CELLULAR RESPONSE OF INSECT CELLS TO VIRUS INFECTION

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ABSTRACT

RNA interference (RNAi) is the dsRNA-triggered gene regulatory mechanism that is evolutionally conserved in most eukaryotic cells. It has been widely used as a powerful tool for functional genomics in various organisms. In flies, mosquitoes or other insect cells, gene functional analysis by RNAi is usually performed through introduced dsRNAs that are synthesized by *in vitro* transcription.

RNAi serves as an important innate immunity against viruses in plants and invertebrates. It has recently been shown that *Aedes albopictus* mosquito C6/36 cells, commonly used for arbovirus propagation, possess an impaired RNAi pathway. In this study, we developed *in vitro* Dicer assay using extracts prepared from mosquito cells. Our results confirmed the inability of C6/36 cells to process dsRNAs into siRNAs, which is consistent with the loss-of-function of Dcr-2 due to a frameshift mutation. However, such a defect could not be complemented by introduction of *Drosophila* Dicer-2. To evaluate the RNAi-based antiviral mechanism in C6/36 cells, we analyzed the replication of a mutant Nodamura virus (NoV) genomic RNA1 of which viral RNAi suppressor B2 is not expressed (NoVR1ΔB2) and cannot accumulate to a detectable level in RNAi-competent cells. In C6/36 cells, the defective RNAi gives rise to complete restoration of NoVR1ΔB2 replication, suggesting that RNAi is the primary antiviral immunity in mosquito cells.

At present, dsRNA, as the trigger of the antiviral RNAi pathway in invertebrate and plants, is the major efficiency limitation factor in RNAi. In this study, a plasmid-based system was developed to express dsRNA intracellular from a DNA cassette containing two convergent T7 promoters in *Drosophila* S2 cells. Efficient knockdown of a
transiently expressed reporter gene or an endogenous gene can be achieved by dsRNA expressed from the system. A random cDNA library was constructed in the dsRNA expression cassette and initial screening led to identification of two host factors that are involved in antiviral response in *Drosophila* S2 cells. The plasmid-based dsRNA expression system provides an alternative tool for functional genomics in *Drosophila* and other insect cells.

**Keywords:** Antiviral, dsRNA, RNAi, Innate Immunity
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CHAPTER 1 INTRODUCTION

1.1 Dengue Virus

Dengue is the most prevalent mosquito-borne viral disease in humans in tropical and subtropical region all over the world. It is caused by dengue virus (DV) which belongs to the genus *Flavivirus* in the family *Flaviviridae*. Transmission of DV to humans is mediated by *Aedes* mosquitoes, including the primary vector *Aedes aegypti* and the secondary vector *Aedes albopictus*. Unlike in human hosts where DV infection is acute and often causes diseases, DV is generally not pathogenic to vector mosquitoes, and able to achieve persistent infection which is required for successful transmission. Mosquitoes get DV after ingestion of blood from infected humans, the virus can replicate in midgut epithelial cells, and then disseminate through haemoceol into salivary glands and other tissues (1). Different from vertebrates, mosquitoes lack the adaptive immune system. The innate immunity is similar to those of vertebrates, it not only confer the defense against bacteria, fungi and protozoa, also against some virus infection (2-4). In addition to physical and chemical barriers that contribute to vector competence, the cellular antiviral response has not yet been well-understood in mosquitoes (1, 5), but some of the cellular pathway such as Toll pathway, Imd pathway and Jak-STAT pathway were involved in antiviral response in different ways (6-11). After a mosquito get the virus from infected people, the mosquito remains persistently infected and can pass the virus to a susceptible individual during next blood feeding. DV is maintained in continuous cycles of human-mosquito-human transmission. Infection of DV is manifested from mild, flu-like symptoms to severe forms, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Dengue is endemic in tropical and sub-tropical regions, where 2.5 billion people living in urban and rural areas are at risk. There are about 390 million
cases of infection each year and about 500,000 cases develop to DHF or DSS (12) with a mortality rate varying from 1% to 5% (13-17). In the United States, *Ae. albopictus* mosquitoes has expanded infestation sites into 36 states in last two decades, resulting in increased risks of dengue infection (18-20). Since the outbreak in Florida and Hawaii in 2011, dengue has appeared more frequently along the U.S.-Mexico border, and threatens to reach a potential epidemic in the U.S. territory, Puerto Rico in 2007 (20-22). Emerging and resurging of dengue is increasingly recognized as a major public health issue in the world. DV has a positive single-stranded RNA genome that is approximately 11,000 nucleotides long and encodes a single open reading frame flanked by 5’- and 3’-noncoding regions. Translation of the single open reading frame produces a polyprotein that can be processed into three structural proteins, including nucleocapsid or core protein (C), a membrane-associated protein (prM), an envelope protein (E), and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (23-25). There are four serotypes of DV (DV-1, DV-2, DV-3 and DV-4), all of which have the potential to cause dengue fever (13). Recently, scientist reported that a fifth dengue virus was discovered in Malaysia, the first new subtype in last fifty years (12). Development of severe DHF and DSS is often associated with sequential infection with a distinct serotype of DV (26), which makes it more challengeable to develop and use vaccines (27, 28). Currently there is no approved drug and vaccine available for treatment and prevention, a few advanced tetravalent vaccines are still on their way which may offer a promising effect in the near future (29-32, 17). While the discovery of the fifth serotype (DV-5?) put new challenge on the vaccine development against all types simultaneously (12).
mosquito breeding sites with applications of effective insecticides was very successful in disruption of transmission of malaria, dengue and yellow fever by 1960s, but failed since 1970s because of complicated reasons, such as loss of financial supports, emergence of insecticide-resistant mosquito populations, environmental issues of insecticides (14) etc. The failure continuation of the vector control program, together with increased global trade, travel, urbanization and climate changes, result in emergence and resurgence of many vector-borne diseases including dengue (14). The problem should be solved. In addition to improve the conventional vector control program and strengthen the efforts to develop vaccines and antiviral drugs, new strategies for interrupting the mosquitoes-human contact are also critically needed, which largely rely on further understanding the mechanisms of dengue virus-mosquito interactions.

1.2 Antiviral immunity in insect cells

1.2.1 RNA interference (RNAi) as antiviral mechanism in insects.

RNA interference (RNAi) is a small RNA-mediated gene regulatory mechanism that is evolutionally conserved in most eukaryotic organisms (33). There are two major classes of small RNAs, short interfering RNAs (siRNAs) and microRNAs (miRNAs) (34). To expand the potent application of RNAi-based antivirals, it is important to understand the underlying mechanisms and the function of RNAi in cell biology. During last few years, accumulated evidence supports the view that RNAi serves as an antiviral defense in invertebrates (71, 67). RNAi is a small RNA-mediated gene regulatory mechanism that is conserved in plants, fungi, worms, insects and mammals (35, 36, 37, 38, 39). In RNAi machinery, a class of 21 to 25 nucleotides (nt) small regulatory RNAs play a major role
in regulating target gene expression (40, 41). These small RNAs can be divided into two groups based on their biogenesis and modes of function: small interfering RNAs (siRNA) and microRNAs (miRNA) (42-44). SiRNAs are processed from double-stranded RNAs (dsRNA) by the RNaseIII family enzyme called Dicer (Dcr) in cytoplasm(51). In plants, the dsRNA triggers may be derived from viral replicative intermediates, direct introduction of exogenous dsRNAs, inverted-repeat transgenes, products of endogenous RNA-dependant RNA polymerase, fold-back structures of RNAs, and transposons. SiRNAs are incorporated into the RNA-induced silencing complex (RISC) that contains the major component Argonaute (AGO) protein, and guide specific cleavage of complementary mRNAs at the central position of a siRNA/mRNA paring (52-54). MiRNAs are chemically similar to siRNAs, but they are encoded by evolutionarily conserved miRNA genes. Long primary transcripts expressed from miRNA genes can be processed into 70-nt long pre-miRNAs, which are exported to the cytoplasm and subsequently diced into ~22 nt miRNAs (45, 46). Mature miRNAs can be loaded into RISC, and direct inhibitory process of target mRNA expression. It’s known that RNAi machinery plays important roles in many biological processes, such as developmental control, physiological regulation, and stress responses. The first experimental evidence that demonstrates the natural antiviral roles of RNAi in animals was established in *Drosophila* cells infected with an insect virus flock house virus (FHV, *Alphanodavirus*, *Nodaviridae*) (64, 60). FHV is both trigger and target of the antiviral RNAi, and B2 protein of FHV is identified as a RNAi suppressor which counteracts such an antiviral response and is required for successful infection (60, 76). Recently, further genetic evidence has demonstrated that RNAi pathway in adult *Drosophila* acts as an innate
immunity against virus infection, and the dsRNAs produced during virus replication are the trigger of RNAi where Dcr2 and AGO2 serve as host sensor and effector of the antiviral immunity (58-61).

In *Drosophila*, there are distinct RNAi components required for production and function of siRNAs and miRNAs (47, 48). Dicer-2 serves as the central engine to sense double-stranded RNA (dsRNA) triggers, and to process them into siRNAs (49). The siRNAs are subsequently loaded onto the RNA induced silencing complex (RISC), and guide the RISC to recognize cognate mRNAs for destruction (50, 51). The target mRNAs is cleaved by Argonaute 2 (Ago2) protein at the central position of a siRNA/mRNA paring (48, 50, 52). For the biogenesis of miRNAs, Pasha and Drosha form the microprocessor that converts primary miRNAs into precursor miRNAs (pre-miRNAs) (53). A complex of Dicer-1 and Loquacious is required for processing of stem-loop structures of pre-miRNAs into mature miRNAs (54-56). AGO1 is involved for mature miRNA production, and also has impacts on miRNA-directed regulation of target mRNA (48). It is currently estimated that expression of at least 30% of all human genes is regulated by miRNAs (57). RNAi has been identified as a component of antiviral immunity in adult *Drosophila* (58-62) and in nematode worms *Caenorhabditis elegans* (62-64). RNAi has also served as an important antiviral defense in fungi (65, 66). In the siRNA pathway, the dsRNA triggers are generally derived from viral infection, direct introduction of exogenous dsRNAs, transcription of an inverted repeat transgene, processing of fold-back structures of RNAs, mobilization of transposable elements, or activities of endogenous RNA-dependant RNA polymerase (RdRP), especially in plants (67). In plants and invertebrates infected with viruses, dsRNAs generated from replicating viruses can be processed by
RNAi machinery, leading to accumulation of viral-specific siRNAs and inhibition of viral replication (67-69, 59, 70-73). Virus-specific RNAi is induced during viral infection, and plays a role in antiviral immunity (59, 74-76). In *Drosophila*, evidence from extensive studies using cultured cells and whole flies strongly supports the concept of antiviral RNAi (78, 59, 75, 79). On the other hand, viruses also evolve strategies to counteract the RNAi-based immunity. Non-structural or structural proteins encoded by a variety of viruses have been identified as viral suppressors of RNAi (VSR) (67, 59, 75-78). Mechanistically, most characterized VSRs suppress RNAi by sequestration of dsRNAs or siRNAs. VSRs bind to dsRNAs or siRNAs in a sequence-independent way, and protect dsRNAs from being processed by Dicer or prevent incorporation of siRNAs into the RISC (75, 78, 62, 79). At the cellular level, the RNAi pathway is an important component of innate antiviral immunity in *Drosophila* (61, 80, 57, 58, 81). Except its role in RNAi, the evolutionarily conserved DExD/H-box helicase Dicer-2 is also involved in controlling an inducible antiviral response in the fat body in *Drosophila* (82, 94). Recently in *Drosophila*, the toll pathway is demonstrated important for an antiviral response against *Drosophila* X virus (DXV), the Imd pathway in antiviral response against Cricket Paralysis virus (CrPV) and Jak-STAT signaling pathway is required but not sufficient for the host defense against *Drosophila* C virus (DCV) (89-91).

The RNAi pathway in mosquitoes was firstly described based on comparative genome analysis of *Aedes aegypti*, *Anopheles gambiae* and *Drosophila melanogaster*. Expressions of mosquito Dcr2, AGO2, AGO3 and AGO4 are required for active RNAi pathway in cultured cells (83, 84). Because many viruses that infect mosquitoes are ssRNA viruses, it is not surprising that those viruses can be targeted by RNAi mechanism.
At the whole organism level, depletion of AGO2 by dsRNA injection led to wide spreading of alphavirus O'nyong-nyong virus in *An. gambiae* mosquitoes (85). Infection of *Ae. aegypti* mosquitoes with Sindbis virus carrying a sequences derived from DENV genome results in resistance to the challenge of homologous DENV (86). Moreover, dsRNAs expressed from inverted-repeat transgenes, which are derived from the DENV-2 genome, can dramatically knock down DENV-2 accumulation in *Ae. albopictus* mosquito cells (87), and also can lead to impaired vector competence for DENV-2 in *Ae. aegypti* mosquitoes (88). It is very clear that viruses could be targeted by RNAi machinery in engineered mosquitoes. In addition to physical and chemical barriers that contribute to vector competence, the cellular antiviral response has not yet been well-understood in mosquitoes (1, 5), while some of the cellular pathway such as Toll pathway, Imd pathway and Jak-STAT pathway were indeed involved in antiviral response against different viruses (6-11), even though many important questions about the cellular responses to viral infection in mosquito cells still remain to be answered.

1.2.2 Other innate immune responses in insects

Innate immunity is an ancient defense that enables multicellular organisms to detect and fight infectious microbes (90). In addition to RNAi, other innate immune response also plays a role in antimicrobial response in *Drosophila*. Such as Toll pathway not only controls resistance to fungal and Gram-positive bacterial infection, but also involved in antiviral response for Drosophila X virus in *Drosophila melanogaster* (91, 92). While Gram-negative bacteria mainly activate the immune deficiency (Imd) pathway, which governs expression of the genes encoding many antibacterial peptides (AMPs) (93). Recent research indicated that Imd pathway is also involved in antiviral immune
responses against Cricket Paralysis virus in *Drosophila* (93). In addition to Toll and Imd pathways, a third, evolutionary conserved innate immunity pathway, Jak-STAT (Janus kinase-signal transducer and activator of transcription) also counteract viral infection in *Drosophila* (90). In this study, they also found that Jak-STAT signaling pathway is required but not sufficient for the antiviral response against Drosophila C virus in *Drosophila* (90), it requires other pathways like Toll pathway and Imd pathway work together to counteract the viral infection. Recent studies demonstrated that the Toll pathway is required for the efficient inhibition of DXV replication and spread during infection in *Drosophila* (6). They also found that DXV and *E. coli* infection induce similar expression levels of the Toll and Imd pathway target genes at different time points, which indicated that both the Toll and Imd signaling pathways are induced upon viral infection. They also found that the expression of any of these AMPs alone is not sufficient to confer viral resistance in *Drosophila*, which suggests that the antiviral response is cellular mediated. In *Ae. aegypti* mosquitoes, the activation of the Toll pathway through RNAi-mediated silencing of the negative regulator Cactus, inhibited first dengue replication in the midgut tissue, while repression of the Toll pathway through gene silencing of MyD88 significantly improved the dengue virus replication. These results demonstrated that the Toll pathway regulates anti-dengue defenses throughout the virus cycle in the mosquito. Also, this inhibition may be crucial for the duration of the extrinsic incubation period of the dengue virus in mosquito (6, 7). This study also indicated that *Ae. aegypti* Toll pathway is universal anti-dengue defense response independent of virus serotype specificity. Dengue infection also can activate Toll pathway, which in turn induces a mechanism that suppress the virus infection (6, 7).
Based on the transcriptional expression profiles of mosquito midguts infected with sindbis (SIN) virus, Sander et al., (2005) hypothesized that early innate immune responses to SIN infection was mediated by Toll pathway, which is later shut-off and the Imd pathway is activated by infection later, which means only Imd pathway but not Toll pathway has an antiviral effect at the level SIN RNA replication (8, 9). The observation of enhanced RNA replication in Drosophila deficient in components of the Imd pathway and increase in Relish dependent transcription in flies challenged with SIN replicon RNA demonstrated that for the first time that Imd/Relish pathway is involved in antiviral defense response (9). Other study also indicated that the Imd pathway is involved in antiviral immune responses to Cricket Paralysis virus (CrPV) infection in Drosophila (93). Recent study demonstrated that the Jak-STAT pathway can inhibit dengue virus infection in Ae. aegypti mosquitoes (10). The dengue infection activates the Jak-STAT immune signaling pathway, which in turn will control the virus replication. Their results suggested that the Jak-STAT pathway is part of the Ae. aegypti mosquito’s anti-dengue defense system and may act independently of the Toll pathway and the RNAi-mediated antiviral defenses (10). While in Drosophila, the Jak-STAT signaling pathway is required in antiviral defense, but not sufficient to activate the antiviral immune response in Drosophila by itself (10).
1.3 Specific Aims of this research
Dengue is one of the most prevalent mosquito-borne diseases in tropical and sub-tropical area. Due to the fact that there is no effective vaccine or antiviral drugs available to counteract the dengue virus infection, the vector control will still be the most efficient strategies in controlling dengue endemic. In the traditional vector controlling strategies, due to environmental concern from the insecticide (14) and insecticide resistance development in insect, the biological control will be the next prevalent strategy for it. In this study, we established plasmid-based dsRNA expression system in insect cells. By applying this system, we can screen insect and host factors that are required for dengue replication and infection. Once repress these factors by RNAi-mediated silencing, we can efficiently restrict the dengue virus replication/infection in transmission vector or host. Also, those genes can be used as potential antiviral drug target in host or biological control target in insect control.

