Modulation of Alphaviruses by Small RNAs

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ABSTRACT

Mosquito-borne diseases remain a significant burden on global public health. Maintenance of mosquito-borne viruses in nature requires a biological transmission cycle that involves alternating virus replication in a susceptible vertebrate and mosquito host. Although infection of the vertebrate host is acute and often associated with disease, continual transmission of these viruses in nature depends on the establishment of a persistent, nonpathogenic infection in the mosquito vector. It is well known that invertebrates rely on small RNA pathways as an adaptive antiviral defense. The canonical antiviral response in these organisms involves dicer enzymes that cleave viral double-stranded RNA replicative intermediates (RIs) into small interfering RNAs (siRNAs; ~21-24 nucleotides). One strand of the siRNA duplex guides the targeting and destruction of complementary viral RNAs when loaded and retained in a multi-protein complex called the RNA-induced silencing complex. Here, we show that mosquito vectors mount a redundant double defense against virus infection mediated by two different small RNA pathways. Specifically, we demonstrate that in addition to a canonical antiviral response mediated by siRNAs, virus infection of the mosquito soma also triggers an antiviral immune pathway directed by ping-pong-dependent PIWI-interacting RNAs (piRNAs; ~24-30 nucleotides). The complexity of mosquito antiviral immunity has important implications for understanding how viruses both induce and modulate RNA-silencing responses in mosquito vectors.

In mammals, viral RIs induce a range of relatively nonspecific antiviral responses. However, it remains unclear if viral RIs also trigger RNA silencing in mammals. Mosquito-borne viruses represent an ideal model for addressing this question as their transmission cycles involve alternating replication in mammalian and invertebrate hosts. Although we report identifying a subset of virus-derived small RNAs that appear to be products of RNA silencing in two mammalian cell lines infected with the mosquito-borne

chikungunya virus (CHIKV), our studies suggest these small RNAs have little biological relevance in combating virus infections. Thus, while the accumulation of virus-derived siRNAs is essential to the survival of mosquitoes infected with CHIKV, they appear to have little functional significance in mammalian antiviral immunity.

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This dissertation is dedicated to my parents Jorge and Lizette Morazzani

Gracias por su apoyo y cariño durante esta nueva etapa de mi vida

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ATTRIBUTION

The dissertation research discussed herein would not have been possible without the help of several individuals. Their names and contributions to each chapter are listed below.

Chapter 2: An Antiviral Immunity Pathway Directed by Ping-Pong-Dependent PIWI-Interacting RNAs in the Mosquito Soma

1. **Michael Wiley.** (Fralin Life Science Institute, Department of Entomology, Virginia Polytechnic Institute and State University): provided technical assistance.
2. **Zach Adelman, Ph.D.** (Fralin Life Science Institute, Department of Entomology, Virginia Polytechnic Institute and State University): assisted in the analysis of small RNA data sets.
3. **Kevin Myles, Ph.D.** (Fralin Life Science Institute, Department of Entomology, Virginia Polytechnic Institute and State University): obtained funding, provided research concept and wrote the manuscript.

Chapter 3: Examining the Role of the B1 *Nodamura Virus* Nonstructural Protein in Mosquitoes

1. **Michael Wiley.** (Fralin Life Science Institute, Department of Entomology, Virginia Polytechnic Institute and State University): provided technical assistance.
2. **Zach Adelman, Ph.D.** (Fralin Life Science Institute, Department of Entomology, Virginia Polytechnic Institute and State University): assisted in the analysis of small RNA data sets.
3. **Kevin Myles, Ph.D.** (Fralin Life Science Institute, Department of Entomology, Virginia Polytechnic Institute and State University): obtained funding, provided research concept and editorial changes.

Chapter 4: Role of virus-derived small RNAs in the antiviral immunity of two mammalian cell lines

1. **Kevin Myles, Ph.D.** (Fralin Life Science Institute, Department of Entomology, Virginia Polytechnic Institute and State University): obtained funding, provided research concept and editorial changes.

CHAPTER 1
LITERATURE REVIEW

Mosquito-borne viruses

Mosquito-borne viruses are the causative agents of several diseases that negatively impact public health and economies on a global scale [1]. Specific examples include dengue virus serotypes 1-4 (DENV 1-4; family *Flaviviridae*, genus *Flavivirus*), yellow fever virus (YFV; family *Flaviviridae*, genus *Flavivirus*) and chikungunya virus (CHIKV; family *Togaviridae*, genus *Alphavirus*) [2]. It is estimated that DENV 1-4 cause approximately 50-100 million cases of dengue fever and several hundred thousand cases of the more severe dengue hemorrhagic fever each year [2, 3]. Yellow fever virus continues to adversely affect as many as 200,000 people each year in tropical regions of Africa and South America, despite an effective vaccine [4]. Chikungunya virus recently re-emerged causing a succession of outbreaks in Eastern Africa and South East Asia with subsequent spread to the European continent [5]. On the island of La Reunion alone, approximately 250,000 cases were reported with more than 25% of the island's inhabitants affected [6]. Chikungunya fever is characterized by a debilitating prolonged arthralgic syndrome that may persist for several weeks or months, causing a significant economic burden to those affected [5]. Although *Aedes aegypti* (*Ae. aegypti*) had previously been the most significant vector of CHIKV, *Aedes albopictus* (*Ae. albopictus*) was the species implicated in the La Reunion outbreak [7, 8]. First introduced to the U.S. in a shipment of used tires from northern Asia in the 1980s, *Ae. albopictus* continues to expand its range throughout the globe [9]. The geographical expansion of *Ae. albopictus* has raised concern that CHIKV may follow the mosquito's spread to the U.S. and other parts of the world [9-13].

Mosquito-borne viruses are maintained in nature through biological transmission cycles that involve alternating virus replication in susceptible vertebrate and mosquito hosts [2]. Although infection of the vertebrate host is acute and often associated with pathology and disease, the establishment of a persistent non-lethal infection in the mosquito host is essential for the continual transmission of these viruses [14]. Accordingly, the more pathogenic the virus is for the mosquito vector, the lower the probability of transmission. Thus, the continual transmission of these viruses requires that infections of the mosquito host are associated with very little fitness cost. An antiviral immune response directed by small RNAs has been shown to

play a role in the mechanism by which the pathogenic outcome of alphavirus infections is modulated in the mosquito [15, 16].

Small RNA pathways

RNA interference (RNAi) is a sequence-specific gene silencing process directed by small RNAs via base-pairing interactions**.** Three major classes of small RNAs have been described in flies: microRNAs (miRNAs), short interfering RNAs (siRNAs), and piwi-interacting RNAs (piRNAs) (Figure 1.1). These small RNA pathways can be distinguished based on the size of their biogenic products and association with specific effector proteins.

MicroRNAs (~22nt in length) are derived from endogenous dsRNAs and regulate host gene expression at the post-transcriptional level. Primary miRNAs (pri-miRNA) are transcribed in the nucleus by RNA polymerase II as imperfectly base paired stem loop structures with single-stranded flanking segments [17, 18]. In *Drosophila*, a 60-70nt pre-miRNA stem loop structure is processed from the pri-miRNA by the nuclease Drosha and the double stranded RNA binding protein Pasha (DGCR8 in mammals) [19]. Pre-miRNAs are exported from the nucleus to the cytoplasm by RanGTP Exportin-5 for further processing [20]. In the cytoplasm, a Dicer enzyme cleaves the pre-miRNAs into miRNA duplexes [21]. The thermodynamic stability at the 5' end of the miRNA duplex determines which miRNA strand will be loaded into an Argonaute-mediated RNA-induced silencing complex (RISC) [20]. The loaded strand provides sequence specificity to the activated miRNA-induced silencing complex (miRISC), while the other strand (the passenger strand or miRNA*) is degraded [20]. Most miRNA binding sites reside in the 3'UTR of the target mRNA. Complementarity with the target RNA is generally limited to nucleotides 2-8 or the "seed sequence" of the miRNA [22]. The short length of the miRNA seed region combined with a lack of perfect complementarity to the target sequence allows a single miRNA to repress the translation of more than one gene, consistent with their role in regulating a wide range of biological processes [23].

Small interfering RNAs (~21nt in length), the canonical products of the RNAi pathway, are generated from long perfectly base-paired dsRNAs [24]. The discovery of endogenous siRNA (endo-siRNA) precursors has led to a recent subdivision of the siRNA pathway (Figure 1.1). Endogenous siRNAs are generated in both gonadal and somatic tissues and are typically derived from three different sources of dsRNA: transposable elements, structural transcripts

(hairpins), or overlapping convergent transcripts. The major role of endo-siRNAs appears to be silencing transposons in both the germ cells and somatic tissues of flies and mammals [25, 26]. Exogenous siRNAs (exo-siRNAs) are derived from exogenous sources of dsRNA (e.g., transgenes, viral replicative intermediates, etc.) and are the mediators of an antiviral RNAi response in invertebrates and plants. Exogenous dsRNA precursors are processed by a Dicer enzyme into siRNA duplexes [27, 28]. In Drosophila, Dicer-2 (Dcr-2) and a dsRNA binding protein, R2D2, direct the siRNA duplex into an siRNA-induced silencing complex (siRISC) [29]. Argonaute-2 (Ago-2), an essential component of the siRISC, cleaves the passenger strand, which is then ejected from the siRISC. The remaining guide strand that is bound to Ago-2 directs the siRISC to complementary RNAs for degradation [30].

Piwi-interacting RNAs (~24-30nt in length) appear to be specific to the germline where they function in repressing transposon activity [31, 32]. The current model of piRNA biogenesis suggests that Dicer is not involved in processing piRNAs [32]. Although the exact source of piRNA precursors is unclear, processed piRNAs map to specific loci, termed piRNA clusters, and genomic intergenic repetitive elements [31, 33]. The piRNA pathway is composed of primary and secondary processing mechanisms [33]. Although the mechanism by which primary piRNAs are processed is not well understood, a model for secondary piRNA biogenesis has been described in *Drosophila* [34, 35]. In the current 'ping-pong' model of piRNA biogenesis, Aubergine (Aub) and Piwi mainly bind antisense piRNAs, while Ago-3 binds sense piRNAs [33]. Piwi-interacting RNAs derived from a specific strand direct the production of piRNAs from the opposite strand, thereby amplifying the population of secondary piRNAs (Figure 1.1) [35, 36].

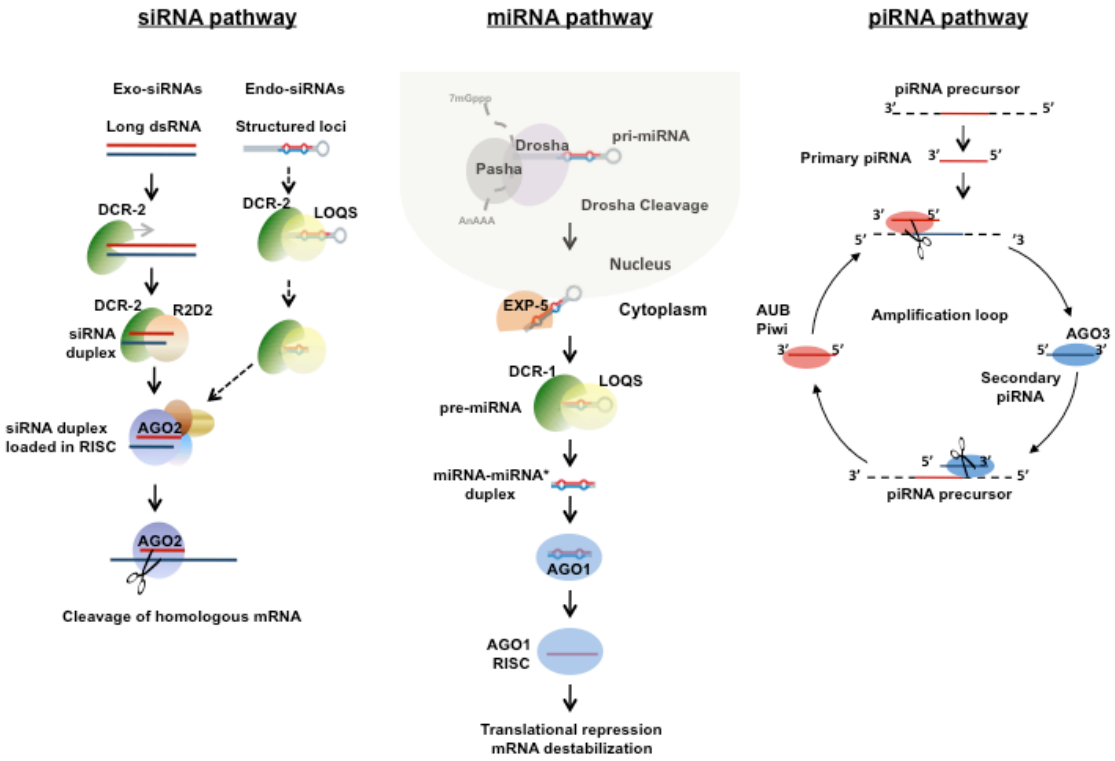

Figure 1.1. Small RNA pathways in flies. Modified from Ghildiyal, M and P.D. Zamore (2009). Three major classes of small RNAs have been described in flies: microRNAs (miRNAs), short interfering RNAs (siRNAs), and piwi-interacting RNAs (piRNAs). These small RNA pathways can be distinguished based on the size of their biogenic products and association with specific effector proteins. MicroRNAs (~22nt in length) are derived from endogenous dsRNAs and regulate host gene expression at the post-transcriptional level. Small interfering RNAs (~21nt in length), the canonical products of the RNAi pathway, are generated from long perfectly base-paired dsRNAs. The discovery of endogenous siRNA (endo-siRNA) precursors has led to a recent subdivision of the siRNA pathway. Piwi-interacting RNAs (~24-30nt in length) appear to be specific to the germline where they function in repressing transposon activity.

Antiviral RNA silencing

Nearly all viruses generate dsRNA, a molecular recognition pattern of RNAi pathways, during viral replication [37]. Double-stranded RNA forms have been identified in each of the three major genera containing arthropod-borne viruses (arboviruses) [38-47]. In invertebrates, viral dsRNA replicative intermediates (RIs) are potent inducers of antiviral RNA silencing. Experiments with *Drosophila melanogaster* loss-of-function mutants indicate that Dcr-2 and Ago-2 are essential components of the antiviral siRNA pathway in flies. Accordingly, in

comparison to wild type flies *Drosophila dcr-2* loss-of-function mutants exhibit increased accumulation of viral RNA and mortality when infected with evolutionarily diverse RNA viruses [48-50].

Studies attempting to exploit an RNAi-based approach to engineer DENV-2 resistance in a mosquito cell line provided one of the earliest indications of a mosquito RNA silencing pathway [51]. Mosquito cells engineered to express a dsRNA inverted repeat corresponding to a portion of the DENV-2 genome were found to be resistant to DENV-2 infection when compared with control cells [51]. Small RNAs (20-30nt) derived from the engineered dsRNA trigger were detected in the DENV-2 resistant cells suggesting that the hairpin structure was processed by Dicer [51]. As a follow-up to this work, *Ae. aegypti* mosquitoes were genetically engineered to express an inverted repeat homologous to a portion of the pre-membrane protein coding region of the DENV-2 RNA genome from a midgut specific promoter [52]. When challenged with an infectious blood meal containing DENV-2, the genetically modified mosquitoes were resistant to virus infection and exhibited reduced levels of virus production [52]. Notably, the DENV-2 resistance phenotype was lost if the RNAi pathway was disrupted [52].

An antiviral silencing response has also been described in *Anopheles gambiae* mosquitoes [53]. *Anopheles gambiae* depleted of Ago-2 exhibit elevated levels of virus replication and dissemination to neighboring tissues when infected with o'nyong nyong virus (ONNV; family *Togaviridae*, genus *Alphavirus*) [53]. More recently, high throughput sequencing technology was used to show that infection of *Ae. aegypti* with Sindbis virus (SINV; family *Togaviridae*, genus *Alphavirus*) results in the accumulation of virus-derived siRNAs predominantly 21nt in length [15]. Suppressing the accumulation of these virus-derived siRNAs using the B2 protein of flock house virus (FHV), a well-characterized suppressor of RNA silencing, dramatically altered the pathogenic outcome of the virus infection in the mosquito host, resulting in rapid and complete mortality [15]. Thus, an antiviral response directed by siRNAs is essential to modulating alphavirus pathogenesis during the course of a natural infection in the mosquito host [15, 54]. Interestingly, depleting Dcr-2 in *Ae. aegypti* does not result in increased mortality following SINV infection [54]. The lack of mortality in mosquitoes depleted of Dcr-2 may be due to functional redundancy in the modulation of virus pathogenesis.

Infection of mosquitoes with viruses of the *Flavivirus* genus has also been shown to trigger an antiviral siRNA response. Infecting *Ae. aegypti* mosquitoes with artificial blood meals

containing DENV-2 and West Nile virus (WNV) results in the accumulation of virus-derived siRNAs [55, 56]. *Aedes aegypti* mosquitoes depleted of R2D2, Ago-2 or Dcr-2 exhibit increased virus replication and shortened extrinsic incubation periods when infected with DENV-2 [55]. Characterization of small RNA populations in *Ae. aegypti* cells infected with DENV-2 and WNV by deep sequencing also reveals an abundant class of 21-nt siRNAs derived from the DENV-2 or WNV genome [56, 57].

Origins of virus-derived siRNAs

Host Dicer proteins are at the vanguard of antiviral immunity directed by small RNAs due to a "sensor" role in detecting the dsRNA associated with viral replication. During viral infections, two major sources of viral dsRNA are potentially accessible to Dicer, highly structured regions present in single-stranded viral genomic mRNAs or dsRNA replicative intermediates (RIs) formed during virus replication (Figure 1.2). Imbalanced synthesis of plus (+) and minus (-) strands is common in the infectious cycles of RNA viruses, usually with the genomic (+) strand produced in greater abundance than its full-length (-) complement [58]. Comparing the abundance of small RNAs in plants infected with several different (+) strand RNA viruses reveals an excess number of siRNAs derived from viral (+) strands, suggesting an origin predominantly from highly structured elements present in single-stranded viral RNAs [59, 60]. Additionally, several hot spots for the production of virus-derived small RNAs from the Cymbidium ringspot virus (CymRSV) genome map to predicted stem-loop structures, providing further support for a single-stranded viral RNA origin [59, 61]. These results have lead to the proposal that pre-miRNA-like structures in viral single-stranded RNAs are the precursors of viral siRNAs [59, 61]. However, infection of other plants, as well as flies and mosquitoes, with several (+) strand RNA viruses generates viral siRNAs from both (+) and (-) strands in proportions that are consistent with a predominantly dsRNA RI origin [60, 62-64]. With regard to alphaviruses, small RNAs sequenced from infected mosquitoes do reveal a significant bias for siRNAs derived from (+) strands, but not in ratios that approximate the imbalances in strand synthesis that have been previously described for alphaviruses [15, 65, 66]. Taken together, these data support an alternate model in which the biogenesis of viral siRNA populations from several (+) strand RNA viruses occurs predominantly from dsRNA viral RIs.

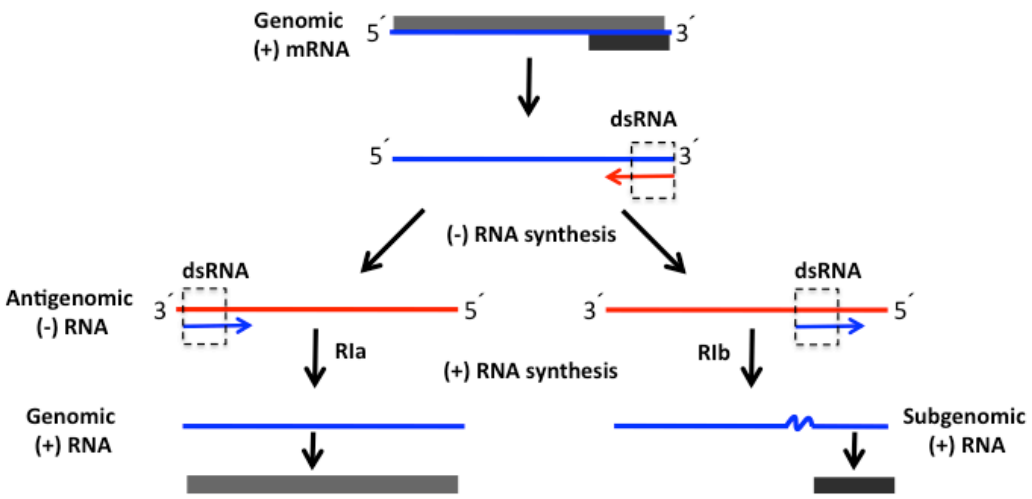

Figure 1.2. Alphavirus replication yields dsRNA replicative intermediates. The genomic RNA of alphaviruses functions as mRNA for the translation of the viral nonstructural proteins and as a template for the synthesis of complementary (-) strand RNAs. The (-) strands then serve as the templates for both the synthesis of new genomic-length (+) strands and a shorter subgenomic length RNA (26 mRNA) that encodes the viral structural genes.

Profiling of virus-derived small RNAs sequenced from infected plant and insect cells reveals an asymmetric distribution across the entire viral genome for many (+) strand RNA viruses [15, 61, 63-65, 67, 68]. Thus, the sequences of viral siRNAs can be assembled into long contiguous fragments providing a novel approach to virus discovery [69]. While distributed across the entire genome, it is clear that some loci in the viral genome are hot spots for viRNA biogenesis [15, 16, 65, 70]. Notably, biological replicate profiles of FHV in *Drosophila* S2 cells and ONNV in mosquitoes are highly reproducible, consistent with a non-random mechanism of siRNA biogenesis [65, 71]. However, siRNA profiles appear to be distinct for particular virus-host combinations [15, 65, 67]. For example, viral siRNA profiles in ONNV-infected *An. gambiae* are distinct from those of SINV-infected *Ae. aegypti* [15, 65]. Similarly, viral siRNAs derived from alphaviruses in mosquitoes do not cluster from any particular region of the viral genome [15, 65, 70], while viral siRNAs generated from FHV in S2 cells cluster near the 5' terminal region [67, 71]. Profiling of viral RNAs sequenced from mosquitoes infected with SINV, ONNV or Semliki Forest virus (SFV; family *Togaviridae*, genus *Alphavirus*) also reveals

no bias towards regions corresponding to the highly abundant 26S subgenomic mRNA, which is also consistent with the processing of viral siRNAs from dsRNA precursors [15, 65, 70].

Diversification of RNAi effector proteins

Genes encoding key protein components of the antiviral siRNA pathway (Dcr-2, Ago-2 and R2D2) have been demonstrated to be among the fastest (top 3%) evolving in the Drosophila genome, suggesting they are under strong positive selection [72]. Thus, invasive nucleic acids, such as viruses and repetitive elements may have led to the diversification of RNAi effector proteins in different organisms in response to an evolutionary arms race. For example, specialized Dicer proteins may have led to a more potent antiviral defense by reducing competition with other small RNA pathways for a limited number of Dicer proteins [31]. Alternatively, specialized Dicer proteins may have arisen in response to rapidly evolving viral strategies to counteract RNA-based immunity [31].

Plant species have evolved to encode four Dicer-like (DCL) enzymes [73]. Plant DCL-1 processes ~22nt miRNAs [18], DCL-2 generates 22nt siRNAs from stress-related natural-antisense transcripts [74], DCL-3 produces 24nt DNA repeat-associated siRNAs that are involved in heterochromatin formation [75], and DCL-4 produces 21nt viral siRNAs that direct the primary antiviral immune response in plants [73]. *Drosophila* species encode two Dicer proteins, Dcr-1 and Dcr-2, which have specialized roles in the production of miRNAs and siRNAs, respectively [76]. Mammals and nematodes encode a single Dicer that is responsible for processing both miRNAs and siRNAs [77-79]. Although Dicer appears to be important to the antiviral immunity of *Caenorhabditis elegans* (*C. elegans*), effective RNA silencing in worms requires the accumulation of secondary siRNAs produced by an RNA-dependent RNA polymerase [80-82]. While mammals lack specialized Dicer proteins, it is unclear if siRNAs direct an antiviral response in these cells [83].

