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RNAi mediated antiviral immunity in Aedes aegypti

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RNAi mediated Antiviral Immunity in Aedes aegypti

by

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[redacted]

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No one navigates his or her graduate career without guidance. I have been fortunate to be educated or assisted by more people than I can count in one sitting. I must thank my mentor Raul Andino. He has always encouraged my curiosity by providing me an environment with incredible colleagues, all of the necessary resources to investigate my thoughts, and discussions that often leave me with new intellectual avenues to venture down. All of this freedom and encouragement of exploration has come at the price of sometimes losing my way, but ultimately, has made me a better scientist.

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RNAi mediated Antiviral Immunity in *Aedes aegypti*

Mark Kunitomi

Abstract

Arboviruses cause an overwhelming number of clinical cases of disease each year around the world. The kinetics of viral replication is of critical importance to the dissemination of virus within the mosquito and ultimately transmission between hosts. Arboviral replication and dissemination is dependent upon their ability to evade the immune response and modulate cell toxicity in two separate hosts. Although there is a tremendous amount of study on the replication of arboviruses in mammalian hosts, there is much less focus on their replication in insects. In mosquitos the siRNA pathway of RNA interference (RNAi) is an indispensible component of the antiviral immune system and a key repressor of viral replication.

In this work, we explore two poorly understood aspects of antiviral RNAi in mosquitoes: systemic dsRNA spread and piRNA mediated immunity. We show that the *Aedes aegypti* cells, Aag2, effectively take up long dsRNA from the extracellular medium to initiate RNAi. Pharmacological and genetic analyses reveal that dsRNA enters the cell via clathrin-mediated endocytosis. Uptake of

exogenous dsRNA directed against Sindbis virus (SINV) inhibits viral replication. However, SINV inhibits RNAi initiated by dsRNA soaking after infection by inhibiting acidification of endosomes. Thus, Sindbis virus may control RNAi antiviral immunity in mosquitoes by suppressing exogenous dsRNA uptake. In addition to its biological role, from a technical point of view the observation that RNAi can be initiated by naked dsRNA in *Aedes aegypti* cells may facilitate studies encompassing a wide variety of biological processes in mosquitoes.

We also show that in infected cells and mosquitos, both virally derived siRNAs (v-siRNAs) and piRNAs (v-piRNAs) are detected in *Aedes aegypti*. Although the piRNA pathway is generally associated with germline defense against selfish genetic elements such as transposons, in *Aedes aegypti* the piRNA pathway mediates antiviral immunity *in vivo* in somatic tissues and characterize the mechanism of v-piRNA biogenesis. We show that both retro-transcription dependent synthesis of viral DNA and Piwi4 are essential for the biogenesis of virally derived small RNAs and that disruption of either causes an increase in viral replication. We propose that the synthesis of DNA from non-retroviral RNA viruses form loci termed Endogenous Viral Elements (EVEs) that are transcribed as piRNA precursors and feed into the ‘Ping-Pong’ mechanism of secondary piRNA synthesis using viral RNA as a target. Our results illustrate a novel somatic function for the piRNA pathway where retro-transcription of viral RNA produces DNA loci that initiate small RNA synthesis to target and degrade RNA viruses. These observations highlight additional complexity to RNAi mediated antiviral immunity in *A. aegypti*.

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Chapter 1:

Introduction

Mosquito transmission of arboviruses such as Dengue Virus (DENV) and Chikungunya Virus (CHIKV) causes widespread and debilitating disease across the globe. Disease in humans can includes severe acute symptoms such as hemorrhagic fever, organ failure, and encephalitis; and yet, mosquitos tolerate high titers of virus in a persistent infection. The mechanisms responsible for tolerance to viral infection in mosquitos are still unclear.

The mosquito vector *Aedes aegypti* is the primary vector of Dengue infection in human populations. Dengue virus infects an estimated 390 million people annually, of which 96 million present symptoms [1]. Infection of *Aedes aegypti* begins in the midgut, following a blood meal from an infected host [2, 3]. From the midgut, the virus disseminates into the hemolymph and infects a wide variety of tissue including the salivary glands[3], from which the virus can be transmitted to the next host during feeding.

The kinetics of viral replication is of critical importance to the dissemination of virus within the mosquito and ultimately transmission between hosts. In mosquitos the siRNA pathway of RNA interference (RNAi) is an indispensible component of the antiviral immune system and a key repressor of viral replication. RNAi clearly plays a cell autonomous antiviral role against arboviral infection in *Aedes aegypti*[4, 5].

RNA interference (RNAi) is a highly conserved mechanism that regulates RNA stability. In plants[6], nematodes[7], and insects[8] RNAi, primarily RNAi mediated by small interfering RNA (siRNA), is a central component of the antiviral immune system.

Generally in insects, the siRNA pathway of RNAi is initiated by long double stranded RNA (dsRNA), which is recognized and cleaved by Dicer-2 into ~21 bp siRNAs[9]. These siRNAs are loaded into Argonaute-2 (Ago2), where one strand is selected to be used as a guide to target complementary single stranded RNAs for degradation[10](Fig. 1). The antiviral RNAi pathway employs long dsRNA viral intermediates of replication to generate siRNAs, which in turn target and degrade the viral genome[8](Fig. 2). This cell autonomous antiviral activity characterizes the primary antiviral immune system in insects[11-13].

However, most multicellular organisms also rely on a systemic response, whereby the initial infected cells elicit a response that can be propagated throughout the organism to block progression of infection. In insects, a systemic RNAi response is essential for effective protection against virus infection[14]. In *Drosophila melanogaster*, the loss of genes required for dsRNA uptake results in hypersensitivity to viral infection characterized by an increase in viral replication and an increase in mortality of infected flies[14]. In *Aedes aegypti* and *Anopheles gambiae* exogenous injected dsRNA inhibits viral replication in a sequence specific manner[11, 12]. How virally derived dsRNA exits an infected cell *in vivo* is unknown; however, it has been demonstrated that a number of insect cell lines and tissues can take up long dsRNA from outside the cell to initiate RNAi[12, 15-17].

The dsRNA uptake pathway has been characterized in *Drosophila melanogaster* S2 cells[15], a hemocyte-like cell line. Clathrin-mediated endocytosis is initiated when extracellular receptors bind to target cargo

molecules. The AP2 complex in conjunction with clathrin and dynamin form a vesicle importing the cargo into the cell[18]. These endosomes mature during intracellular trafficking and are characterized first as early endosomes marked by the presence of Rab5, then as late endosomes marked by Rab7, and ultimately fusing with lysosomes[19]. Endosomes become increasingly acidified along their route by the vacuolar-ATPase (v-ATPase) allowing for the transition from early to late endosomes.

However, it is unclear whether or not RNAi systemic immunity controls viral dissemination in the mosquito, given that dsRNA uptake appears to occur in several tissues *in vivo*[11, 12, 20]. In addition, during infection both virally derived siRNAs (v-siRNAs) and piRNAs (v-piRNAs) are detected signifying additional complexity to antiviral immunity in *Aedes aegypti* mediated by RNAi.

Biogenesis of piRNAs begins in the nucleus with transcription of long single-stranded piRNA precursor RNAs[21](Fig 4). These precursor transcripts undergo a maturation process that includes the binding of a Piwi protein with a bias for association with uridine at the 5’ end (U1), trimming to ~24-30 nt, and 2’-O-methylation of the 3’ end. These antisense primary piRNAs target complementary single stranded RNAs for cleavage by the piwi protein and degradation. This cleavage generates a free 5’ end where Argonatue-3 (Ago3) binds and initiates the maturation of a new sense piRNA that will have a bias for adenosine at the 10th position (A10)[22]. These new piRNAs can then target piRNA precursor transcripts to generate more antisense U1 Piwi bound piRNAs. This ‘Ping-Pong’ mechanism selectively amplifies piRNAs that have

complementary targets. Loaded Piwi proteins also transit to the nucleus where they are thought to define new piRNA clusters in the genome. This complex and poorly understood pathway is primarily utilized to silence selfish genetic elements (primarily transposons) in the germline, but surprisingly, in mosquitos piRNAs have been detected following viral infection[4, 23, 24].