As the primary vector of dengue transmission, Ae. aegypti genome sequences are available, but Aag2, ATC-10 cell lines are hard to be transfected, which makes RNAi-mediated silencing of target gene impossible. Secondary vector, Ae. albopictus C6/36 cell line is transfectable and comparable to Drosophila S2 cells, but no sequence available. While we still can employ Suppression Subtractive Hybridization (SSH) to screen and characterize genes that were differential expressed in response to dengue infection in C6/36 cells. Suppression Subtractive Hybridization (SSH) is PCR-based amplification of only cDNA fragments that differ between a control (driver) and experimental transcriptome. Differences in relative abundance of transcripts are highlighted, as are genetic differences between species. Removal of dsDNA formed by hybridization
between a control and test sample, thus eliminating cDNAs or gDNAs of similar abundance, and retaining differentially expressed, or variable in sequence, transcripts or genomic sequences

1.4.1 Differentially expressed genes in *Ae. albopictus* C6/36 cells in response to dengue infection

Suppression Subtractive Hybridization (SSH) was employed to screen and characterize genes that were differential expressed in *Ae. albopictus* C6/36 cell line in response to dengue virus type 2 (New Guinea-C strain) infection. By this study, we expect to discover some novel insect cellular factors required for dengue virus replication in the mosquito transmission vectors.

1.4.2 Characterization of the antiviral RNAi pathway in *Ae. albopictus* C6/36 cell line

*Ae. albopictus* C6/36 clone is one of the most commonly used mosquito cell lines for the propagation of arboviruses. Compared to original uncloned heterogeneous cells and other cloned cells, C6/36 cell exhibits a high degree of susceptibility to infection by many arboviruses. It’s important to understand the antiviral RNAi pathway in *Ae. albopictus* C6/36 in order to elucidate the reason for its high susceptibility and permissibility to arboviruses infection.

1.4.3 Development of plasmid-based dsRNA expression system in insect cells

Double-stranded RNA (dsRNA) is the trigger of RNA interference (RNAi)-mediated gene regulation. Dicer processes dsRNAs into short interfering RNAs (siRNAs), which are incorporated into the effector RNA induced silencing complex (RISC) and direct degradation of homologous target mRNAs. In plants and invertebrates, the RNAi machinery also acts as an antiviral mechanism through production of viral siRNAs by
Dicer and silencing of replicating viruses. In this study, we try to establish a plasmid-based, high efficiency dsRNA expression system in insect cells that can be used for large scale screening of novel cellular factor required for virus infection/replication.

1.4.4 Construction of dsRNA library and identification of novel genes involved in antiviral response in insect cells

To test the feasibility of the dsRNA expression system for functional genomic analysis, a plasmid-based random cDNA library was constructed by cloning *Drosophila* cDNAs into EcoR I site on pUC/DS. In presence of T7 RNA polymerase, the pUC/DS-based cDNA library is equivalent to a dsRNA library because dsRNAs can be expressed from the inserted cDNAs in transfected cells. As shown before, depletion of the core component (Ago-2) in antiviral RNAi by dsRNA gives rise to rescue of NoVR1GFP, during which GFP presents a visible marker for viral replication. However, it is not known if there is any cellular factor that is not known to be involved in antiviral response but could bring NoVR1GFP to replicate to a significant level when its expression is inhibited. To identify such factors, initial screening was performed using pUC/DS-based dsRNA library, and a B2-null RNA1 mutant of flock house virus (FHV), FHVR1GFP, served as a reporter because of its minimal and low background viral replication compared to NoVR1GFP (62, 76, 94).
CHAPTER 2 MATERIALS AND METHODS

2.1 Cell cultures and transfection

*Drosophila melanogaster* S2 cells, *Ae. albopictus* C6/36 cells, *An. gambiae* 4a-2s4 cells, *Ae. aegypti* Aag2 and ATC-10 cells were passaged and maintained at 27°C in Schneider’s *Drosophila* media (GIBCO) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin and 10% FBS (20% FBS for 4a-2s4). When cells are ready (90-100% confluency), cells were passaged into 24-well plate with concentration of 0.5 ~ 1.0 × 10^6 cells/ml one day before transfection. Transfection was performed using the Lipofectamine™ LTX and Plus™ Reagent (Invitrogen) when the cells reached to 50-80% confluency by following the manufacturer’s instructions. For 24-well plate, 0.5 μg of each plasmid DNA was diluted in 100 μl Opti-MEM®I reduced serum medium. Mix Plus™ reagent gently before use, add 0.5 μl Plus™ reagent for each plasmid into the diluted DNA. Mix gently and incubate for 5 minutes at room temperature. Mix lipofectamine™ LTX gently before use, and add 1.25 μl LTX directly to the diluted DNA. Mix thoroughly. Incubate the mixture 30 minutes at room temperature for the DNA-lipid complexes. The DNA-lipid complexes are stable for 6 hours at room temperature. After the incubation, mix the complexes by pipetting up and down couple times, and then add the complexes dropwise to the well containing cells. Mix gently by rocking the plate back and forth. Incubate the cells at in 27°C incubator for 18-48 hours prior testing for transgene expression efficiency.

2.2 Plasmids and viruses

The eGFP gene was cloned into pIZ/V5 His (Invitrogen) at BamH I/Xho I sites, generating pIZ/eGFP. The cDNA clones for nodamura virus (NoV) wild type (wt) RNA1 and RNA1 B2-deletion mutant (RNA1ΔB2) have been described previously (62). NoV
RNA1 frameshift mutant (RNA1fs) contains a mutation in the ORF of viral RNA-dependent RNA polymerase (RdRP), which abolishes its activity for viral replication. The full-length viral cDNAs were cloned downstream of a T7 promoter, and infectious NoV RNA1 transcripts were synthesized using the mMESSAGE mMACHINE® T7 kit (Ambion) by following the manufacturer’s instructions. The infectious NoV RNA1 clone was linearized by any unique restriction enzyme downstream of the full-length viral cDNAs, after purification by phenol/chloroform (1:1), the linearized plasmid (1 µg) will serve as the transcription template. The transcription reactions need to be assembled at room temperature to avoid the spermidine in the 10 × reaction buffer to coprecipitate the template DNA on ice. After the transcription reaction assembled, the reactions were incubated at 37°C for 2 hours to maximum the yield. Then add 1 µl DNase, mix well and incubate 15 minutes at 37°C to remove the template DNA. After the DNase treatment, the mixtures were phenol: chloroform (1:1) extracted and isopropanol precipitated for the purification purpose. This process will remove all enzyme and most of the free nucleotides from mMESSAGE mMACHINE Kit reactions. Add 295 µl nuclease-free water and 35 µl ammonium acetate stop solution, and mix thoroughly. Extract with an equal volume (350 µl) of phenol/chloroform (1:1) it can be water-saturated, buffer-saturated, or acidic), and then with an equal volume of chloroform. Recover aqueous phase and transfer to new tube, precipitate the RNA by adding equal volume of isopropanol and mixing well. Chill the mixture for at least 15 minutes at -20°C, centrifuge at 4°C for 15 minutes at 13, 500 rpm (Eppendorf, Centrifuge 5804 R, FA-45-30-11 rotor) to pellet the RNA. Discard the supernatant solution, wash the pellet once
with 500 µl 80% ethanol, and resuspend the RNA in a nuclease-free water, store frozen at -70°C for later transfection.

The equal amounts of RNA transcripts (1 µg) were transfected into *D. melanogaster* S2 cells, *Ae. albopictus* C6/36 cells and *An. gambiae* 4a-2s4 cells cultured in 12-well plates respectively using Transmessenger Transfection Reagent (Qiagen). For 24-well plate, on the day of transfection, dilute 1.6 µl Enhancer R in Buffer EC-R. Add 0.8 µg RNA (minimum RNA concentration 0.1 µg/ µl) to make the final volume of 100 µl (Enhancer R + Buffer EC-R + RNA = 100 µl) and mix by vortexing for 10 seconds. 

**Important Note:** 1. Always mix Enhancer R with Buffer EC-R before adding RNA, 2. Always keep the ratio of RNA to Enhancer R constant). Incubate the mixture at room temperature for 5 minutes, then spin down the mixture for a few seconds to collect drops from the top of the tubes. Add 4 µl TransMessenger Transfection Reagent to the RNA-Enhancer R mixture. Mix by pipetting up and down 5 times, or by vortexing for 10 seconds. Incubate the samples for 10 minutes at room temperature to allow transfection-complex formation.

While complex formation takes place, gently aspirate the growth medium from the plate, and carefully wash cells once with sterile 1 × PBS using 1.5 – 2 times the volume of the medium used for cell seeding. Add 100 µl cell growth medium without serum or antibiotics to the tube containing the transfection complexes. Mix by pipetting up and down twice, then immediately add the transfection complexes drop-wise onto the cells. Gently swirl the plate to ensure uniform distribution of the transfection complexes. Incubate cells with the transfection complexes for 3 hours under their normal growth conditions. Remove the complexes from the cells. Wash cells once with 1 × PBS, then add 0.5 ml fresh medium containing serum and antibiotics to the cells. At two days after
transfection, cells were collected and total RNAs were extracted from transfected cells using the Trizol Reagent (Invitrogen).

Plasmid-based dsRNA expression system pUC/DS was generated by inserting the dsRNA expression cassette at the BamH I/Sac I sites in pUC19, which carries two convergent T7 promoters with an EcoR I site in-between and flanked by a T7 terminator and an *E. coli* rrnB T1 transcription terminator sequence. It was generated by a two-step cloning, and each step involves cloning of annealed long oligonucleotides that contain one T7 promoter with a terminator into pUC19 between Sac I and BamH I. EcoR I site was included in the oligonucleotides at the end of T7 promoter sequence, and served as the cloning site for cDNA insertion. T7 RNA polymerase and poly (A) signal was cloned at Hind III/Cla I site to generate pIB/T7. pDS was constructed by inserting the dsRNA expression cassette into pIB/T7 at the Cla I site, right at the downstream of the T7 RNA polymerase gene poly (A) signaling. For *Renilla* reporter system, the *Renilla* luciferase gene was released from pGL4.75 [hRluc/CMV] and cloned into pIZ/V5 His to generate pIZ/Rluc reporter system.

Dengue virus type 2 (DV2) New Guinea C was propagated in Vero cells. The virus was harvested at 7 days post infection and titrated by plaque assay.

*Drosophila* S2 cells were infected with dengue virus type 2 (DV2) New Guinea C strain, and the cells were kept to continuously passage for more than ten times to produce a persistent cell line (S2-DV2). The S2 adapted DV2-NGC virus (DV2-S2) was propagated in C6/36 for two rounds, three days after the second round, collect the virus for S2 cell infection.
Flock house virus (FHV) was collected from S2-FHV persistent cell line, then was propagated in S2 cells for two rounds, three days after the second round propagation, collect FHV for S2 cell treatment.

2.3 Plaque Assay

Vero cells (African green monkey kidney epithelial cells) were use for dengue virus titration. Vero cells were grown in T-75 flasks, in DMEM (Dulbecco’s Modified Eagle Medium) with 10% FBS and 1% antibiotics at 37°C with 5% CO₂ and reseeded every three to four days. When cell90% -100% confluent, trypsinize and seed into two 6-well plates at a density of 250, 000 cells/well (2 ml). Cells were then incubated for a period of 2-3 days until a confluency of 70-80% is obtained. Dilute the dengue virus in successive 10 fold dilutions with virus diluents (same composition that virus was cultured in) for a total of five dilutions (one well leave as mock). Remove most of the culture medium from the vero plates (leaving just enough media to cover the cells without any drying-out occurring, and then add 200 µl of each virus dilution to a corresponding well (mock just add 200 µl virus diluents), and repeat the process on a 2nd plate in order to obtain duplicate dilutions. Place the inoculated plates on an incubated rocker for 1 hour. During the inoculation, prepare enough of the primary nutrient agar overlay to add 4 ml of overlay to each of the wells. Follow the preparation recipe which includes M199 (supplemented with 0.1 gm/L L-glutamine, 100 U/ml Penicillin/100 µg/ml streptomycin, Fungizone, Gentamycin, and 5% FBS), 7.5% sodium bicarbonate, double distilled water, 1% Noble Agar and DEAE. Keep overlay preparation in 56oC waterbath until vero cell/virus incubation is done. Once incubation is completed, add the 4 ml nutrient overlay carefully so as not to disrupt the vero cell monolayer on the plate. Also be careful to
make certain that overlay is at a proper temperature that it will not burn the vero cells, but not cooled enough to begin the hardening process (Typically, the flask is determined to be ready by touching it to the skin of the forearm). After overlay has been applied to every well, place the plate in the incubator (37°C, 5% CO₂) for few hours. Once overlay has hardened, turn the plates upside down and incubate for 7 days undisturbed. Prepare enough of the same overlay preparation, with the substitution of 1% Neutral Red (0.33% Neutral Red stock), in place of the water, to cover each well with 2 ml of agar recipe. Place back in the incubator until new overlay has hardened, and again, turn plates upside down and incubate at 37°C, 5% CO₂ overnight. Begin counting plaques the next day, and every day for the next 3 days or until you have established and endpoint of the plaque formation. Virus tilters will be calculate as follows: [(average number of plaques from replicates) × dilution]/(innoculum volume, ml) = plaque forming units/ml (PFU/ml).

2.4 Luciferase Assay
Renilla luciferase assay was performed using Renilla Luciferase Assay System (Promega) by following the manufacturer’s instructions. At two days after transfection, remove growth medium from the cultured cells and gently apply 400 µl 1 × PBS to rinse the cells, swirl the vessel briefly to remove detached cells and residual growth medium, add 100 µl freshly prepared 1 × Renilla luciferase assay lysis buffer to each well for homogenous lysates preparation. Harvest cells immediately following the addition of Renilla luciferase assay lysis buffer by scraping with a disposable plastic cell lifter or rubber policeman. Transfer the lysate into a tube and lysis cells at room temperature for 15 minutes with consistent rotating the tube on a rotator. The cell lysate can undergo 1 or 2 freeze-thaw cycles to ensure complete lysis of cells before performing the assay. Add 1
volume of 100× *Renilla* Luciferase Substrate to 100 volumes of *Renilla* Luciferase Assay Buffer to prepare *Renilla* luciferase assay reagent right before use. Add 100 µl of Renilla luciferase assay reagent to the 96-well assay plate, then add 20 µl of cell lysate and mix quickly by swirling the plate. Place the plate into Synergy 2 luciferase reading system for the luciferase measurement. (**Note:** Determine the original cell density and transfection parameter to ensure that cells are no more than 95% confluent at the desired time of lysate preparation date.)