Multiple Argonaute proteins have been characterized in plants (ten), fruit flies (five), and humans (eight) [24]. In *A. thaliana*, Ago-1 primarily associates with miRNAs, while Ago-2, Ago-5, and Ago-7 each bind siRNAs in virus-infected cells [84-87]. However, it remains unclear if any of the Ago proteins possess “slicer” activity, leaving uncertain the mechanism by which viral RNA is degraded in these cells [88]. In *Drosophila*, diversification of Ago genes corresponds with functional specialization. Accordingly, siRNAs co-immunoprecipitate (co-IP)

with Ago-2, while miRNAs specifically co-IP with Ago-1 [67, 89]. In mammalian cells, small RNAs associate with all four human Ago (hAgo) proteins, leaving any functional specialization unclear [90-92]. Although hAgo-2 has been demonstrated to have "slicer" activity [90, 91, 93], the specific mechanism by which miRNAs are sorted into specific hAgo proteins remains unknown.

Similar to *Drosophila*, immune-related genes in *An. gambiae* and *Ae. aegypti* are significantly more divergent than other coding sequences in the mosquito genome [94]. While the siRNA pathway has been ascribed an antiviral function in flies [15, 88], a major function of the piRNA pathway appears to be regulating the activity of transposable elements in the germline [32]. While a Piwi clade of Argonaute proteins is comprised of Piwi, Aubergine (AUB), and Argonaute-3 (Ago-3) in Drosophila, there has been a considerable expansion of the PIWI subfamily in the genomes *Ae. aegypti* and *Culex pipiens quinquefasciatus (Cx. pipiens)* (Table 1.1) [95]. As the *Ae. aegypti* and *Cx. pipiens* genomes bear a larger percentage of repetitive elements than the *Drosophila* genome [96, 97], these elements may have provided the original evolutionary pressure for the expansion of PIWI genes in these mosquitoes.

Table 1.1 Expansion of PIWI genes in mosquitoes.

	D. melanogaster	*Ae. aegypti*	Cx. Pipiens
Ago-3	1	1	1
PIWI	1	4	3
AUB	1	3	3

Although functional specialization of small RNA pathways has been described, genetic loss-of-function studies demonstrate redundancy in antiviral responses directed by small RNAs. For example, infection of *A. thaliana* with *Tobacco rattle virus* (TRV) results in the accumulation of 21nt and 24nt viral siRNAs. However, *A. thaliana dcl4* loss-of-function mutants produce 22nt viral siRNAs, the characteristic size for processing by DCL-2, suggesting a hierarchical antiviral activity in which DCL-2 initiates antiviral silencing in the absence of DCL-4 [73]. Therefore, knockout of antiviral silencing in *A. thaliana* requires the loss of both DCL-2

and DCL-4 [73]. Although Dcr-2 has been shown to direct the primary antiviral immune defense in flies, both *Drosophila* X virus and Nora virus fail to exhibit increased virus replication in *dcr-2* loss-of-function mutants [98, 99]. These findings may indicate that viral dsRNA precursors can also be processed by Dcr-1 or another antiviral silencing response; although, other explanations are also possible.

Role of RNAi in virus infections of vertebrate cells

While the siRNA pathway has an important role in the antiviral immunity of invertebrates, it remains unclear if vertebrates also employ RNAi as an antiviral defense. In vitro studies have demonstrated immunization of mammalian cells against several different viruses following pre-transfection with virus-specific siRNAs or small hairpin RNAs [100-105], suggesting that the components required for antiviral RNA silencing are conserved in mammals. Specifically depleting Dicer in mammalian cells infected with different RNA viruses also results in increased virus production and accelerated cell death [106, 107]. However, depleting Dicer also affects the production of miRNAs in these cells, thus, the mechanism responsible for the observed phenotypes is unclear [106]. A recent study using high throughput sequencing technology to characterize small RNA populations in several mammalian cell types infected with different RNA viruses, reportedly identified viral small RNAs that appear to be products of an RNA silencing response [106]. Although the abundance of these viral small RNAs was generally low, specific features distinguished them from the intermediate products of less-specific degradation pathways [106]. For example, viral small RNAs were derived from (+) and (-) strands in approximately equal ratios, consistent with a dsRNA origin [106]. Paring of viral small RNAs in mammalian cells infected with Hepatitis C virus (HCV; family *Flaviviridae*, genus *Hepacivirus*) and Poliovirus into siRNA duplexes with one or two nucleotide overhangs is also consistent with processing by Dicer [106]. Lastly, viral small RNAs were specifically shown to associate with the four mammalian Argonaute proteins [106]. While this study is one of the first to provide evidence of naturally generated viral small RNAs in mammalian cells, it remains unclear if these small RNAs mediate an antiviral RNA silencing response in these cells.

Although an antiviral role for virus-derived small RNAs generated after a natural virus infection has not yet been demonstrated in mammalian cells, virus-encoded miRNAs have been identified in members of the *Herpesvirus*, *Polyomavirus, Adenovirus,* and *Ascovirus* family

[108]. These viruses are all DNA viruses that replicate in the nucleus, which explains the requirement for Drosha and DGCR8 in processing the pre-miRNAs encoded in their genomes. RNA viruses replicating exclusively in the cytoplasm may not be accessible to the nuclear components of the miRNA machinery [108]. Consistent with this, (+) strand RNA viruses have not been found to encode miRNAs [109]. Thus far, the virus encoded miRNAs that have been identified target either cellular genes involved in modulating antiviral immune responses and/or regulatory proteins encoded by the virus [108]. There are also several examples of endogenous miRNAs that affect the replication of viruses. For example, mice depleted of miR-24 and miR-93 are more susceptible to vesicular stomatitis virus (VSV) in comparison to wild type mice infected with the virus [110]. Replication of HCV is stimulated by the expression of the liver-specific miR-122, while decreasing the accumulation of miR-122 represses virus replication [111]. Many other miRNAs have also been shown to be induced in mammalian cells infected with viruses, but the biological significance of these changes remains unclear [108]. It is possible that endogenous levels of miRNAs are manipulated by the virus to create a more favorable environment for replication [108]. These examples clearly illustrate a role for the miRNA machinery in mammalian virus-host interactions.

Other immune responses triggered by dsRNA

Vertebrate innate immune responses recognize a number of pathogen-associated molecular patterns. Viral dsRNA RIs induce interferon (IFN) synthesis [112]. Interferons can be separated into two major classes, type I and type II. The IFN-α/β superfamily are type I IFNs, while IFN-γ is the sole type II IFN [113]. Both classes of IFN are associated with antiviral activity. Although distinct from interferon type I and type II, IFN- (type III) encoded proteins are also associated with antiviral activity [56]. Induction of IFN is transcriptionally regulated by the binding of nuclear factor-κB (NF-κB) and activated interferon regulatory factors (IRFs) to IFN specific promoters [83]. Once transcribed, INF-β binds to the IFN-α/β receptor (IFNAR), triggering the activation of the Janus kinases (JAKs). The JAKs phosphorylate the signal transducers and activators of translation (STATs) activating a signaling cascade [114]. The JAK-STAT signaling cascade induces transcriptional activation of hundreds of IFN-stimulated genes (ISG), eliciting an antiviral state in the cell (Figure 1.3) [115].

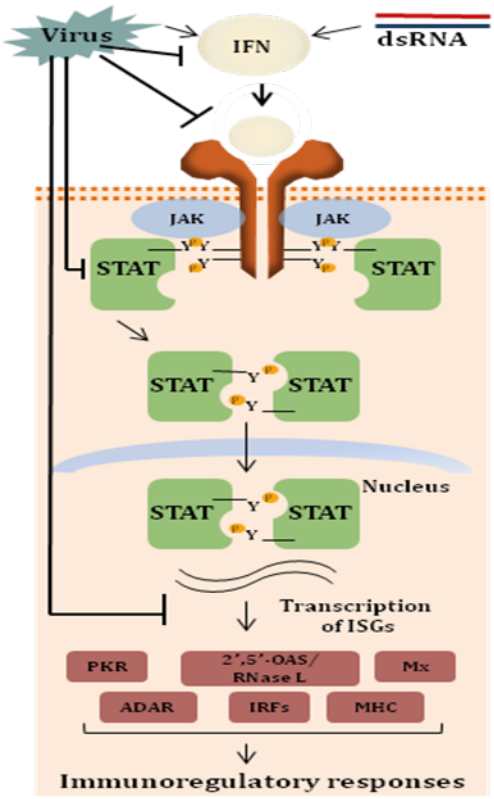

Figure 1.3. Interferon pathway. Modified from Katze *et al*. 2002. Interferon-β binds to the IFN-α/β receptor (IFNAR) triggering the activation of the Janus kinases (JAKs). The JAKs phosphorylate the signal transducers and activators of translation (STATs) activating a signaling cascade. The JAK-STAT signaling cascade induces the transcription of hundreds of antiviral IFN-stimulated genes (ISG), eliciting a cellular antiviral state.

While the Toll family receptors in *Drosophila* regulate anti-fungal and anti-bacterial immunity, the mammalian Toll-like receptor 3 (TLR-3) recognizes viral dsRNA [116]. TLR-3 is primarily associated with endosomes, an optimal location for the detection of viruses using endocytosis as a method of entry into the cell [117]. Activation of TLR-3 by dsRNA induces IFN-β [118]. Retinoic acid inducible gene I (RIG-I) is another receptor that is capable of recognizing dsRNA in mammalian cells [119]. RIG-I contains two N-terminal caspase recruitment domains (CARDs) and a C-terminal helicase domain. Upon dsRNA binding, conformational changes expose the N-terminal CARDs, which signals the activation of interferon regulatory factor 3 (IRF-3) and NF-κB [119]. RNAi-mediated studies confirm a functional role for RIG-1 in antiviral immunity. Cells depleted of RIG-1 were inhibited in their ability to induce type I IFNs in response to viral infection [119, 120]. Melanoma-differentiation-

associated gene 5 (MDA5) is structurally similar to RIG-I and also triggers an antiviral response by recognizing dsRNA [121]. Although both RIG-I and MDA5 induce an antiviral immune response in vertebrates, each receptor recognizes different RNA viruses [121]. RIG-I has been shown to produce IFNs in response to infection with paramyxovirus, influenza virus, and Japanese encephalitis virus, while MDA5 has been shown to be preferentially activated following infection with picornaviruses [121].

Viral dsRNA RIs can also induce many interferon stimulated genes (ISGs) directly, independent of IFNs [116]. Double-stranded RNA-activated protein kinase (PKR) and 2', 5' oligoadenylate synthetase (OAS) are two enzymes directly activated by dsRNA [122]. However, PKR and OAS use distinct mechanisms to inhibit virus replication. Activated PKR phosphorylates the translational initiation factor, eIF-2, resulting in inhibition of cellular protein synthesis [113]. Activation of the endonuclease, RNase L, requires synthesis of 2' 5' oligomers of adenosine by OAS (Figure 1.4) [123]. Unlike small RNA pathways, the OAS/RNAse L pathway results in a non-specific degradation of viral mRNA [124]. In addition to its role in antiviral activity, the OAS/RNase L pathway has recently been linked to antibacterial immunity [125]. The ISG15 gene and members of the ISG56 gene family are also strongly induced by dsRNA in human cells [116]. The human ISG56 gene family is comprised of four members ISG56, ISG54, ISG60, and ISG58 [116]. Products of the ISG56 gene family have been shown to bind to the translation initiation factor eIF-3, resulting in inhibition of protein synthesis [126]. The exact mechanism by which ISG15 functions in antiviral immunity remains elusive [116]. However, studies in mice demonstrated that ISG15 expressed from a chimeric SINV interfered with virus replication and conferred protection against SINV-induced cytopathology [127]. The ability of dsRNA to independently induce several ISGs provides redundancy to the activation of INF-mediated antiviral responses.

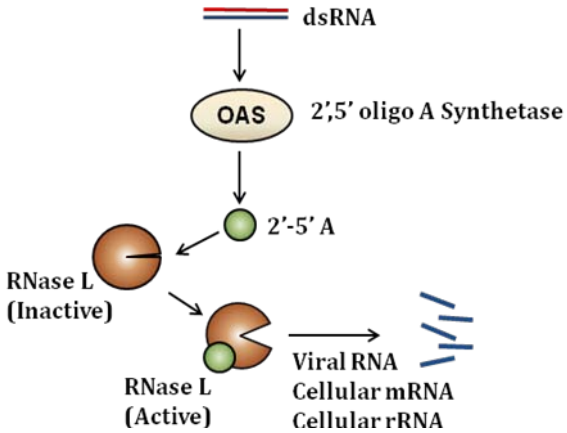

Figure 1.4. OAS/RNase L pathway. RNAse L responds to IFN and dsRNA directly. Activation of the endonuclease RNase L requires synthesis of 2' 5' oligomers of adenosine by OAS. The OAS/RNAse L pathway functions in regulating virus replication by degrading viral mRNA in a non-specific manner. In addition to its role in antiviral immunity, the OAS/RNase L pathway also degrades cellular mRNA and has been recently linked in antibacterial immunity.

Suppressors of RNA silencing

To counteract RNA-based immune responses, various plant and insect viruses have evolved to encode suppressors of RNA silencing (SRS). Viral suppressors differ greatly with respect to sequence, structure and mode of action. Two of the most well characterized viral suppressors are RNA-binding proteins that shield dsRNA produced during viral infection from Dicer processing and RISC assembly. The CymRSV P19 protein dimerizes to form a "molecular caliper" that measures the length of a dsRNA [128, 129]. A P19 dimer exhibits the greatest binding affinity for dsRNAs that are 21nt in length, with progressively weaker binding to shorter or longer dsRNAs [128, 129]. Thus, P19 specifically interferes with the incorporation of siRNA duplexes into the RISC [128, 129]. More recently, P19 was also shown to specifically induce expression of miR168 in CymRSV-infected plants, an activity that appears to be independent of the proteins ability to bind dsRNA [130]. Increased expression of miR168 was shown to negatively regulate AGO1 levels in the virus-infected plants, demonstrating that P19 has multiple suppressor functions [130]. The molecular mechanism by which the flock house virus (FHV) B2 protein mediates suppression is more general. Indiscriminate binding of dsRNA by the B2 protein interferes with the processing of long dsRNAs by Dcr-2 as well as incorporation of siRNA duplexes into the RISC [131-133]. Although dsRNA-binding is essential to the

suppressor activity of these two proteins, it is not yet clear if this is a general feature of most SRS proteins.

For example, the SRS encoded by Cricket paralysis virus (CrPV) does not bind dsRNA or siRNAs, rather the CrPV-1A protein appears to exert an inhibitory effect on RNA silencing through a direct interaction with Ago-2 [134]. Similarly, the P38 capsid and SRS protein encoded by Turnip crinckle virus (TCV) has been shown to directly interact with the *Arabidopsis* AGO1 [135]. In this case, P38 mimics cellular proteins containing glycine/tryptophane (GW/WG) residue repeats [135]. All previously characterized host-encoded GW/WG repeat proteins have been found to promote the assembly and/or action of RNA induced silencing complexes [136]. Viral mimicry of these proteins by P38 is thought to interfere with RISC assembly or function through a tight interaction with Argonaute proteins [135]. As the requirement for GW/WG-rich proteins in RISC assembly and function appears to be widespread in plants and animals, pathogen mimicry of these proteins might prove to be a broadly conserved strategy for modulating RNA-based host immune responses. Indeed, GW/WG motifs have now been identified in a number of plant and vertebrate viruses, some of which correspond to previously identified virulence factors [137]. Other suppressors that interact directly with RNA silencing components include the Cucumber mosaic virus (CMV) 2b protein, the polerovirus P0 protein, and the Cauliflower mosaic virus (CaMV) P6 protein [138].

Some mammalian viruses have also been shown to encode proteins capable of suppressing RNA silencing. However, for many of these proteins the suppressor function has been inferred from studies expressing the putative SRS as an isolated protein with a second reporter construct, an approach that may not accurately reflect the authentic function of the protein during viral infection [138]. Studies of individual viral proteins are unlikely to replicate the spatial and temporal levels of protein expression during an infection. Nor do such studies consider interactions with other viral proteins that might alter function. Finally, the role of the mammalian RNAi machinery in antiviral defense remains unresolved, leaving open the possibility that some of the proteins found to possess suppressor activity in mammalian viruses actually evolved to counter other antiviral responses induced by dsRNA. For example, although the LaCrosse virus (LACV) NSs protein has been shown to inhibit RNA silencing when expressed as an isolated protein, NSs-deficient mutants of LACV replicate as well as the wild-type virus in insect cells [139]. In contrast, NSs-deficient mutants of LACV exhibit growth

deficiencies in INF-competent MEF cells, but replicate to wild-type levels in MEF cells lacking the type I IFN receptor [140]. Similarly, the influenza virus NS1 protein and vaccinia virus E3L protein are known antagonists of PKR function in mammalian cells [141], but also have an inhibitory affect on RNA silencing in plant and insect cells when expressed as individual proteins [142, 143]. Viral SRS proteins likely arose in coevolution with host antiviral immunity and thus might not be present in viruses that do not infect organisms using RNAi as an antiviral defense.

Summary

Advances in our understanding of the RNAi pathway combined with the unprecedented availability of high-throughput sequencing technologies are enabling new investigations into the antiviral immune response of invertebrates. Evidence indicates that RNA silencing plays an important role in the transmission of mosquito-borne viruses [15]. Suppressing the accumulation of viral siRNAs by expressing the FHV B2 protein from recombinant alphaviruses dramatically alters the pathogenic outcome of the virus infection in a mosquito host, resulting in lethal infection [15]. As the potency of the suppressor proteins encoded by some insect viruses have been correlated with the virulence of natural infections, these results do not necessarily preclude the presence of SRS proteins in the genomes of mosquito-borne viruses, particularly those with suppressor activity less potent than that of the FHV B2. However, the results do suggest little evolutionary benefit for mosquito-borne viruses that encode proteins that strongly interfere with RNA silencing. Therefore, it remains unclear how mosquito-borne viruses modulate the vector's RNA-based immune response to establish productive infection. Conversely, an antiviral immune response that is too robust would ultimately result in a non-productive arbovirus infection. Thus, minor alterations in the balance between viral RNA replication and the mosquito's antiviral RNAi response may alter the ability of a mosquito vector to transmit an arbovirus in nature, resulting in either increased or decreased transmission (Figure 1.5). Understanding the specific virus-host interactions involved in maintaining this balance will help us to acquire a broader understanding of the complex transmission dynamics of mosquito-borne viruses in nature.

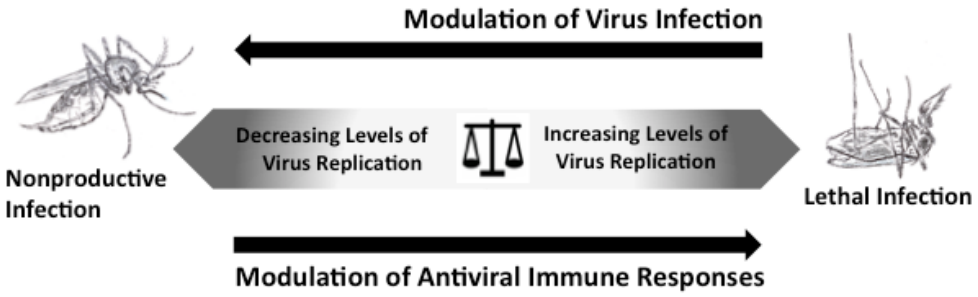

Figure 1.5. Factors influencing the transmission of mosquito-borne viruses in nature. Minor alterations in the balance between viral RNA replication and the mosquito's antiviral RNAi response may alter the ability of mosquito vectors to transmit arboviruses in nature, thus, resulting in either increased or decreased transmission. While an antiviral RNAi response that is too robust may ultimately limit productive arbovirus infections, uncontrolled virus replication results in lethal infection.

CHAPTER 2

AN ANTIVIRAL IMMUNITY PATHWAY DIRECTED BY PING-PONG-DEPENDENT PIWI-INTERACTING RNAS IN THE MOSQUITO SOMA

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ABSTRACT

The natural maintenance cycles of many mosquito-borne pathogens require establishment of persistent non-lethal infections in the invertebrate host. The mechanism by which this occurs is not well understood, but we have previously shown that an antiviral response directed by small interfering RNAs (siRNAs) is important in modulating the pathogenesis of alphavirus infections in the mosquito. Here, we report that alphavirus infection also triggers an antiviral immune pathway directed by ping-pong-dependent PIWI-interacting RNAs (piRNAs) in the mosquito soma, which we demonstrate is capable of modulating the pathogenesis of virus infection in *dicer-2* null mutant mosquito cell lines defective in viral siRNA production. This work establishes a model in which multiple RNA-based immune pathways redundantly modulate alphavirus pathogenesis in mosquito vectors.

INTRODUCTION

In plants and invertebrate animals the double stranded RNA (dsRNA) formed during the replication of RNA viruses is a potent inducer of an antiviral immune response directed by short interfering RNAs (siRNAs) [16]. In flies, exogenous dsRNA is processed by the RNase III enzyme Dicer 2 (Dcr-2) [21]. The resulting siRNA duplexes (~21 nt in length) are then directed into an RNA-induced silencing complex (RISC) [144]. These duplexes contain a guide strand that will provide sequence specificity to the RISC, and a passenger strand that will be removed from the activated RISC. The passenger strand is cleaved by Argonaute 2 (Ago-2), an essential component of the RISC with "slicer" activity [30, 145, 146]. The cleavage products are then removed from the RISC by another endonuclease, C3PO [147]. The remaining guide strand directs the activated RISC to cognate RNAs in the cell resulting in Ago-2-mediated cleavage and sequence specific degradation of the target molecules.

Although other small RNA pathways have not been ascribed an essential antiviral function, the recent identification of virus-derived PIWI-interacting RNAs (piRNAs; 24-30 nt in length) from a *Drosophila* ovary somatic sheet (OSS) cell line suggests an antiviral role for this pathway in the fly ovary [69]. Eukaryotic small RNAs can be distinguished from other non-coding RNAs in the cell by their associations with proteins in the Argonaute (Ago) -family. A Piwi-clade is formed by the Ago proteins Piwi, Aubergine (Aub) and Ago-3. Antisense piRNAs have been found to associate with Piwi and Aub, while sense piRNAs associate with Ago-3 [34, 35]. Mutations in Piwi-clade Ago proteins have previously been shown to increase expression of transposable elements in the fly ovaries [32, 148]. However, no clear role for this pathway has been established outside of the germline. Argonaute 3 and Aub do not appear to be expressed in the *Drosophila* soma [34, 35, 149-151]. While a simplified, alternate version (Piwi-dependent, but Aub- and Ago-3-independent) of the piRNA pathway, called the primary pathway, has been demonstrated in the somatic cells that ensheath the ovary [148, 152], it remains unclear if piRNA pathways operate more broadly in the fly soma. Addressing this question has been complicated by the demonstration that piRNAs play a role in epigenetic repression of transposable elements [153, 154]. A class of small RNA sequences 24-27 nt in length have been mapped to transposons in the soma of Ago-2 mutant flies [155]. Silencing tandem arrays of a *white* transgene in the eyes of wild type flies has also been shown to require functional Piwi and Aub [156]. However, it is

not clear if these represent examples of somatic piRNA production or maternal inheritance of piRNA populations.

Evidence suggests that Dicer is not involved in the production of piRNAs [32]. Most piRNAs are derived from a few genomic loci known as piRNA clusters and tend to be asymmetrical mapping to a single genomic strand in the piRNA cluster [34, 157, 158]. In contrast to the siRNA duplexes generated by Dicer, complementarity between sense and antisense piRNAs is generally limited to 10 nt at the 5' ends [34, 35]. This has led to the 'ping-pong' model of piRNA biogenesis [34, 35]. Many aspects of this model remain theoretical, having been deduced from small RNA profiling studies [34, 35]. In this model, piRNAs derived from a specific strand direct the production of piRNAs from the opposite strand. The cleavage of target strands directed by piRNAs bound to Ago-3 determines the 5' ends of the piRNAs bound by Piwi and Aub, and vice versa [35, 36]. Evidence for this comes from a characteristic U-bias at the 5' end of piRNAs bound by Piwi and Aub that correlates with enrichment for an adenine at the 10^{th} position of the piRNAs bound by Ago-3 [34, 35]. While the factors responsible for processing the primary piRNAs that initiate ping-pong amplification cycles are unknown, as is the location of processing (nucleus or cytoplasm), precursor substrates are believed to be derived from long single-stranded RNAs transcribed from repetitive elements or piRNA clusters [31, 33, 157]. Evidence for this comes from experiments in which a P-element insertion in the *flamenco* piRNA cluster was shown to disrupt the production of piRNAs more than 160 kb downstream of the insertion site [34].

