying protein-

protein interactions (PPIs), requiring minimal prior information of the putative interactors.

Despite being a laborious method, Y2H screenings in this study has proposed a number of highly-suggestive interacting proteins listed in table 3 (page 71). Among the >1300 surviving yeast colonies acquired on low-stringency selecting agars, only ~200 (~15%) colonies were survived on high-stringency selecting agars after the elimination of false-positive interactors. The surviving yeast colonies were subject to plasmid extraction and purification, followed by DNA sequencing for cDNA identifications. Among the proteins identified, CPB1 was subject to downstream assays including functional elucidation studies, due to its versatility especially during pathogenic infections (139, 157, 159, 160, 173), and its unique expression profile in *Ae. aegypti* midgut after blood ingestion (140, 174).

However, a limitation of the Y2H approach is that all primary hits have to be technically verified and biologically evaluated using complementary methods. Chapter 3 and chapter 4 describe the assays conducted to validate CPB1-E interactions, and also the functional assays performed in order to infer the possible outcomes derived from such protein interaction in mosquito midgut upon DENV2 infection. In validating the interactions of CPB1 with DENV E protein, three methods, which are mammalian two-hybrid assay, double immunofluorescence assay, and the most definitive assay of the three, the co-immunoprecipitation assay, were used. However, these assays are time-consuming, costly, and laborious. For example, different versions of plasmid constructs are needed for these assays, the transfection of these plasmids into

each unknown and control samples require optimisations, and each step requires optimisations of culture conditions and optimal sample viewing time points must be determined at each stage of the experiments. Functional studies performed in this study suggested the possible role of CPB1 in *Ae. aegypti* mosquito midgut as a regulatory protein for DENV2 replication and release. The up-regulation of CPB1 expression has been shown to reduce the amount of viral particle released into the extracellular environment, with an observed intracellular accumulation of viral materials (Fig. 5, page 113). This event was speculated on the ability of CPB1 to bind to luminal DENV2 E proteins during viral replication in ER, which may subsequently reduce encapsulation of DENV2 gRNA to form immature viral particles (Fig. 8, page 123).

In conclusion, this study hypothesises that, during a blood meal from a dengue patient, the up-regulation of CPB1 (140) serves as a regulating protein to modulate the intracellular accumulation and extracellular secretion of DENV. The existence of CPB1 leads to intracellular immature DENV or viral genomic RNA accumulation, which reduces the release of infectious viral particles. Therefore, the virus titre is maintained at a low level to minimise infection that might cause devastating damage to the uninfected midgut cells, which in turns protect the vector mosquito from devastating infection. In accordance with previous studies on DENV replication profiles in different mosquito organs (133, 175), including Chikungunya virus (176), low virus titre were recorded in the salivary glands of the mosquito compared to the midgut cells. This explains the significant reduced amount of DENV2 during CPB1 up-regulation in C6/36. The low virus titer is probably insufficient to cause secondary infections in other cell

types of the mosquito host, but strategically tuned to infect salivary glands for further transmission to humans.

The outcomes of this research will contribute to the development of many other vector control strategies. We intend to validate a lot more of these Y2H protein candidates that were discovered and to confirm their interaction with DENV proteins, especially DENV E, prM and NS1 proteins. One of our aims is to use these proteins, especially midgut cellular surface proteins, as a vaccine against midgut proteins of mosquitoes, as one of the innovative tools to control dengue virus dissemination. Instead of viral proteins, these midgut cellular proteins can be made into chimeric vaccines (eg. on yellow fever viral skeleton), to elicit human antibodies against the midgut proteins. During a blood meal of "midgut protein-vaccinated" human, a mosquito ingests the antibodies which theoretically compete for DENV receptors, and subsequently inhibits or halt dengue virus attachment on the midgut surface (although the possibility of the virus using other secondary co-factors for attachment and entry is possible). We believe this methodology can eventually help to reduce dengue virus transmission and possible eradication of DENV in mosquito vectors. This may also eliminate the possibility of antibody-dependent enhancement (ADE) due to secondary DENV infection after vaccination.