**2.5 RNA Extraction, Northern Blot and Small RNA Detection**

Total RNAs were extracted from transfected S2 cells using the Trizol reagent (Invitrogen) following the manufacturer’s instruction. Cells were collected and washed twice in 1 × PBS, pellet by centrifuge, add 1 ml Trizol reagent to each sample and resuspend the cell pellet as soon as possible by vigorously shaking or vortex for one minute, lysis the cell at room temperature for 3 minutes, then add 200 µl chloroform (1/5 volume of Trizol reagent) to each sample, mix well by shaking or vortex for one minute, incubate at room temperature for 3-5 minutes before centrifuge with 12, 000 g for 15 minutes at 4°C for the phase separation. Recover aqueous phase and transfer to new tube, precipitate the RNA by adding isopropanol (70% of aqueous phase or ½ Trizol volume) and mixing well, incubate at room temperature for 10 minutes for RNA precipitation. Spin down sample at 13, 500 rpm for 15 minutes at 4°C to pellet the RNA. Discard the supernatant and wash the RNA pellet with 500 µl 80% ethanol (Prepared with RNase-free water), discard the wash solution and air-dry pellet for 5-10 minutes at room temperature (Do not overdry the pellet or you won’t be able to redissolve it well.),
dissolve pellet in RNase-free water or DEPC-H$_2$O (Note: DEPC inhibits RT reaction). Store frozen at -80°C for later use.

Appropriate amount (10 - 15 μg) of total RNA was loaded into 1 1.5 ml Eppendorf tube and add RNase-free water to final volume of 10 μl (If volume is more than 10 μl, then aliquoted to 1.5 ml Eppendorf tubes and dried in a SpeedVac, redissolve in 10 μl RNase-free water.). Then add 10 μl sample buffer (10 × MOPS buffer/37% formaldehyde/formamide/H$_2$O = 1:1.8:5:2.2), heated to 65°C for 10 minutes and cooled on ice for ~ 2-3 minutes before add 4 μl 6 × RNA loading buffer. Load the mixture on the 1% formaldehyde-denatured RNA gel (Maximal amount is about 25 μl), run the gel in 1 × MOPS buffer at 100 V (FisherBiotech electrophoresis system) for 2-3 hours until the bromophenol blue reached the 2/3 bottom of the gel. Rinse the gel in 10 × SSC buffer for 10 minutes. RNAs were transferred to a Hybond™-N+ membrane with Trans-BLOT SD Semi-dry transfer cell (BIO-RAD). Then the membrane was briefly washed in 10 × SSC, and fixed in a Gene Linker UV chamber (Spectrolinker™, 180mj/cm$^2$). To certify equal loading of RNA samples, the membrane was stained with 0.2% methylene blue to estimate the amount of RNA by visualization. Then the pre-hybridization was performed in the PerfectHyb™ Hybridization Buffer (Sigma) at 65°C for at least 1 hour to overnight (94) in a hybridization shaker (Amersham Pharmacia Biotech). $[^{32}P]$-dCTP labeled DNA fragment with the random primer DNA labeling Kit (TakaRa) were used as probe. Labeled probes were boiled at 100°C for 5 minutes and cooled on ice for 2-3 minutes before adding to pre-hybridization mix, and incubated for 8 hours to overnight.
For small RNA detection, Appropriate amount (20-30 µg) RNAs were mixed with formamide (1/2 the RNA volume), then denatured at 95°C for 5 minutes, quickly chilled on ice, add 6 × RNA loading buffer, after that the samples are ready for loading. RNA samples were loaded on 15% Urea-PAGE gel in 0.5 × TBE buffer at 200V until bromophenol blue reached the bottom of the gel (about 3 -4 hours). To certify equal loading of RNA samples, the gel was stained in 1 µg/ml EB solution in 0.5 × TBE buffer to estimate the amount of RNA by visualization. After that, RNAs were transferred from gel to a Hybond™-N+ membrane in 0.5 × TBE buffer at 3 mA/cm² for 30 minutes with Trans-BLOT SD Semi-dry transfer cell (BIO-RAD). Rinse the membrane in 0.5 × TBE buffer, fixed in a Gene Linker UV chamber (Spectrolinker™, 180mj/cm²) and store at 4°C until use.

2.6 Denaturing urea polyacrylamide gel electrophoresis (Urea-PAGE)

For small RNA detection, prepare 15% denaturing urea polyacrylamide (Acrylamide:Bis-Acrylamide = 19:1) gel in a total volume of 45 ml: combine 4.5 ml of 5 × TBE and 10 ml H₂O, dissolve 18.9 g urea by continuously stirring with a small magnetic bar, then add 16.9 ml of acrylamide 40% solution (Acrylamide: Bis-Acrylamide 19:1) to make the total volume up to 45 ml. Add 240 µl of 10% Ammonium persulfate and 10 µl TEMED. Immediately pour gel with a 25 ml serological pipette, place the comb and keep it horizontal at room temperature for about 30 minutes until polymerized. Pre-run the gel in 0.5 × TBE for 30 minutes at 200 volts, rinse the well with 0.5 × TBE using a 30 ml syringe, and load the heat-denatured RNA sample prepared as described above. The [³²P]-labeled siRNA or DNA oligonucleotide is loaded as a marker. Run gel at 200 volts until bromophenol blue reaches about 2/3 of gel (siRNAs should migrate between the
bromophenol blue and xylene cyanol FF). Carefully remove the gel from the glass plate, drain off the extra buffer from the gel and stain the total RNA with EB staining reagent in 0.5 × TBE buffer as loading control. Transferred RNAs from gel to a Hybond™-N+ nylon membrane in a 0.5 × TBE buffer at 3 mA/cm² for 30 minutes with a Semi-Dry Transfer Cell (BIO-RAD), rinse the membrane with 0.5 × TBE, then UV crosslink the membrane at 150 mJoule (Membrane can be stored at 4°C until use). Pre-hybridization was performed at (37-42°C) for at least one hour, then [γ-32P]-ATP labeled oligonucleotide probe will be added to the pre-hybridization mix, and continue the incubation for 8 hours to overnight, and after three serial wash steps (once in 2 × SSC/0.1% SDS, twice in 0.2 × SSC/0.1% SDS, each wash 30 mins at RT to 37°C), expose the membrane to a sheet of X-ray film (BioMax MS Film, Kodak) or a screen of PhosphorImager and check results. Exposure time will depend on the strength of radioactive signal on the gel.

2.7 Quantitative Real-time RT-PCR
Total RNAs (1 µg) from Drosophila S2 cells were reverse transcribed into cDNA using MMuLV Reverse Transcriptase (New England Biolabs) following the manufacturer’s protocol. Quantitative real-time PCR was performed on Mastercycler® ep realplex (Eppendorf) with KAPA SYBR Fast Universal qPCR Kit (KAPA Biosystems). All samples were analyzed in triplicate. The raw data were uploaded on pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php with the free software RT2 Profile PCR Array Data Analysis Version 3.5. The p values are calculated based on a Student’s t-test of the replicate 2^(ΔΔCt) values for each gene in the control group and treatment groups.
2.8 Construction of a plasmid-based dsRNA library

Purification of mRNAs from total RNAs was performed using the mRNA isolation kit (Roche). First and second strand cDNA synthesis were carried out using M-MuLV Reverse Transcriptase and the Second Strand cDNA Synthesis (New England Biolabs) following the manufacturer’s instructions. After digestion of cDNAs with Rsa I, the cDNAs were separated on 1% agarose gel electrophoresis and the fragments with the length of 400-900 bp were recovered. To prepare the library vector, pUC/DS was digested with EcoR I and blunted using DNA Polymerase I, Large (Klenow) Fragment (New England Biolabs), followed by treatment with calf intestinal phosphatase – Alkaline Phosphatase (AP) (Roche Applied Science). Ligation of cDNA with the vector pUC/DS was performed using T4 DNA ligase (Roche Applied Science), and the ligation product was transformed into *E. coli* DH5α/Top10 competent cells. PCR-based screening with M13 forward and reverse primers was used to verify ligation and cloning efficiency, and 100 positive clones that contain cDNA inserts were selected for plasmid preparation and initial screening.

2.9 Establishment of S2 cell line that is persistently infected with dengue virus

*Drosophila* S2 cells were infected with dengue virus (DV) type 2 New Guinea C strain, and the cells were kept to continuously passage for more than ten times to produce a persistent cell line (S2-DV2). The infection rate in S2-DV2 was confirmed by indirect immunofluorescent assay (IFA), and around 90%-100% of S2 cells are infected.
2.10 Synthesis of radioactive labeled dsRNAs by \textit{in vitro} transcription

Ambion’s MEGAScript® T7 Kit provides an efficient tool to produce dsRNAs with high yield and quality. According to manufacturer’s instruction, dsRNAs are synthesized using the DNA template with two convergent T7 promoters flanking at both ends and labeling of dsRNAs is carried out by including \([\alpha^{-32}P] UTP\) in \textit{in vitro} transcription reaction. The reaction assembles and purification will be the same as mRNA synthesis with mMESSAGE mMACHINE Kit with slice difference. The UTP concentration will be only 1/40 of other NTPs, with \([\alpha^{-32}P] UTP\) substitute the rest UTP to label the synthesized dsRNA.

2.11 \textit{In vitro} assay for Dicer activity using extracts from \textit{Drosophila S2 cells and mosquito cells}

Processing of dsRNA into siRNAs by Dicer was first established by Hannon and colleagues (51, 53). Crude and purified extracts prepared from \textit{Drosophila S2 cell} have been widely used in biochemical analysis of RNAi machinery. In our study, we adopted the same method with some modification. Each cell lines were passaged into two 100-mm cell culture dishes with complete Schneider's \textit{Drosophila} medium at a concentration of 1 ×10^6 cells/ml, and incubate at 28ºC. At 3 days after passage, collect cell culture into 50-ml centrifuge tubes. Centrifuge at 1000 g for 3 minutes at room temperature to pellet the cells, discard the supernatants (medium). Gently resuspend the cell pellets in 10 ml 1 × PBS to wash the cell twice, resuspend the cell pellets in 4 ml of ice-cold Hypotonic buffer, and combine all cell suspensions into one 50-ml centrifuge tube. Centrifuge at 1000 g for 5 minutes at 4ºC, remove the supernatant. Estimate the volume of the cell pellet, and add 50%-80% volume of ice-cold Hypotonic buffer without KCl. Gently resuspend the cells, transfer the cell suspension into a cold Dounce tissue homogenizer.
(Type B, 2 ml, Kontes), and keep on ice for 10 minutes. Slowly dounce 30 times, and transfer the lysate to a 1.5-ml microcentrifuge tube. Centrifuge at 20,000 g at 4°C for 20 mins. Carefully transfer the supernatant into a 1.5-ml microcentrifuge tube, and do not touch the pellets. Add glycerol to a final concentration of 10%, store the S2 extract at -80 ºC for in vitro dicer assay.

The dsRNA corresponding to the 500-bp of the 5’-end eGFP was uniformly labeled with [α-32P] UTP by using Ambion’s MEGAScript® T7 Kit, and incubated with cell extracts prepared from cultured cells as described previously (95). After incubation at 30°C for 2 hours, the RNAs in the reaction were purified and analyzed by 15% Urea-polyacrylamide gel electrophoresis (Urea-PAGE) followed by direct autoradiography. The siRNA of eGFP purchased from Qiagen was labeled with [γ-32P] ATP using T4 polynucleotide kinase and used as siRNA size marker.

2.12 Immunoprecipitation

*Drosophila* S2 cells and *Ae. albopictus* C6/36 cells transfected with pIZ/FLAG-DmDcr2 were collected at two days after transfection, and lysis in 1× Lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.1 % Triton-X-100, pH7.4) at room temperature for 15 minutes. Clarify lysate of denatured protein and cell debris by centrifugation for 10 minutes 8, 200 g at 2-8°C. FLAG-tagged *Drosophila* Dcr-2 (FLAG-DmDcr2) was immunoprecipitated from cell extracts using anti-FLAG M2 affinity gel (Sigma). Before use, carefully mix EZview Red ANTI-FLAG M2 Affinity Gel beads until completely and uniformly suspended. After that, immediately aliquot 40 µl of the 50% slurry into a clean 1.5 ml microcentrifuge tube on ice. The beads were washed/equilibrated twice with 500 µl 1× TBS (50 mM Tris-HCl, 150 mM NaCl, pH7.4), add 200 – 1000 µl cell lysates
supernatant to the washed resin. Gently shake all samples and control on a roller shaker for 1-2 hours at 2 – 8°C. The binding step may be extended overnight to ensure maximum binding. Pellet the beads by centrifugation 8, 200 g for 30 seconds, remove the supernatant by pipette. Wash the bead pellet twice with 500 µl 1 × TBS (50 mM Tris-HCl, 150 mM NaCl, pH7.4), and place on ice for next step. Add 100 µl 3 × FLAG elution solution (150 ng/µl in 1 × TBS [50 mM Tris-HCl, 100 mM NaCl, pH7.4]) to each sample and control resin. Incubate the samples and control with gentle shaking for 30 minutes at 2 – 8°C. Pellet the resin for 30 seconds at 8, 200 g, transfer the supernatant to fresh test tube for further analysis. *In vitro* analysis of dsRNA processing using purified FLAG-DmDcr2 was performed as described in 2.11 (95).

2.13 Western Blotting and Antibodies
Protein samples were separated on a 12% SDS-PAGE (Acrylamide:Bis-Acrylamide = 29:1) and transferred to a Hybond™-P nylon membrane. The membranes were blocked in 1 × TBST wash buffer containing 1% nonfat dried milk at room temperature for overnight, then incubated with the primary antibody, anti-FLAG mouse monoclonal antibody (1 µg/ml) at room temperature for 1 hour, then wash three times with 1 × TBST wash buffer (15 mins per wash) and then incubated with HRP-conjugated secondary antibody (0.25 µg/ml) - goat anti-mouse radish peroxidase (Thermo Scientific) at room temperature for 2 hours, with three washes same as after the primary incubation, the membrane is ready for signal detection. Signal was detected by using Piece® ECL Western Blotting Substrate (Thermo Scientific) as recommended by the manufacturer. Mix detection reagents 1 and 2 at 1:1 ratio (The amount of detection reagent mix need to cover the membrane surface at least) and add it to the blot. Incubate the blot at room
temperature for 1 minute. Drain excess reagent, cover blot with a clear plastic sheet protector or clear plastic wrap and signal was detected by GENE GNOME SYN GENE BIO IMAGINE system.
CHAPTER 3 DIFFERENTIALLY EXPRESSED GENES IN Aedes albopictus C6/36 IN RESPONSE TO DENGUE INFECTION

3.1 Introduction
As the most prevalent mosquito-borne viral disease in humans in tropical and sub-tropical region all over the world, dengue is transmitted to humans by Ae. mosquitoes, including the primary vector Ae. aegypti and the secondary vector Ae. albopictus. Unlike in human hosts where dengue infection is acute and often causes diseases, it is generally not pathogenic to vector mosquitoes, while able to achieve persistent infection which is required for successful transmission. So, in Aedes mosquitoes, there must have some cellular genes that are required for dengue infection/replication. The genome sequence of Ae. aegypti was released recently (96), which makes large scale gene functional characterization possible. Unfortunately, the Ae. aegypti cell lines such as Aag2, ATC-10 are hard to be transfected, which makes the functional verification by RNAi-mediated silencing impossible mission. While Ae. albopictus C6/36 cell line is transfetcable and the efficiency is comparable to Drosophila S2 cells (Figure 9A). Even some high through-out strategies such as microarray analysis etc was excluded due to the absence of genome sequence, we still can employ Suppression Subtractive Hybridization (SSH) to screen and characterize genes that were differential expressed in response to dengue virus type 2 (New Guinea-C strain) infection in Ae. albopictus C6/36 cell line.