Many mosquito-borne viruses are associated with human and animal diseases. Most of these viruses have RNA genomes and are classified in one of three genera: *alphavirus*, *flavivirus* or *bunyavirus*. Although virus infections of the vertebrate host are acute and often associated with disease, the maintenance of these pathogens in nature generally requires the establishment of a persistent non-lethal infection in the insect host. The mechanism by which this occurs is not well understood, but we have previously shown that an antiviral response directed by siRNAs is important to the pathogenic outcome of alphavirus infections in the mosquito [15]. Expression of the heterologous flock house virus (FHV) B2 protein from a recombinant alphavirus increases virus replication in infected mosquitoes resulting in dramatic mortality [15]. The FHV B2 is a well characterized dsRNA-binding protein that interferes with the access of siRNA pathway protein components to the dsRNA-by-products of virus replication [131, 133, 159]. The

pathogenesis associated with infection of mosquitoes with recombinant alphaviruses expressing B2 proteins is similar to an enhanced disease phenotype that has been described in *dcr-2* null mutant flies infected with RNA viruses [15, 48-50]. However, increased mortality is not observed when culicine mosquitoes in which Dcr-2 levels have been depleted with dsRNA are infected with alphaviruses [54, 160]. This led us to hypothesize that culicine mosquitoes posses a Dcr-2-independent antiviral pathway susceptible to B2-mediated interference. To investigate this we undertook a comprehensive survey of virus-derived small RNAs in culicine mosquitoes and cells infected with chikungunya virus (CHIKV; Genus: *Alphavirus*). Our results indicate the presence of an antiviral immune pathway directed by ping-pong-dependent piRNAs in the soma of culicine mosquitoes. We also demonstrate modulation of alphavirus infections in cell lines with defective siRNA production by an antiviral piRNA pathway. Collectively, our results support a model in which there is functional-redundancy in RNA-based immune pathways modulating alphavirus pathogenesis in the soma of disease vector mosquitoes.

MATERIALS AND METHODS

Cell culture. We cultured C6/36, u4.4, and CCL-125 cells in DMEM supplemented with FBS. C7-10 cells were cultured as described in [161]. Aag2 cells were cultured in Schneider's Drosophila medium (Lonza) supplemented with FBS. We cultured all cells at 28°C and 5% CO_2.

Recombinant virus production and infections. We created p3'dsCHIK by inserting a duplicate subgenomic promoter and multiple cloning site (MCS) immediately downstream of the E1 structural protein coding sequence of pCHIKic. Heterologous sequences were inserted into the MCS of p3'dsCHIK. Recombinant viruses were rescued as described [15]. One to two-day-old *A. aegypti* (Liverpool) and *A. albopictus* (Wise) were injected in the thorax with 10^4 pfu of virus. Three to five-day-old *D. melanogaster* (Oregon-R) were injected in the thorax with 10^4 pfu of virus using a Nanoject II (Drummond Scientific). Cells were infected at a multiplicity of infection (MOI) of 0.05.

RNA isolation and detection. We extracted RNA with Tri Reagent RT (Molecular Research Center). We performed northern blotting with a DNA fragment corresponding to the CHIKV 3' UTR that we ^{32}P-labeled using the Amersham Megaprime™ DNA Labeling System (GE Healthcare). We analyzed CHIKV (+) strand RNA levels using strand-specific quantitative real-time PCR (ssqPCR) and a Taqman assay (Life Technologies) as described [162].

Small RNA library preparation, deep sequencing and analysis. We prepared libraries from total RNA isolated from cultured mosquito cells, and adult female mosquitoes and flies four days after infection with CHIKV using Illumina's small RNA sample prep kit according to the manufacturer's instructions, with the exception that we recovered small RNAs that were 18 to 35-nt in size by PAGE. We sequenced libraries using the Illumina GAII. We analyzed sequence data sets as described [15]. Briefly, reads were stripped of adaptor sequences and mapped to the CHIKV genome (strain 37997) using parameters that did not permit mismatches. The abundance of small RNAs mapping to the CHIKV genome in each library were normalized and compared by expressing as a percentage of the total reads in the same size class, except when expressed relative to viral mRNA copy numbers. In which case, viral small RNAs were normalized by the

total number of 18-30nt reads, excluding non-coding RNAs such as rRNAs, tRNAs and snRNAs, in the smallest sequence set. A detailed analysis of the small RNAs sequenced from each library is found in (Appendix A Table 2.1). We generated Weblogo 3 (http://weblogo.threeplusone.com/) plots to analyze the frequency of nucleotide usage in small RNA reads of an identical size class.

Cloning, sequencing and analysis. We synthesized cDNA from total RNA isolated from cultured mosquito cells and adult mosquitoes using RT-PCR and an oligo-dT primer. We amplified the cDNA products of the RT-PCR reactions using PCR and *dcr-2* specific primers. Overlapping amplicons were assembled into complete clones of the *dcr-2* mRNAs. We generated a consensus for the transcripts in each sample by comparing the sequences of three complete *dcr-2* clones. Consensus sequences for the *A. albopictus* and C6/36 *dcr-2* transcripts were further augmented with deep sequencing reads generated from 454 and mRNA-Seq whole transcriptome analysis. After combining reads from all sources the *A. albopictus* and C6/36 *dcr-2* transcripts were sequenced at minimum 10x coverage. We genotyped regions of C6/36 and C7-10 genomic DNA suspected of containing indels using PCR and *dcr-2* specific primers. The homozygosity of the deletions was confirmed by comparing the sequences in 24 clones (12 per cell line) derived from the amplified DNA products. We performed 5' and 3' RACE on *dcr-2* transcripts from *A. albopictus* and C6/36 cells using the First Choice RLM-RACE kit (Ambion) according to the manufacturer's instructions. We verified 5' and 3' ends using the sequences of the cloned RACE products. Sequencing of the *A. albopictus dcr-2* RACE products revealed two different mRNA isoforms with alternative 5' UTR sequences. We identified and compared protein domains in Dcr-2 sequences using the Pfam protein database [163] (e-value of $1x10^{-9}$).

Accession numbers. Dicer-2 sequences have been deposited with NCBI under the following accession numbers: JF819820, JF819821, JF819822, JF819823, JF819824.

RESULTS

Production of virus-derived ping-pong-dependent piRNAs in the mosquito soma

To investigate the small RNA populations present in *Aedes aegypti* and *Aedes albopictus* mosquitoes infected with CHIKV, we prepared cDNA libraries from the ~18-35-nt fraction of total RNA and sequenced on an Illumina GA-II platform. A detailed analysis of the sequenced virus-derived small RNAs in each library is presented in Table 2.1. As expected, viral siRNAs were the predominant small RNA species perfectly matching the CHIKV genome in both mosquitoes (Figure 2.1A and B). In the infected *A. aegypti* ~10% of the total 21-nt small RNAs were derived from the virus, while in the infected *A. albopictus* ~23% of the total 21-nt small RNAs were of viral origin (Figure 2.1A and B). In each of the infected mosquito species virus replication positively correlated with the accumulation of viral siRNAs, with higher levels of viral RNA resulting in more abundant viral siRNAs (Figure 2.2A). Despite the observed differences in virus replication, the production of virus was similar in both mosquito species suggesting that the greater accumulation of viral siRNAs in *A. albopictus* resulted in more robust modulation of the virus infection (Figure 2.2B).

The first indication that the infected mosquito species also produced an abundant class of viral piRNAs were broad peaks in the size distributions of 24-30-nt reads mapping to the virus in the respective small RNA libraries (Figure 2.1A and B). Imbalanced synthesis of (+) and (-) strands is a common trait among RNA viruses, with genomic strands typically produced in greater abundance than their counter parts. The genomic (+) strand (49S) RNA of an alphavirus serves both as mRNA and as a template for the synthesis of complementary (-) strand RNA. Viral (-) strands then serve as templates for the synthesis of new genomic-length (+) strand RNAs, as well as for shorter subgenomic (+) strand (26S) RNAs that encode the viruses structural genes. We have previously shown that alphavirus-derived siRNAs in infected mosquitoes do not cluster from any particular region of the virus genome and are generated from (+) and (-) strands in proportions that suggest the primary Dcr-2 substrates for siRNA biogenesis are the viral dsRNA replicative intermediates (RIs) formed during replication [15, 65]. In contrast, 23-30-nt viral piRNAs in each of the infected mosquito species clustered near the 5′ termini of the genomic and subgenomic (+) strands, and consistent with previous descriptions of asymmetry in piRNAs derived from transposable elements (TEs), were almost exclusively

derived from virus (+) strands (Figure 2.1A and B). Overall, these results suggest that the biogenesis of viral piRNAs is occurring primarily from the 49S and 26S virus mRNAs (Figure 2.1A and B).

Distinct piRNA pathways operate in the germline and somatic compartments of the *Drosophila* ovary [148, 152]. In the germline, piRNAs tend to exhibit a 5' uridine and an adenine at the 10^{th} nt position, suggesting secondary amplification by a ping-pong-dependent mechanism [34, 35]. In somatic ovarian cells, piRNAs also exhibit a 5' U bias but without a predominant nucleotide at the 10^{th} nt position, suggesting biogenesis by a ping-pong-independent primary pathway [148, 151, 152]. Previous descriptions of viral piRNAs in *Drosophila* OSS cells [69] indicate a strong preference for a 5' U but no bias at the 10^{th} nt position, suggesting these are products of a primary pathway [148, 151, 152]. The viral piRNAs indentified in this study exhibit a strong 5' U and A10 signature suggesting they are products of a ping-pong-dependent pathway (Figure 2.1A and B). However, as we prepared our cDNA libraries from whole mosquitoes the source (germline or soma) of viral piRNA production was unclear. To determine the source of viral piRNA production, we sequenced small RNA populations in *A. albopictus* infected with CHIKV from which the ovaries had been removed. The 5-fold enrichment of viral piRNAs exhibiting a 5' U and A10 signature that was observed in these samples suggests the presence of a ping-pong-dependant antiviral piRNA pathway in the mosquito soma (Figure 2.1B and C).

Despite recent progress in the application of next-generation sequencing, studying small RNA pathways in non-model organisms presents many challenges. To assess the feasibility of employing a fruit fly model to study the biogenesis and effector mechanisms of viral piRNAs, we sequenced small RNAs from *Drosophila melanogaster* infected with CHIKV. In contrast to the two mosquito species used here, we were unable to obtain evidence for the accumulation of viral piRNAs in infected fruit flies (Figure 2.2 C). The ovaries of the mosquito have been shown to be largely refractory to alphavirus infection [164], and production of TE-derived piRNAs has not been demonstrated in somatic cells outside of the *Drosophila* ovaries. Therefore, it is possible that CHIKV tissue tropisms preclude robust generation of viral piRNAs in the fruit fly. Alternatively, detection may have been hindered by appreciable differences in the kinetics of virus replication in mosquitoes and fruit flies, respectively (Figure 2.2A and B). Regardless of

the explanation, an apparent lack of viral piRNA production in *D. melanogaster* demonstrates the limited feasibility of using a fruit fly model to study mosquito antiviral piRNA pathways.

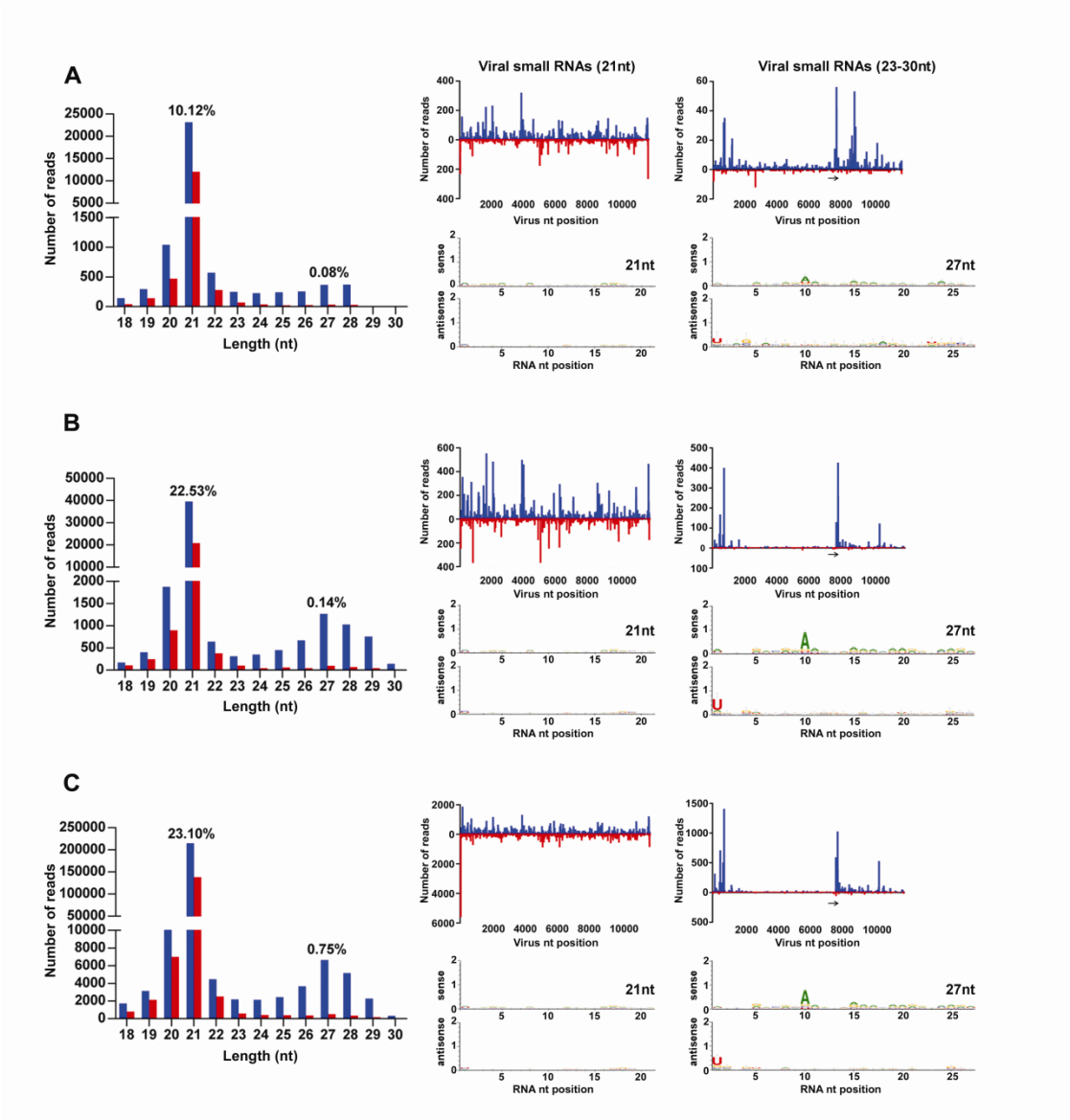

Figure 2.1. Expression of viral piRNAs in the mosquito soma. (A) Size distribution, density plots, and nucleotide analysis of virus-derived small RNAs in *A. aegypti*, (B) *A. albopictus* and (C) head and thorax of *A. albopictus* infected with CHIKV. Blue bars indicate small RNAs derived from virus (+) strands and red bars indicate small RNAs derived from virus (-) strands. Normalized percentages of specific viral small RNA size classes are indicated above. Arrows denote approximate start of 26S mRNA.

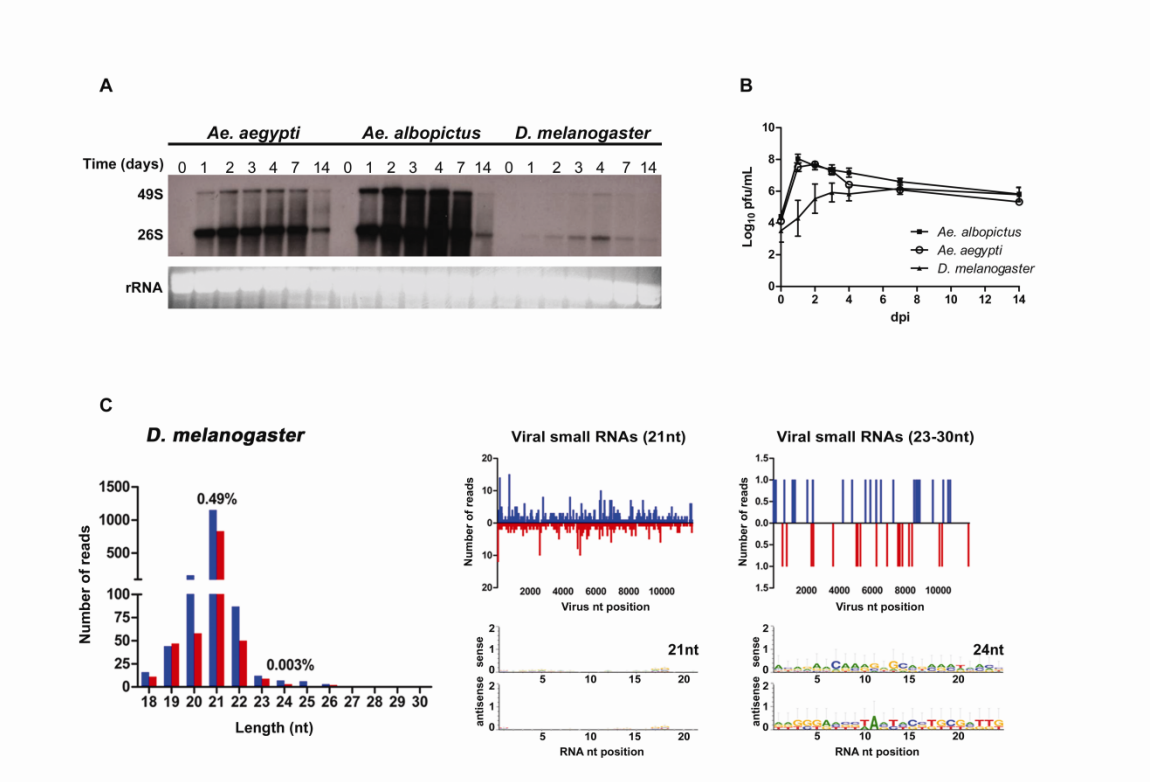

Figure 2.2. Replication of CHIKV in mosquitoes and fruit flies. (A) Northern blot detection 49S genomic and 26S subgenomic viral RNA in mosquitoes and flies injected with CHIKV. (B) Virus accumulation in mosquitoes and flies injected with CHIKV. (C) Size distribution, density plots, and nucleotide analysis of virus-derived small RNAs in *D. melanogaster* infected with CHIKV. Blue bars indicate small RNAs derived from virus (+) strands and red bars indicate small RNAs derived from virus (-) strands. Normalized percentages of specific viral small RNA size classes are indicated above.

Increased production of virus-derived piRNAs during pathogenic virus infections

We next examined the production of viral piRNAs during pathogenic virus infections of *A. albopictus.* Mosquitoes were infected with a recombinant CHIKV expressing either the Nodamura virus (NoV) B2 or FHV B2 under the control of a second subgenomic promoter. A double subgenomic CHIKV containing an untranslatable NoV B2 (ΔB2; $B2^{-}B1^{-}$) that has been described previously [165] served as a control virus. As expected, expression of the NoV or FHV B2 by CHIKV decreased production of viral siRNAs in comparison with levels present in mosquitoes infected with the control virus (Figure 2.3A, B and C). However, 21-nt viral siRNAs were more abundant in mosquitoes infected with CHIKV-B2 (FHV) than in those infected with CHIKV-B2 (NoV), suggesting that FHV B2 is a less effective suppressor of RNA silencing than is NoV B2 (Figure 2.3B and C). Survival curves for mosquitoes infected with CHIKV- ΔB2

were indistinguishable from those of mock infected mosquitoes (Figure 2.3D). Although infection with CHIKV-B2 (FHV) increased mortality, the weaker suppressor activity of FHV B2 was associated with a less virulent infection than was expression of NoV B2 by the virus (Figure 2.3D). Analysis of viral piRNAs revealed increased production in mosquitoes infected with recombinant viruses expressing B2 proteins when compared to levels in mosquitoes infected with the control virus (Figure 2.3A, B and C). Notably, the highest levels of viral piRNA production were observed in mosquitoes infected with CHIKV-B2 (NoV) suggesting an inverse relationship between the abundance of viral siRNAs and viral piRNAs (Figure 2.3A, B and C).

To examine the relationship between virus replication and the production of virus-derived small RNAs we performed strand-specific quantitative real-time PCR (ssqPCR) analysis on the total RNA used for small RNA library preparation (Figure 2.4). In the presence of B2-mediated interference, the accumulation of viral siRNAs negatively correlated with virus replication, with decreased production of viral siRNAs resulting in higher levels of viral mRNA (Figure 2.3A, B, C and 2.4). Next, we used our ssqPCR results to determine the average number of virus (+) strands per virus-derived small RNA in the mosquitoes infected with recombinant viruses. As expected, infection with recombinant viruses expressing B2 proteins was associated with an increased number of virus (+) strands per 21-nt viral siRNA when compared to mosquitoes infected with the control virus (Figure 2.3E). The magnitude of the increase correlated with the effectiveness of the B2 protein, with the more pathogenic CHIKV-B2 (NoV) having the highest number of virus (+) strands per viral siRNA (Figure 2.3E). In contrast with the accumulation of viral siRNAs in infected mosquitoes, the abundance of viral piRNAs positively correlated with expression of viral mRNA, with higher levels of viral mRNA resulting in increased production of viral piRNAs (Figure 2.3A, B, C and 2.4). However, similar to the antiviral siRNA pathway, infection with recombinant viruses expressing B2 proteins was associated with an increased number of virus (+) strands per 27- or 28-nt viral piRNA when compared to mosquitoes infected with the control virus, suggesting that in the presence of the dsRNA-binding proteins production of viral piRNAs do not rise to levels sufficient to modulate pathogenesis (Figure 2.3F). Thus, these results are consistent with our hypothesis of a Dcr-2-independent antiviral pathway susceptible to B2-mediated interference.

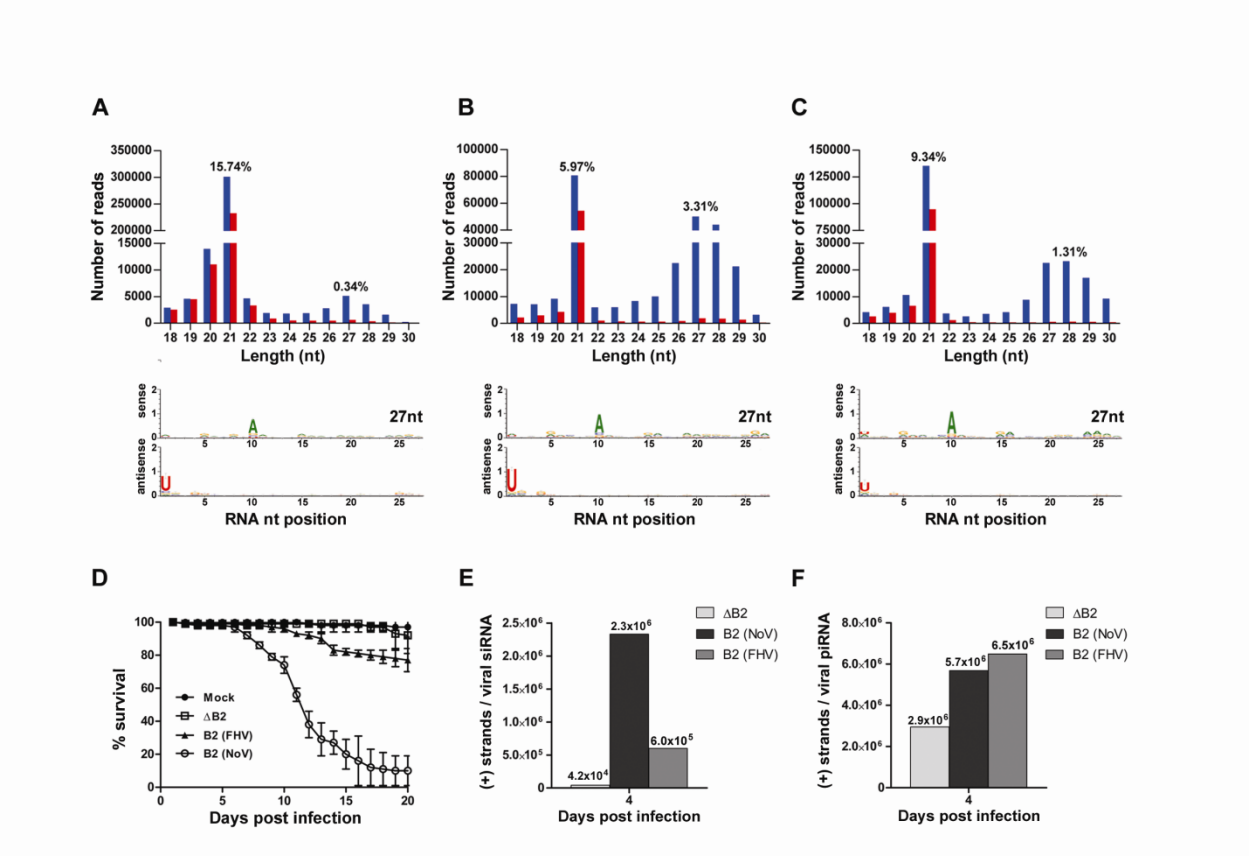

Figure 2.3 Expression of viral piRNAs increases during pathogenic virus infection. (A) Size distribution and nucleotide analysis of virus-derived small RNAs in the head and thorax of *A. albopictus* infected with CHIKV-ΔB2, (B) CHIKV-B2 (NoV), or (C) CHIKV-B2 (FHV). Blue bars indicate small RNAs derived from virus (+) strands and red bars indicate small RNAs derived from virus (-) strands. Normalized percentages of specific viral small RNA size classes are indicated above. (D) Survival of *A. albopictus* after infection with recombinant CHIK viruses and mock injection. Error bars indicate the standard deviation among triplicate cohorts (n = 90). (E and F) Strand-specific quantitative real-time PCR analysis of CHIKV (+) strands in total RNA used for small RNA library preparation; plotted as the number of copies per virus-derived small RNA..