In this dissertation

We examined the ability of *Aedes aegypti* Aag2 cells to take up dsRNA to establish an antiviral response. We used a combination of biochemical, cell biological and genetic approaches to establish that Aag2 cells take up exogenous dsRNA by clathrin-mediated endocytosis. Exogenous dsRNA inhibits virus replication, in a sequence-specific manner, but virus infection by Sindbis virus blocks this dsRNA entry by inhibiting endosome acidification and maturation.

We demonstrate that the piRNA pathway in mosquitos mediates antiviral immunity *in vivo* in somatic tissues and characterize the mechanism of v-piRNA biogenesis. We show that both retro-transcription dependent synthesis of viral DNA and Piwi4 are essential for the biogenesis of virally derived small RNAs and that disruption of either causes an increase in viral replication. We propose that the synthesis of DNA from non-retroviral RNA viruses form loci termed Endogenous Viral Elements (EVEs) that are transcribed as piRNA precursors and feed into the ping-pong mechanism of secondary piRNA synthesis using viral RNA as a target. Our results illustrate a novel somatic function for the piRNA pathway where retro-transcription of viral RNA produces DNA loci that initiate small RNA synthesis to target and degrade RNA viruses.

Taken together, we have characterized aspects of the relationship between RNAi mediated mosquito antiviral immunity and arboviral infection and replication. We show that antiviral RNAi in mosquitoes has similarities to that in

Drosophila melanogaster (systemic RNAi) and also more complex (piRNA mediated immunity).

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Figure 1

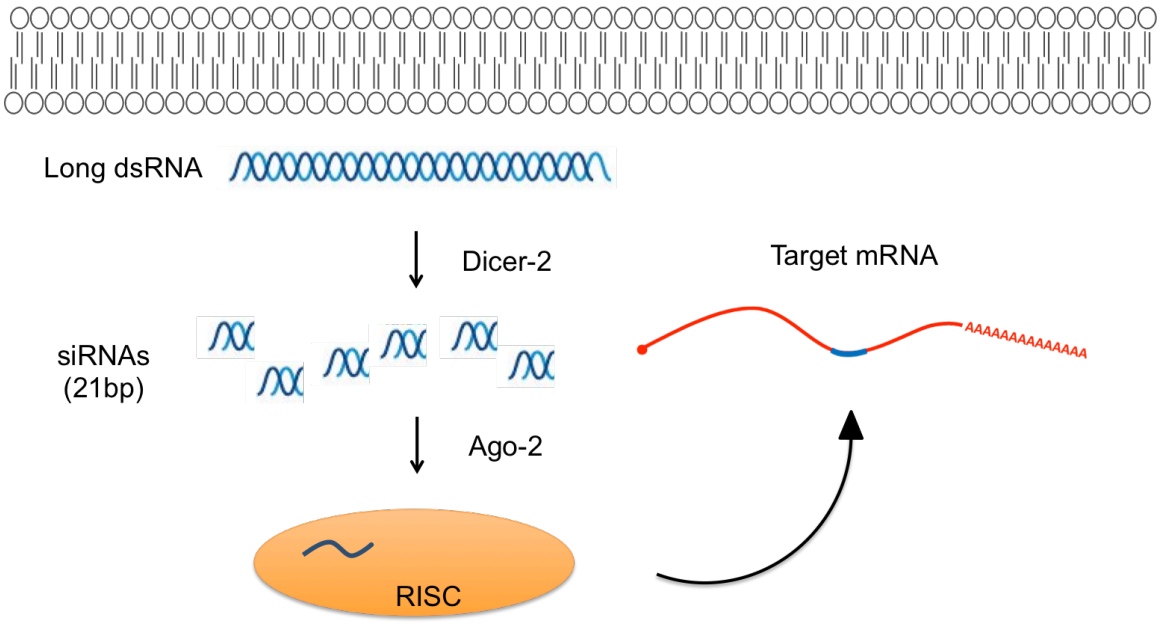


Fig 1 | The siRNA pathway in insects.

In insects, the siRNA pathway of RNAi is initiated by long dsRNA, which is recognized and cleaved by Dicer-2 into ~21 bp siRNAs. These siRNAs are loaded into Ago2, where one strand is selected to be used as a guide to target complementary single stranded RNAs for degradation.

Figure 2

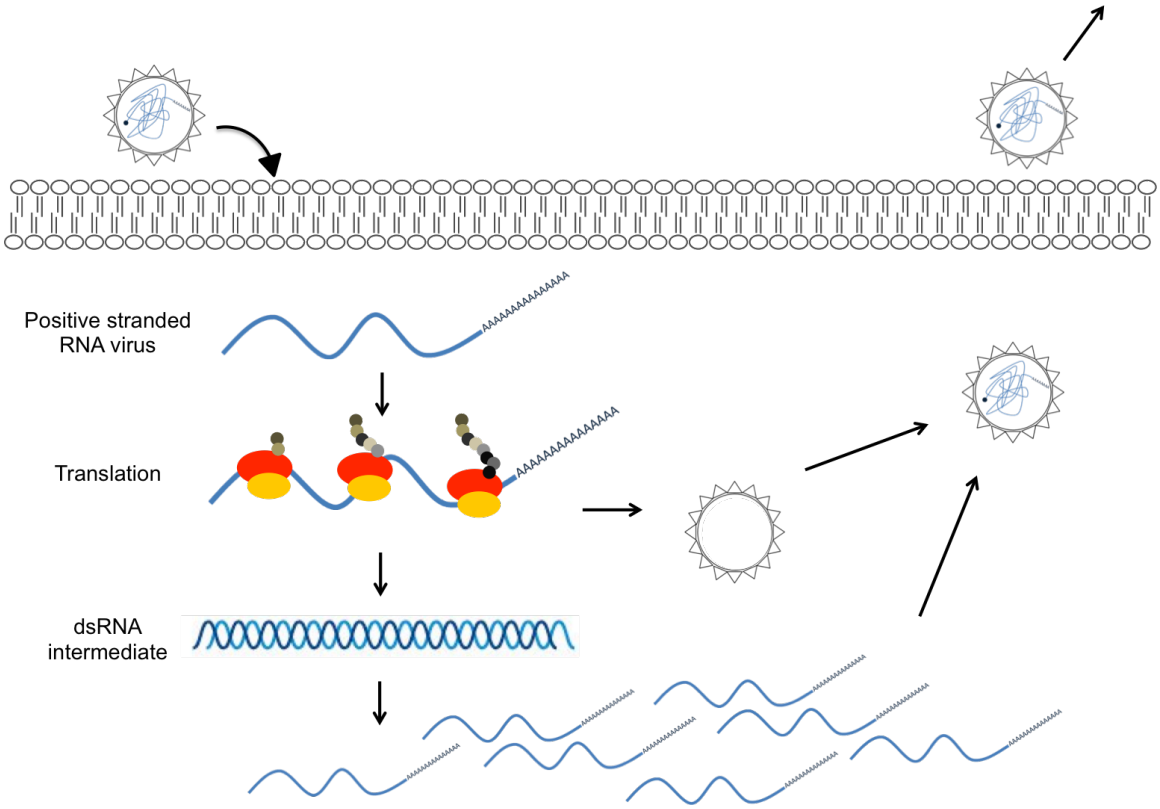


Fig 2 | Generic positive stranded RNA virus life cycle.

The virus enters the cell via receptor-mediated endocytosis. Upon entrance into the cell the viral positive strand RNA is released into the cytoplasm. The RNA is translated via host machinery. During viral replication the positive strand RNA is used as a template to form a complimentary negative strand forming a dsRNA intermediate. The dsRNA intermediate is a molecular signature of non-self to the host. The negative strand is then used as a template to produce positive strand genomes. Newly synthesized positive strand genomes are then packaged into virions for release from the cell.