Subtractive hybridization is a technology that allows for PCR-based amplification of only cDNA fragments that differ between a control (driver) and experimental transcriptome. Differences in relative abundance of transcripts are highlighted, as are genetic differences between species. The technique relies on the removal of dsDNA formed by hybridization between a control and test sample, thus eliminating cDNAs or gDNAs of similar
abundance, and retaining differentially expressed, or variable in sequence, transcripts or genomic sequences. In this study, we employed SSH to screen and characterize genes that were differential expressed in response to DV2-NGC infection in Ae. albopictus C6/36 cell line.
3.2 Results

3.2.1 Flow chart of Suppression Subtractive Hybridization (SSH)

**cDNA synthesis**
cDNAs prepared from control (cDNA-A) and virus-infected (cDNA-B) *Ae. albopictus* ~

**Rsa I digestion**
The two cDNA populations (A and B) are separately digested to obtain 0.1-2kb blunt-ended molecules

**Adapter ligation**
Forward subtraction: cDNA-B as tester; reverse subtraction: cDNA-A as tester

**First hybridization and second hybridization**

**First and second amplification by suppression PCR**

**Subtractive cDNAs**
Differentially expressed sequences are enriched

**EcoR I adaptor ligation**

**Cloning of cDNAs into pUC/DS or pDS vector (up- or down-regulated genes)**

**E.coli transformation of cDNA library**

**Colony picking and transfer to membrane for Dot blot analysis**

**Sequencing the differentially expressed clones**

**Blastp against *Aedes aegypti* to find the homology of differentially expressed clones**

**Functional characterization of differentially expressed clones**
3.2.2 Differential expressed cellular genes in response to DENV-2 infection in *Ae. albopictus* C6/36 cell line

**Figure 1.** Accumulation of viral genomic RNAs in C6/36 cells infected with DV2 NGC. The cells were infected with dengue virus type 2 (DV2) (New Guinea C strain) at an MOI of 1. Samples were collected at indicated time points (days) post infection (dpi). Total RNAs were extracted using Trizol Reagent. Northern hybridization for detection of viral RNAs was done with the probe of $^{32}$P-labeled NS2B cDNA of DV2 NGC.
Figure 2. Flow chart for screening mosquito cellular genes that are affected by DV2 infection.
Figure 3. Differentially expressed mosquito cellular genes in response to DV-2 infection in *Ae. albopictus* C6/36 cells. ◊, Up-regulated by DV2 infection; ◇, Down-regulated by DV-2 infection.
**Table 1. Ae. albopictus** cellular genes upregulated by DV-2 infection in C6/36 cells

<table>
<thead>
<tr>
<th>Clone</th>
<th>Description (BLASTp results)</th>
<th>Expression</th>
<th>Function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td><em>Culex quinquefasciatus</em> conserved hypothetical protein</td>
<td>+</td>
<td>Unknown</td>
<td>N/A</td>
</tr>
<tr>
<td>F2</td>
<td><em>Aedes aegypti</em> protein transport protein sec23</td>
<td>+</td>
<td>Transport protein from ER to Golgi</td>
<td>Hicke et al., 1989, 1992; Cai et al., 2007</td>
</tr>
<tr>
<td>F5</td>
<td><em>Aedes aegypti</em> translation initiation factor 5C</td>
<td>+</td>
<td>Initiation translation from mRNA into polypeptide</td>
<td>Dong et al., 2009</td>
</tr>
<tr>
<td>F25</td>
<td><em>Aedes aegypti</em> cytochrome c oxidase subunit iv</td>
<td>+</td>
<td>Catalyzes the reduction of oxygen to water</td>
<td>Ho et al., 1995</td>
</tr>
<tr>
<td>F27</td>
<td><em>Aedes aegypti</em> vacuolar ATP synthase subunit ac39</td>
<td>+</td>
<td>Acidify a range of intracellular organelles</td>
<td>Mellman, 1992; Dow, 1999</td>
</tr>
<tr>
<td>F47</td>
<td><em>Culex quinquefasciatus</em> histone deacetylase 3</td>
<td>+</td>
<td>Transcription regulation</td>
<td>Atkinson et al., 2007</td>
</tr>
<tr>
<td>F55</td>
<td><em>Aedes aegypti</em> epoxide hydrolase</td>
<td>+</td>
<td>Cis-stilbene-oxide hydrolase activity</td>
<td>Borovsky et al., 2002</td>
</tr>
<tr>
<td>F100</td>
<td><em>Aedes aegypti</em> high density lipoprotein binding protein / vigilin</td>
<td>+</td>
<td>RNA binding</td>
<td>Nene et al., 2007</td>
</tr>
<tr>
<td>F227</td>
<td><em>Aedes aegypti</em> peroxiredoxin 6, prx-6</td>
<td>+</td>
<td>Antioxidant processes</td>
<td>David et al., 2007</td>
</tr>
<tr>
<td>F248</td>
<td><em>Aedes aegypti</em> dihydrolipoamide dehydrogenase</td>
<td>+</td>
<td>Flavin-dependent oxidoreductase</td>
<td>Carothers et al., 1989</td>
</tr>
<tr>
<td>F272</td>
<td><em>Aedes aegypti</em> DNA topoisomerase/gyrase</td>
<td>+</td>
<td>Control of topological states of DNA by transient breakage and subsequent rejoining of DNA strands</td>
<td>Nene et al., 2007</td>
</tr>
</tbody>
</table>

**Note:** +, up-regulated.
Table 2. *Ae. albopictus* cellular genes down-regulated by DV-2 infection in C6/36 cells

<table>
<thead>
<tr>
<th>Clone</th>
<th><strong>Description</strong> (BLASTp results)</th>
<th><strong>Expression</strong></th>
<th><strong>Function</strong></th>
<th><strong>References</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>R23</td>
<td><em>Aedes aegypti</em> cytidylate kinase</td>
<td>–</td>
<td>DNA replication and repair</td>
<td>Sanchez and Muller, 1998</td>
</tr>
<tr>
<td>R47</td>
<td><em>Aedes aegypti</em> fatty acid synthase</td>
<td>–</td>
<td>Promote viral replication</td>
<td>Perera et al., 2012</td>
</tr>
<tr>
<td>R102</td>
<td><em>Aedes aegypti</em> leucine aminopeptidase</td>
<td>–</td>
<td>Protein turnover, defense and cell redox status</td>
<td>Blot and Scornik, 1991; Cappiello et al., 2006</td>
</tr>
<tr>
<td>R110</td>
<td><em>Aedes aegypti</em> farnesyl-pyrophosphate synthetase</td>
<td>–</td>
<td>Unknown</td>
<td>N/A</td>
</tr>
<tr>
<td>R123</td>
<td><em>Aedes aegypti</em> p15-2a protein, putative</td>
<td>–</td>
<td>Unknown</td>
<td>N/A</td>
</tr>
<tr>
<td>R144</td>
<td><em>Aedes aegypti</em> gtpase_rho</td>
<td>–</td>
<td>Cell proliferation, apoptosis, gene expression</td>
<td>Ridley, 2001; Boureux et al., 2007; Bustelo et al., 2007</td>
</tr>
<tr>
<td>R220</td>
<td><em>Aedes aegypti</em> polyadenylate-binding protein</td>
<td>–</td>
<td>Binds 3’ poly (A) tail and interacts with 5’cap-binding eIF4G</td>
<td>Blobel, 1973; Imataka et al., 1998; Le et al., 1997</td>
</tr>
<tr>
<td>R19</td>
<td><em>Aedes aegypti</em> prolyl endopeptidase (prolyl oligopeptidase)</td>
<td>–</td>
<td>Unknown</td>
<td>N/A</td>
</tr>
<tr>
<td>R20</td>
<td><em>Aedes aegypti</em> dimeric dihydrodiol dehydrogenase</td>
<td>–</td>
<td>Unknown</td>
<td>N/A</td>
</tr>
</tbody>
</table>

**Note:** –, down-regulated.
3.3 Discussion

**Figure 4.** The Flavivirus Life Cycle (Mukhopadhyay et al., Nature Reviews Microbiology, 2005).

Viruses belonging to the family Flaviviridae are considered class 4 viruses within the Baltimore classification scheme. Viruses in this class are positive sense, single stranded RNA viruses which replicate their genome through a partially double-stranded intermediate form. The life-cycle of Flaviviridae has been divided into several stages including: virus attachment and entry, virus fusion and disassembly in the endosome, protein translation & polyprotein processing on membranes, viral RNA replication on membrane, immature virus assembly and budding into ER (Endoplasmic reticulum), virus mature & Furin cleavage of prM, and finally mature virus release (Figure 4).
Kuhn and his collaborators found that when *Ae. albopictus* C6/36 cells were infected with dengue virus, several types of lipids, including phosphatidylcholine, lysophospholipids, sphingomyelin, and ceramide, increase two- to threefold compared to levels in uninfected cells (97). These lipids promote viral replication, change the curvature and permeability of membranes, and control signaling pathways involved in membrane fusion, fission, and cytoskeletal reorganization. To replicate in mosquito cells, DV depends on the host enzymes, including fatty acid synthase (FAS). Dengue viral replication is reduced about 1000-fold when FAS inhibitor-C75 is added to cells that are infected with DV (97), which is consistent with our results (Table 1), FAS is positive regulator of dengue virus replication in C6/36 cells.

In *Saccharomyces cerevisiae*, protein transport protein sec23 is component of the coat protein complex II (COPII) which promotes the formation of transport vesicles from the endoplasmic reticulum (ER). Sec23p is required for transport of secretory, plasma membrane, and vacuolar proteins from ER to the Golgi complex (98-100), which process is required for dengue virus replication (Figure 4), mosquito Sec23p may implicate in dengue virus budding into ER and transport from ER to Golgi for the virus maturation.

The peroxiredoxin (Prx) family is composed of antioxidant proteins ubiquitously found in prokaryotic and eukaryotic (yeast, plant and animal) species (101-103). Peroxiredoxin 6 (prx-6) is a 1-cysteine peroxiredoxin involved in antioxidant processes (104). David’s lab found that prx-6 can be used as physiological and genetic indicator of multiple environmental stress response in pacific oyster (104). In our study, we find that prx-6 is induced by dengue virus infection in *Ae. albopictus* C6/36 cells, which is consistent with their results, prx-6 as the host stress response factors.
The V-ATPases are ubiquitous among eukaryotes (105,106). Originally identified in plant and yeast vacuoles, they are known to acidify a range of intracellular organelles (107, 108). Dengue requires different pH environment for certain stages in its life cycle (Figure 4). From our study in C6/36 cells, we noticed that vacuolar ATP synthase subunit ac39 was induced by dengue virus infection. Maybe, the dengue virus takes advantage of this protein to maintain the pH environment in intracellular organelles such ER, Golgi etc for its viral particle assemble, budding and maturation stages in its life cycle.

Poly (A) binding protein 1 (PABP1) plays an important role in host protein translation. PABP1 binds the 3’ poly (A) tail found on most host mRNAs and interacts with the 5’ cap-binding eIF4F complex (109-111). Interactions between PABP1 and the eIF4F complex member eIF4G improve host protein translation by increasing eIF4F-cap binding affinity, enhancing the efficiency of ribosome initiation complex formation, and increasing the rate of protein translation (112-115). Many viruses have developed protein translation strategies that allow them to translate viral proteins independently of PABP1. Many of these viruses have been shown to perturb PABP1’s location and/or function, creating a cellular environment in which viral protein translation is favored. For example, virus may cleavage PABP1, inhibiting translation of host mRNA (116, 117), or some viral proteins can competitively bind to PABP1, leading to a decrease in PABP1’s interaction with binding partners eIF4G and PABP-interacting protein 2 (118), or relocates PABP1 partially to the nucleus during virus infection (118-122) to shutoff the host protein synthesis while favorite viral protein translation (123).
The Rho family of GTPases is a family of small (~21 kDa) signaling G protein (more specific, a GTPase), and is a subfamily of the Ras superfamily. The members of the Rho GTPase family have been shown to regulate many aspects of intracellular actin dynamics, and are found in all eukaryotic organisms including yeasts and some plants. Rho proteins are found to play a role in cell proliferation, apoptosis, gene expression, and multiple other common cellular functions (124-126). It is indicated in our study that GTPase-Rho is inhibited by dengue virus in C6/36 cells (Table 1). It’s possible that dengue virus interferes with the host apoptosis process to create cellular environment for viral spread to the neighbor cells and establish the system infection in host cells.

3.4 Conclusions

- Using SSH, 150 clones are identified differentially expressed upon DV2 infection;

- Out of these clones, 30 differentially expressed sequence are identified to be up-regulated by DV2 infection;

- While 36 differentially expressed sequences are down-regulated by DV2 infection, in which FAS is found required by DV2 replication.
CHAPTER 4 CHARACTERIZATION OF THE ANTIVIRAL RNAI PATHWAY IN Aedes albopictus C6/36 CELL LINE

4.1 Introduction

Ae. albopictus C6/36 clone is one of the most commonly used mosquito cell lines for the propagation of arboviruses. Compared to original uncloned heterogeneous cells and other cloned cells, C6/36 cell exhibits a high degree of susceptibility to infection by many arboviruses, such as dengue virus, St. Louis encephalitis virus, and Sindbis virus, and allows viruses to grow to a significantly higher yield (33, 34). Since virus adsorption is not responsible for the high permissibility observed in C6/36 cells, it is suggested that C6/36 cells might lack certain cellular regulatory mechanisms that restrict virus replication (33).

Recently, the fact that RNAi plays a potent role in antiviral immunity has been well documented in Drosophila melanogaster and mosquito cells (58-62). RNAi is a small RNA-mediated gene regulatory mechanism that is conserved in eukaryotic cells. Small RNAs, including small interfering RNAs (siRNAs) and microRNAs (miRNAs), are 20-24 nucleotides (nt) long and regulate target gene expression in a sequence-specific manner (63). Production of siRNAs is mediated through the processing of long double-stranded RNAs (dsRNA) by an RNAIII-like enzyme Dicer, while miRNAs are generated from RNA precursors that have a characteristic stem-loop structure. SiRNAs and miRNAs can be incorporated into the RNA-induced silencing complex (RISC) and direct homologous target mRNA for degradation or translation inhibition (63, 64). In D. melanogaster, there are distinct RNAi pathways in which Dicer-1 is required for miRNA biogenesis and Dicer-2 is required for siRNA production (65). However, comparative genomic analysis suggested that mosquitoes might also be equipped with similar RNAi
machinery as *D. melanogaster* (62, 66). In the RNAi-mediated antiviral mechanism, Dicer recognizes dsRNAs generated from replicating viruses, processes dsRNAs into viral specific siRNAs and establishes the sequence-specific antiviral response (58, 64, 57, 127). To counteract such a defense, viruses have also evolved to encode a viral suppressor of RNAi pathway (127).

In *Ae. albopictus* C6/36 cells, conflicting results about RNAi-mediated antiviral immunity have been observed. DsRNA is able to knockdown transient gene expression and inhibit viral replication although it is not as efficient as that in *Drosophila* cells (129, 130) and RNAi competent mosquito cells (Figure 4). However, the absence of virus-derived siRNAs in West Nile virus-infected C6/36 cells argued that RNAi-mediated antiviral mechanism might be not induced (131). In this study, we find that the Dicer-2 function is defect in *Ae. albopictus* C6/36 cells.
4.2 Results

4.2.1 DsRNA-mediated silencing of transient gene expression is defective in Ae. albopictus C6/36 cells

Figure 5. DsRNA-mediated silencing of transient gene expression in Ae. albopictus C6/36 cells and An. gambiae 4a-2s4 cells. Cells were transfected with pIZ/GFP plus a 500-bp dsRNA that targets Renilla luciferase (RL), firefly luciferase (FL) or GFP (GFP). Accumulation of GFP mRNA in C6/36 (A) and 4a-2s4 (B) was analyzed by Northern blot. 1. Mock, 2. pIZ/eGFP, 3. pIZ/eGFP + dsRluc RNA, 4. pIZ/eGFP + dsFluc RNA, 5. pIZ/eGFP + dsGFP RNA.
**Figure 6.** DsRNA-mediated silencing of transient gene expression in *Ae. albopictus* C6/36 cells and *An. gambiae* 4a-2s4 cells (Northern Blot Analysis). Cells were transfected with pIZ/GFP plus a 500-bp dsRNA that targets *Renilla* luciferase (RL), firefly luciferase (FL) or GFP (GFP). Accumulation of GFP mRNA in C6/36 (A) and 4a-2s4 (B) was analyzed by Northern blot. 1. Mock, 2. pIZ/eGFP, 3. pIZ/eGFP + dsRLuc RNA, 4. pIZ/eGFP + dsFluc RNA, 5. pIZ/eGFP + dsGFP RNA.