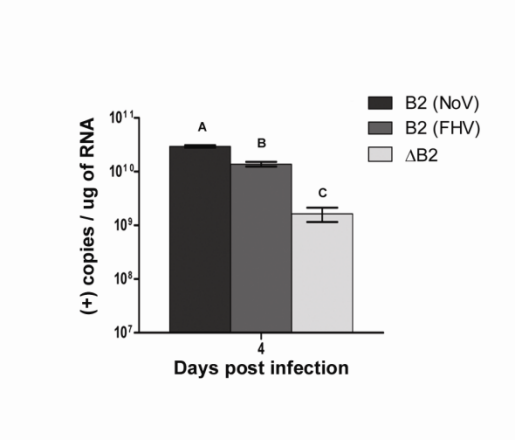

Figure 2.4. Expression of viral mRNA in the mosquito soma. Strand-specific quantitative real-time PCR analysis of CHIKV (+) strands in total RNA used for small RNA library preparation. Levels not connected by same letter are significantly different ($P < 0.0001$).

Identification of *dcr-2* null mutant mosquito cell lines

We sequenced and analyzed small RNA populations in three *A. albopictus* (C6/36, u4.4, and C7-10) and two *A. aegypti* (Aag-2, and CCL-125) continuous cell lines infected with CHIKV. Aag-2 cells originate from a cell line derived from embryos [166]. CCL-125, C6/36, u4.4, and C7-10 cells all originate from cell lines prepared from homogenized mosquito larvae [167-170]. The CCL-125 and Aag-2 cell lines are non-clonal populations and likely represent an amalgamation of various embryonic or larval tissue types [166, 170]. The C6/36, u4.4, and C7-10 cell lines are subclones derived from an original larval cell line, but the specific tissues from which they were generated are unknown [168, 169, 171].

We identified 21-nt small RNAs derived from CHIKV in each mosquito cell line (Figure 2.5A, B, C and S3). However, this size class was much less prominent in the libraries prepared from C6/36 and C7-10 cells than in those prepared from u4.4, Aag2 and CCL-125 cells (Figure 2.5A, B, C and 2.6). Just as we had observed in adult mosquitoes, a prominent class of 23-30-nt small RNAs derived from the virus was evident in each cell line (Figure 2.5A, B, C and 2.6). The clustering and asymmetry of these small RNAs suggests that each of the mosquito cell lines used in this study has an antiviral piRNA pathway. Given the indeterminate origin of these cell lines, it is possible that some of the mosquito cells are analogous to a *Drosophila* OSS cell line from which viral piRNAs have been previously described [69]. However, viral piRNAs identified in each of the mosquito cell lines exhibited strong 5' U and A10 signatures (Figure 2.5A, B, C and 2.6), suggesting they are products of a ping-pong-dependent pathway rather than the primary products described in OSS cells [148, 151, 152].

In contrast to other cell lines used in this study, the 24-30-nt viral piRNAs were the predominant small RNAs derived from CHIKV in C6/36 and C7-10 cells. Analysis of 21-nt reads mapping to the virus in C6/36 and C7-10 cells revealed clustering, asymmetry, and a ping-pong signature not present in same size reads cloned from any other cell line, suggesting that the 21-nt small RNAs derived from the virus in these cells are a subset of viral piRNAs (Figure 2.5A, B, C and 2.6). To confirm that siRNA processing was defective in the C7-10 and C6/36 cell lines we sequenced the complete *A. albopictus,* u4.4, C6/36 and C7-10 *dcr*-2 open reading frames (ORFs). The *A. albopictus* Dcr-2 contains a DExH-box domain, a domain of unknown function (DUF), a PAZ domain, and two RNase III domains (Figure 2.5D). The u4.4 Dcr-2

differed with the *A. albopictus* sequence at two conservative amino acid substitutions. In contrast, the C7-10 Dcr-2 contained an in-frame deletion of 33 amino acids (del 33) between the DUF and PAZ domains (Figure 2.5D). Consistent with a *dcr-2* null phenotype, the deletion was determined to be homozygous by genotyping the region of genomic DNA containing the indel identified in the C7-10 *dcr-2* ORF. Although the 33 amino acid deletion does not occur within an identifiable domain structure, it may prevent the protein from assuming a functional three-dimensional configuration. Genotyping a region of C6/36 genomic DNA containing a single nucleotide deletion identified in the C6/36 *dcr-2* ORF revealed a homozygous frameshift mutation (FS −1) that results in a premature termination codon. The 821 amino acid protein that is predicted to be translated lacks a portion of the PAZ domain as well as both RNAse III domains, which are consistent with a *dcr-2* null phenotype (Figure 2.5D). Together, these data indicate that homozygous deletions identified in the *dcr-2* alleles of the C7-10 and C6/36 cell lines are null mutations that abrogate viral siRNA production. (Additional details regarding the characterization of the continuous cell lines used in this study are provided in supplementary text.)

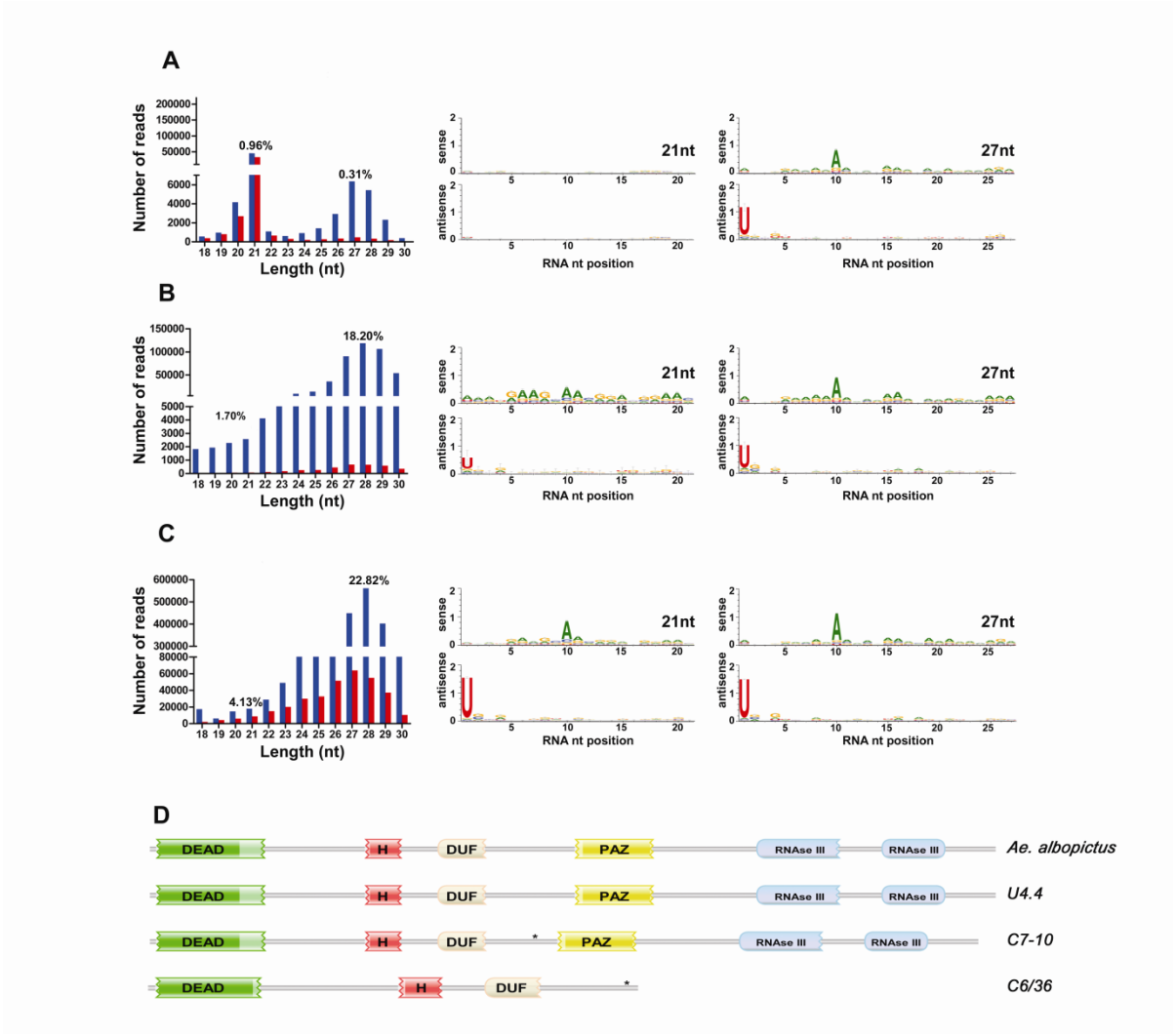

Figure 2.5. Identification of *dcr-2* null mutant mosquito cell lines. (A) Size distribution and nucleotide analysis of virus-derived small RNAs in u4.4 cells, (B) *dcr-2*$^{FS-1}$ (C6/36) cells and (C) *dcr-2*$^{del\ 33}$ (C7-10) cells infected with CHIKV. Blue bars indicate small RNAs derived from virus (+) strands and red bars indicate small RNAs derived from virus (-) strands. Normalized percentages of specific viral small RNA size classes are indicated above. (D) Schematics indicating Dcr-2 domains. The *A. albopictus* Dcr-2 contains a DExH/D protein family domain (DEAD) and helicase conserved C-terminal domain (H); a domain of unknown function (DUF); a PAZ domain; and tandem RNase III domains. Asterisks indicate locations of deletions in *dcr-2* sequences.

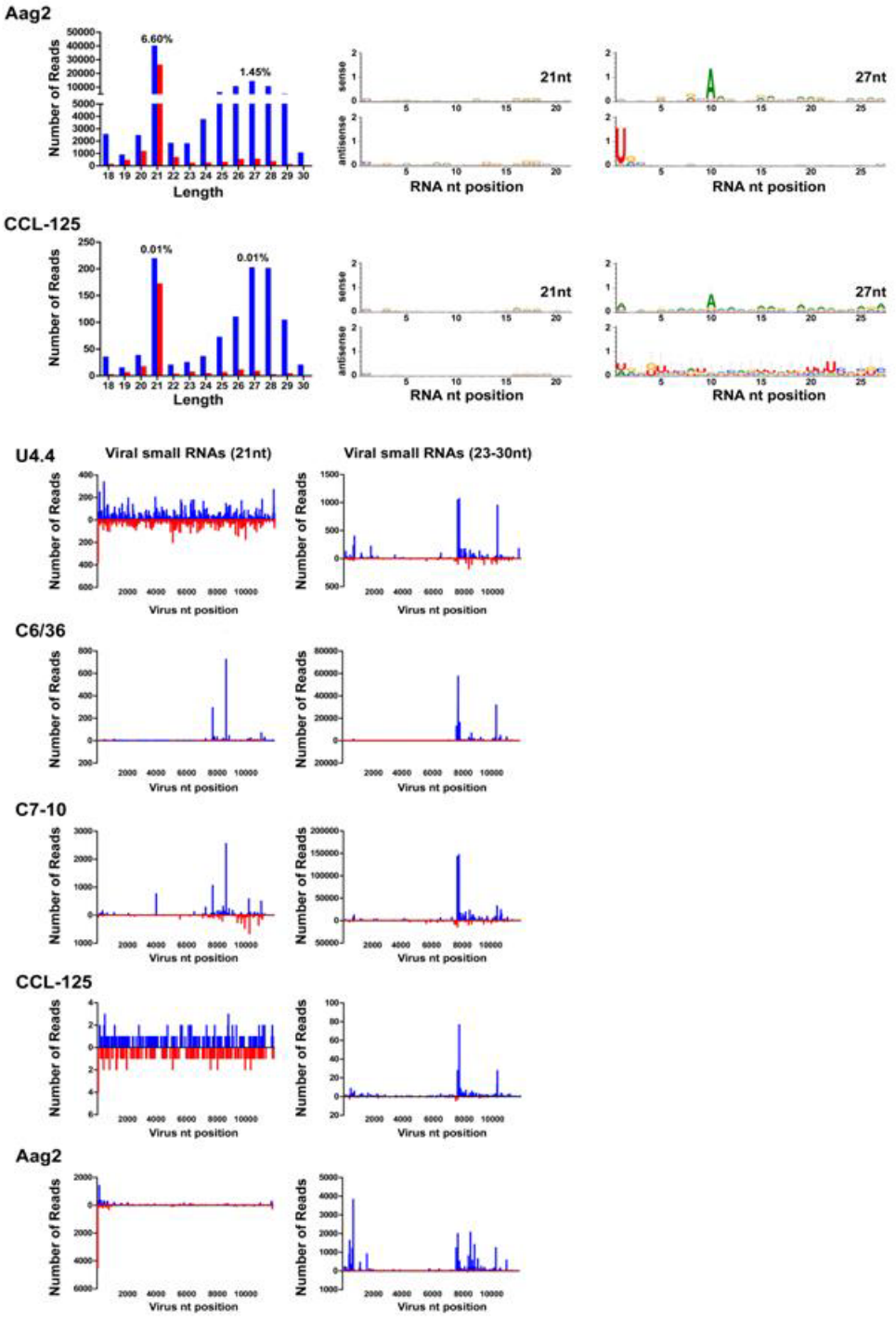

Figure 2.6. Expression of virus-derived small RNAs in continuous mosquito cell lines. Size distribution, density plots, and nucleotide analysis of virus-derived small RNAs in mosquito cell lines infected with CHIKV. Blue bars indicate small RNAs derived from virus (+) strands and red bars indicate small RNAs derived from virus (-) strands. Normalized percentages of specific viral small RNA size classes are indicated above.

Virus-derived piRNAs direct an antiviral response that modulates alphavirus pathogenesis in *dcr-2* null mutant mosquito cells

We have previously demonstrated severe cytopathology in C6/36 cells infected with a recombinant Sindbis virus (SINV; the prototype alphavirus) expressing the FHV B2 [15]. Similar cytopathological changes were not observed in cells infected with a recombinant SINV expressing a C44Y FHV B2 mutant protein or with a wild-type SINV [15]. The C44 residue of FHV B2 is important to the protein's suppressor activity due to its role in binding dsRNA [159]. Mutant C44A and C44S B2 proteins exhibit reduced dsRNA binding affinity [159], and the C44Y B2 is associated with decreased suppressor activity [15]. To determine if the antiviral piRNA pathway present in *dcr-2*$^{FS-1}$ (C6/36) cells modulates the pathogenesis of CHIKV, we infected *dcr-2*$^{FS-1}$ (C6/36) cells with recombinant viruses expressing either the FHV B2 or C44Y B2. In contrast to our previous results with SINV-B2 (C44Y), infection of *dcr-2*$^{FS-1}$ (C6/36) cells with CHIKV-B2 (C44Y) caused mild non-lethal cytopathology, from which the cells ultimately recovered (Figure 2.7D). Importantly, more severe cytopathological changes were observed during infection with CHIKV-B2 (FHV), demonstrating decreased immune modulation in these cells (Figure 2.7D). The enhanced pathogenicity of recombinant CHIKV expressing the wild type B2 protein correlated with decreased production of viral piRNAs in comparison to cells infected with CHIKV expressing the C44Y FHV B2, suggesting that the antiviral piRNA pathway in *dcr-2*$^{FS-1}$ (C6/36) cells is sensitive to FHV B2-mediated suppression (Figure 2.7A and B). This was confirmed by an increase in the number of virus (+) strands per 28-nt piRNA in cells infected with CHIKV-B2 (FHV) when compared to cells infected with CHIKV-B2 (C44Y) (Figure 2.7C). Consistent with the weaker suppressor activity of FHV B2, infection with CHIKV-B2 (FHV) did not result in complete destruction of the cell monolayer. Therefore, we infected *dcr-2*$^{FS-1}$ (C6/36) cells with recombinant CHIKV expressing the stronger NoV B2 suppressor. Recombinant virus containing the untranslatable ΔB2 served as a control. Although we again observed mild cytopathology in cells infected with CHIKV- ΔB2, infection with recombinant virus expressing the NoV B2 suppressor resulted in more extensive destruction of the cell monolayer (Figure 2.7D). Similar results were observed during infection of *dcr-2*$^{del\ 33}$ (C7-10) cells with CHIKV-B2 (NoV) and CHIKV- ΔB2 (Figure 2.8). Taken together, these data demonstrate modulation of alphavirus pathogenesis in cell lines with defective siRNA production by an antiviral piRNA pathway.

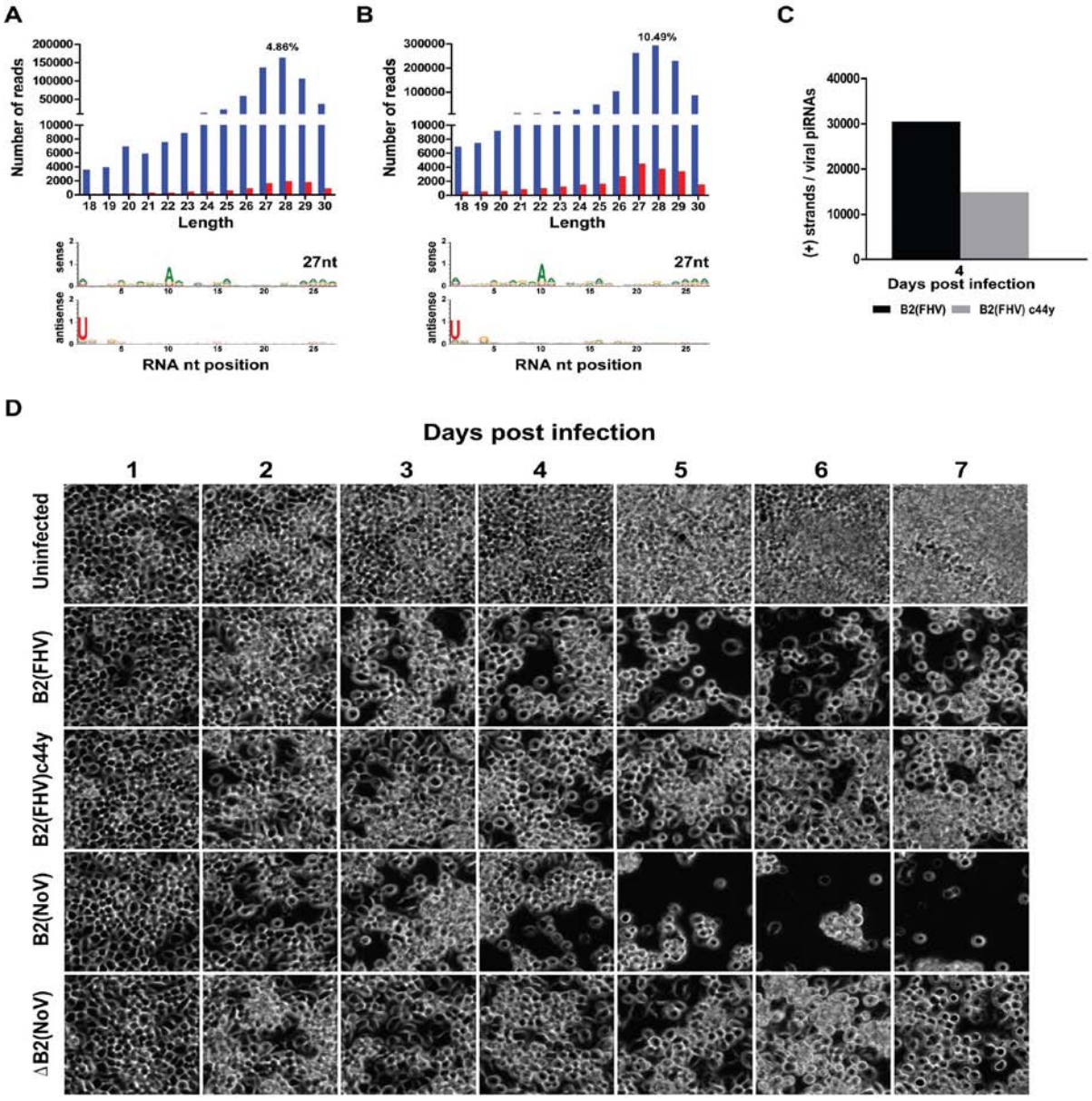

Figure 2.7. Suppression of the antiviral piRNA pathway in *dcr-2*$^{FS-1}$ cells by a dsRNA-binding protein. (A) Size distribution and nucleotide analysis of virus-derived small RNAs in *dcr-2*$^{FS-1}$ cells infected with CHIKV-B2 (FHV) or (B) CHIKV-B2 (C44Y). Blue bars indicate small RNAs derived from virus (+) strands and red bars indicate small RNAs derived from virus (-) strands. Normalized percentages of specific viral small RNA size classes are indicated above. (C) Strand-specific quantitative real-time PCR analysis of CHIKV (+) strands in total RNA used for small RNA library preparation; plotted as the number of copies per virus-derived small RNA. (D) Modulation of alphavirus infection by an antiviral piRNA pathway in *dcr-2*$^{FS-1}$ (C6/36) cells. Time course of cytopathology in *dcr-2*$^{FS-1}$ (C6/36) cells infected with recombinant CHIK viruses (20X magnification).

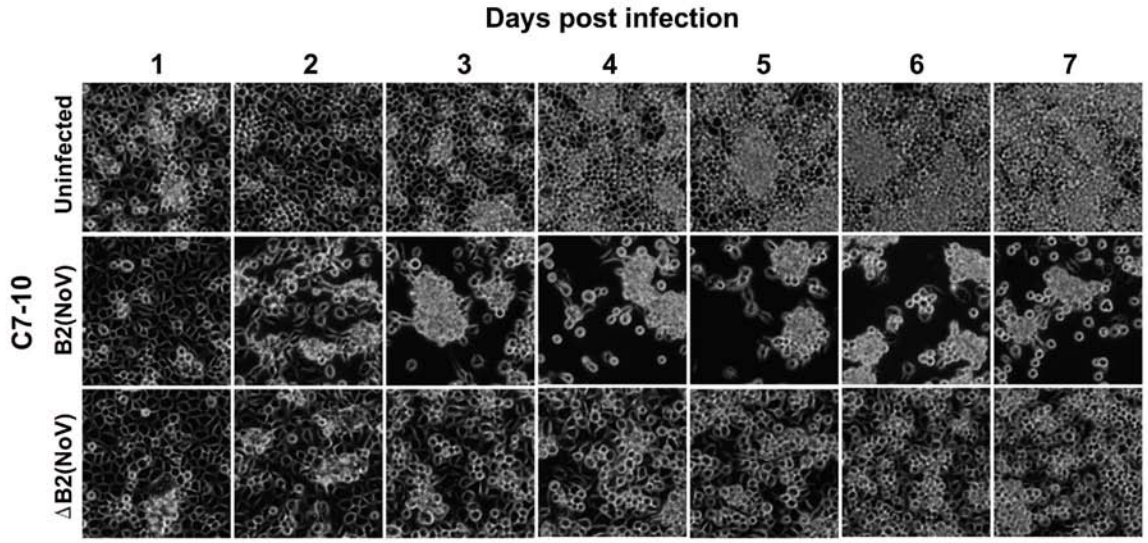

Figure 2.8 Modulation of alphavirus infection by an antiviral piRNA pathway in *dcr-2*$^{del\ 33}$ (C7-10) cells. Time course of cytopathology in *dcr-2*$^{del\ 33}$ (C7-10) cells infected with recombinant CHIK viruses (20X magnification).