Figure 3

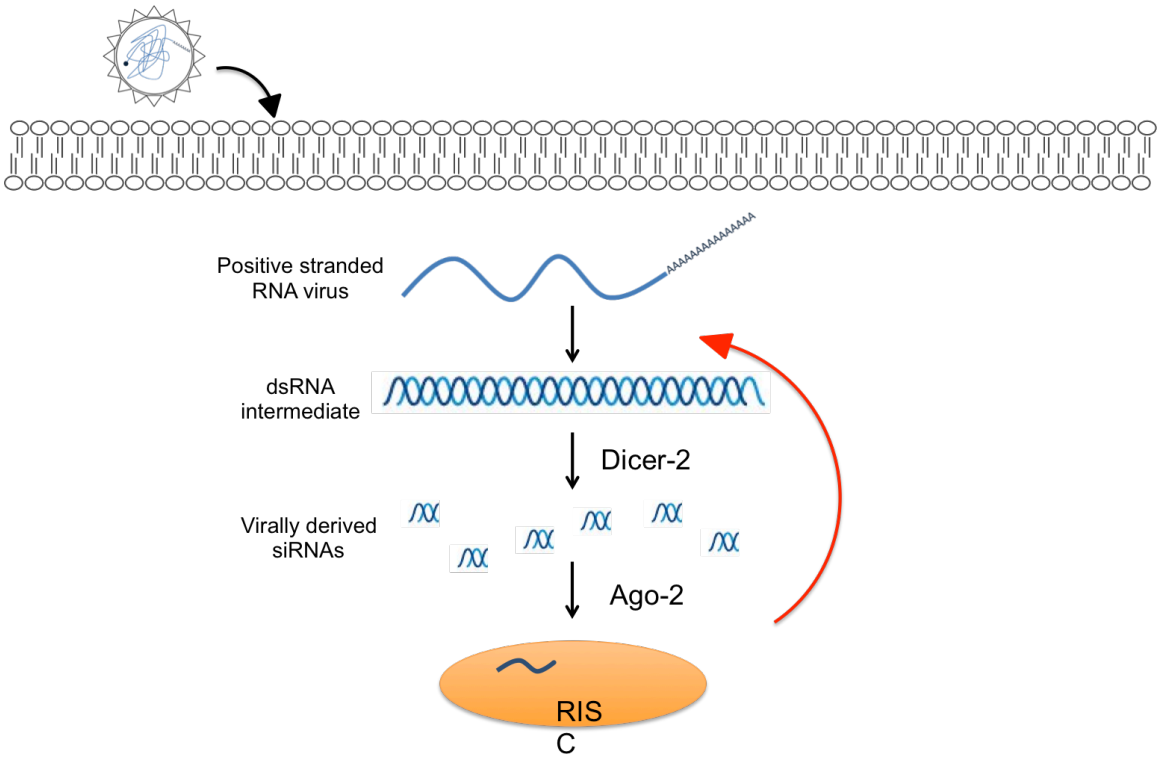
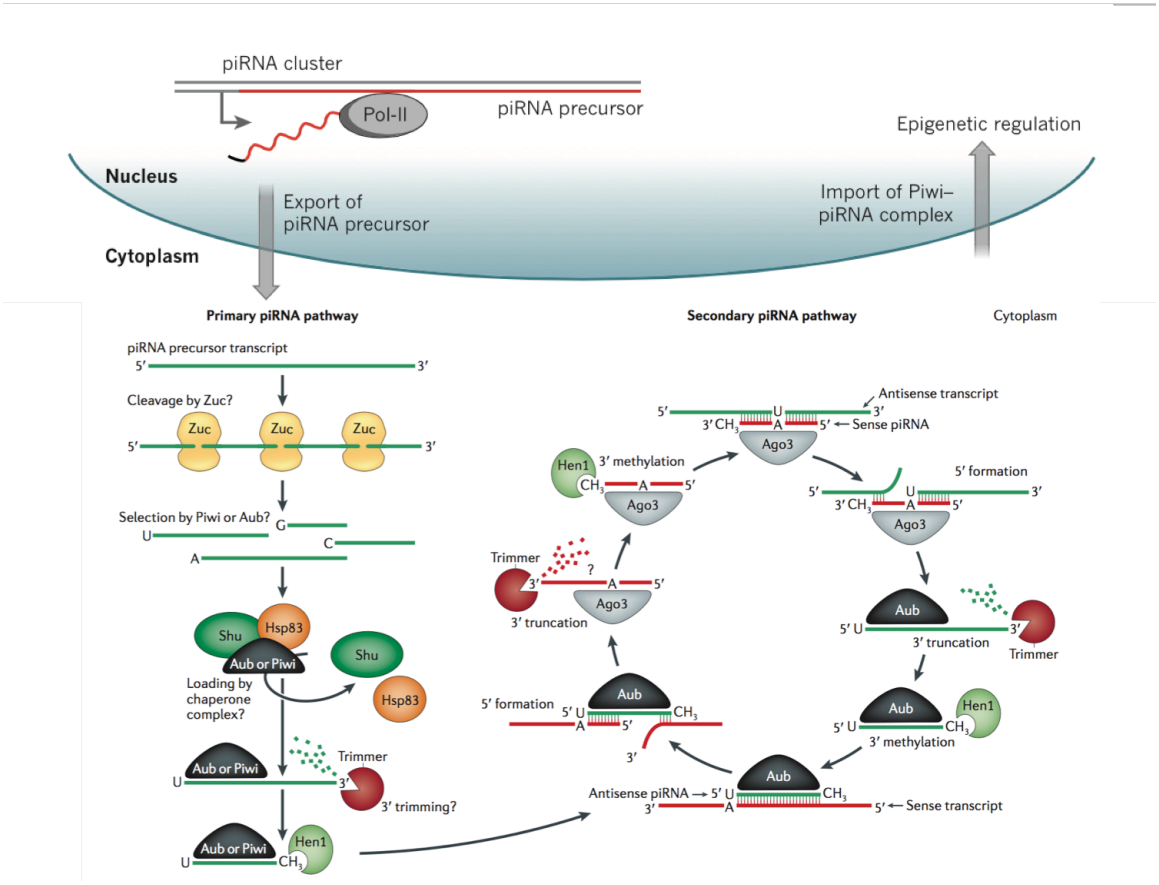


Fig 3 | Antiviral RNAi in insects.

The antiviral RNAi pathway employs long dsRNA viral intermediates of replication to generate virally derived siRNAs (v-siRNAs). These v-siRNAs are loaded into Ago2, where one strand is selectively retained to be used as a guide to target the viral genome for degradation. Thus viral replication is inhibited at two levels, the degradation of both the dsRNA intermediate and genomic RNA.

Figure 4



Adapted from Lutejin 2013

Fig 4 | The piRNA pathway.

Biogenesis of piRNAs begins in the nucleus with transcription of long single-stranded piRNA precursor RNAs. These precursor transcripts undergo a maturation process that includes the binding of a Piwi protein with a bias for association with uridine at the 5' end (U1), trimming to ~24-30 nt, and 2'-O-methylation of the 3' end. These antisense primary piRNAs target complementary single stranded RNAs for cleavage by the piwi protein and degradation. This cleavage generates a free 5' end where Ago3 binds and

initiates the maturation of a new sense piRNA that will have a bias for adenosine at the 10th position (A10). These new piRNAs can then target piRNA precursor transcripts to generate more antisense U1 Piwi bound piRNAs. This ‘Ping-Pong’ mechanism selectively amplifies piRNAs that have complementary targets. Loaded Piwi proteins also transit to the nucleus where they are thought to define new piRNA clusters in the genome.