DsRNA-mediated silencing of transient gene expression can efficiently knock-down eGFP reporter gene expression in *An. gambiae* 4a-2s4 cells (Figure 5B & Figure 6B), but not in *Ae. albopictus* C6/36 cells (Figure 5A & Figure 6A). This indicated that the RNAi-mediated target gene silencing (dsRNA as a trigger) is defective in C6/36 cells in early steps of the siRNA pathway.
4.2.2 Cell extract from *Ae. albopictus* C6/36 cells does not contain the dicer activity for processing dsRNAs into siRNAs

Dicer cleaves dsRNAs, producing siRNAs that mediated regulation of target gene expression. Presence of Dicer enzymatic activity has been widely analyzed by *in vitro* biochemical analysis using crude or purified cell extracts. In the model system *Drosophila melanogaster*, *in vitro* biochemical analysis has been widely used as a tool to study mechanisms underlying RNAi and virus-RNAi interactions. Using crude or purified extracts prepared from *Drosophila* S2 cells, major components of the RNAi machinery, such as Dicer-2, Ago-2, and R2D2, were identified and their functions have been illustrated (58-62, 133, 134). To analyze the defect of C6/36 cells in processing dsRNAs into siRNAs, we prepared cell extracts from cultured C6/36 cells and performed *in vitro* analysis for Dicer activity (95). As controls, cell extracts were also prepared from *Drosophila* S2 cells, *Ae. aegypti* Aag2 and ATC-10 cells, in which RNAi machinery is believed to be intact (136-138). As expected, S2 cell extract efficiently cleaved uniformly [*^{32}P-UTP*]-radiolabeled 500 bp dsRNAs and generated 21-nt siRNAs (Figure 7, lane 7). Similarly, extracts from Aag2 and ATC-10 cells also possess a comparable capacity to process dsRNAs into siRNAs (Figure 7, lane 2 and lane 5), which demonstrates presence of functional RNAi in these cells. In contrast, incubation of dsRNAs with the extract from C6/36 cells did not produce any trace of siRNAs (Figure 7, lane 9), indicating the direct correlation of complete loss of capacity in siRNA generation with the mutation in Dcr-2. In this research, we performed Dicer activity assay using extracts prepared from *Drosophila* S2 cells, *Ae. albopictus* C6/36 cells, *Ae. aegypti* ATC-10 cells and Aag2 cells. All cell extracts except C6/36 one can process [*^{32}P-UTP*]-dsRNA into siRNAs (Figure 7), which means C6/36 cells have a dysfunctional RNAi pathway. Further
investigation indicates that the Dicer-2 gene in C6/36 cells have a single nucleotide deletion at 2460 nt in Dcr-2 ORF, translation the C6/36 Dicer-2 into an attenuated dicer protein of 819 AA (Compared to 1659 AA full length dicer-2) without RNaseIII domains (135), which may account for the RNAi dysfunction in in vitro dicer assay of C6/36 cell lines and high replication level of some arboviruses in C6/36 cell compared to other RNAi-intact mosquito cell lines like Aag2, ATC-10.
Figure 7. *In vitro* analysis of Dicer activity using extracts prepared from different cell lines. [$^{32}$P-UTP] Radiolabeled GFP dsRNAs were incubated with cell extracts prepared from *Ae. albopictus* C6/36 cells, *Drosophila* S2 cells, *Ae. aegypti* Aag2 and ATC-10 cells respectively. The processed dsRNAs were purified from the reaction and analyzed on a 15% Urea-PAGE. The input dsRNAs and the siRNA products generated by Dicer are indicated by arrows. M, radiolabeled siRNA of eGFP.
4.2.3 Replication of B2-null mutant of NoV RNA1 is completely restored in C6/36 cells

Nodamura virus (NoV), a member of Nodaviruses, has two positive single-stranded genomic RNAs. RNA1 (NoVR1) encodes a viral RdRP and is able to self-replicate while RNA2 is responsible for capsid synthesis. The genomic RNA1 also contains a small overlapping gene (B2), which is expressed from the subgenomic RNA (RNA3). It has been shown that NoVB2 functions as a potent RNAi suppressor (Figure 8A) (76). Inhibition of RNAi by B2 is mediated by its binding to dsRNA, making the dsRNA unavailable for cleavage by Dicer (132). In Drosophila S2 cells, replication of NoVR1 induces a strong RNAi-based antiviral response. In the absence of B2 expression, the B2-null mutant of NoVR1 (NoVR1ΔB2) (Figure 8A) lost the ability to inhibit RNAi and could not replicate to a detectable level (94, 62), which makes NoVR1ΔB2 a unique reporter for functional analysis of cellular antiviral RNAi. To functionally evaluate the defect of RNAi-based antiviral mechanism in C6/36 cells, we examined replication of NoVR1, NoVR1ΔB2, and a RNA1 mutant (NoVR1fs) (Figure 8A) that has a frameshift mutation in the ORF of viral RdRP (62). For purpose of comparison to C6/36 cells, cells with competent RNAi, including Drosophila S2 cells and An. gambiae 4a-2s4 cells (62), were also included in this study and were transfected with infectious RNA transcripts synthesized from NoV RNA1 cDNA clones using T7 RNA polymerase. As expected, NoVR1 can replicates efficiently in S2 and 4a-2s4 cells while replication of NoVR1ΔB2 were not detected because of absence of RNAi suppressor B2 (Figure 8B). The genomic RNA1 detected in the cells transfected with NoVR1ΔB2 represents the RNA transcripts introduced by transfection, because NoVR1fs also gave a similar signal and no subgenomic RNA3 was accumulated.
Ae. albopictus C6/36 cells have a dysfunctional RNAi, and are impaired at the step of processing dsRNAs into siRNAs, at which the suppressor of NoVB2 also acts in RNAi pathway. In this case, the B2 suppressor could become dispensable for NoVR1 in C6/36 cells and replication of NoVR1ΔB2 would be expected to be comparable to NoVR1. Northern blot analysis confirmed our speculation and showed that accumulation of NoVR1ΔB2 was indeed comparable to NoVR1 while replication of RNA1fs remained undetectable (Figure 8B). Our study provided the first functional analysis of the defective antiviral RNAi in C6/36 cells. In this study, we showed that dysfunctional RNAi completely restores replication of NoVR1ΔB2 in C6/36 cells. In the absence of RNAi suppressor NoVB2, NoVR1ΔB2 can replicate as efficiently as NoVR1, indicating that NoVB2 becomes dispensable for self-replication of NoVR1 in cells in which Dcr-2 is defective, which suggests that RNAi is the primary antiviral immunity in mosquito cells and the Dcr2-independent viral small RNAs in C6/36 cells are not able to mediate an antiviral response. Because of the defect in the primary antiviral mechanism, C6/36 cells could be used as a unique system for studying the role of other cellular responses on viral infection, including Toll, Imd and Jak-STAT pathways as described in Drosophila (90, 91, 93).
Figure 8. Functional analysis of RNAi-based antiviral immunity in C6/36 cells. (A) Genome organization and gene expression of NoV RNA1, NoVR1ΔB2, and NoVR1FS. The overlapping gene B2 is translated from a subgenomic RNA, RNA3. (B) Rescue of B2-null mutant of NoV RNA1 in C6/36 cells. Infectious NoVR1 transcripts were synthesized from the full-length cDNA clones of wild-type NoV RNA1 (R1) and RNA1 B2-null mutant (ΔB2) and NoV RNA1 frameshift mutant (fs), and the transcripts were transfected into Ae. albopictus C6/36 cells, Drosophila S2 cells and An. gambiae 4a-2s4 cells. At two days after transfection, total RNAs were extracted from transfected cells for Northern Blotting and probed by NoVRNA3 cDNA that was randomly labeled by [α-32P]-dCTP.
In fruit flies and mosquitoes, RNA interference (RNAi) functions as a natural antiviral immunity through recognizing double-stranded RNAs (dsRNAs) produced from a replicating virus and subsequently generating functional viral specific small interfering RNAs (siRNAs). In this study, we report that *Ae. albopictus* C6/36 mosquito cells exhibit inability to initiate dsRNA-mediated inhibition of transient gene expression, which is associated with defective Dicer activity in dsRNA processing. Such a compromised RNAi machinery in C6/36 cells leads to promoted replication of nodamura virus (NoV) RNA1 in the absence of the essential B2 protein, a suppressor of RNAi. We also found that direct introduction of siRNAs is still able to knockdown the expression of target genes (Figure 9), and on the other hand, the pathway for the biogenesis of microRNAs (miRNAs) still remains functional (Figure 10) in *Ae. albopictus* C6/36 cells. These results demonstrate that C6/36 cells lose the capacity to process dsRNAs into siRNAs, indicating the presence of a defective RNAi-based antiviral immunity. This feature of C6/36 cells might contribute to its high degree of permissibility to many arboviruses.
4.2.4 Inhibition of transient gene expression by siRNAs in *Ae. albopictus* C6/36 cells

**Figure 9.** Inhibition of transient gene expression by siRNAs in *Ae. albopictus* C6/36 cells. (A), *An. gambiae* 4a-2s4 (B) and *Drosophila* S2 cells (C). Cells were transfected with pIZ/eGFP plus either GFP siRNA (GFP), control siRNA (Ctr) or mock (-). Specific cleavage of GFP mRNA by siGFP was detected by Northern blot hybridization. The mRNA of GFP and the 5’ end cleavage product by siGFP are indicated.
4.2.5 Biogenesis of miRNAs is still functional in C6/36 cells

![Figure 10](image)

**Figure 10.** MiRNAs biosynthesis in *Ae. albopictus* C6/36 cells, and *Ae. aegypti* ATC-10 cells and *An. gambiae* 4a-2s4 cells. Twenty microgram (20 μg) of total RNAs extracted from *Ae. albopictus* C6/36, *Ae. aegypti* ATC-10 and *An. gambiae* 4a-2s4 cells were run on a 15% Urea-PAGE. The miRNAs were detected by [γ-32P]-ATP-labeled DNA oligonucleotides that are complementary to *Ae. albopictus* miR-275 or miR-184.
4.2.6 *Drosophila* Dcr-2 cannot reconstitute dsRNA processing activity in transfected C6/36 cells

To investigate if the defective Dcr-2 in C6/36 cells can be complemented by introduction of a functional Dcr-2, we created a construct expressing a *Drosophila* Dcr-2 that is tagged with 3×FLAG at N-terminal (FLAG-DmDcr2) based on the backbone of pIZ/V5-His (Figure 11A) (139). As shown in Figure 11A, the OpIE2 promoter can drive efficient expression of the reporter GFP gene from pIZ/eGFP in both S2 cells and C6/36 cells, indicating that pIZ vector is suitable for expression of DmDcr2 in C6/36 cells. To analyze *Drosophila* Dcr-2 activity in C6/36 cells, we prepared extracts from the cells transfected with pIZ/FLAG-DmDcr2 and performed *in vitro* assay as did in Figure 7. The result showed that activity in processing of dsRNAs into siRNAs was not restored in C6/36 cells after introduction of *Drosophila* Dcr-2 (Figure 11B). This was further confirmed with failed siRNA production from dsRNAs by FLAG-DmDcr2 pull-downed from the transfected cells (Figure 11C).
Figure 11. Expression of *Drosophila* Dcr-2 in C6/36 cells cannot complement the defective Dcr-2. (A) Schematic presentation of expression vector for *Drosophila* Dcr-2 (DmDcr2), pIZ/FLAG-DmDcr2. Green fluorescence showed efficient expression pIZ/eGFP in *Ae. albopictus* C6/36 cells compared to that in *Drosophila* S2 cells. (B) *In vitro* analysis of Dicer activity in C6/36 cells transfected with pIZ/FLAG-DmDcr2. Cell extracts were prepared at 2 days after transfection, and *in vitro* assay for dsRNA processing was done as described above. (C) *In vitro* analysis of dsRNA processing using purified FLAG-DmDcr2. The cell lysates were prepared from S2 cells and C6/36 cells transfected with pIZ/FLAG-DmDcr2, and FLAG-DmDcr22 was immunoprecipitated using anti-FLAG M2 affinity gel by following the manufacturer’s instruction.
4.3 Conclusions

- DsRNA-mediated gene regulation is defective in C6/36 cells, which may contribute to the high permissibility of C6/36 cells to infection by many arboviruses;

- In the absence of functional Dicer activity, siRNAs are still able to incorporate into RISC, which can execute specific degradation of complementary mRNAs;

- MiRNA processing is still functional in C6/36 cells, suggesting that the defect in dsRNA processing might be derived from the disruption of Ae. albopictus Dicer-2 activity, but not Dicer-1.

- Using Nodamura virus as a model, we functionally analyzed the defect of C6/36 in RNAi-mediated antiviral immunity, demonstrating that RNAi pathway is the primary antiviral mechanism in mosquito cells.
CHAPTER 5 DEVELOPMENT OF A PLASMID-BASED DSRNA EXPRESSION SYSTEM IN INSECT CELLS

5.1 Introduction

RNAi-based functional genomic studies in *Drosophila* cells are usually carried out by dsRNA library that targets all of annotated genes in the genome (140). The dsRNA are *in vitro* synthesized by T7 RNA polymerase from individual DNA template carrying convergent T7 promoters. However, genome-wide RNAi has not been established in mosquito and other insect cells because of lack of an effective tool, a dsRNA library. For most laboratories, it is difficult to develop their own dsRNA libraries because the whole process involves broad bioinformatics supports for design and selection of target sequences, large-scale preparation DNA template by RT-PCR/PCR, dsRNA synthesis from each template by *in vitro* transcription, which is labour-intensive and also at a high cost. Production of long dsRNAs *in vivo* can be achieved by either transcription from long inverted-repeat sequences or from the sequences that carrying convergent promoters (141). Because of instability of long inverted-repeats in plasmids, the strategy with convergent promoters is more often used. Expression of dsRNA from two opposing T7 promoters can be efficiently mediated by T7 RNA polymerase in bacteria and trypanosomes, which has been successfully used for genome-wide RNAi in *C. elegans* and trypanosomes (142, 143). In plants, a viral vector-based random cDNA library has been constructed and successfully used for functional studies of disease resistance pathway by virus-induced gene silencing (144), demonstrating that such a random cDNA-based RNAi library can also be used to elucidate a gene function or cellular pathway by a forward screening. However, plasmid based dsRNA expression has not been established for functional genomic studies in insect cells. In this study, we
developed a plasmid-based dsRNA expression system, in which dsRNA is intracellularly synthesized from a dsRNA expression cassette by T7 RNA polymerase. Since the IE2 promoter of Orgyia pseudotsugata nucleopolyhedrovirus mediates the constitutive expression of T7 RNA polymerase, this system could allow for production of dsRNA and analysis of gene function not only in *Drosophila* S2 cells but also in mosquito and other insect cells. Using this dsRNA expression system, the plasmid-based dsRNA library directly constructed from a random cDNA pool will provide a powerful and alternative strategy for functional genomic studies at various focuses in insect cells, such as innate immunity, particular cellular pathway, interaction of insect cells with pathogens, and functional analysis of genes that are differentially expressed under different conditions. This system would be especially useful for functional genomics in insect cells which genome sequence is not fully available.
5.2 Results

5.2.1 Intracellular dsRNA expressed from PCR fragments mediates efficient RNAi

dsRNA Production

Plasmid-based dsRNA Expression

Figure 12. Schematic representation of the plasmid constructs for dsRNA intracellular expression. (A) The plasmid (pIB/T7) is used for expression of T7 RNA polymerase (Kohl et al., 2004). POpIE2, the baculovirus Orgyia pseudotsugata immediate-early promoter. OpIE2 pA, OpIE2 polyadenylation sequence. (B) PCR products with convergent T7 promoters used for synthesis of GOI dsRNA by T7 RNA polymerase in vitro. (C) DNA template target gene dsRNA was cloned into pUC19, generating pUC/DS-GOI (GOI: Gene of interest).
5.2.2 RNAi-mediated silencing of GFP reporter gene by intracellular expressed dsRNA from PCR-based template in Drosophila S2 cells

In fly and mosquito cells, dsRNAs used in gene function analysis are synthesized by *in vitro* transcription from dsRNA template, a PCR fragment that contains two convergent promoters flanking the cDNA sequence from the gene of interest. To analyze RNAi efficacy of dsRNAs that are intracellularly expressed, *Drosophila* S2 cells were co-transfected by a plasmid expressing T7 RNA polymerase (pIB/T7) (145, Figure 12A), together with a GFP reporter (pMT/GFP, Figure 13B) and a PCR template of GFP dsRNA possessing opposing T7 promoters at 5’ end of each DNA strand, as shown in Figure 12B. At sixteen hours post transfection (hpt), GFP expression driven by the metallothionein (MT) promoter from pMT/eGFP was induced by addition of CuSO4 (Final concentration of 0.5 mM). Efficient knockdown of transient expression of the reporter GFP by dsRNA intracellularly transcribed from the PCR template was achieved (Figure 13B). Our result indicates that T7 RNA polymerase is able to drive expression of a gene with a T7 promoter in S2 cells, of which activity is also found in mosquito and mammalian cells (145-147), suggesting that dsRNA template could be directly used in RNAi analysis in *Drosophila* and mosquito cells.
Figure 13. RNAi-mediated silencing of GFP reporter gene by intracellular expressed dsRNA from PCR-based template in Drosophila S2 cells. Drosophila S2 cells in a 24-well plate were transfected with different combinations as indicated. Induction of pMT/GFP was done using 0.5 mM of CuSO$_4$ at 16 hours after transfection. The fluorescence (a) and light (b) images of cells were captured under a fluorescence microscope at 2 days after induction. 1. pMT/GFP + dsGFP RNA, 2. pMT/GFP, 3. pMT/GFP + dsGFP Template, 4. pMT/GFP + dsLacZ Template.
5.2.3 RNAi-mediated silencing of GFP reporter gene by intracellular expressed dsRNA from pUC/DS in Drosophila S2 cells

DsRNA template that is originally used for synthesis of dsRNA by *in vitro* transcription (Figure 13A) can efficiently knockdown target gene expression when introduced into the cells in which T7 RNA polymerase is expressed. But, for each individual target, corresponding dsRNA template needs to be amplified with a pair of long specific primers containing T7 promoter sequence. To simplify primer design for dsRNA template and broaden its application, a universal dsRNA expression cassette pUC/DS bearing the sequence elements as indicated in Figure 14A was constructed. The resultant cassette has convergent T7 promoters with an EcoRI site in-between and flanked by a T7 terminator and an *E.coli* rrnB T1 transcription terminator sequence. It was cloned into pUC19 at BamHI and SacI site, generating the dsRNA expression vector pUC/DS, which could be used for expression of dsRNA corresponding to any sequence inserted at EcoRI site.