SUPPLEMENTARY TEXT

Assembly of small RNAs to examine virus populations in cultured mosquito cell lines

The ability to assemble cloned viral siRNA sequences into long contiguous fragments has provided a novel approach to virus discovery by deep sequencing [69]. We utilized this approach to determine if cultured mosquito cells, adult mosquitoes, and adult flies had any established persistent viral infections. For assembly of small RNA reads we used the Velvet program [172] with a k-mer of 17 and a minimum contig length of 100 as described previously [69]. To identify virus-specific contigs we searched the non-redundant protein databases of the National Center for Biotechnology Information (NCBI) by BLASTX. Our results revealed mix infection of several mosquito cell lines by viruses of various genome types. Analysis of small RNA sequence contigs confirm a persistent infection of C6/36 and Aag2 cells by C6/36 densovirus and cell fusion agent virus, respectively [57, 173]. A large percentage of small RNA sequence contigs from u4.4 cells also exhibited partial identity with Drosophila X virus (DXV) suggesting these cells are persistently infected with a previously unidentified member of the *Birnaviridae*. Other than those of CHIKV, virus-specific contigs were not identified in the C7-10 and CCL-125 cell lines, or in *A. albopictus, A. aegypti* and *D. melanogaster*.

DISCUSSION

Antiviral pathways initiated by viral RNA have been shown in a diverse array of organisms to require amplification to mount an effective immune response. For example, effective RNA-based antiviral immunity in *C. elegans* requires amplification of the viral siRNAs generated from dsRNA RIs by an endogenous RNA-dependent RNA polymerase (RdRP) activity [16]. Similarly, some plants are thought to require RdRP-dependant production of secondary siRNAs in order to mount an effective antiviral immune response [63, 174]. However, RdRP-dependant production of secondary viral siRNAs has not been demonstrated in flies. In this study, we used massively parallel sequencing to survey small RNA populations in mosquitoes and cells from two different culicine species infected with CHIKV. In addition to 21-nt viral siRNAs, our analysis revealed widespread and abundant production of virus-derived piRNAs in mosquito soma outside the ovaries. Similar to other piRNAs that have been described previously, those in the mosquito exhibit strand asymmetry, in this case corresponding to virus (+) strands. However, in contrast to previous descriptions of viral piRNAs in an OSS cell line [69], those identified in the mosquito soma contain a preference for a 5' uridine and an adenine at nucleotide position 10 suggesting a ping-pong amplification mechanism.

We have previously shown that the pathogenic effects of alphavirus infection are modulated in the mosquito vector by an RNA silencing response directed by 21-nt siRNAs. However, we demonstrate here modulation of alphavirus pathogenesis in *dcr-2* null mutant cell lines unable to process dsRNA into siRNAs. Analysis of the small RNAs present in *dcr-2* null mutant cells infected with CHKV revealed piRNAs derived from the virus. Similar to viral piRNAs described in the mosquito soma, those in *dcr-2* null mutant cells exhibited strand asymmetry corresponding to virus (+) strands and signatures of ping-pong amplification. It has been proposed that TE-derived piRNAs are products of long, single-stranded RNA precursors transcribed from piRNA clusters [31, 33, 157]. Suppressing the accumulation of viral piRNAs in *dcr-2*$^{FS-1}$ (C6/36) cells infected with CHIKV expressing the FHV B2 protein resulted in elevated levels of viral RNA and severe cytopathology. In contrast, viral pathogenesis was effectively modulated in *dcr-2*$^{FS-1}$ (C6/36) cells infected with CHIKV expressing a C44Y B2 mutant. As the C44 residue in the FHV B2 has previously been shown to be important in binding dsRNA, these results suggest that dsRNA serves as the precursor substrate of primary viral piRNAs.

Imbalanced synthesis of (-) and (+) strands is a common feature of RNA viruses, typically with production of the genomic strand being greater than its full-length complement. Alphaviruses are thought to synthesize (-) strand RNAs for a limited duration of time early in the infection [66], presumably establishing an upper limit on the number of dsRNA RIs present in the cell. However, production of the (+) single-stranded genomic (49S) and subgenomic (26S) RNAs continues much longer. The 49S and 26S RNAs ultimately become the predominant virus-specific RNA species present in cells infected with an alphavirus [175]. The alphavirus 49S and 26S RNAs have 5' -m^7G cap structures and 3' -poly(A) tails and are completely dependent on the cellular machinery for translation [176]. Despite this similarity with cellular mRNAs, our results demonstrate that the piRNA-based immune response present in culicine mosquitoes predominately targets viral mRNAs. The innate immune systems of insects rely on ancient and well conserved signal transduction pathways to mount an effective response to microbial pathogens. The immune receptors that activate these pathways have evolved the ability to distinguish pathogen from self by recognizing a limited number of broadly conserved pathogen-associated molecular patterns, essential to the survival of the invading microbe but not found in the host. Pattern recognition molecules include lipopolysaccharides, teichoic acids, mannans, and viral dsRNA. It is not uncommon for some of these molecules to activate multiple signal transduction pathways in the infected host. Collectively, our findings support a model in which antiviral siRNA and piRNA biogenesis pathways compete for a limited number of precursor dsRNA RIs in the infected cell (Figure 2.9). While recognition of dsRNA activates both pathways, they diverge with the amplification of secondary piRNAs that are preferentially generated from viral mRNAs (Figure 2.9). The interdependence of these two RNA-based immune pathways on a common precursor substrate for the production of primary effector molecules also suggests a mechanism for the coordination of otherwise compartmentalized antiviral responses. Under conditions in which dsRNA is efficiently processed by Dcr-2, relatively small quantities of the precursor substrate may enter the piRNA biogenesis pathway, augmenting the primary antiviral response directed by viral siRNAs through ping-pong amplification. However, effective modulation of virus pathogenesis by an antiviral piRNA pathway in *dcr-2* null mutant cells unable to process dsRNA to siRNAs reveals that viral piRNAs are also capable of directing a robust immune response as viral dsRNA becomes more abundant. While the siRNA pathway appears to be the primary antiviral defense in mosquito

cells, the normally subordinate antiviral activity of the piRNA pathway comes to the forefront when Dcr-2 is inactivated. Our model has similarities with the hierarchical action of antiviral defenses demonstrated in some plants; however, in those cases functional-redundancy is provided by a duplication of Dicer-like proteins [73].

To counteract RNA silencing immune pathways the genomes of many plant and insect viruses encode proteins that suppress RNA silencing [88], suggesting that these virulence factors confer an evolutionary advantage to the pathogen. However, it remains unclear if mosquito-borne viruses, which also infect vertebrate hosts, encode similar proteins. The continual transmission of mosquito-borne viruses depends on the establishment of a non-lethal infection in the invertebrate host. This suggests that these viruses would derive little evolutionary benefit from encoding proteins that strongly suppress both arms of the mosquito's RNA-based antiviral immune response. Indeed, our studies with recombinant viruses expressing B2 proteins, which we have shown suppress both the antiviral siRNA and piRNA pathway, confirm this. Nevertheless, our work has also shown that the antiviral defenses of the mosquito are more complex than previously thought, which has important implications for understanding the evolutionary arms race between host and pathogen in these important vectors of disease.

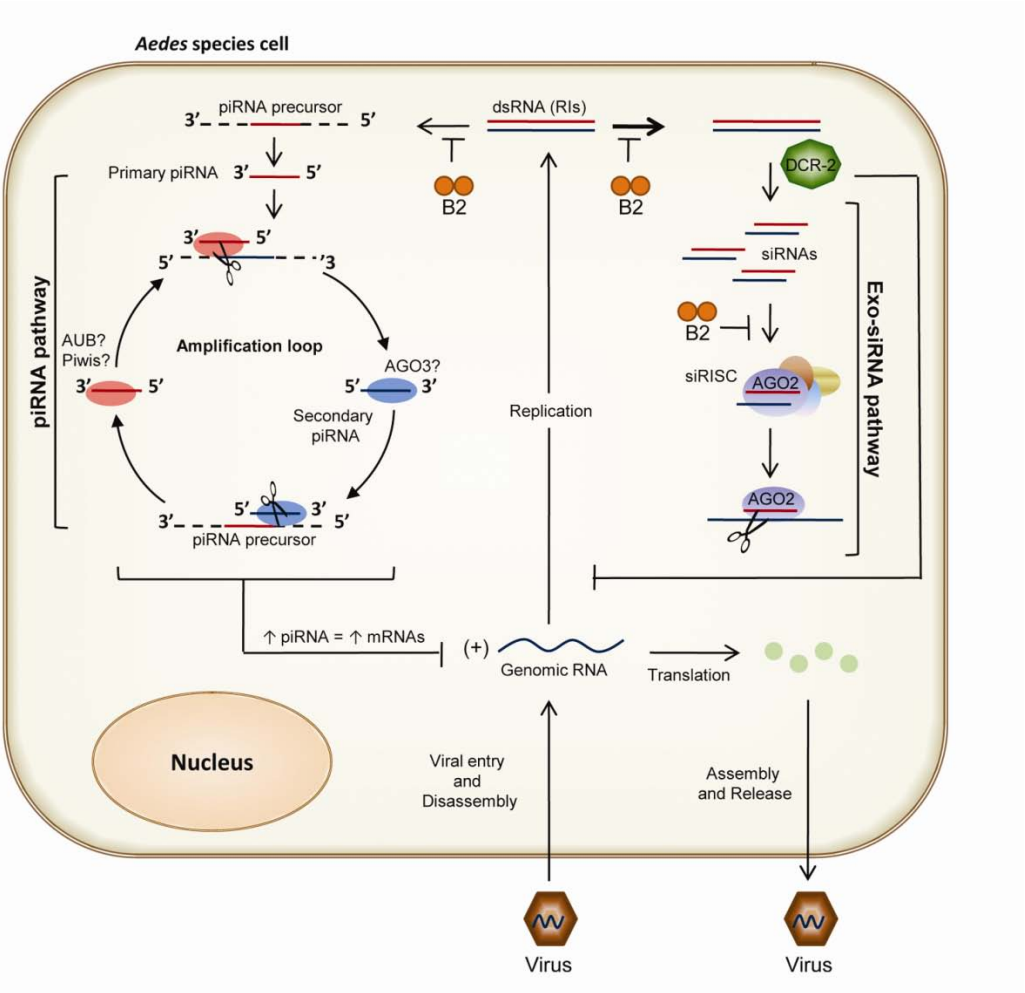

Figure 2.9. Functional-redundancy in RNA-based immune pathways modulating alphavirus pathogenesis in the mosquito soma. Following entry and uncoating, the genomic (+) strand RNA of an alphavirus serves both as mRNA and as a template for the synthesis of complementary (-) strand RNA. The viral (-) strands then serve as templates for the synthesis of new genomic-length (+) strand RNAs, as well as for shorter subgenomic (+) strand RNAs (26S mRNA) that encode the virus' structural genes. Alphaviruses are thought to synthesize (-) strand RNAs for a limited duration of time early in the infection, establishing an upper limit on the number of dsRNA RIs present in the cell. However, production of the (+) single-stranded genomic (49S) and subgenomic (26S) RNAs continues much longer, ultimately becoming the predominant virus-specific RNAs present in the cell. Our findings support a model in which antiviral siRNA and piRNA biogenesis pathways compete for a limited number of precursor dsRNA RIs in the infected cell. While recognition of dsRNA activates both pathways, secondary piRNAs are preferentially generated from viral mRNAs. Efficient processing of dsRNA RIs by Dcr-2 restricts the amount of precursor substrate available to enter the piRNA biogenesis pathway. The B2 protein binds both siRNA duplexes and long dsRNAs preventing the protein components of antiviral pathways access to dsRNAs, but inhibition is not absolute. Elevated levels of viral replication increase the abundance of 49S and 26S mRNA precursor substrates available for production of secondary piRNAs.

CHAPTER 3

EXAMINING THE ROLE OF THE B1 *NODAMURA VIRUS* NONSTRUCTURAL PROTEIN IN MOSQUITOES

E.M. Morazzani, M.W. Wiley, Z.N. Adelman, and K.M. Myles

ABSTRACT

Plants and invertebrates employ RNA silencing to combat virus infection. As a counter-defense mechanism several plant and insect viruses have evolved to encode suppressors of RNA silencing (SRS). During infection, *Nodamura virus* (NoV) expresses two small proteins, B1 and B2. While the NoV B2 protein has been characterized as a suppressor of RNA silencing, the function of the NoV B1 protein has not been determined. We investigated the role of the NoV B1 protein as a suppressor of the siRNA and/or a newly discovered antiviral piRNA pathway. Our results demonstrate that unlike the NoV B2 protein, the NoV B1 protein does not appear to function as a suppressor of RNA silencing in mosquitoes.

INTRODUCTION

It is well established that both plants and invertebrate organisms employ small RNA pathways as an antiviral defense mechanism [16]. The canonical antiviral response in these organisms involves Dicer enzymes that cleave viral replicative intermediates into small RNA duplexes termed short interfering RNAs (siRNAs; ~21-24 nucleotides) [16]. While one strand of the siRNA duplex guides the silencing of complementary target viral RNAs when loaded in an Argonaute-mediated RNA-induced silencing complex (RISC), the passenger strand is quickly discarded [30]. In plants, multiple Dicer-like enzymes are involved in a complex and redundant antiviral response [73]. Until now, RNA-based immunity in animals was thought to be less complex and less redundant due to the presence of an antiviral small RNA pathway with a single dedicated Dicer enzyme. However, we have demonstrated that culicine mosquito vectors have evolved a redundant antiviral defense directed by two different small RNA pathways. Specifically, we have shown that in addition to the canonical antiviral response mediated by siRNAs [15, 65], ping-pong-dependent Piwi-interacting RNAs (piRNAs; ~24-30 nucleotides) also accumulate in response to virus infection of the mosquito soma. The complexity of mosquito antiviral immunity has important implications for understanding how viruses modulate RNA-silencing responses in mosquito vectors.

Suppressors of RNA silencing (SRS) have been identified in several viruses that infect invertebrate hosts [177]. The most well-characterized SRS identified in an animal virus thus far is the B2 protein of flock house virus (FHV; family *Nodaviridae,* genus *Alphanodavirus*) [178]. A protein of similar function is also present in the distantly related Nodamura virus (NoV; family *Nodaviridae*, genus *Alphanodavirus*) [177]. Unlike other alphanodaviruses, NoV was first isolated from female *Culex tritaeniorhynchus* mosquitoes and can lethally infect both insects and mammals [179-181]. Nodamura virus has a bipartite genome comprised of two (+) strand RNA molecules, RNA1 and RNA2. RNA1 encodes the RNA-dependent RNA polymerase (RdRP) protein A, and RNA2 encodes the capsid precursor protein. Notably, RNA1 can self-replicate independent of RNA2. The nonstructural proteins, B1 and B2, are translated from RNA3, a subgenomic mRNA templated by RNA1. The NoV RNA3 contains three AUG codons. The first and second AUG codons initiate protein synthesis of two forms of B2. B1 is synthesized from the third AUG codon in an overlapping reading frame [165]. All three proteins, B1 and the two

forms of B2, have been detected in cells transfected with NoV RNA1 [165]. While NoV B2 is a known suppressor of RNA silencing, the function of NoV B1 remains unclear. As multiple SRS proteins have been identified in the genomes of some viruses [182], we used high-throughput sequencing to investigate a potential role for the NoV B1 as a SRS protein. However, our results do not support a role for the NoV B1 in RNA silencing.

MATERIALS AND METHODS

Recombinant virus production and infections

The p3'dsCHIK was created by inserting a duplicate subgenomic promoter and multiple cloning site (MCS) immediately downstream of the E1 structural protein coding sequence of pCHIKic. Heterologous sequences were inserted into the MCS of p3'dsCHIK. Recombinant viruses were harvested as described [15]. Nodamura B2/B1 point mutations were introduced by PCR amplification using specific primers containing the desired point mutations. One to two-day-old *Ae. albopictus* (Wise) were injected in the thorax with 10^4 pfu of virus.

RNA isolation and detection

RNA was extracted with Tri Reagent RT (Molecular Research Center) following manufactures instructions. CHIKV (+) strand RNA levels were analyzed using strand-specific quantitative real-time PCR (ssqPCR) and a Taqman assay (Life Technologies) as described [162]. A Student's paired *t*-test was used for the statistical analysis of ssqPCR results for different pairs at each time point.

Small RNA library preparation, deep sequencing and analysis

Small RNA libraries were prepared from total RNA isolated from adult female mosquitoes four days after infection with CHIKV using Illumina's small RNA sample prep kit according to the manufacturer's instructions, with the exception that we recovered small RNAs that were 18 to 35-nt in size by PAGE. Small RNA libraries were sequenced using the Illumina GAII. Sequence data sets were analyzed as described [15]. Briefly, reads were stripped of adaptor sequences and mapped to the CHIKV genome (strain 37997) using parameters that did not permit mismatches. The abundance of small RNAs mapping to the CHIKV genome in each library were normalized and compared by expressing as a percentage of the total reads in the same size class, except when expressed relative to viral mRNA copy numbers. In which case, viral small RNAs were normalized by the total number of 18-30-nt reads, excluding non-coding RNAs such as rRNAs, tRNAs and snRNAs, in the smallest sequence set. A detailed analysis of the small RNAs sequenced from each library is found in (Appendix A Table 3.1).

RESULTS

Expressing B1 from a recombinant CHIKV does not inhibit the production of viral siRNAs or piRNAs in infected *Ae. albopictus*

To determine if the NoV B1 protein functions as a suppressor of antiviral pathways directed by small RNAs, we examined the production of viral siRNAs and piRNAs during the infection of *Aedes albopictus* (*Ae. albopictus*) with recombinant chikungunya viruses (CHIKVs) expressing several different NoV B2/B1 mutants. To distinguish between a well-established suppressor activity present in the NoV B2 protein and the potential suppressor activity of the NoV B1 protein, we introduced a series of point mutations in the overlapping reading frames of B2 and B1 (Figure 3.1). A double subgenomic CHIKV containing untranslatable versions of both reading frames (dsCHIKV B2--/B1-) served as a control [165]. As expected, decreased production of viral siRNAs was observed in mosquitoes infected with recombinant viruses expressing a functional B2 protein (dsCHIKV B2++/B1+ or dsCHIKV B2++/B1-), when compared to mosquitoes infected with the control virus (Figure 3.2). Johnson *et al.* previously reported increased levels of B2 in mammalian cells transfected with NoV RNA1 (B2++/B1-) compared to wild type NoV RNA1 (B2++/B1+), suggesting that the expression levels of B2 may be regulated by B1 [165]. Accordingly, 21-nt viral siRNAs were less abundant in mosquitoes infected with dsCHIKV B2++/B1- than in those infected with dsCHIKV B2++/B1+, consistent with a potential regulatory role for B1 (Figure 3.2A and C). Importantly, the abundance of viral siRNAs did not decrease relative to the control in mosquitoes infected with dsCHIKV B2--/B1+ (Figure 3.2B and D). Nor did viral piRNA levels decrease in these mosquitoes (Figure 3.2B and D). These results indicate that the accumulation of viral siRNAs and piRNAs are not affected by the expression of the NoV B1 protein in *Ae. albopictus* infected with CHIKV.

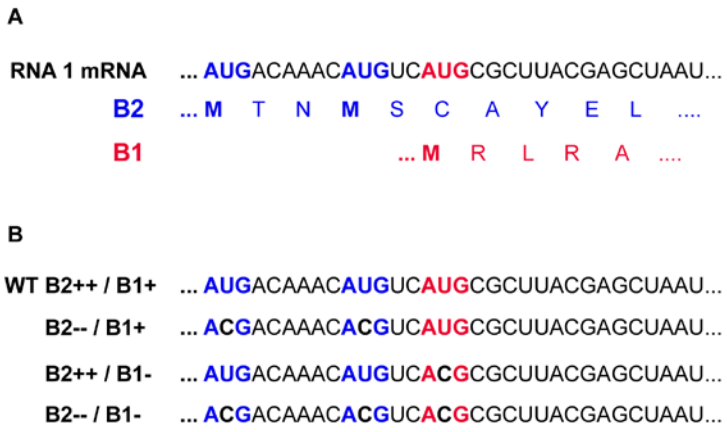

Figure 3.1. Point mutations in the B1 and B2 ORFs. (A) NoV RNA 3 ORFs encoding the B2 and B1 nonstructural proteins. (B) Point mutations introduced into the B2 and B1 ORFs to abolish translation. Blue letters indicate start codons present in the B2 ORF. Red letters indicate the start codon present in the overlapping reading frame encoding B1.

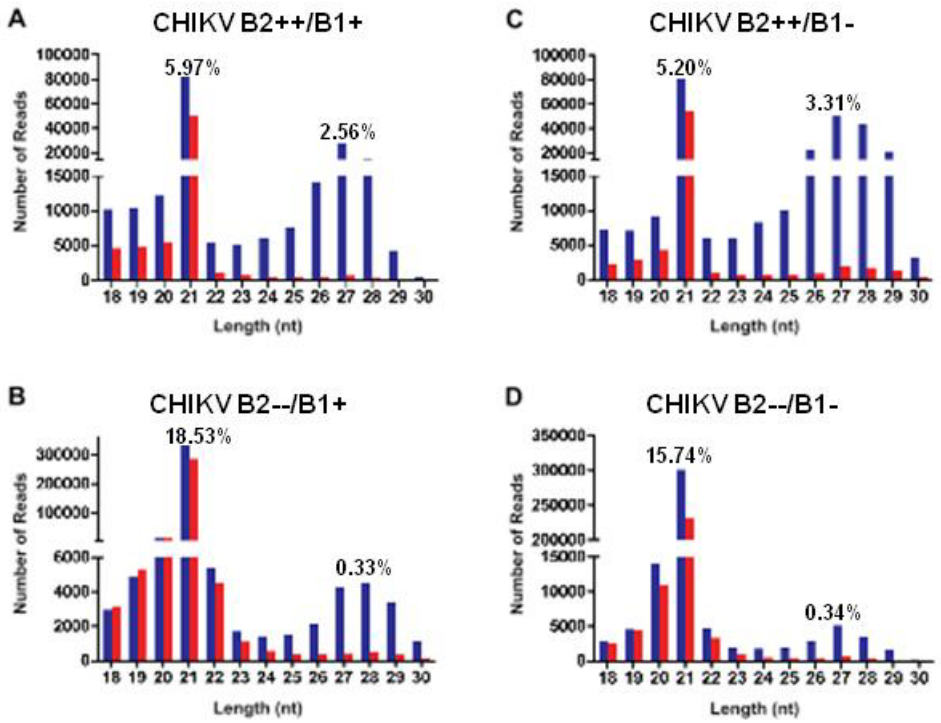

Figure 3.2 Effect of the NoV B1 protein on the accumulation of viral siRNAs and piRNAs in *Ae. albopictus*. Size distribution of virus-derived small RNAs in *Ae. albopictus* infected with (A) dsCHIKV B2++/B1+, (B) dsCHIKV B2--/B1+, (C) dsCHIKV B2--/B1+, or (D) dsCHIKV B2--/B1-. Blue bars indicate small RNAs derived from virus (+) strands and red bars indicate those derived from (-) strands. Normalized percentages of specific viral small RNA size classes are shown above.

Modulation of pathogenesis in *Ae. albopictus* infected with recombinant CHIK viruses expressing B1.