Chapter 2:

Clathrin mediated endocytosis of dsRNA by *Aedes aegypti* Aag2 cells establishes effective antiviral immunity against infection

ABSTRACT

Uptake of double stranded RNA (dsRNA) is required for effective RNA interference (RNAi) mediated antiviral immunity in *Drosophila melanogaster*. Here, we show that the *Aedes aegypti* cells, Aag2, also effectively take up long dsRNA from the extracellular medium to initiate RNAi. Pharmacological and genetic analyses reveal that dsRNA enters the cell via clathrin-mediated endocytosis. Uptake of exogenous dsRNA directed against Sindbis virus (SINV) inhibits viral replication when dsRNA is introduced prior to and concurrent to infection. However, dsRNA is inefficient when introduced following infection. Sindbis virus infection inhibits RNAi initiated by dsRNA soaking, but not transfected dsRNA, against a luciferase reporter gene. Viral infection prevents dsRNA uptake by inhibiting acidification of endosomes. Thus, Sindbis virus may control RNAi antiviral immunity in mosquitoes by suppressing exogenous dsRNA uptake.

INTRODUCTION

RNA interference (RNAi) is a highly conserved mechanism that regulates RNA stability. In plants[6], nematodes[7], and insects[8] RNAi, primarily RNAi mediated by small interfering RNA (siRNA), is a central component of the antiviral immune system. In insects, the siRNA pathway of RNAi is initiated by long double stranded RNA (dsRNA), which is recognized and cleaved by Dicer-2

into ~21 bp siRNAs[9]. These siRNAs are loaded into Argonaute-2 (Ago2), where one strand is selected to be used as a guide to target complementary single stranded RNAs for degradation[10]. The antiviral RNAi pathway employs long dsRNA viral intermediates of replication to generate siRNAs, which in turn target and degrade the viral genome[8]. This cell autonomous antiviral activity characterizes the primary antiviral immune system in insects[11-13].

Importantly, most multicellular organisms also rely on a systemic response, whereby the initial infected cells elicit a response that can be propagated throughout the organism to block progression of infection. In insects, a systemic RNAi response is essential for effective protection against virus infection[14]. In *Drosophila melanogaster*, the loss of genes required for dsRNA uptake results in hypersensitivity to viral infection characterized by an increase in viral replication and an increase in mortality of infected flies[14]. In *Aedes aegypti* and *Anopheles gambiae* exogenous injected dsRNA inhibits viral replication in a sequence specific manner[11, 12]. How virally derived dsRNA is released from infected cells is unknown; however, a number of insect cell lines and tissues can uptake long dsRNA from outside the cell to initiate RNAi[12, 15-17].

The dsRNA uptake pathway was initially characterized in *Drosophila melanogaster* S2 cells[15], a hemocyte-like cell line. Clathrin-mediated endocytosis is initiated when extracellular receptors bind to target cargo molecules. The AP2 complex in conjunction with clathrin and dynamin form a vesicle importing the cargo into the cell[18]. These endosomes mature during intracellular trafficking and are characterized first as early endosomes marked by

the presence of Rab5, then as late endosomes marked by Rab7, and ultimately fuse with lysosomes[19]. Endosomes become increasingly acidified along their route by the vacuolar-ATPase (v-ATPase) allowing for the transition from early to late endosomes.

The mosquito vector *Aedes aegypti* is the primary vector of Dengue infection in human populations. Dengue virus infects an estimated 390 million people annually, of which 96 million present symptoms [1]. Infection of *Aedes aegypti* begins in the midgut, following a blood meal from an infected host [2, 3]. From the midgut, the virus disseminates into the hemolymph and infects a wide variety of tissue including the salivary glands[3], from which the virus can be transmitted to the next host during feeding. RNAi clearly plays a cell autonomous antiviral role against Dengue virus infection in *Aedes aegypti*[4, 5]. Even though dsRNA uptake appears to occur in several tissues *in vivo*[11, 12, 20], it is unclear whether or not RNAi systemic immunity controls viral dissemination in the mosquito.

Here we examine the ability of *Aedes aegypti* Aag2 cells to take up dsRNA to establish an antiviral response. We used a combination of biochemical, cell biological and genetic approaches to establish that Aag2 cells take up exogenous dsRNA by clathrin-mediated endocytosis. Exogenous dsRNA inhibits virus replication, in a sequence-specific manner, but virus infection by Sindbis virus blocks this dsRNA entry by inhibiting endosome acidification and maturation.

RESULTS

Aag2 cells uptake dsRNA. To determine whether or not Aag2 cells uptake dsRNA, we added dsRNA targeting Firefly luciferase (dsFluc) to the culture media (soaking) for 16 hours prior to transfection with transfection with Fluc and Renilla luciferase (Rluc) expression vectors. Under these conditions, Aag2 cells appear to uptake dsRNA over a wide range of concentrations as Firefly luciferase reporter expression is severely reduced in a dsRNA dose-dependent manner (Fig. 1A).

We next examined the kinetics of dsRNA uptake by incubating Aag2 cells with Fluc dsRNA for various periods of time before the dsRNA was washed off. We observed significant knockdown of the luciferase reporter in cells soaked in dsRNA for as little as 30 minutes before washing with knockdown increasing with increased incubation time (Fig. 1B).

To determine the optimal length of dsRNA for uptake, Aag2 cells were soaked with dsRNAs (500 ng/ml) targeting Firefly luciferase with a range of lengths from 21-600 bp (Fig. 1C). Each dsRNA was also transfected into Aag2 cell (bypassing the need for uptake) to control for their ability to initiate RNAi (Fig. 1D). While transfected dsRNA silences luciferase activity irrespective of length, we observed a correlation between dsRNA length and knockdown efficiency by soaking. Long dsRNA are most effective to silence luciferase activity compared to shorter dsRNAs. siRNAs are unable to silence luciferase following soaking (Fig. 1C). Furthermore, fluorescent dsRNA is rapidly internalized by Aag2 cells

and accumulates in vesicle-like structures (Fig. 1E). These data suggest that Aag2, as previously demonstrated for *Drosophila melanogaster* S2 cells[15], are able to take up exogenous dsRNA to initiate an RNAi response.

Pharmacological inhibition of endocytosis blocks cellular uptake of exogenous dsRNA. In *Drosophila melanogaster* S2 cells, the endocytic pathway mediates dsRNA uptake[15]. To assess the role of the endocytic pathway in Aag2 dsRNA uptake we inhibited the endocytic pathway with two specific inhibitors. Bafillomycin-A1 (Baf-A1) and Dynasore were tested for their effect on RNAi silencing initiated by dsRNA soaking.

We examined the effect of preventing acidification of the endosome by inhibiting v-ATPase. Aag2 cells were pre-treated for 1 hour with Baf-A1, a drug that inhibits maturation of endosomes by targeting v-ATPase. These cells were then soaked with Fluc dsRNA for 30 minutes. Following dsRNA soaking, dsRNA was washed off and replaced with fresh media with Baf-A1 (Fig. 3A). Baf-A1 treatment significantly decreases silencing of the Firefly luciferase reporter (Fig. 3B). These results are consistent with the proposed mechanism of action of Baf-A1, as the drug should not prevent entry of dsRNA cargo into endosomes; but rather, prevent the maturation of these endosomes and cargo release.

We also tested the effect of inhibiting vesicle formation at the plasma membrane. Aag2 cells were treated with Dynasore, a drug that inhibits dynamin, a key factor of endocytic vesicle formation at the plasma membrane, 1 hour prior to Fluc dsRNA soaking. Following treatment, both the drug and dsRNA were

washed off. Dynasore treatment results in a small but statistically significant reduction in silencing (Fig. 3C). It is possible that inhibition of dynamin does not reduce dsRNA uptake completely because dsRNA may remain bound to the cell surface receptor and following removal of the drug dsRNA could be internalized. We were unable to continue treatment with Dynasore because prolong incubations with Dynasore are toxic to Aag2 cells.