Addition of T7 transcriptional termination sequences at each end of the dsRNA template is due to possible read-through of bidirectional transcription by T7 RNA polymerase beyond the DNA template of dsRNA, which may affect dsRNA formation and efficiency of RNAi. It has been shown that the rrnB T1 terminator can stop the transcription mediated by T7 RNA polymerase (148, 149). To test production of dsRNA from pUC/DS in presence of pIB/T7 and its efficiency in RNAi, 500 bp GFP sequence was cloned into pUC/DS at EcoRI site, producing pUC/DS-GFP. As a control, pUC/DS-lacZ was also constructed by inserting 500 bp LacZ sequence into pUC/DS. Co-transfection was performed in S2 cells by combining pUC/DS-GFP, pIB/T7 and pMT/GFP together. As shown in Figure 14B, dsRNA expressed from the plasmid pUC/DS-GFP was very
efficient in inhibition of transient GFP expression, which is similar to the effect mediated by GFP dsRNA intracellular expressed from PCR-template (Figure 14B). From this experiment, it is confirmed that dsRNA expressed from the plasmid pUC/DS-GFP was very efficient in inhibition of transient expression of reporter GFP gene (Figure 14B).
Figure 14. RNAi-mediated silencing of GFP reporter gene by intracellular expressed dsRNA from pUC/DS in *Drosophila* S2 cells. (A) Schematic representation of pUC/DS, pIB/T7 and pMT/GFP reporter system. (B) *Drosophila* S2 cells in a 24-well plate were transfected with different plasmid combinations as indicated. Induction of pMT/GFP was done using 0.5 mM of CuSO$_4$ at 16 hours after transfection. The fluorescence (a) and light (b) images of cells were captured under a fluorescence microscope at 2 days after induction. 1. Mock; 2. pMT/GFP + pIB/T7; 3. pMT/GFP + pIB/T7 + DNA template fragment for GFP dsRNA; 4. pMT/GFP + pIB/T7 + DNA template for LacZ dsRNA; 5. pMT/GFP + pIB/T7 + GFP dsRNA; 6. pMT/GFP + pIB/T7 + pUC/DS-GFP-a; 7. pMT/GFP + pIB/T7 + pUC/DS-GFP-b; 8. pMT/GFP + pIB/T7 + pUC/DS-LacZ.

Note: pUC/DS-GFP-a and pUC/DS-GFP-b are different clones tested in the assay.
5.2.4 Silencing of transiently expressed Renilla luciferase reporters by dsRNA expressed from pUC/DS system in Drosophila S2 cells

To further verify the RNAi efficiency of dsRNA expression vector, pUC/DS-Rluc was constructed by cloning 500 bp of cDNA of Renilla luciferase (Rluc) gene into the pUC/DS. The pUC/DS-Rluc was co-transfected with pIB/T7 and renilla luciferase reporter system pIZ/Rluc or pIB/Rluc-CP. When performing luciferase assays, measurements on pIZ/Rluc reporter system are made on the total accumulated reporter protein within cells. While pIZ/Rluc-CP is destabilized luciferase reporter by genetically fusing two protein degradation sequences, CL1 and PEST, to the luciferase gene. Due to an increased rate of degradation, these destabilized reporters respond faster and often display a greater magnitude of response to rapid transcriptional events and are therefore called the Rapid Response™ Reporters. A consequence of inclusion of the degradation sequences is that the destabilized luciferase proteins do not accumulate in the cell to the same extent as the nondestabilized luciferase-containing controls. As a result, destabilized reporter proteins typically generate lower signal intensities.

The intracellularly expressed Rluc dsRNA from pUC/DS-Rluc could efficiently knockdown Renilla luciferase reporter pIZ/Rluc by ~70% (Figure 15A) in transfected S2 cells compared to control dsRNA of LacZ produced from pUC/DS-LacZ or pIB/Rluc-CP reporter by ~90% (Figure 15B). Therefore, this plasmid-based dsRNA expression system has provided a convenient tool for gene function analysis, but also could be used as a platform to construct a library of dsRNA expression for functional genomics.

Note: The RlucCP reporter gene contains two protein destabilization sequences, CL1 and PEST. The protein encoded by RlucCP responds more quickly and with greater magnitude to changes in transcriptional activity than the Rluc gene, its more stable counterpart.
5.2.5 **DsRNAs Intracellular expressed from PCR fragments trigger efficient silencing of endogenous gene expression**

We have demonstrated that intracellular expressed dsRNA from pUC/DS can efficiently knockdown transient expression of target reporter genes in *Drosophila* S2 cells, implicating that it could be also effective in regulating an endogenous gene. To confirm this, Nodamura virus (NoV)-based reporter was used in our assay (150). NoV is a positive ssRNA virus that belongs to *Alphanodavirus, Nodaviridae*, in which flock house virus (FHV) is also a member. NoV and FHV have a similar genome organization, and the genomic RNA1 is self-replicable and encodes a viral RNA polymerase and B2 (Figure 12A) (146, 147, 151, 152). It is known that B2 is a suppressor of antiviral silencing. NoVR1GFP, a mutant of NoV RNA1 in which B2 gene is replaced by GFP coding sequences (Figure 11A), cannot accumulate to a detectable level due to induction of strong antiviral RNAi. In *Drosophila*, RNAi has been identified as an antiviral immunity (67, 95). Viral dsRNAs derived from replicating viruses are recognized and processed by Dicer-2 into viral specific siRNA, which is incorporated into the effector complex RISC and direct target mRNA cleavage by endonuclease argonaute 2 (Ago2), which is the major component of RISC (71). When Ago2 is silenced with dsRNA, the host RNAi pathway gets disrupted, and mutant virus like NoVR1GFP can replicate independent of B2 expression (94, 76). In this assay, S2 cells were transfected with a plasmid combination of pIZ/NoVR1GFP, pIB/T7 and DmAgo2 dsRNA. As controls, LacZ dsRNA template was included in the co-transfection to replace dsDmAgo2 template. NoVR1GFP could be efficiently rescued by intracellularly expressed Ago2 dsRNA as indicated by green fluorescence expressed from replicating NoVR1GFP (Figure 16B), suggesting efficient knockdown the endogenous Ago2.
Figure 16. DsRNAs Intracellular expressed from PCR fragments trigger efficient silencing of endogenous gene expression in Drosophila S2 cells. (A) Schematic representation of Nodamura Virus reporter system NoVR1GFP (Replace the BamH I–Sac I fragment of RNA1 with the coding sequence for GFP). (B) Cells in a 24-well plate were transfected with different plasmid combinations as indicated. The fluorescence (a) and light (b) images of cells were captured under a fluorescence microscope at 1 day after transfection. 1. pIZ/NoVR1GFP + pIB/T7 + dsLacZ RNA template; 2. pIZ/NoVR1GFP + pIB/T7 + dsDmAgo2 Template.
5.2.6 Knockdown of endogenous gene expression by dsRNAs expressed from pUC/DS system in Drosophila S2 cells

Intracellular expressed dsRNA with introduced T7 RNA polymerase from PCR-based dsRNA template (Figure 16A) can efficiently knockdown endogenous target gene expression (Figure 16B). To verify the efficiency of intracellular expressed dsRNA from pUC/DS system in silencing endogenous target genes, 500 bp *Drosophila* Ago2 sequence was cloned into pUC/DS at EcoR I site, producing pUC/DS-DmAgo2. As a control, pUC/DS-lacZ was also constructed by inserting 500 bp LacZ sequence into pUC/DS.

In this assay, S2 cells were transfected with a plasmid combination of pIZ/NoVR1GFP, pIB/T7 and dsRNA expression vector pUD/DS-DmAgo2. As controls, pUC/DS-LacZ was included in the co-transfection to replace pUD/DS-DmAgo2. NoVR1GFP could be efficiently rescued by intracellularly expressed Ago2 dsRNA as indicated by green fluorescence expressed from replicating NoVR1GFP (Figure 17B), suggesting efficient knockdown the endogenous Ago2.
Figure 17. Knockdown of endogenous gene expression by dsRNAs expressed from pUC/DS system in Drosophila S2 cells. (A) Schematic representation of pUC/DS and pIB/T7 plasmids and NoVRaGFP reporter system. (B) Efficient knockdown of endogenous target by intracellular expressed dsRNAs. Drosophila S2 cells in a 24-well plate were transfected with different plasmid combinations as indicated. The fluorescence (a) and light (b) images of cells were captured under a fluorescence microscope at 1 day after transfection. 1. pIZ/NoVR1GFP; 2. pIZ/NoVR1GFP + dsDmAgo2 RNA; 3. pIZ/NoVR1GFP + pIB/T7 + pUC/DS-LacZ; 4. pIZ/NoVR1GFP + pIB/T7 + pUC/DS-DmAgo2.
5.2.7 Simplified plasmid-based dsRNA expression system in Drosophila S2 cells

For this plasmid-based dsRNA expression system pUC/DS, pIB/T7 is required to be co-transfected with pUC/DS for production of T7 RNA polymerase. In order to improve transfection efficiency and simplify the procedure, one-plasmid system was developed by cloning the dsRNA expression cassette into pIB/T7 at the Cla I site, generating dsRNA expression vector pDS, as shown in (Figure 18A). In S2 cells co-transfected with pDS/GFP and pMT/GFP, GFP expression could be efficiently down regulated as found in cells expressing pUC/DS-GFP (Figure 18B), indicating pDS is an improved dsRNA expression system with similar efficiency as pUC/DS system. The single plasmid contains both T7 RNA polymerase gene and its dsRNA template, which can lead to increased efficiency in transfection and dsRNA production. Another advantage using pDS in loss-of-function assays is that the backbone of pDS, pIB/V5-His (Invitrogen), encodes a blasticidin-resistant gene which can be used for selection of cells stably expressing specific dsRNAs (persistent cell lines) (145). This is especially important for studying some genes usually not very sensitive to transiently expressed dsRNAs.
Figure 18. Efficiency of two-plasmid system (pUC/DS) and single-plasmid system (pDS) in silencing transient expressed reporter. (A) Schematic representation of dsRNA expression systems, pUC/DS and pDS. (B) Efficient knockdown of GFP reporter expression by intracellular expressed dsRNAs in Drosophila S2 cells. Cells in a 24-well plate were transfected with different plasmid combinations as indicated. Induction of pMT/GFP was done using 0.5 mM of CuSO₄ at 16 hours after transfection. The fluorescence (a) and light (b) images of cells were captured under a fluorescence microscope at 2 days post induction. 1. pMT/GFP; 2. pMT/GFP + dsGFP RNA; 3. pMT/GFP + pIB/T7; 4. pMT/GFP + pIB/T7 + pUC/DS; 5. pMT/GFP + pIB/T7 + pUC/DS-Rluc; 6. pMT/GFP + pIB/T7 + pUC/DS-GFPa; 7. pMT/GFP + pIB/T7 + pUC/DS-GFPb; 8. pMT/GFP + pDS; 9. pMT/GFP + pDS/Rluc; 10. pMT/GFP + pDS/GFP.

Note: pUC/DS-GFPa and pUC/DS-GFPb are different clones tested in the assay.
5.2.8 Knockdown of endogenous gene expression by intracellular expressed dsRNAs from pDS system in Drosophila S2 cells

From last experiment, intracellular expressed dsRNA from pDS can knockdown transient GFP expression as efficient as pUC/DS system (Figure 19B). Furthermore, we analyzed the efficiency of intracellular expressed dsRNAs from pDS in silencing the major component gene, Ago-2, in Drosophila RNAi pathway to rescue the NoVR1GFP mutant virus replication in S2 cells. Our data indicated that intracellular dsDmAgo2 RNA expressed from pDS can completely restore the NoVR1GFP mutant virus replication (Figure 19B). Same as two-plasmid based system pUC/DS, single plasmid system pDS can efficiently knockdown exogenous as well as endogenous gene expression in Drosophila S2 cells.
Figure 19. Knockdown of endogenous gene expression by intracellular expressed dsRNAs from pDS system in *Drosophila* S2 cells. (A) Schematic representation of pDS plasmid, NoVR1GFP reporter. (B) Efficient knockdown of endogenous agonaute 2 expression by dsRNAs expressed with pDS system in *Drosophila* S2 cells. 1. pIZ/NoVR1GFP + pDS, 2. pIZ/NoVR1GFP + pDS/DmAgo2, 3. pIZ/NoVR1GFP + pDS/Rluc.
5.2.9 Efficiency of pUC/DS and pDS in silencing endogenous target genes expression in Drosophila S2 cells

Furthermore, we analyzed and compared the efficiency of dsRNAs expressed intracellularly from pUC/DS and pDS in silencing of major components in Drosophila RNAi pathway, including Ago-2, Dcr-2 and Ago-1, Dcr-1. Ago-1 is required for maturation and production of microRNAs and not essential for antiviral response (153, 154). Virus replication (Figure 20) and Northern blotting analysis (Figure 21) indicated that intracellular expressed dsRNAs of Ago-2 from pUC/DS (pUC/DS-DmAgo2) and pDS (pDS/DmAgo2) led to efficient rescue of NoVR1GFP replication to a level that is similar to that from directly transfected dsRNA (Figure 20). In contrast, NoVR1GFP still remained at the basal level of replication in cells transfected with empty vector, pUC/DS-DmAgo1 and pDS/DmAgo1 (Figure 20). Results indicated that pUC/DS and pDS systems possess similar ability in knockdown of a target gene, both transiently expressed and endogenous target genes. From those experiments, we verified that pUC/DS and pDS can be used for functional analysis of cellular genes in Drosophila, mosquito (An. gambiae cell 4a-2s4 cell, Data not shown), and other insect cells (Sf21, Data not shown). Plasmid-based dsRNA expression systems, pUC/DS and pDS, can be used as a RNAi-based forward or reverse genetic tool to analyze cellular response to virus infection or elucidate specific cellular pathway in insect cells.
Figure 20. Efficiency of pUC/DS and pDS in silencing endogenous gene expression in *Drosophila* S2 cell. Cells in a 24-well plate were transfected with different plasmid combinations as indicated. The fluorescence (a) and light (b) images of cells were captured under a fluorescence microscope at 2 days after transfection. 1. pIZ/NoVR1GFP; 2. pIZ/NoVR1GFP + pIZ/NoVB2; 3. pIZ/NoVR1GFP + dsDmAgo2-1 RNA; 4. pIZ/NoVR1GFP + dsDmAgo2-2 RNA; 5. pIZ/NoVR1GFP + pIB/T7 + pUC/DS; 6. pIZ/NoVR1GFP + pIB/T7 + pUC/DS-DmAgo1; 7. pIZ/NoVR1GFP + pIB/T7 + pUC/DS-DmAgo2-1; 8. pIZ/NoVR1GFP + pIB/T7 + pUC/DS-DmAgo2-2; 9. pIZ/NoVR1GFP + pIB/T7 + pUC/DS-DmDcr1; 10. pIZ/NoVR1GFP + pIB/T7 + pUC/DS-DmDcr2; 11.
pIZ/NoVR1GFP + dsRluc RNA; 12. pIZ/NoVR1GFP + pIB/T7 + pUC/DS-Rluc; 13. pIZ/NoVR1GFP + pDS; 14. pIZ/NoVR1GFP + pDS/DmAgo1; 15. pIZ/NoVR1GFP + pDS/DmAgo2-1; 16. pIZ/NoVR1GFP + pDS/DmAgo2-2; 17. pIZ/NoVR1GFP + pDS/DmDcr1; 18. pIZ/NoVR1GFP + pDS/DmDcr2; 19. pIZ/NoVR1GFP + pDS/Rluc; 20. mock.