To examine the relationship between virus replication and the production of virus-derived small RNAs in mosquitoes infected with CHIKV expressing a B1 protein, we performed strand-specific quantitative real time PCR (ssqPCR) analysis on the total RNA used for small RNA library preparation (Figure 3.3A). As expected, decreased production of viral siRNAs in mosquitoes infected with CHIK viruses expressing a functional B2 protein resulted in increased levels of viral mRNA (Figure 3.3A). In contrast, viral mRNA did not increase when only B1 was expressed (Figure 3.3A). Using our ssqPCR results, we determined the average number of virus (+) strands per virus-derived small RNA in the mosquitoes infected with recombinant CHIK viruses. While an increased number of virus (+) strands per 21-nt viral siRNA was associated

with infection with recombinant viruses expressing a functional B2 protein (dsCHIKV B2++/B1+ or dsCHIKV B2++/B1-), we did not detect an increased number of (+) strands per viral siRNAs in mosquitoes solely expressing the NoV B1 protein (Figure 3.3B). Similarly, no increase in the number of virus (+) strands per 27-28nt viral piRNA was observed in mosquitoes infected with dsCHIKV B2--/B1+ (Figure 3.3C). Next, we examined if expressing the NoV B1 affects the pathogenic outcome of CHIKV infection in mosquitoes. As expected, infection with recombinant CHIK viruses expressing a functional B2 protein resulted in increased mortality (Figure 3.3D). In contrast, survival curves for mosquitoes infected with dsCHIKV B2--/B1+ were indistinguishable from those of mock injected mosquitoes, suggesting that the expression of the B1 protein had little effect on the pathogenic outcome of virus infection (Figure 3.3D).

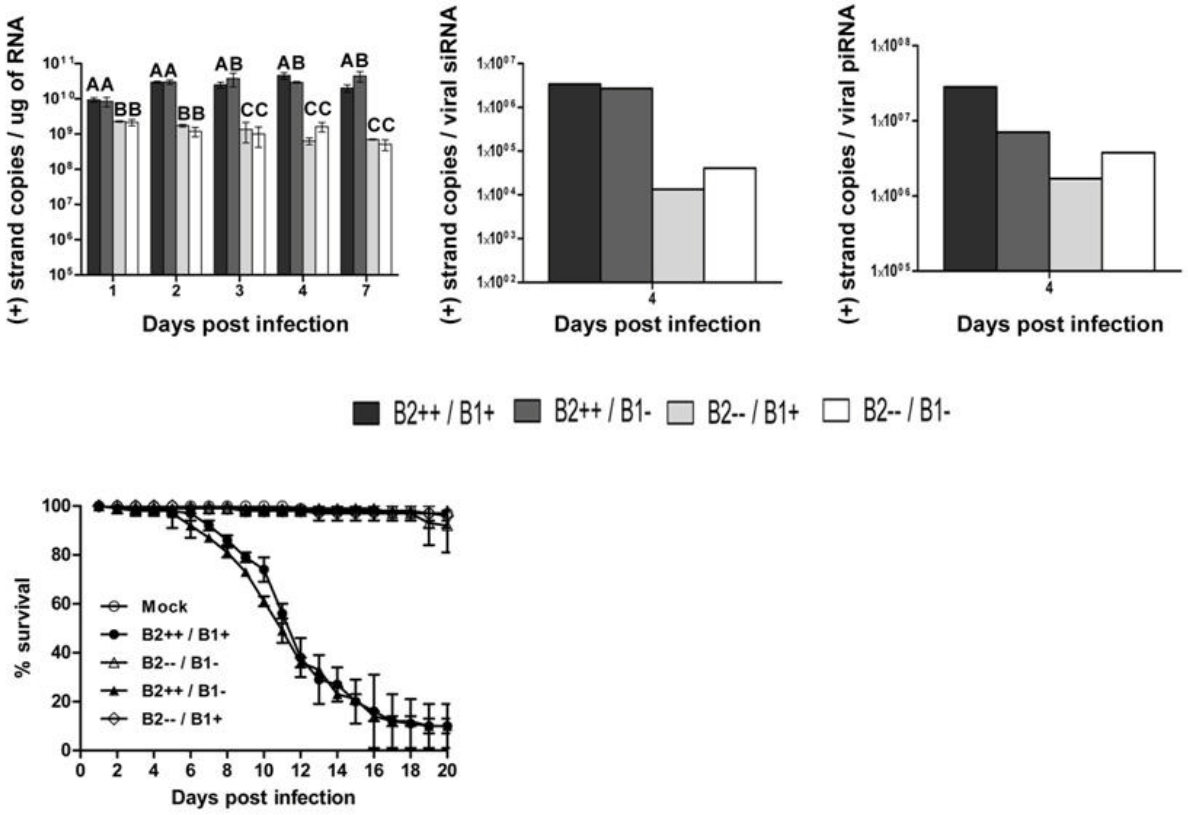

Figure 3.3. Effects of B1 expression on viral RNA levels and mortality. (A) Strand-specific quantitative real-time PCR analysis of CHIKV (+) strands in total RNA used for small RNA library preparation; (B) plotted as the number of (+) strand copies per virus-derived siRNAs or (C) piRNAs. Levels not connected by same letter are significantly different for each time point. (D) Survival of *Ae. albopictus* after infection with recombinant CHIKVs and mock injection. Error bars indicate the standard deviation among triplicate cohorts (n=90).

DISCUSSION

To counteract antiviral RNA silencing some viruses have evolved to encode multiple suppressors of RNA silencing (SRS). During infection, NoV expresses two small nonstructural proteins, B1 and B2 from subgenomic RNA3. It is well known that the B2 protein of NoV functions as a suppressor of RNA silencing. More recently, we have shown that B2 also interferes with viral piRNA production in mosquitoes infected with CHIKV. However, the function of the NoV nonstructural B1 protein has yet to be determined. To examine if the NoV B1 protein also functions as a suppressor of antiviral small RNA pathways in the mosquito, we infected *Ae. albopictus* with recombinant CHIK viruses expressing several different NoV B2/B1 mutants. While the expression of B1 did not directly interfere with the production of viral small RNAs, it does appear to regulate levels of the B2 suppressor protein when expressed from an overlapping reading frame. Consistent with this, western blot analysis demonstrate increased levels of B2 protein in mammalian cells infected with NoV defective in B1 production [165].

The *Nodaviridae* family is comprised of two genera: *Alphanodavirus* and *Betanodavirus.* While betanodaviruses predominantly infect fish, alphanodaviruses predominantly infect insects [183, 184]. However, NoV is unique among alphanodaviruses in that it also infects mammals [180]. Though the subgenomic RNA3 of all viruses identified in the *Nodaviridae* family encode a B2 protein, some alphanodaviruses and betanodaviruses lack a B1 ORF [185]. For example, the RNA3 of Boolarra virus (BoV; family *Nodaviridae,* genus *Alphanodavirus*), Striped jack nervous necrosis virus (SJNNV; family *Nodaviridae,* genus *Betanodavirus*), and Atlantic halibut nodavirus (AHNV; family *Nodaviridae,* a tentative *Betanodavirus*) encodes only the non-structural B2 protein [186, 187]. Interestingly, even though the B1 ORF is present in Pariacoto virus (PaV; family *Nodaviridae,* genus *Alphanodavirus*), the B1 protein was undetectable following virus replication according to the methods used [188]. In addition to NoV there is experimental evidence for B1 and B2 protein production in cells infected with FHV or Black beetle virus (BBV; family *Nodaviridae,* genus *Alphanodavirus*) [165, 189-191]. Notably in comparison to B2, B1 is produced in much lower abundance in mammalian cells infected with NoV [165]. A sub-optimal translation initiation context for the B1 start site has been proposed to explain the difference in B1 and B2 protein abundance [165].

Based on work by Johnson *et al.* NoV replication in mammalian cells does not require the synthesis of B1 or B2 [165]. Consistent with this, FHV deficient in B1 and B2 production replicates to wild type levels in mammalian and yeast cells [192-194]. In Drosophila cells however, while the B2 protein is essential for FHV replication [15, 195], the B1 protein is dispensable to virus replication [191]. Nevertheless it is important to note that it is still unclear whether mammals also employ RNA silencing as an antiviral defense, which may provide an explanation as to why B2, a suppressor or RNA silencing, is dispensable for NoV replication in mammalian cells. The dispensability of the NoV and FHV B1 protein and the absence of a B1 ORF in the genome of other alphanodaviruses, suggests little evolutionary benefit for these viruses to encode a B1 protein. Since B1 is encoded in the same reading frame as the RNA-dependent RNA polymerase (RdRP) protein A, mutations along the B1 coding region may be limited even under strong selective pressures.

Functional studies of the distantly related non-structural proteins of betanodaviruses may provide insight on the role of the B1 protein of alphanodaviruses. For example, similar to the distantly related B2 protein of alphanodaviruses, the B2 protein of several betanodaviruses is essential to viral RNA accumulation in several cell types [185, 186, 196]. Nevertheless, a functional study on the B1 protein of redspotted grouper nervous necrosis virus (RGNNV; family *Nodaviridae,* genus *Betanodavirus*) suggests the RGNNV B1 protein plays a role in modulating pathogenesis in fish infected with RGNNV [197]. Specifically that work demonstrates that the B1 protein of RGNNV is expressed early during infection and modulates cell death as an anti-necrotic death factor [197]. Even though our data does not suggest a direct role for the NoV B1 protein as an antagonist of RNA silencing, it is possible that the NoV B1 also modulates pathogenesis. Consistent with this B1 appears to indirectly modulate pathogenesis by limiting the suppressor activity of the B2 protein. However it remains to be determined whether the NoV B1 protein has a direct antagonistic role in insect or mammalian antiviral immunity.

CHAPTER 4

ROLE OF VIRUS-DERIVED SMALL RNAS IN THE ANTIVIRAL IMMUNITY OF TWO MAMMALIAN CELL LINES

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Manuscript in preparation for submission

ABSTRACT

In plants and invertebrates, dsRNAs formed during the replication of RNA viruses are potent inducers of RNA silencing. RNA silencing is a process by which viral dsRNA is cleaved into siRNAs and homologous RNA transcripts degraded. In mammals, viral dsRNA induces a range of relatively nonspecific responses that degrade both cellular and viral mRNAs. However, it remains unclear if viral dsRNA also triggers RNA silencing in mammalian cells. Mosquito-borne viruses represent an ideal model for addressing this question as transmission cycles involve alternating replication in mammalian and invertebrate hosts. Although we report identifying a subset of virus-derived small RNAs that appear to be products of Dicer cleavage in two mammalian cell lines naturally infected with chikungunya virus, our studies suggest these small RNAs have little biological relevance in combating virus infections. This raises questions about the pathways employed during experimental targeting of viruses with RNA interference in mammalian cells.

INTRODUCTION

In plants and invertebrates, dsRNA replicative intermediates (RIs) formed during virus replication induce an antiviral RNA interference (RNAi) response [198]. RNAi is a process whereby intracellular dsRNA is cleaved by the RNase III enzyme Dicer into short interfering RNAs (siRNAs; ~21 nt in length) and homologous mRNA transcripts silenced [21]. The specificity of the antiviral response is determined by the siRNA sequence bound to an Argonaute protein, a key component of the RNA induced silencing complex (RISC) [31]. Though much of the RNAi machinery appears to be conserved in mammalian cells, it remains unclear whether RNA silencing functions as a natural antiviral immune response in mammals [33, 105, 199-202]. Similar to flies, endogenous siRNAs derived from various genomic loci have been described in mouse oocytes and embryonic stem cells, suggesting that the mammalian Dicer is able to process long dsRNAs (reviewed in ref [33], [108]). Although mammalian cells also appear to have an antiviral siRNA pathway, evidence for this comes primarily through the use of experimentally introduced small RNAs or dsRNAs that resemble microRNA (miRNA) intermediates [201, 202].

Addressing whether or not mammals employ the RNAi machinery as a natural antiviral defense is complicated by the fact that viral dsRNA (> 30bp) induces a range of relatively nonspecific antiviral responses in vertebrate cells, many of which are also capable of degrading mRNAs into smaller RNA products. For example, viral infection of vertebrate cells induces interferon (IFN), leading to the transcription of a large number of antiviral IFN-stimulating genes (ISGs) and the establishment of an antiviral state in the cell (for review see [203]). Several ISGs can also be induced directly, independent of IFN [116]. Double-stranded RNA-activated protein kinase (PKR) and 2',5' oligoadenylate synthetase (OAS) are two enzymes that can be directly activated by dsRNA to inhibit virus replication [122]. Activated PKR phosphorylates the translational initiation factor eIF-2, resulting in inhibition of protein synthesis [113]. OAS synthesizes 2'5' oligomers of adenosine activating the endonuclease RNase L [123], which degrades mRNA in the infected cell [204]. Thus, one or more antiviral responses may have supplanted the role of RNAi in mammalian immunity.

In the somatic cells of flies, two classes of small RNAs predominate, miRNAs (22-nt in length) derived from the cleavage of endogenous dsRNAs [24], and siRNAs that can be derived from both endogenous (e.g., transposons, convergent transcripts, stem-loop structures, etc.) and

exogenous (e.g., transgenes, viral replicative intermediates, etc.) sources of dsRNA [24, 31]. These small RNAs are processed by two distinct Dicer proteins; Dicer-1 is involved in the production of miRNAs, while Dicer-2 is dedicated to siRNA pathways [76]. Functional specialization is also present in the Argonaute-mediated effector complexes of the fly, with Argonaute-1 (Ago1) acting in the miRNA-RISC, and Argonuate-2 (Ago2) providing "slicer" activity to the siRNA-RISC [89]. Partitioning between Ago1 and Ago2 appears to be determined by the structure, length and first nucleotide of the small RNA duplex [205]. In contrast with flies, mammals have a single Dicer that processes both miRNAs and siRNAs, suggesting less diversification of the RNAi machinery, and possibly greater overlap between the vertebrate pathways [77-79, 106].

Previous studies establishing the role of the RNAi pathway as an antiviral defense in invertebrate organisms provide a comparative framework for determining if mammals employ RNA silencing in antiviral immunity [15, 65, 67, 206]. Mosquito-borne viruses are ideal for addressing this question as their biological transmission cycles require alternating virus replication in a susceptible vertebrate and invertebrate host. Chikungunya virus (CHIKV) is an alphavirus with a positive-sense (+), non-segmented single-stranded RNA genome ~12 kb in length [58]. The 5' two-thirds of the genome encodes the nonstructural or replicase proteins, while the 3' one-third encodes the structural genes [58]. During alphavirus replication, genomic RNA is transcribed into full-length minus strand RNA copies [58]. The structural genes are translated from a subgenomic mRNA (26S RNA) transcribed from an internal promoter present in the full-length minus sense RNA copy [58]. Minus sense RNA copies also function as templates for the production of new genomes (49S RNA). Hence, dsRNA replicative intermediates are produced during alphavirus replication [42, 178]. During epidemics, transmission of CHIKV is thought to occur in a human-mosquito-human cycle [5].

To determine if mammals naturally employ the RNAi machinery as an antiviral defense we infected HEK-293 and MEF cell lines with the mosquito-borne CHIKV and characterized the populations of small RNAs present in the cells. Although we report identifying a subset of virus-derived small RNAs that appear to be products of Dicer cleavage in these two mammalian cell lines, our studies suggest little to no biological relevance in antiviral immunity.

MATERIALS AND METHODS

Cell culture

Human embryonic kidney cells (HEK-293) (ATCC) were cultured in DMEM supplemented with FBS and antibiotics. Mouse embryonic fibroblast (MEF) cells were cultured in RPMI-1640 medium (Lonza) supplemented with FBS and antibiotics. All cells were cultured at 37°C and 5% CO_2.

siRNA transfections

HEK-293 cells were plated on a 6-well plate in DMEM containing 10% FBS without antibiotics. MEF cells were plated on 12-well plate in RPMI-1640 medium containing 10% FBS without antibiotics. After 24hrs, cells (40-60% confluent) were transfected with a validated RNase L *Silencer* siRNA (Ambion), CHIKV-specific custom *Silencer* siRNA (Ambion), *Silencer* GAPDH control siRNA (Ambion), or a *Silencer* negative control siRNA (Ambion) using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. Relative gene expression was analyzed using pre-designed GAPDH, RNase L and CHIKV Taqman gene expression assays (Ambion) 24-48 hrs post transfection according to the manufacturer's instructions.

Recombinant virus production and infections

The p3'dsCHIK was created by inserting a duplicate subgenomic promoter and multiple cloning site (MCS) immediately downstream of the E1 structural protein coding sequence of pCHIKic. B2 and GFP sequences were inserted into the MCS of p3'dsCHIK. Recombinant viruses were rescued as described [15]. Cells were infected at a multiplicity of infection (MOI) of 0.05 or 1, see Table 4.1 for details.

RNA isolation and detection

Total RNA was extracted with Tri Reagent RT (Molecular Research Center) following manufacturer's instructions. CHIKV (+) strand RNA levels were analyzed using a strand-specific quantitative real-time PCR (ssqPCR) Taqman assay (Life Technologies) as described [162]. A Student's *t*-test was used for the statistical analysis of ssqPCR results.

Small RNA library preparation, deep sequencing and analysis

Total RNA was isolated from cultured mammalian cells 24 or 48hrs after infection with CHIKVs using the *mir*Vana™ miRNA Isolation Kit (Ambion) according to the manufacturer's instructions. Small RNA libraries were prepared from total RNA using Illumina's small RNA sample prep kit according to the manufacturer's instructions. Libraries were sequenced using the Illumina GAII. Analysis of sequence data sets was as described [15] with the following exceptions. Reads stripped of adaptor sequences were mapped to the CHIKV genome (strain 37997). To compare the abundance of viral small RNAs among datasets, strand-specific viral small RNAs of the same size were expressed as a percentage of the total sense or antisense small RNA reads. When plotted relative to viral mRNA copy numbers, viral small RNAs were normalized based on the total number of 21-22 nt reads, excluding non-coding RNAs, in the smallest sequence set. A detailed analysis of the small RNAs sequenced from each library is found in Appendix A Table 4.1.

RESULTS

Production of virus-derived small RNAs in HEK-293 cells infected with CHIKV.

We characterized small RNA populations (18-24 nt) from chikungunya virus (CHIKV) infected human embryonic kidney cells (HEK-293) by deep sequencing. From a total of 4,720,770 small RNA reads (18-24 nt) sequenced, 214,692 reads perfectly matched the CHIKV genome (Table 4.1). The majority of virus-derived small RNAs mapped to CHIKV positive strands, with no clear peak at 21 or 22-nt, the canonical size for products of Dicer cleavage (Figure 4.1A). The sense (+) viral small RNAs were generated predominately from the last one-third of the viral genome, which corresponds to the highly abundant 26S mRNA (Figure 4.1B). The strong strand bias and lack of a predominant nucleotide size class suggested that the majority of these viral small RNAs were not being generated by an RNA silencing response. Activated RNase L is also capable of producing small RNA cleavage products in mammalian cells [207]. RNase L cleavage occurs predominantly at the 3' side of UU or UA sequences [207]. Nucleotide analysis revealed a strong preference for a UU or UA at the 3' end of sequenced (+) viral small RNAs, suggesting that they are mainly products of RNase L cleavage (Figure 4.2A).

Since RNase L cleavage tends to be exclusive to single-stranded RNAs [204], we decided to examine more closely the viral small RNAs mapping to the CHIKV negative strands (commonly found in dsRNA replicative intermediates) for evidence of Dicer cleavage. Although present in relatively low abundance, the antisense (-) viral small RNAs were predominately 21-22 nt in length and exhibited a U bias at position 1, which is consistent with Dicer processing in mammals [208, 209] (Figure 4.1A and 4.2C). A strong UU or UA bias was also observed at the 3' end of the putative Dicer cleavage products, primarily generated from two specific loci corresponding to CHIKV nucleotides 19-40 and 7496-7517, respectively (Figure 4.1B and 4.2B). However, whether or not the observed 3' bias is a legitimate feature of these small RNAs remains unclear due to the limited number of loci from which they were generated. Interestingly, these two loci have been identified as conserved sequence elements (CSE) in alphavirus genomes [58]. The CSE contained within the first 45 nucleotides of the viral genome forms a structured element [58]. The complementary sequence in the minus-strand is the promoter for plus-strand RNA (49S) synthesis [210]. The other CSE corresponds to the promoter sequence for transcription of 26S mRNAs [58]. Notably, both CSEs are positioned near the 5' ends of the

alphavirus 49S and 26S mRNAs, respectively. Similar clustering of viral siRNAs has been reported near the 5' terminal regions of the flock house virus (FHV) RNA1 and RNA2 in a *Drosophila* cell line [211]. Though the majority of CHIKV-derived small RNAs sequenced in HEK-293 cells appear to be products of RNase L cleavage, characterization of a less abundant population of (-) viral small RNAs appears to indicate that the CHIKV genome is also targeted by a Dicer-mediated RNAi response.

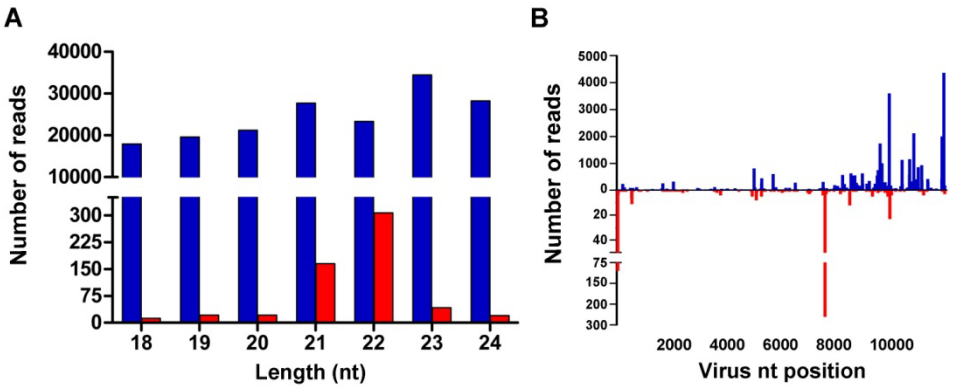
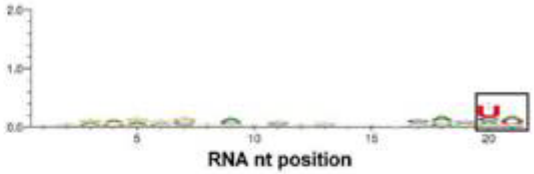


Figure 4.1. Characterization of virus-derived small RNAs in HEK-293 cells. (A) Distribution by length (nt) of viral small RNAs in HEK-293 cells infected with CHIKV. (B) Density plot of 21-22 nt viral small RNA-generating loci in CHIKV. Vertical axis denotes the number of viral small RNA start positions per loci. Blue and red bars indicate small RNAs derived from CHIKV (+) and (-) strands, respectively.

A

(+) viral small RNAs U U/A bias at the 3'end

	293	293 (-) control	293 RL siRNA	MEF	MEF *RNase L -/-*
18	48%, 74%	37%, 67%	29%, 62%	18%, 57%	23%, 61%
19	46%, 72%	37%, 66%	23%, 60%	15%, 62%	24%, 66%
20	43%, 75%	38%, 69%	26%, 58%	16%, 53%	25%, 61%
21	63%, 77%	58%, 74%	39%, 66%	20%, 59%	21%, 64%
22	44%, 74%	36%, 69%	24%, 63%	16%, 62%	22%, 72%
23	65%, 83%	54%, 75%	34%, 64%	14%, 69%	22%, 69%
24	63%, 79%	58%, 74%	36%, 63%	18%, 61%	23%, 70%



B

(-) viral small RNAs U U/A bias at the 3'end

	293	293 (-) control	293 RL siRNA	MEF	MEF *RNase L -/-*
18	8%, 75%	15%, 54%	11%, 56%	8%, 32%	17%, 33%
19	29%, 76%	33%, 78%	8%, 77%	16%, 49%	15%, 48%
20	24%, 95%	10%, 50%	0%, 78%	19%, 72%	24%, 75%
21	73%, 92%	72%, 74%	69%, 86%	58%, 66%	63%, 68%
22	81%, 90%	72%, 88%	88%, 94%	60%, 74%	47%, 59%
23	64%, 88%	50%, 75%	65%, 78%	45%, 87%	23%, 68%
24	64%, 88%	44%, 56%	0%, 100%	29%, 57%	20%, 61%

C

(-) viral small RNAs U bias at the 5'end

	293	293 (-) control	293 RL siRNA	MEF	MEF *RNase L -/-*
18	0%	8%	0%	6%	15%
19	29%	33%	0%	16%	24%
20	38%	10%	22%	11%	17%
21	47%	56%	47%	16%	12%
22	62%	59%	68%	46%	26%
23	40%	50%	35%	43%	30%
24	25%	44%	25%	14%	35%

Figure 4.2. Nucleotide analysis of virus-derived small RNAs (A) Sequence bias represented as a percentage for a UU or UA at the 3' end of the total small RNAs derived from CHIKV (+) strands or (B) CHIKV (-) strands. (C) Sequence bias represented as a percentage for a U at position 1 of the total small RNAs derived from CHIKV (-) strands.