Genetic analysis of dsRNA uptake. Our pharmacological studies suggest that the endocytic pathway is necessary for dsRNA uptake in Aag2 cells. In order to validate this assay, we targeted Ago2 to silence the siRNA pathway of RNAi. Soaking of Aag2 cells with dsRNA targeting Ago2 reduced Ago2 mRNA by ~90% relative to control dsRNA treatment (Fig. 2B). Aag2 cells depleted of Ago2 mRNA are significantly impaired in their ability to silence firefly luciferase activity over several concentrations of dsRNA (Fig 2C). Thus, depletion of the RNAi machinery utilizing the RNAi machinery can be assessed by this assay. To confirm and extend these results, we down regulated the expression of a number of candidate genes encoding *Aedes aegypti* proteins involved in clathrin-mediated endocytosis. We tested multiple genes known to form complexes required for clathrin-mediated endocytosis such as the AP2 complex and the HOPs complex (VPS33, 18, 41, and 11). Aag2 cells were initially soaked in long dsRNA to knock down each candidate gene. Following a three-day incubation to allow for mRNA knockdown and protein turnover we added dsRNA targeting Firefly luciferase or control dsRNA (targeting eGFP) for 6 hours. The dsRNA was

then washed off and the cells were transfected with Firefly and Renilla luciferase expression vectors. For each candidate dsRNA treatment Firefly activity was normalized by Renilla and then the dsFluc condition was normalized to the control treatment.

As a positive control, we targeted Ago2 to silence the siRNA pathway of RNAi. Soaking of Aag2 cells with dsRNA targeting Ago2 reduced Ago2 mRNA by ~90% relative to control dsRNA treatment (Fig. 3D). Pre-treatment with dsRNA targeting many of these genes results in significant suppression of RNAi induced by dsRNA soaking including each member of the two complexes targeted (Fig. 3D). Additionally, knockdown of the v-ATPase also significantly reduced dsRNA uptake initiated RNAi in a similar manner to its pharmacological inhibition by Baf-A1. Knockdown of genes that are involved in vesicular transport outside of with the endocytic pathway, such as the AP1 complex, had no effect on dsRNA uptake and initiation of RNAi (Fig. 3D). Knockdown of LAMP1 also did not affect dsRNA uptake initiated RNAi, indicating that the dsRNA exits endosomes prior to fusion with the lysosome (Fig. 3D). Our analysis indicates that dsRNA enters into the cells by a clathrin-mediated endocytosis. As a control, we transfected Fluc dsRNA directly into the cells (bypassing the uptake machinery). Firefly luciferase reporter activity was unaffected by knockdown of these genes when dsRNA is transfected into cells indicating that they are solely required for dsRNA uptake (Fig, 3E). In contrast, in cells treated with Ago2 dsRNA there is a significant suppression of RNAi initiated both by soaking and transfection of the dsRNA (Fig. 3D,E).

Pre-treatment of Aag2 cells with dsRNA directed against viruses. Uptake of dsRNA plays a critical role in systemic immunity in *Drosophila melanogaster*[14]. Both *Drosophila melanogaster* and *Aedes aegypti* pre-injected with dsRNA targeting a virus show reduced viral replication during infection[11, 12, 14].

To test the hypothesis that pre-treatment of cells with dsRNA can protect from viral infection, we soaked Aag2 cells with dsRNA (500 ng/ml) targeting the region of Sindbis virus (SINV) that encodes nsp4 for 72 hours prior to infection with a recombinant SINV encoding eGFP[25]. Sindbis virus is a member of the *Alphavirus* subfamily of *Togaviridae*, a virus family characterized as having positive sense, non-segmented, single stranded genomes[26]. High concentration of dsRNA reduces viral replication to undetectable levels as shown by microscopy and western blot (Fig. 4A and B). This indicates that dsRNA uptake mediated vaccination results in a uniform phenotype as all of the cells treated were protected. We next utilized a Cell Imager platform (Cellinsight) to analyze the number of cells infected (GFP positive cells) and magnitude of infection (GFP intensity per infected cell) in Aag2 cells pretreated with Sindbis dsRNA. Treatment with dsRNA decreased the number of infected cells in a dose dependent manner (Fig. 4C). In addition, of the cells infected (GFP positive), we observed a decrease in the average intensity of GFP per infected cell (Fig. 4D).

We further determined whether the protective effect of dsRNA treatment could be overcome by the multiplicity of infection. Aag2 cells treated with 500 ng/ml of SINV dsRNA for 72 hours were infected with SINV (Fig. 5A) at a MOI of

either 0.1 (Fig 5B) or 10 (Fig 5C). The MOI of infection did not greatly affect the effect on virus replications as both infections were significantly inhibited by several orders of magnitude.

To ensure that is a general phenomenon, we soaked cells with dsRNA directed against either SINV or DENV, infected with either virus, and then quantified percentage knockdown of genome relative to control dsRNA. Pre-treatment with dsRNA against either virus severely reduced the genome copy number (Fig. 6). We also determined whether external dsRNA treatment could reduce infection of persistent viral infections on endogenous viruses. Aag2 cells are persistently infected with Cell fusing agent virus (CFAV), and presumably the innate antiviral RNAi machinery controls this virus. Strikingly, treatment of Aag2 cells with dsRNA targeting the region encoding NS4 and NS5 of CFAV reduces viral genome RNA levels by ~70% (Fig. 6). Our data show that treatment with exogenous dsRNA targeting a viral genomic RNA can reduce viral replication when introduced both before and after infection.

Suppression of dsRNA uptake by Sindbis virus infection. We next examined the effect dsRNA treatment on inhibition of viral replication over time. We infected Aag2 cells with SINV and soaked the cells in SINV dsRNA either at the time of infection or at several time points after infection (Fig. 7A). SINV replication was effectively inhibited when treated with cognate dsRNA concurrent to infection (Fig 7A), but at time points later in the infection, inhibition by dsRNA soaking is

severely reduced (Fig 7A). In contrast Dengue virus and Cell fusing agent virus (CFAV) were unable to block exogenous dsRNA initiated silencing.

To test the effect of SINV on RNAi initiated by dsRNA soaking, we infected Aag2 cells with SINV and soaked the cells with dsRNA targeting Fluc either at the time of infection or at several time points after infection (Fig. 7B). Silencing of the Fluc reporter was inhibited by SINV infection starting at 1 dpi and this inhibition increased throughout the time course. In contrast, if the dsRNA uptake pathway is bypassed by transfecting dsRNA directly into the cell, SINV has no effect on Fluc silencing (Fig. 7B). This suggests that SINV infection inhibits uptake of dsRNA without affecting the core RNAi function.

To further analyze the mechanism by which SINV infection the suppresses RNAi silencing we soaked Aag2 cells infected with SINV with dextran labeled by Tetramethyl Rhodamine and Fluorocein to monitor endocytosis and endosome acidification[27] (Fig. 7C,D). As acidity increases, FITC signal relative to Rhodamine signal decreases (Fig. 7C). Pretreatment with Baf-A1 inhibits endosome acidification and, therefore, increases FITC/Rhodamine signal ratio (Fig. 7D). Infection with SINV also increases FITC/Rhodamine signal, indicating that SINV inhibits endosome acidification and, consequently, maturation and trafficking.

DISCUSSION

We have used a combination of molecular biology and pharmacological techniques to show that Aag2 cells utilize clathrin-mediated endocytosis to take up long dsRNA and initiate RNAi and have further characterized the kinetics and efficiency of RNAi initiated by long dsRNA. Double-stranded RNA enters the cell at the plasma membrane in a clathrin, dynamin, and AP2 complex dependent manner. Transport of dsRNA requires the early endosome (rab5), the v-ATPase complex, and the HOPs complex. The requirement for these genes strongly implicates a classical mode of clathrin-mediated endocytosis as the means to transport long dsRNA into the cell. The requirements for late endosome and v-ATPase components indicate that dsRNA is not released until late in the endocytic pathway (see scheme in Fig. 7C). A similar uptake mechanism was previously identified in *Drosophila melanogaster*[15], and it seems that this process is evolutionarily conserved suggesting that dsRNA uptake is central to efficient antiviral defense in insects.