Note: pUC/DS-DmAgo2-1 and pUC/DS-DmAgo2-2 target different regions of the DmAgo2 coding sequences.
Figure 21. Efficiency of pUC/DS and pDS in silencing endogenous gene expression in *Drosophila* S2 cells (Northern blot analysis). Cells in a 24-well plate were transfected with different plasmid combinations as indicated. Samples were collected at 2 days after transfection for RNA Extraction. Northern Blot was performed with [α-32P]-dCTP labeled GFP as probe. 1. pIZ/NoVR1GFP; 2. pIZ/NoVR1GFP + pIZ/NoVB2; 3. pIZ/NoVR1GFP + dsDmAgo2-1 RNA; 4. pIZ/NoVR1GFP + dsDmAgo2-2 RNA; 5. pIZ/NoVR1GFP + pIB/T7 + pUC/DS; 6. pIZ/NoVR1GFP + pIB/T7 + pUC/DS-DmAgo1; 7. pIZ/NoVR1GFP + pIB/T7 + pUC/DS-DmAgo2-1; 8. pIZ/NoVR1GFP + pIB/T7 + pUC/DS-DmAgo2-2; 9. pIZ/NoVR1GFP + pIB/T7 + pUC/DS-DmDcr1; 10. pIZ/NoVR1GFP + pIB/T7 + pUC/DS-DmDcr2; 11. pIZ/NoVR1GFP + dsRluc RNA; 12. pIZ/NoVR1GFP + pIB/T7 + pUC/DS-Rluc; 13. pIZ/NoVR1GFP + pDS; 14. pIZ/NoVR1GFP + pDS/DmAgo1; 15. pIZ/NoVR1GFP + pDS/DmAgo2-1; 16. pIZ/NoVR1GFP + pDS/DmAgo2-2; 17. pIZ/NoVR1GFP + pDS/DmDcr1; 18. pIZ/NoVR1GFP + pDS/DmDcr2; 19. pIZ/NoVR1GFP + pDS/Rluc; 20. mock.

Note: pUC/DS-DmAgo2-1 and pUC/DS-DmAgo2-2 target different regions of the DmAgo2 coding sequences.
5.3 Conclusions

- Plasmid-based dsRNA expression systems, pUC/DS and pDS, have been established in *Drosophila* cells;

- pUC/DS and pDS systems have similar efficiency in silencing exogenous and endogenous targets;

- pUC/DS and pDS can be used for functional analysis of cellular genes in *Drosophila*, mosquito, and other insect cells;

- pUC/DS and pDS can be used as a RNAi-based forward or reverse genetic tool to analyze cellular response to virus infection or elucidate specific cellular pathway in insect cells.
CHAPTER 6 CONSTRUCTION OF RANDOM DSRNA LIBRARY AND IDENTIFICATION OF NOVEL CELLULAR GENES INVOLVED IN ANTIVIRAL IMMUNE RESPONSE IN INSECT CELLS

6.1 Introduction

RNAi serves as an important innate immunity against viruses in plants, fungi, bacteria, flies, mosquitoes and other insect cells. As the most commonly used arbovirus propagation cell line, *Ae. albopictus* mosquito C6/36 cell line has recently been shown have a dysfunctional RNAi pathway, and is impaired at the step of processing dsRNAs into siRNAs. Due to defect RNAi pathway in C6/36 cells, the dsRNA expression system does not work very well in it. Also, we found that other *Ae.* mosquito cell lines such as *Ae. aegypti* Aag2 and ATC-10 cells are nontransfectable, so we need to find a cell line that is RNAi competent and easy transfected to screen for the cellular factors required for dengue virus infection/replication by RNAi-mediated silencing of the target genes. As known, *Drosophila* S2 cell is well established RNAi competent cell line, and it can be infected by well-adapted dengue virus (155). In this study, a random cDNA library was constructed in the dsRNA expression cassette and initial screening led to identification of two host factors that are involved in antiviral response in *Drosophila* S2 cells. With comparative genomics, we also tested the homologs of those novel factors in *Ae. aegypti* Aag2 cell line to see whether those dengue virus host factors are implicated in the antiviral pathway in the same way as in *Drosophila* S2 cells.
6.2 Results
6.2.1 Genome-wide RNAi screening for cellular factors that affect virus replication in *Drosophila* S2 cells

Over 100 pUC/DS-cDNA clones were randomly selected and plasmids were isolated, followed by co-transfection of S2 cells with pIZ/NoVR1GFP. Plasmids pUC/DS-DmAgo2 and pUC/DS-LacZ were used as a positive and negative control, respectively. There were two potential positive hits (Figure 22) observed in the initial screen, which are further confirmed by secondary co-transfection assay, followed by Northern blotting analysis (Figure 23) for detection of mutant virus reporter FHVR1GFP replication. DNA sequencing revealed that DmUnc119 (*Unc*coordinated 119, Unc119) and DmNFAT (*nuclear factor of activated T cells, NFAT*) are the factors in *Drosophila* cells whose depletions by RNAi-mediated silencing can rescue flock house mutant virus FHVR1GFP and may be involved in antiviral mechanism in a manner not described before. In order to verify the involvement of Unc119 and NFAT in antiviral response, a *Drosophila* S2 cell line persistently infected with Dengue virus type 2 (DV2) New Guinea C strain (S2-DV2) was established by continuous passage of virus-infected cells. DsRNA templates were PCR-amplified from pUC/DS-DmUnc119 and pUC/DS-DmNFAT using the T7 primer, and dsRNAs were synthesized by *in vitro* transcription. In S2-DV2 cells transfected with dsRNA targeting Unc119 and NFAT, DV2 replication were significantly enhanced by 3.7-fold (Figure 24) and 8.7-fold (Figure 28) compared to control lacZ dsRNA treatment, suggesting that DmUnc119 and DmNFAT could confer a general state of antiviral response in S2 cells.
Figure 22. Genome-wide RNAi screening for cellular factors that affect virus replication in *Drosophila* S2 cells. (A) Schematic representative of the pUC/DS-S2cDNA library co-transfection with pMT/FHVR1GFP reporter system. (B) Cells in a 24-well plate were transfected with different plasmid combinations as indicated. Induction of pMT/FHVR1GFP was done using 0.5 mM of CuSO₄ at 16 hours after transfection. The fluorescence (a) and light (b) images of cells were captured under a fluorescence
microscope at 2 days after induction. 1. Mock; 2. pMT/FHVR1GFP + pIB/T7; 3. pMT/FHVR1GFP + pIB/T7 + pUC/DS-LacZ; 4. pMT/FHVR1GFP + pIB/T7 + pUC/DS-DmAgo2; 5. pMT/FHVR1GFP + pIB/T7 + pUC/DS-DmUnc119; 6. pMT/FHVR1GFP + pIB/T7 + pUC/DS-DmNFAT.

Figure 23. Effect of silencing DmUnc119 and DmNFAT on host antiviral response in Drosophila S2 cells. Cells in a 24-well plate were transfected with different plasmid combinations as indicated. Induction of pMT/FHVR1GFP was done using 0.5 mM of CuSO₄ at 16 hours after transfection. Samples were collected at two days after induction for RNA extraction. Northern blot was performed with [α-³²P]-dCTP labeled GFP as probe. 1. Mock; 2. pMT/FHVR1GFP + pIB/T7; 3. pMT/FHVR1GFP + pIB/T7 + pUC/DS-LacZ; 4. pMT/FHVR1GFP + pIB/T7 + pUC/DS-DmAgo2; 5. pMT/FHVR1GFP + pIB/T7 + pUC/DS-DmUnc119; 6. pMT/FHVR1GFP + pIB/T7 + pUC/DS-DmNFAT.
6.2.2 Effect of DmUnc119 dsRNA treatment on DV2 replication in S2-DV2 cell line

*Drosophila* actin 5C gene was used as endogenous reference gene when analyzing *Drosophila* Unc119 and NFAT gene transcription level (mRNA level) after different treatments. *Ae. aegypti* NFAT and Unc119 were analyzed by quantitative Real-time RT-PCR with *Ae. aegypti* act1 gene as endogenous reference gene. When cells were challenged with different viruses such as flock house virus and dengue virus type 2 (New Guinea-C strain), the virus replication level was also detected by qRT-PCR with specific primers (Table 3).

**Table 3.** Quantitative Real-time RT-PCR primers used to detect target gene transcription (mRNA) in *Drosophila* S2 cell and *Ae. aegypti* Aag2 cells.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>qRT-PCR primer sequences (5’-3’)</th>
<th>PCR Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DmAct5c</td>
<td>F: TTACTCTTTCAACCACCACCAGCTGA  R: TGCTCAAAGTGAGGGCAACAT</td>
<td>84</td>
</tr>
<tr>
<td>DmNFAT</td>
<td>F: CCACCAACACCAGCTACTATG        R: CTGCTCCTGGGTGTGGTTATAAG</td>
<td>111</td>
</tr>
<tr>
<td>DmUnc119</td>
<td>F: TGATCGAAGCCACTCTCTC          R: CGTAGATGTGCTCAACCGTATT</td>
<td>102</td>
</tr>
<tr>
<td>AaAct1</td>
<td>F: GCTCCACCAGAACTACTAC          R: ATCGTACTCTGCTTTGGAAATC</td>
<td>99</td>
</tr>
<tr>
<td>AaNFAT</td>
<td>F: CCGACGATTAGACGACCTATTACT     R: TCCCTGTCTCCTGCTGTAACCTA</td>
<td>94</td>
</tr>
<tr>
<td>AaUnc119</td>
<td>F: CATCCCACCAGATCTAGTAAC        R: CGCGTAGTCTGCTTGTTAT</td>
<td>112</td>
</tr>
<tr>
<td>D2/NGC</td>
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<td>94</td>
</tr>
<tr>
<td>FHVR1</td>
<td>F: CACATGCGTCCAGTAGTTA          R: GCTCCACCGCTAACCCTATATATT</td>
<td>116</td>
</tr>
</tbody>
</table>

**Note:** F: Forward primer; R: Reverse primer.
**Figure 24.** Effect of DmUnc119 dsRNA treatment on DV2 replication in S2-DV2 persistent cell line. S2-DV2 cells in 24-wells plate are transfected by DmUnc119 dsRNA and control dsRNA (LacZ), at different time points (dpt), cells are collected for RNA extraction and qRT-PCR analysis.

The p values are calculated based on a Student’s t-test of the replicate values in the control group and treatment groups, and p < 0.01 is indicated by *. 
6.2.3 Effect of FHV infection on DmUnc119 transcription in Drosophila S2 cells

**Figure 25.** Effect of FHV infection on DmUnc119 transcription in *Drosophila* S2 cells. S2 cells were infected with Flock house virus (FHV) of MOI 1.0. At different time point, infected cells are collected for RNA extraction and qRT-PCR assay.

The p values are calculated based on a Student’s t-test of the replicate values in the control group and treatment groups, and p < 0.05 are indicated with *.
6.2.4 Effect of DV2 infection on AaUnc119 transcription in Ae. aegypti Aag2 cells

Figure 26. Effect of DV2 infection on AaUnc119 transcription in Ae. aegypti Aag2 cells.

Note: Aag2 cells were infected with DV2 of MOI 1.0. At different times, infected cells are collected for RNA extraction and qRT-PCR assay.

The p values are calculated based on a Student’s t-test of the replicate values in the control group and treatment groups, and p < 0.05 are indicated with *. 
6.2.5 Effect of over-expression of DmUnc119 on DV2 replication in Drosophila S2 cells

![Overexpression-Western Blot Analysis](image)

**Figure 27.** Effect of over-expression of DmUnc119 on DV2 replication in *Drosophila* S2 cells. *Drosophila* cells in 24-wells plate are transfected with over-expression plasmid, 16 hours post transfection, cells are infected with DV2-S2 virus, 3 days after infection, cells are collected for RNA extraction and qRT-PCR assay. (A) Western blot verification of over-expression, (B) DmUnc119 transcription by qRT-PCR, (C) DV2 replication in target genes over-expressed cells by qRT-PCR.

**Note:** The p values are calculated based on a Student’s t-test of the replicate values in the control group and treatment groups, and p < 0.001 are indicated with *. 

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6.2.6 Effect of DmNFAT dsRNA treatment on DV2 virus replication in S2-DV2 cell line

Figure 28. Effect of DmNFAT dsRNA treatment on DV2 virus replication in S2-DV2 cell line. S2-DV2 cells in 24-wells plate are transfected by DmNFAT dsRNA and control dsRNA (LacZ), at different time points (dpt), cells are collected for RNA extraction and qRT-PCR analysis.

Note: The p values are calculated based on a Student’s t-test of the replicate values in the control group and treatment groups, and p < 0.01 are indicated with *, p<0.001 by **.
6.2.7 Effect of FHV infection on DmNFAT transcription in Drosophila S2 cells

**Figure 29.** Effect of FHV infection on DmNFAT transcription in *Drosophila* S2 cells. S2 cells were infected with Flock house virus (FHV) of MOI 1.0. At different time points, mock and infected cells are collected for RNA extraction and qRT-PCR analysis.

**Note:** The p values are calculated based on a Student’s t-test of the replicate values in the control group and treatment groups, and p < 0.05 are indicated with *.
**6.2.8 Effect of DV2 infection on AaNFAT transcription in *Ae. aegypti* Aag2 cells**

**Figure 30.** Effect of DV2 infection on AaNFAT transcription in *Ae. aegypti* Aag2 cells. Note: Aag2 cells were infected with DV2 of MOI 1.0. At different time points, mock and infected cells are collected for RNA extraction and qRT-PCR analysis.