Expressing a strong suppressor of RNA silencing in HEK-293

As the majority of viral small RNAs generated in CHIKV infected HEK-293 cells appeared to be products of RNase L cleavage, we next examined small RNA populations from *RNase L* silenced HEK-293 cells infected with CHIKV. Using a sequence-specific siRNA, levels of RNase L mRNA were reduced by 90% relative to a control siRNA (Figure 4.4A). Depleting RNase L in CHIKV infected HEK-293 cells decreased production of (+) viral small RNAs. However, the largest decrease occurred in the 21-nt size class (Figure 4.3A and B, Table 4.1 Appendix A). In contrast, lower levels of RNase L did not alter the abundance of (-) viral small RNAs in the infected cells (Figure 4.3A and B, Table 4.1 Appendix A). While a weaker 3' UU or UA bias was observed in small RNAs derived from virus (+) strands in the infected *RNase L* silenced HEK-293 cells, the depletion of RNase L did not appear to affect the 3' bias in (-) viral small RNAs (Figure 4.2A and B). Taken together, these results suggest that small RNAs derived from CHIKV (-) strands in infected HEK-293 cells are products of an RNA silencing pathway. Although a subset of small RNAs derived from virus (+) strands may also be products of Dicer cleavage, we are unable to distinguish these from other small RNAs produced from low levels of activated RNase L in the cell or other non-specific degradation pathways.

Processing of viral dsRNA by Dicer is not sufficient to restrict virus replication in Ago-deficient flies [49, 50, 211]. However, silencing Dicer in mammalian cells has been shown to disrupt miRNA processing, necessitating an alternative approach to investigate the role of HEK-293 Dicer cleavage products in directing an antiviral response [77, 106, 212, 213]. The B2 protein of Nodamura virus (NoV) is a well characterized suppressor of RNA silencing that associates with viral dsRNA replicative intermediates and siRNA duplexes, shielding them from Dicer processing and RISC assembly [214]. Expressing strong suppressors of RNA silencing in cells in which the RNAi machinery does not play a major role in antiviral defense has been shown to have little effect on virus replication [134]. Thus, to determine if the HEK-293 Dicer cleavage products are directing an antiviral defense in these cells, we expressed the NoV B2 protein from a recombinant CHIKV. Cells infected with a recombinant CHIKV expressing a mutant (F49S) NoV B2 deficient in its ability to bind dsRNA, served as controls. Levels of viral mRNA in HEK-293 cells infected with dsCHIKV-B2 or dsCHIKV-B2 (F49S) were compared by strand-specific quantitative real-time PCR (ssqPCR). Lower levels of viral mRNA were observed in cells infected with dsCHIKV-B2 when compared to cells infected with the control virus,

suggesting that HEK-293 Dicer cleavage products have little effect on virus replication (Figure 4.3D). We also compared the levels of viral mRNA in the *RNase L* silenced HEK-293 cells infected with CHIKV. In contrast to the results obtained in cells expressing B2, viral mRNA increased in comparison to cells treated with a control siRNA (Figure 4.3C, 4.4A-C), confirming the role of RNase L in mammalian antiviral immunity.

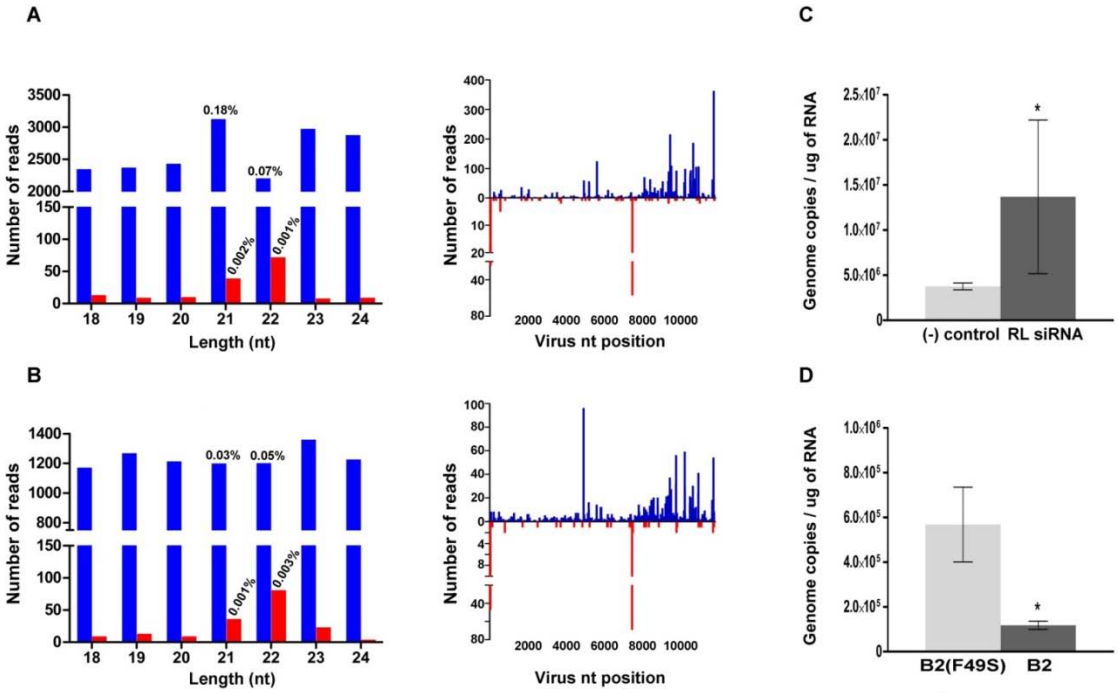


Figure 4.3 Viral RNA levels do not increase in HEK-293 cells infected with dsCHIKV-B2. Size distribution and density plots of virus-derived small RNAs in (A) HEK-293 cells treated with the control siRNA and (B) *RNase L* siRNA. Vertical axis denotes the number of viral small RNA start positions per loci. Blue and red bars indicate small RNAs derived from CHIKV (+) and (-) strands, respectively. Normalized percentages of specific viral small RNA size classes are indicated above. (C) Strand-specific quantitative real-time PCR analysis of CHIKV (+) strands copies per ug of RNA from HEK-293 cells treated with the *RNase L* or control siRNA. (D) Strand-specific quantitative real-time PCR analysis of CHIKV (+) strands copies per ug of RNA from HEK-293 cells infected with dsCHIK-B2 or dsCHIKV-B2(F49S).

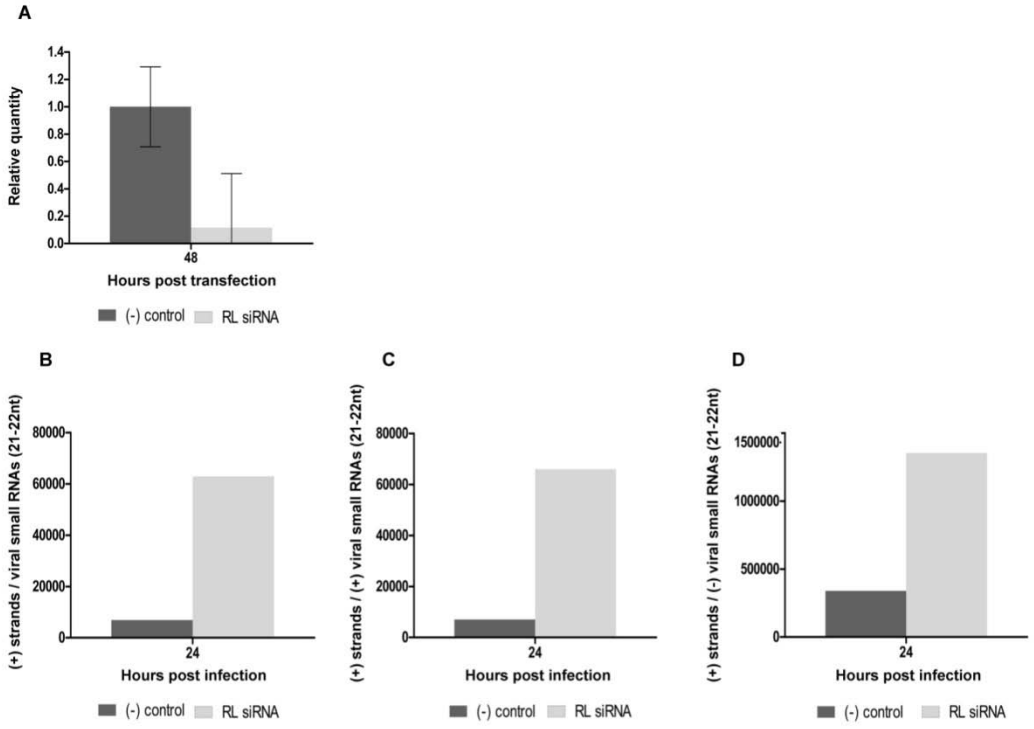


Figure 4.4. RNase L silencing enhances CHIKV replication in HEK-293 cells. (A) Quantitative real-time PCR analysis of RNase L expression after siRNA-mediated interference. (B) Strand-specific quantitative real-time PCR analysis of CHIKV (+) strands plotted as the number of copies per total virus-derived small RNAs, (C) (+) virus-derived small RNAs, and (D) (-) virus-derived small RNAs.

Production of virus-derived small RNAs in MEF cells infected with CHIKV

To determine if the RNAi machinery has been conserved in MEF cells we transfected them with a sequence-specific siRNA targeting CHIKV prior to infection with the virus. In cells transfected with the CHIKV-specific siRNA we observed significantly reduced levels of viral mRNA and virus production, when compared with cells treated with the non-specific control siRNA (Figure 4.4C-F). These results demonstrate modulation of CHIKV replication in MEF cells by an RNA silencing response following the experimental introduction of a virus-specific siRNA. Thus, we next investigated the role of RNAi as a natural antiviral immune defense in MEF cells by sequencing and characterizing small RNAs from infected cells. Of ~23 million total small RNA reads (18-24 nt) sequenced in CHIKV-infected MEF cells, 4,734 perfectly matched the CHIKV genome (Table 4.1). As observed in HEK-293 cells, the majority of viral small RNAs generated in MEF cells were derived from CHIKV (+) strands, with no clear peak in

the 21 or 22-nt size class. However, (+) viral small RNAs from CHIKV infected MEF cells failed to cluster in the 26S mRNA region and also lacked any bias at the 3' end (Figure 4.5A and 4.2A). However, the majority of (-) viral small RNAs were 21-22 nt in length and predominantly derived from CSEs in the genome of CHIKV, consistent with our previous results in HEK-293 cells (Figure 4.1B, 4.5A). Taken together, these results suggest that viral dsRNA is processed by Dicer in MEF cells infected with CHIKV.

Unlike HEK-293 cells, the majority of the viral small RNAs sequenced in CHIKV infected MEF cells lacked many of the hallmarks of RNase L cleavage. Thus, we examined small RNA populations from MEF *RNase L -/-* cells infected with CHIKV. In the absence of RNase L, (+) viral small RNAs accumulated to higher levels and with clear peaks at 21 and 22-nt (Figure 4.5B) [15]. The abundance of (-) viral small RNAs also increased in the absence of RNase L, suggesting that Dicer cleavage products were enriched MEF *RNase L-/-* cells infected with CHIKV (Figure 4.5B). When virus-derived siRNAs are prevented from accumulating in mosquito cells infected with an alphavirus, increased levels of viral RNA and cytopathology are observed [15]. Again confirming the role of RNase L in mammalian antiviral immunity, RNase L-deficient MEF cells exhibited increased levels of viral RNA and cytopathology, in comparison to wild type MEF cells infected with CHIKV (Figure 4.5C, 4.6G and 4.6I-J). This occurs despite an increased abundance of Dicer cleavage products, suggesting that these products have little role in restricting virus replication.

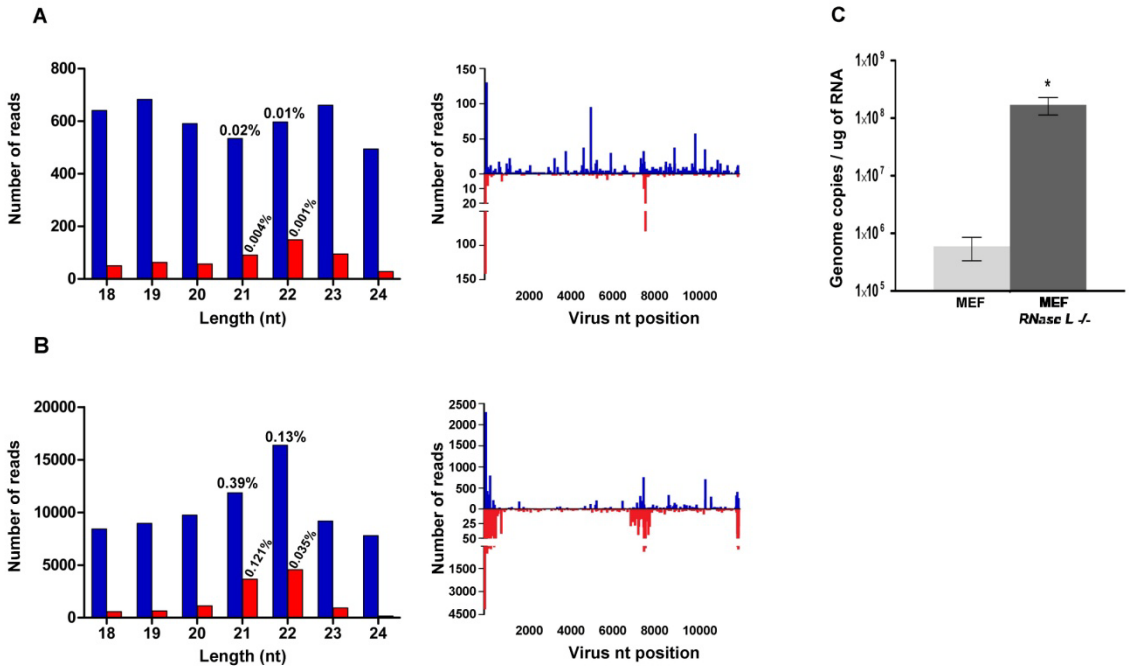


Figure 4.5. Characterization of virus-derived small RNAs in MEF and MEF *RNase L -/-* cells. Size distribution and density plots of virus-derived small RNAs in (A) MEF and (B) MEF *RNase L -/-* cells. Vertical axis denotes the number of viral small RNA start positions per loci. Blue and red bars indicate small RNAs derived from CHIKV (+) and (-) strands, respectively. Normalized percentages of specific viral small RNA size classes are indicated above. (C) Strand-specific quantitative real-time PCR analysis of CHIKV (+) strands copies per ug of RNA from MEF and MEF *RNase L -/-* cells.

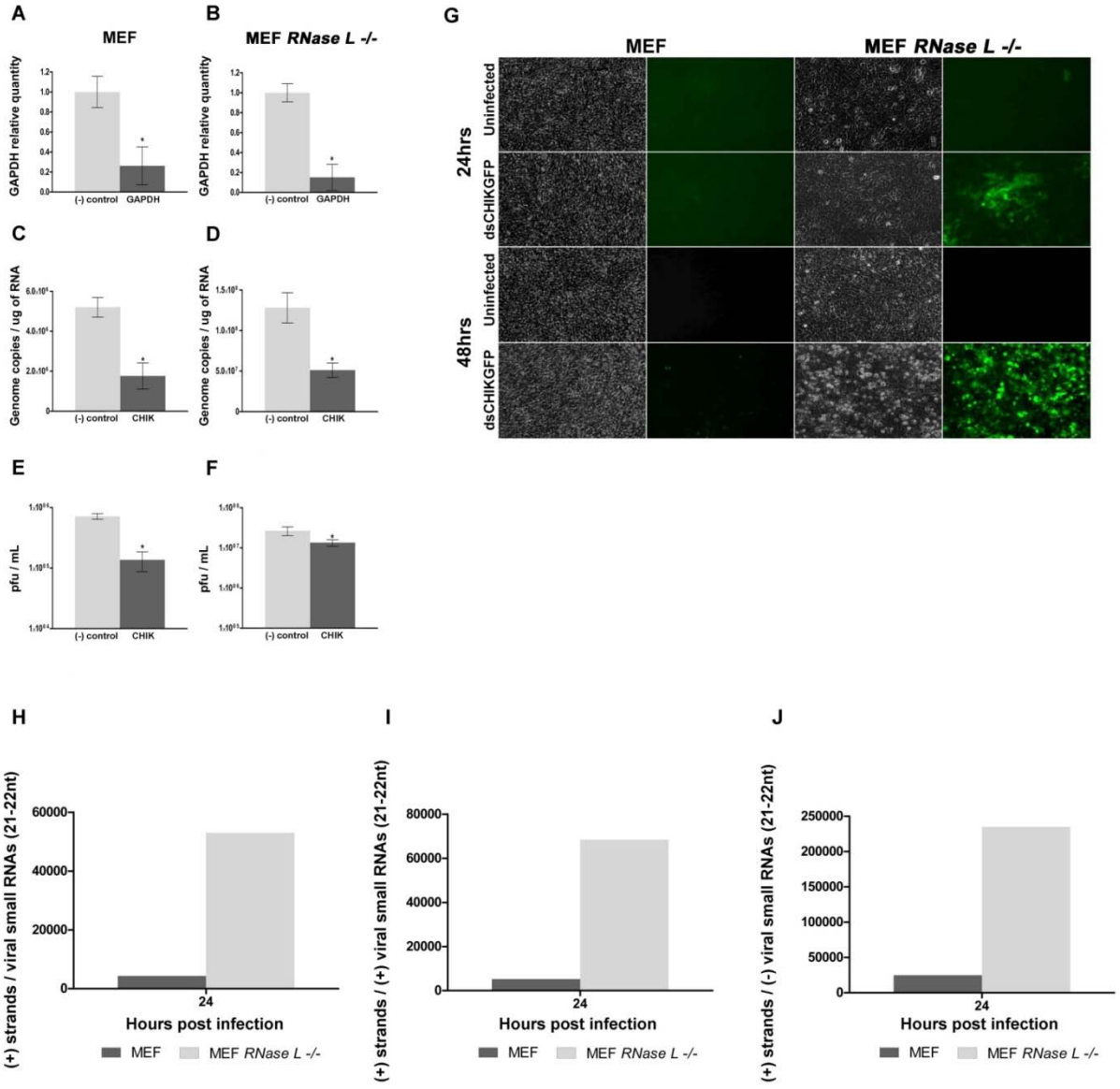


Figure 4.6. The RNase L pathway modulates CHIKV infection in MEF cells. (A-F) Quantitative real-time PCR analysis of mRNA expression after siRNA-mediated interference in MEF and MEF RNase L -/- cells. (G) Cytopathology in MEF and MEF RNase L -/- cells infected with a double subgenomic CHIKV virus containing GFP (20X magnification). (H) Strand-specific quantitative real-time PCR analysis of CHIKV (+) strands plotted as the number of copies per total virus-derived small RNAs, (I) (+) virus-derived small RNAs, and (J) (-) virus-derived small RNAs.

Expressing a strong suppressor of RNA silencing in MEF *RNase L-/-* cells

We next examined, whether the Dicer cleavage products in RNase L-deficient MEF cells were susceptible to B2-mediated suppression. Small RNA populations were sequenced and characterized from MEF *RNase L -/-* cells infected with recombinant CHIK viruses expressing NoV B2 or containing an untranslatable NoV B2 (ΔB2; $B2^{-}B1^{-}$) described previously [165]. Accumulation of (-) viral small RNAs (21-22 nt) was suppressed in MEF *RNase L -/-* cells infected with dsCHIKV-B2, in comparison to cells infected with dsCHIKV- ΔB2, confirming that the MEF Dicer cleavage products are susceptible to suppression by B2 (Figure 4.7A and B, Table 4.1). Interestingly, the abundance of (+) viral small RNAs was not affected by the expression of B2, suggesting these are not products of an RNA silencing response, but an alternate pathway capable of generating small RNA products (Figure 4.7A and B, Table 4.1).

Next, we investigated the role of the MEF Dicer cleavage products in modulating virus replication in MEF *RNase L -/-* cells. We compared the levels of (+) strand viral RNA in MEF *RNase L -/-* infected with dsCHIKV-B2 or dsCHIKV-ΔB2. Expression of B2 did not increase CHIKV replication in MEF *RNase L -/-* cells, in comparison to cells infected with dsCHIKV-ΔB2 (Figure 4.3C and 4.7C) [15]. Taken together, our results indicate that mammalian cells retain the ability to generate virus-derived Dicer products during a natural infection, but these products do not appear to have biological relevance in mammalian antiviral immunity.

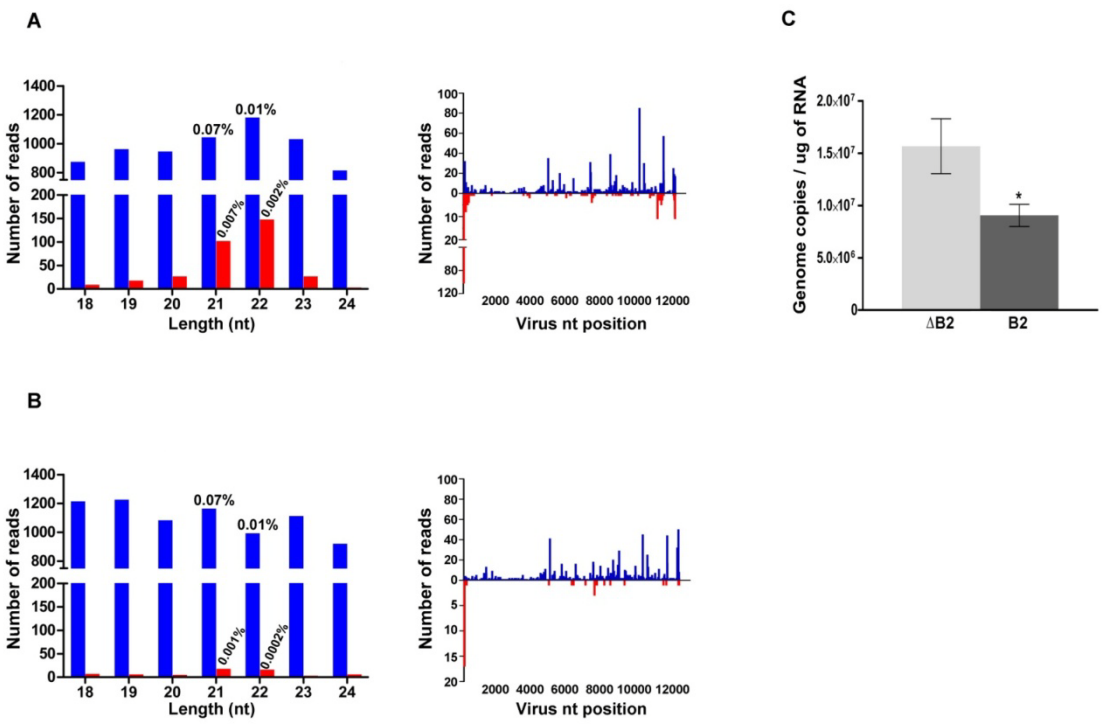


Figure 4.7. B2 expression suppresses the accumulation of (-) viral small RNAs in CHIKV-infected MEF *RNase L-/-* cells. Size distribution and density plots of virus-derived small RNAs in MEF *RNase L -/-* cells infected with (A) dsCHIKV-B2 or (B) dsCHIKV-ΔB2. Vertical axis denotes the number of viral small RNA start positions per loci. Blue and red bars indicate small RNAs derived from CHIKV (+) and (-) strands, respectively. Normalized percentages of specific viral small RNA size classes are indicated above. (C) Strand-specific quantitative real-time PCR analysis of CHIKV (+) strands copies per ug of RNA from MEF *RNase L -/-* cells infected with dsCHIKV-B2 or dsCHIKV-ΔB2.