Aag2 cells can uptake dsRNA to protect these cells from viral infection. Soaking Aag2 cells with dsRNA targeting Sindbis-eGFP virus prior to infection significantly reduces eGFP signal compared to controls and suggest a physiological effect of dsRNA uptake. In addition, our data suggest that uptake of dsRNA targeting a virus concurrent to or after infection can alter the balance of this infection in Aag2 cells by reducing the level of viral genomic RNA copies. We conclude that the effect of dsRNA treatment post infection depends upon the

timing of treatment relative to the rate of viral replication.

While our study demonstrates that mosquito cells, like Aag2, can utilize clathrin-mediated endocytosis to take up naked dsRNA from the extracellular milieu, there are still many questions that remain in relation to the role of RNAi initiated by dsRNA uptake and systemic spread of RNAi in vivo. It is not known if or how virally derived dsRNA can exit infected cells *in vivo* to initiate spread of RNAi. The observations of dsRNA uptake in mosquitos and that mosquito cell lines rarely display cell lysis following viral infection[28] leads us to speculate that there must be an active mechanism for dsRNA release that acts in concert with dsRNA uptake.

RNAi is a major component of the antiviral immune system in the mosquito as it is in other insects as well as plants and worms. However, many viruses that infect either insects or plants (or both) encode suppressors of RNAi[29-31]. Thus, the relationship between RNAi and viral RNAi suppressors exerts selective pressure upon both host and virus. The result of this selective pressure leads to reciprocal adaptation in the form of an evolutionary ‘arms race’. In mosquitos, this selective pressure has led to ‘rapid, positive, diversifying selection’ of RNAi genes[32]. How do arboviruses withstand this selective pressure? There are indications of RNAi suppressors encoded by Arboviruses[33, 34]; however, these suppressors have yet to be examined using live virus. It is generally accepted that *Alphaviruses*, such as SINV, do not encode RNAi suppressors. Our results suggest that, in contrast to the general accepted view, SINV infection effectively suppress RNAi by blocking dsRNA

uptake. Thus, SINV may control antiviral immunity by inhibiting systemic but not cell autonomous RNAi. This observation is akin to inhibition of innate immunity in vertebrates by blocking the signaling in the interferon pathway[35].

Our study on dsRNA uptake and antiviral immunity in Aag2 cells in conjunction with the observation that injection of naked dsRNA can inhibit viral replication *in vivo* in *Aedes aegypti* point to a similar form of systemic RNAi as in *Drosophila melanogaster*. Formal proof that dsRNA uptake is necessary for antiviral immunity in adult *Aedes aegypti* and identification of the tissues that participate in this immunity warrants additional studies to forward our understanding of antiviral immunity in *Aedes aegypti*.

MATERIALS AND METHODS

Cells culture and viral propagation. *Aedes aegypti* Aag2 cells were cultured at 28 °C without CO_2 in Schneider's *Drosophila* medium (GIBCO-Invitrogen), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1X non-essential amino acids (NEAA, UCSF Cell Culture Facility, 100X stock is 0.1 μM filtered, 10 mM each of Glycine, L-Alanine, L-Asparagine, L-Aspartic acid, L-Glutamic Acid, L-Proline, and L-Serine in de-ionized water), and 1X Penicillin-Streptomycin-Glutamine (Pen/Strep/G, 100X = 10,000 units of penicillin, 10,000 μg of streptomycin, and 29.2 mg/ml of L-glutamine, Gibco).

Human Huh7 and African green monkey Vero cells were cultured at 37 °C with 5% CO_2 in Minimum Essential Media with 10% heat-inactivated FBS, 1X NEAA, 1X sodium pyruvate (100X = 100 mM, Gibco), 1X Pen/Strep/G.

Sindbis viral stocks were produced by infecting Vero cells at low MOI (below 1) in MEM with 2% heat-inactivated FBS. After CPE was observed (~72 hours post infection), the supernatant centrifuged at 3,000 g for 10 min, passed through a .45 μm filter, supplemented with 10% glycerol, flash frozen, and stored at -80 °C.

Dengue viral stocks (Dengue-2 Jamaica 1409) were produced as described above except that Huh7 cells were used for propagation and the viral stock was supplemented with 20% FBS instead of glycerol.

Virus titration. Virus was titrated by plaque assay by infecting confluent monolayers of Vero cells with serial dilutions of virus.

Cells were incubated under an agarose layer for 2 to 3 days at 37°C before being fixed in 2% formaldehyde and stained with crystal violet solution (0.2% crystal violet, and 20% ethanol).
Viral titers were calculated, taking into account plaque numbers and the dilution factor.

dsRNA preparation. PCR utilizing primers including the T7 RNA polymerase promoter were used to amplify *in vitro* templates for RNA synthesis using Phusion polymerase (NEB). Manufacturer's recommendations were used for the

concentrations of all reagents in the PCR. Primers were synthesized by *Integrated DNA Technologies*, Inc. (IDT). The thermocycling protocol is as follows: 98°C 2:00, (98°C 0:15, 65°C 0:15, 72°C 0:45, these three cycles were repeated 10X with a lowering of the anneal temperature by 1°C per cycle), (98°C 0:15, 60°C 0:15, 72°C 0:45, these three steps were repeated 30X), 72°C 2:00. For a 100 µl in vitro transcription reaction (IVT) the composition was as follows: 30 µl of PCR product, 20 µl 5X IVT buffer (400 mM Hepes, 120 mM $MgCl_2$, 10 mM Spermidine, 200 mM DTT), 16 µl 25 mM rNTPs, and 1 unit of T7 RNA polymerase (made in-house). IVT reaction was incubated at 37°C for 3-6 hours and then 1 µl of DNase-I (NEB) was added and the reaction was further incubated at 37°C for 30 min. The RNA was purified by phenol-chloroform-isoamyl alcohol followed by isopropanol precipitation.

Products were quantified using a Nanodrop (Thermo Scientific) and analyzed by agarose gel electrophoresis to ensure integrity and correct size.

dsRNA soaking. Monolayers of Aag2 cells were seeded into 96-well plates (40,000 cells per well) in 100 µl of complete media (Schneider's *Drosophila* medium, 10% FBS, 1X NEAA, and 1X Pen/Strep/G) and allowed to attach overnight. Prior to dsRNA soaking, cells were washed once with Phosphate Buffered Solution w/o calcium or magnesium (dPBS, 0.1 µM filtered, 0.2g/L KH_2PO_4, 2.16g/L Na_2HPO_4, 0.2g/L KCl, 8.0g/L NaCl). Cells were soaked in dsRNA in minimal media (Schneider's *Drosophila* medium, 0.5% FBS, 1%

NEAA, and 1% Pen/Strep/G) for the time indicated by the experiment. All incubations were performed at 28 °C without CO_2.

Luciferase assays. Cells were soaked in dsRNA for the indicated period of time using the dsRNA soaking method above. Prior to transfection, cells were washed 3X with dPBS, and then put into complete media. Cells were transfected with plasmids encoding Firefly (pAc Fluc) and Renilla luciferase (pUb Rluc) with Effectene (Qiagen) using the manufacturer's instructions with the following modification: 200 ng pAc Fluc and 50 ng pUb Rluc were used per transfection with a ratio of 1 µl effectene / 250 ng plasmid DNA. Firefly and *Renilla luciferase* sequences from the plasmids pGL3 and pRL-CMV (Promega) were cloned into pAc/V5-HisB (Invitrogen) and pIEX pUb ([36]) respectively.