**Note:** The p values are calculated based on a Student’s t-test of the replicate values in the control group and treatment groups, and p < 0.01 are indicated with **. 
6.2.9 DmNFAT regulated by DYRKs and phosphoprotein phosphatases

Gwack et al found that DYRK-family kinases are regulators of NFAT in Drosophila by genome-wide RNAi screen (172, Figure 31). In this study, the dsRNA-mediated silencing of DYRK genes indicated that DYRK2 and DYRK3 are required for DV2 replication in Drosophila S2 cells (Figure 32A), whereas DYRK1 is the restriction factor for DV2 replication in S2 cells (Figure 32A). In this study, we found that in Ae. aegypti Aag2 cells, AaNFAT (Figure 25) and AaUnc119 (Figure 26) participated in antiviral pathway similar as in Drosophila S2 cells. In human resting cells, NFAT proteins are heavily phosphorylated and reside in the cytoplasm, when cells exposed to stimuli such as Ca\(^{2+}\) levels rise, they will get dephosphorylated by the calmodulin-dependent phosphatase calcineurin and translocate to the nucleus (173). In Drosophila, 18-22 PPP genes generated from a core set of 8 indispensible phosphatases in most insect cells. Based on the primary structures, the PPP enzymes are divided into 5 subgroups: (i) The type 1 or PPP1 subfamily, including Pp1-96A, Pp1-9C, Pp1-13C, Pp1-87B, Pp1-9C, Ppγ-55A, PpN-56A, PpD5, PpD6, Pp1-γ1, Pp1-γ2 (174). (ii) The calcineurin/Pp2B/PPP3 Ca\(^{2+}\)-regulated protein phosphatases, including three closely related isoforms, CanA1, Pp2B-14D, CanA-14F. (iii) The type 2 or (PPP2-4-6) phosphatases, Pp2A (a.k.a. microtubule star, mts) (175), Pp4-19C (176), PpV (177) and Pp4-like (178). (iv) PPP5 subgroup, PpD3. (v) PPP7 subgroup, retinal degeneration C (rdgC), Ca-calmodulin regulated protein phosphatase protecting retina from light-induced degeneration (179, 180). In this study, we try to figure out how these PPPs participate in the NFAT regulatory pathway and which step they will step in for the dephosphorylation (Figure 31). Our results indicated that PPPs are localized in cytoplasm (Figure 35), where they function to
dephosphorylate NFAT and activate its translocation to nucleus for the re-phosphorylation by DYRK1, activate its regulatory function on downstream genes, which will affect virus replication in host cells.

**Figure 31:** DmNFAT regulated by DYRKs and phosphoprotein phosphatases (PPPs).
Figure 32. Effect of RNAi-mediated silencing of DmDYRK1, DmDYRK2 and DYRK3 on DV2-S2 replication and DmNFAT transcription (mRNA) in *Drosophila* S2 cells. (A) Effect of dsRNA-mediated DmDYRKs silencing on DV2-S2 replication in *Drosophila* S2 cells, (B) Effect of dsRNA-mediated DmDYRKs silencing on DmNFAT transcription (mRNA).

Note: The p values are calculated based on a Student’s t-test of the replicate values in the control group and treatment groups, and p < 0.05 are indicated with *.
Figure 33. Effect of DmDYRK1 (A) DmDYRK2 and DmDYRK3 (B) silencing on the DmNFAT regulation and DV2 replication in *Drosophila* S2 cells.
Figure 34. Effect of RNAi-mediated silencing of *Drosophila* Phosphoprotein phosphatases (PPPs) on DV2-S2 replication and DmNFAT transcription (mRNA) in *Drosophila* S2 cells. (A) Effect of dsRNA-mediated DmPPPs silencing on DV2-S2 replication in *Drosophila* S2 cells, (B) Effect of dsRNA-mediated DmPPPs silencing on DmNFAT transcription (mRNA).

Note: The p values are calculated based on a Student’s t-test of the replicate values in the control group and treatment groups, and p < 0.01 are indicated with *.
Figure 35. Effect of DmPPPs silencing on the DmNFAT regulation and DV2 replication in Drosophila S2 cells.
6.3 Conclusions

- DsRNA expression system can be used in analysis gene function in insect cells.
  providing an alternative tool for functional genomics in *Drosophila* and other
  insect cells;

- DmUnc119 and DmNFAT are implicated in antiviral response in *Drosophila S2*
  cells;

- AaUnc119 and AaNFAT are involved in antiviral immune response in *Ae. aegypti*
  Aag2 cell;

- DmDYRK2 and DmDYRK3 are responsible for phosphorylation of cytoplasmic
  NFAT, which will let the NFAT reside in the cytoplasm, while PPPs counteract
  the function of DmDYRK2 and DmDYRK3 to dephosphorylation of NFAT and
  promote its translocation from cytoplasm to nucleus;

- In nucleus, DmDYRK1 is responsible for the re-phosphorylate nuclear NFAT to
  activate the downstream gene expression.
CHAPTER 7 DISCUSSIONS

RNAi is the major innate immunity against viruses in flies and mosquitoes. The sensory protein Dicer processes dsRNAs derived from virus replication into siRNAs and initiates the sequence-specific antiviral response. In this study, we established in vitro Dicer assay with the cell extracts prepared from mosquito cells, and confirmed the absence of Dicer enzymatic activity in C6/36 cells (Figure 7). Using Nodamura virus as a model, we functionally analyzed the defect of C6/36 in RNAi-based antiviral immunity, demonstrating that RNAi pathway is the primary antiviral mechanism in mosquito cells.

In this study, we demonstrated that dsRNA-mediated gene regulation is defective in C6/36 cells, in which the capacity of the cell extract to process dsRNAs into siRNAs in C6/36 cells is compromised. As known, *Ae. albopictus* C6/36 cell line is the most prevalently used cells for flavivirus propagation due to its high susceptibility and permissibility to the arbovirus infection. The defective RNAi-mediated antiviral immunity found in this study may contribute to the high permissibility of C6/36 cells to infection by many arboviruses. On the other hand, we also found that in the absence of functional Dicer activity, siRNAs are still able to incorporate into RISC, which can execute specific degradation of homologous mRNAs (Figure 9). At the same time, microRNA processing is still functional normally in C6/36 cells (Figure 10), suggesting that the defect in dsRNA processing might be derived from the disruption of *Ae. albopictus* Dicer-2 activity. Our C6/36 and *Ae. albopictus* Dicer-2 full length cloning indicated that there is a single nucleotide deletion at 2460 nt (G) in C6/36 Dicer-2 ORF which attenuated the dicer protein in the C6/36 cell line at 819 AA compared to the 1659 AA wild type dicer-2 protein in *Ae. albopictus* (Data not shown). Based on the domain
structures of representative members of Dicer-2 protein, the RNaseIII domain usually located at the C-terminal (156), which is absent from C6/36 Dicer-2 protein. So the truncated C6/36 Dicer-2 protein lost the ability to bind and process the dsRNA into siRNA required for target mRNA degrading. Our results also indicated that the defective Dcr-2 in C6/36 cells cannot be complemented by introduction of a functional Dcr-2, DmDcr2 (Figure 11B). This was further confirmed with failed siRNA production from dsRNAs by FLAG-DmDcr2 pull-downed from the transfected cells (Figure 11C). These results indicated that in *Ae. albopictus* C6/36 cells, there is some unknown mechanism that restricts the normal expression of wild type Dicer-2. To address this issue, further experiments need to be carefully designed in future.

RNAi has been used as a powerful tool for gene loss-of-function studies, leading to uncover novel gene functions in many biological processes in plants and animals. The systems for RNAi-based functional genomic analysis have been established in plants, worms, flies, mice and humans (144, 157-160). Various strategies are being used in different organisms in term of development of RNAi libraries, including the nature of effector RNAs (long dsRNA, siRNA or short-hairpin RNA) and the delivery of the effector RNAs (synthesized *in vitro* or expressed from a vector *in vivo*). Selection of RNAi library in functional genomics will largely depend on the cell/organism to work with, the purpose of screening, and the cost issue.

In insect cells, dsRNAs used for gene functional studies are usually *in vitro* transcribed by T7 RNA polymerase from DNA template that contains two convergent T7 promoters (140). Production of long dsRNAs *in vivo* can be achieved by either transcription from long inverted-repeat sequences or from the sequences that carrying convergent promoters
Because of instability of long inverted-repeats in plasmids, the strategy with convergent promoters is more often used. Expression of dsRNA from two opposing T7 promoters can be efficiently mediated by T7 RNA polymerase in bacteria and trypanosomes, which has been successfully used for genome-wide RNAi in C. elegans and trypanosomes (142, 143). However, plasmid based dsRNA expression has not been established for functional genomic studies in insect cells. In this study, a plasmid-based dsRNA expression system was developed and could serve as useful tool for functional genomics in insect cells. We have demonstrated that dsRNA intracellularly expressed from plasmid-based dsRNA expression cassette can efficiently knockdown transient expression of target reporter genes (Figure 13, 14, 15) and also is effective in regulating an endogenous gene (Figure 16 and 17) in Drosophila S2 cells. Furthermore, we analyzed and compared the efficiency of dsRNAs expressed from pUC/DS and pDS (Figure 18, 20 and 21) in regulation of major components in Drosophila RNAi pathway, including Ago-2 and Ago-1, in which Ago-2 is responsible for siRNA production, while Ago-1 is required for maturation and production of microRNAs and not essential for antiviral response (153, 154). Results indicated that pUC/DS and pDS systems possess similar ability in knockdown of a target gene, either transiently expressed or endogenous. So, the plasmid-based dsRNA expression system was successfully established in Drosophila S2 cell line. Next, we try to apply this system to discover novel components in antiviral RNAi pathway in Drosophila S2 cells. From the initial screening, two potential candidates DmUnc119 and DmNFAT (Figure 22, 23) were discovered. RNAi-mediated silencing of DmUnc119 and DmNFAT can efficiently rescue the mutant flock.
house virus FHVR1GFP replication (Figure 22, 23), which means that these two genes may be involved in antiviral responses in *Drosophila* cells in unknown way.

**Unc**oordinated 119 (Unc119) family of neural protein is involved in neuron and nervous system development in humans, *Drosophila*, *C. elegans* (161) as well as in Zebrafish (162). The unc-119 genes are conserved both in function and structure. The carboxyl terminus of unc-119 is indispensable for its conserved function across different species. Studies demonstrated that HRG4/HsUnc-119 and DmUnc-119 can efficiently rescue the defects from *unc-119* null mutant in *C. elegans* (161). In addition to the conserved function of Unc-119, in *C. elegans*, unc-119 is involved in feeding, chemosensation, and locomotion of the worm (163) and T cell activation (164). Recent study demonstrates that Unc-119 is required for G protein trafficking in sensory neurons in *C. elegans* (165).

Also, some research indicates that Unc119 is implicated in myofibroblast differentiation through the activation of Fyn and the p38 MAPK (mitogen-activated protein kinase) pathway (166), which is also involved in insect immunity, as well as environmental stresses response in *Drosophila*. Innate immunity as the dominant immunity in plants, fungi, insects and in primitive multicellular organisms, play vital role in antimicrobial defense process. Even though, DmUnc119 most like is involved in neurons and nervous system development, it is still potential implicated in RNAi-mediated antiviral pathway in some way, which is consistent with our results (Figure 22 & 23). In S2-DV2 persistent cell line, silencing of DmUnc119 can improve the DV2 replication significantly by 3.7-(Figure 24) folds compared to control group, indicating that DmUnc119 may participate in antiviral immune response in *Drosophila* S2 cells. As known, in addition to RNAi antiviral response, other cellular pathway like Toll pathway, Imd pathway, and Jak-STAT
pathway are also involved in antiviral immune responses in insect and mosquito cells. In this study, we also found DmUnc119 is up-regulated by FHV virus infection at different time points (Figure 25). Also, when *Ae. aegypti* Aag2 cells were challenged with DV2-NGC (MOI 1.0), it induced the *Aedes* homolog of DmNFAT, AaNFAT transcription at different time point after infection (Figure 26). This finding indicated that in mosquito cell, AaUnc119 are implicated in the antiviral response same as in *Drosophila* S2 cells.

NFAT (nuclear factor of activated T cells) transcription factors play a critical role in inducible gene transcription during the immune response (167-170). Despite their name, NFAT proteins are expressed not only in T cells, but also in other classes of immune-system cells. In human, there are five Nuclear Factors of Activated T-cells, NFAT1-5, while in *Drosophila* NFAT gene encodes the only homolog dNFAT with highest similarity to hNFAT5. Silencing of DmNFAT by dsRNA will improve DV2 replication by 1.6 folds at one day post transfection and 8.7 folds higher at two days post transfection (Figure 28) in S2-DV2 persistent cell line. Loss-of-function study of dNFAT in *Drosophila* indicated that dNFAT contributes significantly to salt stress response (171).

In this study, we also found that DmNFAT is up-regulated by FHV virus infection in *Drosophila* S2 cells at different time points (Figure 29). Same as Unc119, the Aedes homolog, AaNFAT is up-regulated upon DV2 virus infection in Aag2 cells (Figure 30). It’s possible the DmNFAT is involved in universal pathway against stress response like salt stress, virus infection etc. and may be same case in *Aedes* mosquito cells.

Recent study with genome wide RNAi screening identifies DYRK (dual-specificity tyrosine-phosphorylation regulated kinases)-family kinases as regulators of NFAT (172. Figure 31). This study indicates that DYRK is key kinase that regulates NFAT1
phosphorylation status. While different DYRK genes do not all function at the same way. DYRK2-DYRK3-DYRK4, localized to the cytoplasm, will function primarily as ‘maintenance’ kinases that sustain the phosphorylation status of cytoplasmic NFAT in resting cells, whereas DYRK1A and DYRK1B, localized to nucleus, will re-phosphorylate nuclear NFAT and promote its nuclear export (181). In this study, the dsRNA-mediated silencing of DYRK genes indicated that DmDYRK2 and DmDYRK3 are restricted factors of activated DmNFAT in nucleus, required for DV2-S2 replication in Drosophila S2 cells (Figure 32, Figure 33B), whereas DmDYRK1 is positive regulator of activated DmNFAT in nucleus, involved in antiviral response against DV2-S2 replication in S2 cells (Figure 32, Figure 33A). Chow et al. reported that NFAT transcription factor contributes to the cytokine interleukin-2 (IL-2) expression by binding to its promoter region. Their data demonstrated that NFAT transcription factor is involved in IL-2 gene expression and therefore play significant role in the initiation of immune response (182). As known, innate immunity is an ancient defense that enables multicellular organisms to detect and fight infectious microbes (90). In addition to RNA interfering (RNAi), other innate immunity also plays a role in antimicrobial response in Drosophila. Such as Toll pathway not only controls resistance to fungal and Gram-positive bacterial infection, but also involved in antiviral response in Drosophila melanogaster (91, 92). While Gram-negative bacteria mainly activate the immune deficiency (Imd) pathway, which governs expression of the genes encoding many antibacterial peptides (93). Recent research indicated that Imd pathway is also involved in antiviral immune responses in Drosophila (93). In addition to Toll and Imd pathways, a third, evolutionary conserved innate immunity pathway, Jak-STAT (Janus kinase-
signal transducer and activator of transcription) also counteract viral infection in mosquito (10) and *Drosophila* (90). In our study, we find out that dNFAT is involved in antiviral pathway, could rescue flock house mutant virus to comparable level to wild type virus without B2 suppressor. High potentially, the dNFAT is implicated in the RNAi-mediated antiviral pathway in *Drosophila*. Phosphotrotein phosphatases (PPPs) are important regulatory enzymes present in all eukaryotic organisms. PPPs cooperate with kinases to ensure phosphorylation - dephosphorylation regulatory cycle reversible. In *Drosophila*, 18-22 PPP genes generated from a core set of 8 indispensible phosphatases in most insect cells. Based on the primary structures, the PPP enzymes divided into 5 subgroups (176). In this study, we randomly pick up one or two members from each subgroup to test how they cooperate with DYRKs to maintain the NFAT phosphorylation-dephosphorylation status balance in *Drosophila* S2 cells. Those selected PPPs are list as follows:

- The type 1 or PPP1 subfamily, including *Pp1-13C, Pp1-87B*,
- The calcineurin/Pp2B/PPP3 Ca^{2+}-regulated protein phosphatases, *CaNa*,
- The type 2 or (PPP2-4-6) phosphatases, *Pp2A* (a.k.a. *microtubule star, mts*) (175), *Pp4-19C* (176), *PpV* (177),
- PPP5 subgroup, *PpD3*,
- PPP7 subgroup, *retinal degeneration C (rdgC)*, Ca-calmodulin regulated protein phosphatase protecting retina from light-induced degeneration (178, 180).

From the quantitative RT-PCR data, the PPPs function as counteract to DmDYRK2 and DmDYRK3 in cytoplasm to dephosphorylate the NFAT and motivated its translocation to
nucleus for the rephosphorylation by DmDYRK1 to activate the NFAT gene, which is involved in antiviral response in our results. So when those PPPs gene are silenced by dsRNA, the NFAT activated form (NFAT-(P)) will get down-regulated in nucleus, which will favor the virus replication (Figure 34A & Figure 35) without affect the NFAT transcription level (Figure 34B). From these results we can conclude that these PPPs function mainly in cytoplasm to activate NFAT and its translocation to nucleus (Figure 35).
APPENDIX

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Cleavage of poly(A) binding protein by enterovirus proteases concurrent with inhibition of translation


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