5.2 Future studies

Several approaches can be done in future to strengthen the work in this study:

- Reverse genetics

Reverse genetics allow researchers to study the phenotypic outcomes of a specific gene using point mutagenesis, directed deletion, or gene suppression (e.g. siRNA). The success in the construction of recombinant dengue virus-like particles (VLP) in *Pichia pastoris* exhibited immunological properties *in vivo* (177). This technique sheds light on point mutagenesis study to create dengue VLPs which are prone or devoid of the ability to interact with CPB1. This, in turn, further refines the molecular interaction of CPB1-E, while allowing researchers to study the importance of such interaction in *Ae. aegypti* midgut cells during DENV infections.

- Protein down-regulation

Mosquito cell line C6/36 was reported to have dysfunctional antiviral RNAi response (167). This was also shown in this study where custom-made siRNA against CPB1 expression in the C6/36 cell line did not exhibit CPB1 gene suppression. However, mosquito protein down-regulation assay was successfully conducted in adult *Ae. aegypti* using long dsRNA targeting the desired gene (178). Hence, in the future experiments, dsRNA can be constructed using gene-specific primers, with T7 promoter sequence included in both the forward and reverse primers. PCR products are subjected to dsRNA production using T7 Megascript kit (or other kits with similar function). dsRNA can be transfected into different

mosquito cell lines or *in vivo* thorax injection into adult mosquito for further studies.

- Cryo-electron microscopy

The first structure of a flavivirus was determined by using cryo-electron microscopy (cryo-EM) (38). Since then, many researchers acquired the technology to study the morphological changes of DENV particles, and its tropism during viral infections (37, 78, 179-181). In future, cryo-EM can be applied in this study to observe the virus particle changes and accumulation during infection, with excessive CPB1 in the host cells. It is expected that DENV2 accumulation is observed in the enlarged ER (ER stress) of the host cells.

- Live-cell imaging

Live-cell imaging to observe DENV tropism in tissue culture cell line was performed before (182). To apply the technique into this study, the ER of insect/mammalian cell lines will first be stained using ER-Tracker™ Blue-White DPX (or other similar products). Meanwhile, DENV2 can be labelled (e.g. DiD), and infect the host cells to observe the viral tropism. This technology allows the visualisation of DENV2 infection pathway in live cells, including the cells with up-regulated CPB1 expression.

- Single-cell RNA quantification

The double IFA results (Fig. 2, page 109) show that not all the midgut cells are susceptible to DENV2 infection. This phenomenon leads to a speculation where only a certain cell types found in *Ae. aegypti* midgut contribute to DENV2 infection, multiplication, and release. To quantitate the portion of DENV2-

susceptible midgut cells, some single-cell analyses technologies, such as C_1™ Single-Cell Auto Prep System (Fluidigm Corporation, USA), allow bench top automation of the isolation, lysis, and pre-amplification of single cells for downstream qPCR analyses. This assay allows the researcher to quantify the portion of the midgut cell types that are involved in DENV2 replication during infection in *Ae. aegypti*, thus comprehends the understandings of interactions between DENV2 and *Ae. aegypti*.

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List of publications:

- **Hong-Wai Tham**, Vinod RMT Balasubramaniam, Miaw-Fang Chew, Hamdan Ahmad, Sharifah Syed Hassan (2014). "Protein-protein interactions between *Ae. aegypti* midgut and dengue virus 2: two-hybrid screens using the midgut cDNA library". Accepted for publication in the *Journal of Infection in Developing Countries* (JIDC) (manuscript number: 6422, accepted for publication).
- **Tham H-W**, Balasubramaniam V, Tejo B, Ahmad H, & Hassan S (2014) CPB1 of *Aedes aegypti* Interacts with DENV2 E Protein and Regulates Intracellular Viral Accumulation and Release from Midgut Cells. *Viruses* 6(12):5028-5046
- Vinod RMT Balasubramaniam, **Tham H Wai**, Abdul R Omar, Iekhsan Othman, Sharifah S Hassan (2012) "Cellular transcripts of chicken brain tissues in response to H5N1 and Newcastle disease virus infection". *Virology Journal* 2012, 9:53 (doi:10.1186/1743-422X-9-53).
- Balasubramaniam VRMT, **Hong Wai T**, Ario Tejo B, Omar AR, Syed Hassan S (2013) "Highly Pathogenic Avian Influenza Virus Nucleoprotein Interacts with TREX Complex Adaptor Protein Aly/REF". *PLoS ONE* 8(9): e72429. doi:10.1371/journal.pone.0072429

Conferences attended/Peer reviewed proceedings published:

- Presented a poster in *International Postgraduate Conference on Biotechnology 2011* (IPCB, 2011), Universiti Terengganu Malaysia, Institute of Marine Biotechnology, on 15th to 17th December 2011.

"Expression and purification of dengue virus non-structural protein 1 (NS1), roles in disease manipulation and pathogenesis"

Tham Hong Wai, Vinod RMT Balasubramaniam, Hamdan Bin Ahmad, Iekhsan Bin Othman, Sharifah Syed Hassan

- Author in poster presentation in *International Postgraduate Conference on Biotechnology 2011* (IPCB, 2011), Universiti Terengganu Malaysia, Institute of Marine Biotechnology, on 15th to 17th December 2011. "Cellular transcripts of chicken brain tissues in response to H5N1 infection"

  Vinod RMT Balasubramaniam, Sharifah S Hassan, **Tham H Wai**, Abdul R Omar, Iekhsan Othman

- Presented a poster in *International Meeting on Emerging Diseases and Surveillance* (IMED, 2013), Vienna, Austria, on 15th to 18th February 2013. "Discovery of novel mosquito-dengue viral protein interactions: Construction of adult *Ae. aegypti* cDNA library for use in yeast two–hybrid system"

  Tham Hong Wai, Vinod RMT Balasubramaniam, Hamdan Ahmad, Sharifah Syed Hassan

- Presented a poster in *The Malaysian Society for Biochemistry & Molecular Biology* (MSBMB, 2013), Putrajaya Marriott Hotel & Spa, Malaysia, on 28th & 29th August 2013. "Construction of whole adult *Aedes aegypti* cDNA library for use in yeast two hybrid system in dengue research"

  Tham Hong Wai, Vinod RMT Balasubramaniam, Hamdan Ahmad, Sharifah Syed Hassan

- Author in poster presentation in *The Malaysian Society for Biochemistry & Molecular Biology* (MSBMB, 2013), Putrajaya Marriott Hotel & Spa, Malaysia, on 28th & 29th August 2013. "Highly pathogenic avian influenza nucleoprotein interacts with TREX complex adaptor protein Aly/REF"

  Vinod RMT Balasubramaniam, **Hong Wai Tham**, Bimo Ario Tejo, Abdul Rahman Omar, Sharifah S Hassan

- Presented a poster in 21st Annual Scientific Meeting of the Malaysian Society for Molecular Biology & Biotechnology (MSMBB, 2014), Monash University Malaysia, on 1st to 3rd October 2014. "Discovery of novel mosquito-dengue viral protein interactions: Construction of *Aedes aegypti* midgut cDNA library for use in yeast two–hybrid system"

  Tham Hong Wai, Vinod RMT Balasubramaniam, Hamdan Ahmad, Sharifah Syed Hassan

- Author in poster presentation in 21st Annual Scientific Meeting of the Malaysian Society for Molecular Biology & Biotechnology (MSMBB, 2014), Monash University Malaysia, on 1st to 3rd October 2014. "Expression and one-step purification of recombinant dengue virus type 2 (DENV-2) envelope domain (III) protein"

  Chew Miaw Fang, **Tham Hong Wai**, Sharifah Syed Hassan