24 Hours post transfection, cells were lysed in 50 µl passive lysis buffer (Promega), and Firefly and Renilla luciferase activity was determined from 10 µl of lysate using a dual luciferase reporter assay system using the manufacturer's instructions (Promega) and analyzed on an Ultra-evolution plate reader (Tecan) using an integration time of 100 ms.

Pharmacological inhibition of the endocytic pathway was performed as above except Bafilomycin A1 (Baf-A1) or Dynasore (Sigma Aldrich) was added at the concentration indicated to the culture media 1 hour prior to dsRNA treatment.

The genetic analysis of dsRNA uptake candidates was performed as above with the following exceptions: 30,000 cells were seeded per well, cells were treated with the initial dsRNA (targeting the candidate gene) for 72 hours,

washed 3X with dPBS, before the addition of the secondary dsRNA (targeting the reporter).

qPCR Analysis. Total RNA was extracted using TRIzol (Life Technologies). cDNA synthesis was performed using the iScript cDNA synthesis kit (Bio-Rad). Specific genes or viral genomes were analyzed using SYBR green methods on a CFX-Connect (Bio-Rad). All genes / viruses tested for relative quantitation were normalized to RP49 expression. Relative quantitation was calculated by the $2^{-\Delta\Delta CT}$ method[37]. Absolute quantitation was calculated using a standard of known quantity.

Western blot. Cells were washed twice with ice-cold dPBS and then lysed in NP-40 buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.5% NP40) for 30 min at 4 °C and then centrifuged at max speed (16,000 rcf) for 15 minutes. The supernatant was mixed in equal volume with 2X loading buffer (Bio-Rad).

Lysates were run on a 12% polyacrylamide-SDS gel in running buffer (30 mM Tris, 200 mM glycine, and 0.1% SDS) at 200 V for 45 min.

Proteins were electrophoretically transferred to an Immobilon-P^{SQ} PVDF membrane (Millipore) for 1 h at 100 V in transfer buffer (30 mM Tris, 200 mM glycine, and 20% methanol).

Membranes were blocked for 1 h at room temperature in Tris Buffered Saline with Tween (TBST, 400 mM Tris, 1.4 mM NaCl, and 0.1% Tween-20, pH 7.6) with 5% non-fat milk and then probed with either anti-eGFP or anti-Actin (Santa

Cruz Biotechnology) primary antibodies in TBST with 5% non-fat milk followed by HRP conjugated anti-rabbit secondary antibody (GE Healthcare) in TBST with 2.5% non-fat milk.

Microscopy. Monolayers of Aag2 cells were seeded into poly-lysine treated 96-well glass bottom plates (40,000 cells per well) in 100 µl of complete media and allowed to attach overnight. Prior to infection, cells were washed with dPBS, infected with SINV for 1 hour in minimal media, washed 3X with dPBS, and then put into minimal media. Cells were incubated with 2.5 mg/ml TMR-FITC-Dextran for 30 min in minimal media, washed 3X with dPBS, and put into complete media. Four hour after Dextran treatment, images of cells were taken using a Leica fluorescent microscope using Volocity software (PerkinElmer).

Statistical analysis. Values were expressed as the means +/- standard deviation, and statistical analysis was performed with Microsoft Excel using an unpaired, two-tailed Student's t-test to determine significance. Differences were considered significant at $P < 0.01$.

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Figure 1

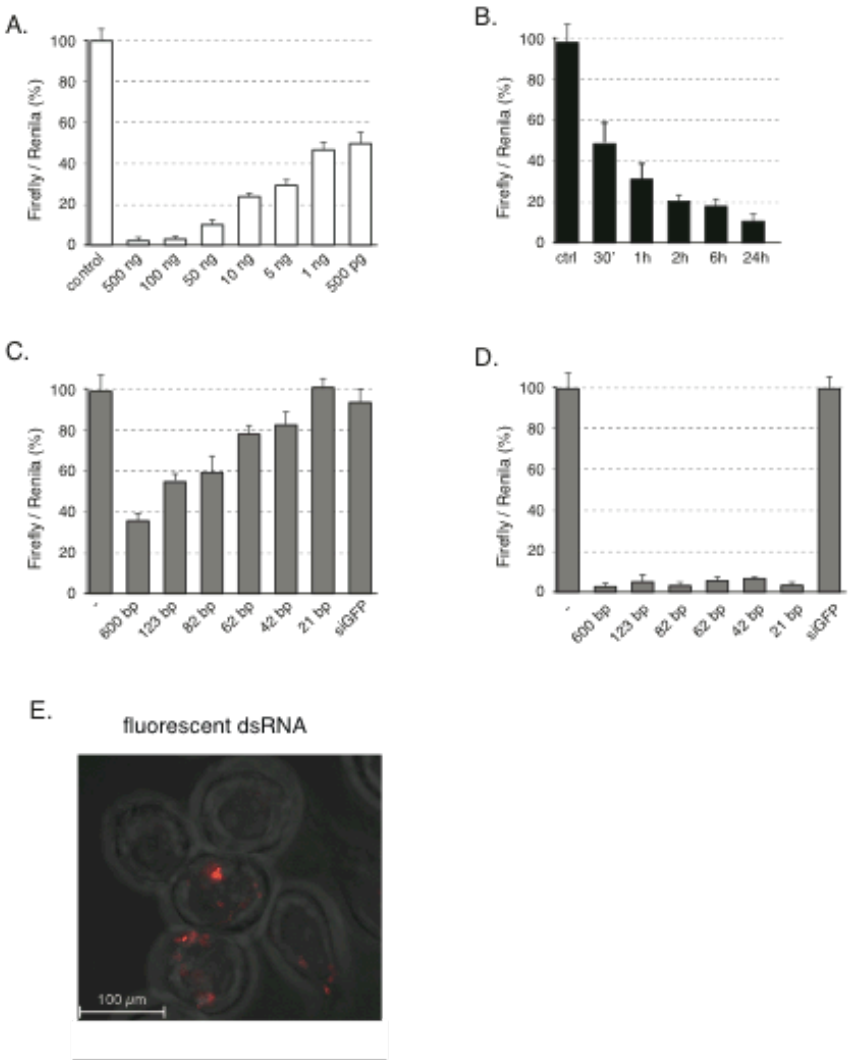

Fig. 1 | Characterization of dsRNA uptake in Aag2 cells.

Silencing of Firefly luciferase expression by dsRNA soaking is concentration dependent. (A) Aag2 cells were soaked in decreasing concentrations (ng/mL) of dsRNA overnight, washed, and then transfected with Firefly and Renilla expression plasmids. Luciferase activity was measured after 16 hours and is expressed as percentage relative to dsRNA control condition of Firefly / Renilla

values. (B) Time course comparing silencing of *luciferase* after soaking of Aag2 cells in 500 ng/ml of dsRNA. The experiment was performed as described in (A), but dsRNA was washed away after the indicated incubation times. (C, D) Silencing of Firefly luciferase expression after exposure of Aag2 cells to dsRNA either by transfection (1 ng/well) (C) or by soaking (500 ng/ml) (D). Luciferase activity after treatment (soaking or transfection overnight) with specific dsRNA of different sizes was compared with treatment with a non-specific control dsRNA. (E) Aag2 cells internalize Cy3-labelled dsRNA. 1 hour after incubation the dsRNA is internalized but remains in small punctate structures. Optical sections were deconvolved and flattened into a two-dimensional projection for presentation. For all the above experiments, results represent the average and standard deviation of 6 replicates.