DISCUSSION

It is well established that RNA silencing functions as a conserved antiviral immune response in invertebrate organisms, however, less clear is the role of this pathway in vertebrate antiviral immunity. Several studies have previously shown that the RNAi-machinery present in mammalian cells can mediate an antiviral response [215]. Consistent with this, transfection of a virus-specific siRNA effectively modulated the replication of the mosquito-borne CHIKV in infected HEK-293 and MEF cells. Control siRNAs had no effect on virus replication suggesting that the antiviral response induced in these cells was independent of ISGs like PKR and RNAse L. Indeed, transfection of the CHIKV-specific siRNA into MEF cells deficient in RNase L also effectively inhibited virus replication. These and other similar results raise the possibility that RNAi can be exploited as an effective therapeutic strategy to control the pathogenesis associated with viral infections. However, it remains unclear if RNA silencing functions as a natural antiviral defense in mammals. To investigate this question, we characterized small RNA populations (18-24 nt) in HEK-293 and MEF cells infected with CHIKV. Similar to a previous study that characterized small RNA populations in several different mammalian host systems infected with various RNA viruses, we identified virus-derived small RNAs that appear to be products of Dicer cleavage in both the HEK-293 and MEF cells infected with CHIKV [106]. Whereas infection of mosquitoes with CHIKV generates viral siRNAs that are predominantly 21-nt in length, map the length of the viral genome, and have only a slight bias for viral (+) strands (unpublished data), virus-derived small RNAs that appear to be products of Dicer cleavage in CHIKV-infected HEK-293 and MEF cells are 21-22 nt, largely restricted to two genomic loci, and exhibit an almost complete bias for CHIKV (-) strands. Taken together, these results indicate that small RNAs are naturally generated from viral dsRNA precursors in HEK-293 and MEF cells infected with CHIKV. However, the small RNAs generated in these cells appear to be distinct from those produced in the invertebrate host, possibly suggesting a biogenic origin from viral dsRNA precursors different from those targeted by the RNA silencing pathway of the mosquito.

In invertebrate organisms, the abundance of virus-derived siRNAs has been shown to positively correlate with the efficiency of RNA silencing [16]. For example, the pathogenesis of CHIKV is effectively modulated in *Aedes albopictus* mosquitoes in which ~4.8% of the total

sequenced small RNAs (18-24 nt) are siRNAs derived from the virus (unpublished results). By contrast, increased virus replication is accompanied by significant pathogenesis in mosquitoes infected with a recombinant CHIKV expressing the NoV B2 protein in which the abundance of viral siRNAs decreases to ~1.0% of total sequenced small RNAs (18-24 nt; unpublished results). Suppressing the accumulation of virus-derived Dicer products in HEK-293 and MEF cells infected with CHIKV did not increase virus replication or pathogenesis, suggesting little role in modulating the viral infection in these cells. Consistent with this was the relatively low abundance of virus-derived Dicer products in the HEK-293 and MEF cells infected with CHIKV, which ranged from 0.01% to 0.001% of the total sequenced small RNAs (18-24 nt). Although the abundance of virus-derived Dicer products increased in response to higher levels of CHIKV replication in MEF *RNase L -/-* cells, the small RNAs continued to have little effect on the accumulation of viral RNA in these cells. Overall, these results suggest a limited role for RNAi as a natural antiviral defense in mammalian cells.

As viral dsRNA RIs (>30bp) [202] formed during alphavirus replication induce a range of antiviral immune responses in mammalian cells, it was anticipated that many of the small RNAs sequenced in HEK-293 and MEF cells infected with CHIKV would be the intermediate products of interferon-mediated degradation pathways. Indeed, the majority of virus-derived small RNAs sequenced in HEK-293 and MEF cells infected with CHIKV appear to result from the cleavage of viral RNA by RNase L. These small RNAs have no predominant length, cluster in the region of the CHIKV genome corresponding to the 26S mRNA, and exhibit a complete bias for virus (+) strands. Infection of MEF cells deficient in RNase L with CHIKV increased virus replication and pathogenesis in comparison to wt cells. Similarly, depletion of RNase L in HEK-293 cells prior to infection with CHIKV significantly increased virus replication. These findings are consistent with previous reports of RNase L-mediated antiviral immunity, and suggest a role in mammalian cells that is analogous to the antiviral response directed by siRNAs in invertebrate organisms [15, 53]. Thus, RNase L may have supplanted RNA silencing as a natural RNA-based immune response in mammalian cells.

Although the experimental introduction of virus-specific dsRNA molecules resembling miRNA precursors and fully processed siRNAs have been shown to induce an artificial antiviral response in mammalian cells [202], it remains unclear if the virus-derived Dicer products identified here and in a previous study enter any specific biogenesis pathway. Several factors

contribute to uncertainty about an antiviral function for mammalian siRNA pathways. Mammalian cells lack specialized Dicers, instead encoding a single enzyme that processes both miRNA and siRNA precursors [24]. Overlap in the processing of miRNA and siRNA precursors presents significant challenges to assigning a specific pathway an antiviral function. Although eukaryotic small RNAs can often be distinguished by specific associations with proteins in the Argonaute (Ago) –family, mammalian Ago proteins also appear to lack specialization in these interactions. Indeed, Parameswaran *et al.* reported the non-specific association of virus derived small RNAs with all four Argonaute family proteins in a mammalian cell line transfected with a hepatitis C replicon, which precluded the authors from identifying a specific biogenesis pathway [106]. Although the entanglement of mammalian small RNA pathways also prohibits us from identifying a specific biogenesis pathway, virus-derived Dicer products in HEK-293 and MEF cells infected with CHIKV exhibit several features that are consistent with the potential involvement of the miRNA machinery. For example, unlike virus-derived siRNAs detected in mosquitoes infected with CHIKV, virus-derived Dicer cleavage products in HEK-293 and MEF cells are predominately 22-nt, generated from structured regions in the CHIKV genome, and exhibit a 5' U bias, all of which are also hallmarks of miRNAs.

Our results raise an important question; if mammalian cells lack an antiviral immune response that is directed by naturally occurring virus-derived small RNAs, then by what mechanism are experimentally introduced virus-specific dsRNA molecules and processed siRNAs acting to establish the robust antiviral effect reported here and in previous studies? If the introduction of such molecules into mammalian cells is redirecting the miRNA pathway to an antiviral function, this may have important consequences for the diverse cellular processes in which miRNAs are normally involved. Thus, a major challenge in the future will be addressing this question. The answer might have large implications for the future potential of RNAi-based antiviral therapeutics.

CHAPTER 5

SUMMARY

A distinguishing characteristic of alphavirus infections of the mosquito host is the establishment of a persistent, nonpathogenic state. Typically, virus replication increases to high levels during an initial ‘‘acute’’ phase of infection in susceptible cells and tissues, but is then modulated to lower levels during the persistent phase of the infection. The exact mechanism by which virus replication is modulated in mosquito cells is unknown. We have previously demonstrated the importance of RNA silencing in the mechanism by which alphaviruses establish a persistent infection in a mosquito vector [15]. Suppressing the accumulation of virus-derived siRNAs using the B2 protein of flock house virus, a well-characterized suppressor of RNA silencing, dramatically alters the pathogenic outcome of the virus infection in the mosquito host, resulting in rapid and complete mortality [15].

For this project, we undertook a comprehensive survey of virus-derived small RNAs in culicine mosquitoes and cells infected with CHIKV. Although piRNAs have previously only been identified in cells associated with the ovaries, our results indicate the presence of a novel antiviral immune pathway directed by piRNAs in the soma of culicine mosquitoes. We also demonstrate modulation of alphavirus infections in cell lines with defective siRNA production by an antiviral piRNA pathway. Collectively, our results support a model in which there is functional-redundancy in RNA-based immune pathways modulating alphavirus pathogenesis in the soma of disease vector mosquitoes. It remains unclear how mosquito-borne viruses modulate the vector’s complex RNA-based immune responses to establish productive infection. However, our results suggest little evolutionary benefit for mosquito-borne viruses encoding proteins that strongly interfere with RNA silencing.

Advances in our understanding of small RNA pathways, combined with an unprecedented availability of high-throughput sequencing technologies, enabled our investigations into the antiviral immune responses of both mammals and mosquitoes. The dsRNA formed during the replication of RNA viruses is a potent inducer of RNA silencing in the mosquito. In mammals, viral dsRNA induces a range of relatively nonspecific antiviral responses. However, it remains unclear if viral dsRNA also triggers RNA silencing in

mammalian cells. Because maintenance of mosquito-borne viruses in nature requires a biological transmission cycle that involves alternating virus replication in a susceptible vertebrate and invertebrate host, these viruses represented an ideal system for addressing this question. Although we identified a subset of virus-derived small RNAs that appear to be products of RNA silencing in two mammalian cell lines naturally infected with CHIKV, our studies suggest that these small RNAs have little biological relevance in combating virus infections. Thus, while the accumulation of virus-derived siRNAs is essential to the survival of mosquitoes infected with CHIKV, they appear to have little functional significance in mammalian antiviral immunity.

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APPENDIX A: Small RNA analysis summary

Table 2.1. Analysis of the sequenced virus-derived small RNAs

***Ae. aegypti* WB CHIK**

	18	19	20	21	22	23	24	25	26	27	28	29	30
Sense vRNAs	144	294	1040	23145	571	247	226	240	255	367	371	/	/
Antisense vRNAs	41	141	470	12050	279	72	39	25	26	36	30	/	/
Ratio s/as vRNAs	3.51	2.09	2.21	1.92	2.05	3.43	5.79	9.60	9.81	10.19	12.37	/	/
% of small RNAs = vRNAs	0.99%	2.22%	2.80%	10.12%	0.06%	0.18%	0.23%	0.23%	0.12%	0.08%	0.08%	/	/

***Ae. albopictus* WB CHIK**

	18	19	20	21	22	23	24	25	26	27	28	29	30
Sense vRNAs	173	402	1878	39544	643	312	350	448	669	1266	1026	758	140
Antisense vRNAs	107	249	896	20786	376	103	44	62	45	98	69	45	15
Ratio s/as vRNAs	1.62	1.61	2.10	1.90	1.71	3.03	7.95	7.23	14.87	12.92	14.87	16.84	8.75
% of small RNAs = vRNAs	0.97%	2.53%	6.43%	22.53%	0.12%	0.32%	0.32%	0.37%	0.20%	0.14%	0.10%	0.11%	0.08%

***D. melanogaster* WB CHIK**

	18	19	20	21	22	23	24	25	26	27	28	29	30
Sense vRNAs	16	44	162	1150	87	12	7	6	3	1	/	/	/
Antisense vRNAs	11	47	58	830	50	9	3	1	2	/	/	/	/
Ratio s/as vRNAs	1.45	0.94	2.79	1.39	1.74	1.33	2.33	6.00	1.50	/	/	/	/
% of small RNAs = vRNAs	0.02%	0.05%	0.10%	0.01%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	/	/	/

***Ae. albopictus* HT CHIK**

	18	19	20	21	22	23	24	25	26	27	28	29	30
Sense vRNAs	1715	3136	11650	214553	4460	2165	2122	2430	3666	6647	5150	2263	324
Antisense vRNAs	809	2126	6999	138446	2503	600	431	385	374	518	353	169	34
Ratio s/as vRNAs	2.12	1.48	1.66	1.55	1.78	3.61	4.92	6.31	9.80	12.83	14.59	13.39	9.53
% of small RNAs = vRNAs	1.28%	2.98%	6.59%	23.10%	0.21%	0.47%	0.78%	1.05%	0.96%	0.75%	0.75%	0.69%	0.51%

***Ae. albopictus* HT CHIK B2(NoV)**

	18	19	20	21	22	23	24	25	26	27	28	29	30
Sense vRNAs	7268	7082	9215	80790	5970	6022	8339	10094	22438	49968	43940	21262	3216
Antisense vRNAs	2202	2958	4261	54324	1061	742	659	657	893	1899	1743	1374	321
Ratio s/as vRNAs	3.30	2.39	2.16	1.49	5.63	8.12	12.65	15.36	25.13	26.31	25.21	15.47	10.02
% of small RNAs = vRNAs	0.93%	3.69%	1.76%	5.20%	0.11%	0.74%	1.51%	2.60%	3.52%	3.31%	2.96%	2.00%	1.42%

***Ae. albopictus* HT CHIK ΔB2**

	18	19	20	21	22	23	24	25	26	27	28	29	30
Sense vRNAs	2900	4581	13989	301109	4685	1925	1837	1904	2807	5148	3546	1614	219
Antisense vRNAs	2540	4536	11043	232666	3322	900	532	492	487	623	392	172	44
Ratio s/as vRNAs	1.14	1.01	1.27	1.29	1.41	2.14	3.45	3.87	5.76	8.26	9.05	9.38	4.98
% of small RNAs = vRNAs	0.51%	3.31%	3.35%	15.74%	0.09%	0.25%	0.44%	0.56%	0.45%	0.34%	0.36%	0.31%	0.32%

***Ae. albopictus* HT CHIK B2 (FHV)**

	18	19	20	21	22	23	24	25	26	27	28	29	30
Sense vRNAs	4222	6137	10647	135258	3728	2645	3557	4202	8863	22642	23246	17025	9300
Antisense vRNAs	2621	3974	6598	94738	1243	489	379	405	400	630	674	636	463
Ratio s/as vRNAs	1.61	1.54	1.61	1.42	3.00	5.41	9.39	10.38	22.16	35.94	34.49	26.77	20.09
% of small RNAs = vRNAs	3.10%	4.06%	2.18%	9.34%	0.07%	0.36%	0.85%	1.10%	1.32%	1.34%	1.31%	0.80%	0.88%

C6/36 CHIK

	18	19	20	21	22	23	24	25	26	27	28	29	30
Sense vRNAs	1823	1934	2285	2576	4124	5182	9283	13980	36006	90462	118779	106466	54004
Antisense vRNAs	20	52	67	80	117	175	256	265	458	672	663	590	356
Ratio s/as vRNAs	91.15	37.19	34.10	32.20	35.25	29.61	36.26	52.75	78.62	134.62	179.15	180.45	151.70
% of small RNAs = vRNAs	2.44%	5.05%	4.46%	1.70%	1.42%	3.02%	6.55%	10.88%	15.78%	16.02%	18.20%	14.46%	13.03%

C6/36 CHIK B2 (FHV) c44y

	18	19	20	21	22	23	24	25	26	27	28	29	30
Sense vRNAs	6951	7482	9219	13683	12746	20713	27094	48616	104012	262437	293263	229694	86949
Antisense vRNAs	576	580	631	907	1031	1244	1539	1673	2724	4538	3811	3445	1559
Ratio s/as vRNAs	12.07	12.90	14.61	15.09	12.36	16.65	17.60	29.06	38.18	57.83	76.95	66.67	55.77
% of small RNAs = vRNAs	0.89%	2.71%	2.12%	2.33%	1.77%	3.88%	4.66%	6.59%	8.97%	9.41%	10.49%	7.41%	5.17%

C6/36 CHIK B2 (FHV)

	18	19	20	21	22	23	24	25	26	27	28	29	30
Sense vRNAs	3636	3975	6943	5917	7586	8891	13271	21851	58411	136532	163454	106322	36904
Antisense vRNAs	143	158	244	312	316	497	490	620	949	1693	1979	1806	922
Ratio s/as vRNAs	25.43	25.16	28.45	18.96	24.01	17.89	27.08	35.24	61.55	80.65	82.59	58.87	40.03
% of small RNAs = vRNAs	0.30%	1.41%	1.60%	0.61%	0.46%	0.90%	0.67%	1.87%	4.20%	4.06%	4.86%	2.69%	2.24%

U4.4 CHIK

	18	19	20	21	22	23	24	25	26	27	28	29	30
Sense vRNAs	563	966	4161	47185	1087	610	911	1418	2923	6349	5428	2316	387
Antisense vRNAs	382	795	2698	32841	667	307	194	266	342	475	340	177	56
Ratio s/as vRNAs	1.47	1.22	1.54	1.44	1.63	1.99	4.70	5.33	8.55	13.37	15.96	13.08	6.91
% of small RNAs = vRNAs	0.18%	0.47%	0.64%	0.96%	0.18%	0.13%	0.15%	0.23%	0.31%	0.31%	0.35%	0.27%	0.19%

C7-10 CHIK

	18	19	20	21	22	23	24	25	26	27	28	29	30
Sense vRNAs	17433	6125	14652	17982	28791	48942	81772	110430	193590	448762	561413	402234	109193
Antisense vRNAs	2072	4213	5967	8693	14887	20127	29966	32663	51542	63967	55047	37292	10428
Ratio s/as vRNAs	8.41	1.45	2.46	2.07	1.93	2.43	2.73	3.38	3.76	7.02	10.20	10.79	10.47
% of small RNAs = vRNAs	2.52%	8.45%	8.99%	4.13%	3.55%	6.50%	11.75%	14.92%	20.10%	19.28%	22.82%	17.65%	15.50%

Aag2 CHIK

	18	19	20	21	22	23	24	25	26	27	28	29	30
Sense vRNAs	2576	918	2490	40273	1859	1824	3779	6349	10710	14418	10718	5164	1084
Antisense vRNAs	167	477	1190	26325	705	256	269	333	552	577	382	151	51
Ratio s/as vRNAs	15.43	1.92	2.09	1.53	2.64	7.13	14.05	19.07	19.40	24.99	28.06	34.20	21.25
% of small RNAs = vRNAs	5.31%	2.62%	2.82%	6.60%	0.60%	0.54%	1.05%	1.30%	1.45%	1.45%	1.67%	1.45%	1.66%

CCL-125 CHIK

	18	19	20	21	22	23	24	25	26	27	28	29	30
Sense vRNAs	36	16	39	220	21	26	37	73	111	203	202	105	21
Antisense vRNAs	3	7	18	173	4	8	5	7	12	9	3	5	1
Ratio s/as vRNAs	12.00	2.29	2.17	1.27	5.25	3.25	7.40	10.43	9.25	22.56	67.33	21.00	21.00
% of small RNAs = vRNAs	0.01%	0.01%	0.01%	0.01%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.02%	0.03%	0.04%

Table 3.1. Analysis of the sequenced virus-derived small RNAs

***Ae. albopictus* HT CHIK B2++ / B1+**

	18	19	20	21	22	23	24	25	26	27	28	29	30
Sense vRNAs	10239	10299	12136	85587	5346	5188	5957	7571	14004	27816	15916	4250	456
Antisense vRNAs	4650	4893	5539	49791	1129	623	380	401	453	604	272	131	24
Ratio s/as vRNAs	2.20	2.10	2.19	1.72	4.74	8.33	15.68	18.88	30.91	46.05	58.51	32.44	19.00
% of small RNAs = vRNAs	1.20%	2.51%	0.62%	5.97%	0.17%	0.62%	0.99%	1.39%	2.03%	2.56%	3.07%	2.16%	1.30%

***Ae. albopictus* HT CHIK B2++ / B1-**

	18	19	20	21	22	23	24	25	26	27	28	29	30
Sense vRNAs	7268	7082	9215	80790	5970	6022	8339	10094	22438	49968	43940	21262	3216
Antisense vRNAs	2202	2958	4261	54324	1061	742	659	657	893	1899	1743	1374	321
Ratio s/as vRNAs	3.30	2.39	2.16	1.49	5.63	8.12	12.65	15.36	25.13	26.31	25.21	15.47	10.02
% of small RNAs = vRNAs	0.93%	3.69%	1.76%	5.20%	0.11%	0.74%	1.51%	2.60%	3.52%	3.31%	2.96%	2.00%	1.42%

***Ae. albopictus* HT CHIK B2-- / B1+**

	18	19	20	21	22	23	24	25	26	27	28	29	30
Sense vRNAs	2967	4837	17167	352165	5409	1711	1446	1535	2099	4253	4539	3370	1126
Antisense vRNAs	3177	5318	14114	285533	4551	1084	554	421	387	427	466	343	163
Ratio s/as vRNAs	0.93	0.91	1.22	1.23	1.19	1.58	2.61	3.65	5.42	9.96	9.74	9.83	6.91
% of small RNAs = vRNAs	0.68%	3.11%	4.18%	18.53%	0.14%	0.25%	0.41%	0.52%	0.39%	0.32%	0.33%	0.20%	0.17%

***Ae. albopictus* HT CHIK B2-- / B1-**

	18	19	20	21	22	23	24	25	26	27	28	29	30
Sense vRNAs	2900	4581	13989	301109	4685	1925	1837	1904	2807	5148	3546	1614	219
Antisense vRNAs	2540	4536	11043	232666	3322	900	532	492	487	623	392	172	44
Ratio s/as vRNAs	1.14	1.01	1.27	1.29	1.41	2.14	3.45	3.87	5.76	8.26	9.05	9.38	4.98
% of small RNAs = vRNAs	0.51%	3.31%	3.35%	15.74%	0.09%	0.25%	0.44%	0.56%	0.45%	0.34%	0.36%	0.31%	0.32%

Table 4.1. Analysis of the sequenced virus-derived small RNAs

293 CHIK 48hrs (MOI = 0.05)

	18	19	20	21	22	23	24
Sense vRNAs	17939	19597	21208	27695	23341	34486	28229
Antisense vRNAs	12	21	21	165	307	42	20
Ratio s/as vRNAs	1494.92	933.19	1009.90	167.85	76.03	821.10	1411.45
% of sense small RNAs = vRNAs	4.31%	9.57%	10.21%	7.94%	1.17%	3.86%	7.53%
% of antisense small RNAs = vRNAs	0.003%	0.01%	0.01%	0.047%	0.015%	0.005%	0.005%

293 (-) control siRNA CHIK 24hrs (MOI=1)

	18	19	20	21	22	23	24
Sense vRNAs	2346	2372	2431	3125	2205	2973	2876
Antisense vRNAs	13	9	10	39	72	8	9
Ratio s/as vRNAs	180.46	263.56	243.10	80.13	30.63	371.63	319.56
% of sense small RNAs = vRNAs	0.15%	0.12%	0.15%	0.03%	0.05%	0.13%	0.27%
% of antisense small RNAs = vRNAs	0.001%	0.001%	0.001%	0.001%	0.003%	0.002%	0.001%

293 RL siRNA CHIK 24hrs (MOI=1)

	18	19	20	21	22	23	24
Sense vRNAs	1171	1268	1213	1199	1201	1360	1226
Antisense vRNAs	9	13	9	36	81	23	4
Ratio s/as vRNAs	130.11	97.54	134.78	33.31	14.83	59.13	306.5
% of sense small RNAs = vRNAs	0.21%	0.13%	0.27%	0.18%	0.07%	0.19%	0.43%
% of antisense small RNAs = vRNAs	0.001%	0.001%	0.001%	0.002%	0.001%	0.001%	0.001%

MEF CHIK 24hrs (MOI=0.05)

	18	19	20	21	22	23	24
Sense vRNAs	641	683	591	534	597	661	494
Antisense vRNAs	50	63	57	91	149	95	28
Ratio s/as vRNAs	12.82	10.84	10.37	5.87	4.0	6.96	17.64
% of sense small RNAs = vRNAs	0.05%	0.13%	0.04%	0.02%	0.01%	0.02%	0.04%
% of antisense small RNAs = vRNAs	0.004%	0.012%	0.003%	0.004%	0.001%	0.002%	0.002%

MEF *RNase L* -/- CHIK 24hrs (MOI=0.05)

	18	19	20	21	22	23	24
Sense vRNAs	8429	8961	9737	11862	16371	9177	7799
Antisense vRNAs	577	640	1118	3652	4566	920	150
Ratio s/as vRNAs	14.61	14.00	8.71	3.25	3.59	9.98	51.99
% of sense small RNAs = vRNAs	0.49%	1.34%	0.59%	0.39%	0.13%	0.23%	0.45%
% of antisense small RNAs = vRNAs	0.033%	0.095%	0.067%	0.121%	0.035%	0.023%	0.009%

MEF *RNase L* -/- CHIK ΔB2 24hrs (MOI=0.05)

	18	19	20	21	22	23	24
Sense vRNAs	875	962	946	1045	1182	1032	815
Antisense vRNAs	9	18	27	102	148	27	3
Ratio s/as vRNAs	97.22	53.44	35.04	10.25	7.99	38.22	271.67
% of sense small RNAs = vRNAs	0.34%	0.11%	0.12%	0.07%	0.01%	0.05%	0.10%
% of antisense small RNAs = vRNAs	0.003%	0.002%	0.003%	0.007%	0.002%	0.001%	0.0004%

MEF *RNase L* -/- CHIK B2 24hrs (MOI=0.05)

	18	19	20	21	22	23	24
Sense vRNAs	1214	1226	1083	1164	993	1112	920
Antisense vRNAs	7	6	5	18	16	3	6
Ratio s/as vRNAs	173.43	204.33	216.60	64.67	62.06	370.67	153.33
% of sense small RNAs = vRNAs	0.34%	0.10%	0.10%	0.07%	0.01%	0.04%	0.10%
% of antisense small RNAs = vRNAs	0.002%	0.001%	0.001%	0.001%	0.0002%	0.0001%	0.0007%