Figure 2

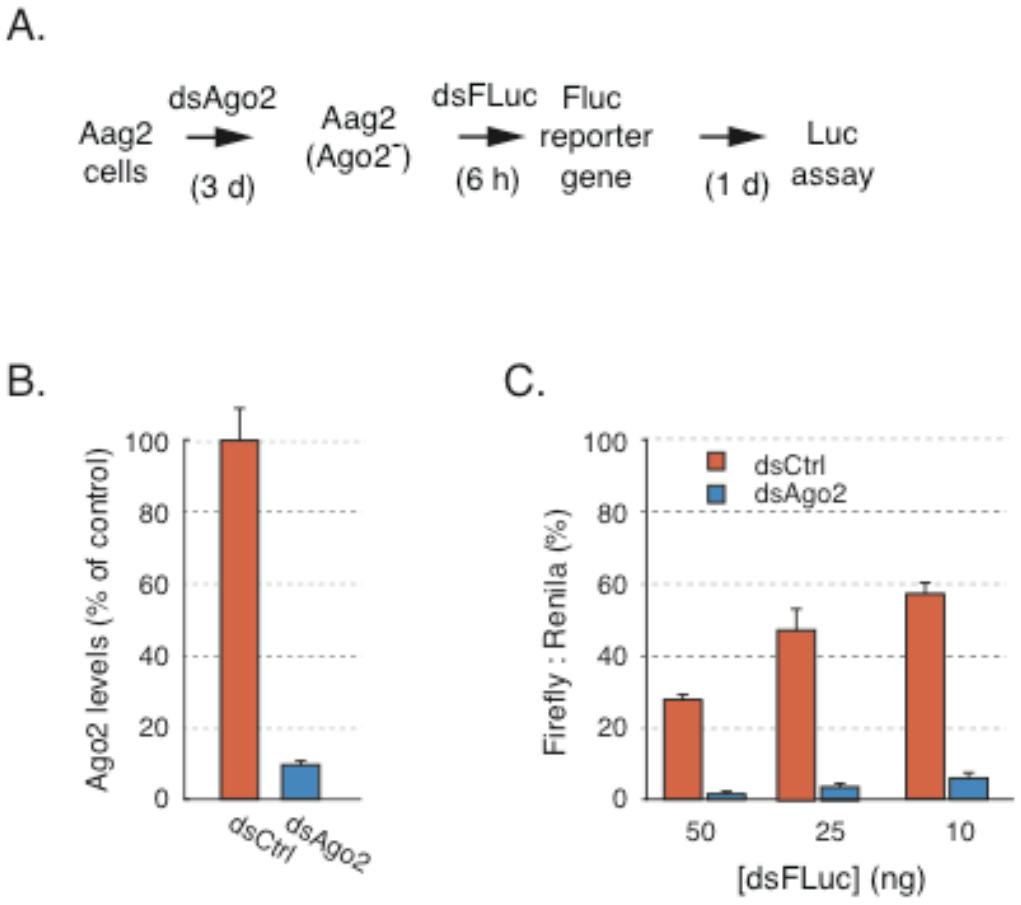

Fig. 2 | Validation of genetic screen assay in Aag2 cells.

(A) Schematic description of experimental design. (B) Targeting Ago2 by dsRNA soaking reduces Ago2 mRNA. Aag2 cells were soaked in Ago2 dsRNA for 3 days before RNA extraction and RT-qPCR. Ago2 expression is shown as the mean of relative quantitation normalized to RP49. The control dsRNA treatment is set at 100%. (C) Aag2 cells depleted of Ago2 in A display a reduced ability to implement RNAi. Following soaking with 500 ng/ml of Ago2 dsRNA, Aag2 cells were soaked in 50 ng/ml of Fluc dsRNA, washed, and then transfected with

Firefly and Renilla expression plasmids or co-transfected with dsRNA and expression plasmids. . Luciferase activity was measured after 24 hours and is expressed as percentage relative to dsRNA control condition of Firefly / Renilla values. For all the above experiments, results represent the average and standard deviation of 6 replicates.

Figure 3

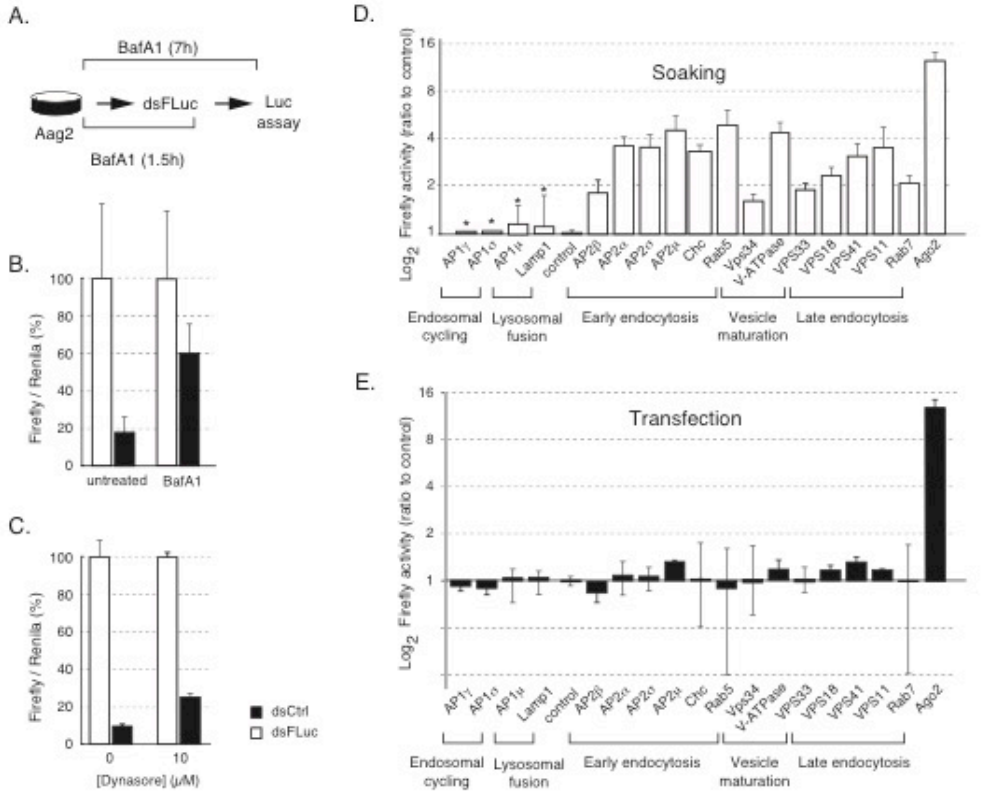

Fig. 3 | Pharmacological and genetic analysis of the dsRNA uptake pathway.

(A) Schematic description of experimental design. (B) Inhibition of v-ATPase by Baf-A1 inhibits silencing of Firefly luciferase by dsRNA soaking. Aag2 cells were incubated with Baf-A1 (200 μM) for 1 hour prior to addition of dsRNA. Following a 30-minute incubation with 500 ng/ml of Fluc dsRNA, dsRNA was washed off and replaced with fresh complete media with Baf-A1 and then transfected with Firefly and Renilla expression plasmids. Luciferase activity was measured after 16 hours and is expressed as percentage relative to dsRNA control condition of Firefly/Renilla values. (C) Inhibition of dynamin inhibits silencing of Firefly luciferase by dsRNA soaking. Aag2 cells were incubated with Dynasore for 1

hour prior to addition of dsRNA. Following a 30-minute incubation, drug and dsRNA were washed off and replaced with fresh complete media, and transfected with Firefly and Renilla expression plasmids. Luciferase activity was measured 16 hours after transfection and is expressed as Firefly:Renilla ratios normalized to the non-specific control dsRNA treatment. Components of clathrin-mediated endocytosis and vesicular transport were depleted by dsRNA soaking for 3 days (500 ng/ml). Following depletion, Aag2 cells were either soaked in 50 ng/ml of Fluc dsRNA for 6 hours, washed, and then transfected with Firefly and Renilla expression plasmids (D) or co-transfected with 1 ng of Fluc dsRNA and expression plasmids (E). For all the above experiments, results represent the average and standard deviation of 6 replicates. All conditions show statistically significant differences ($p < 0.01$, T-test) from the control unless marked by an asterisk.

Figure 4

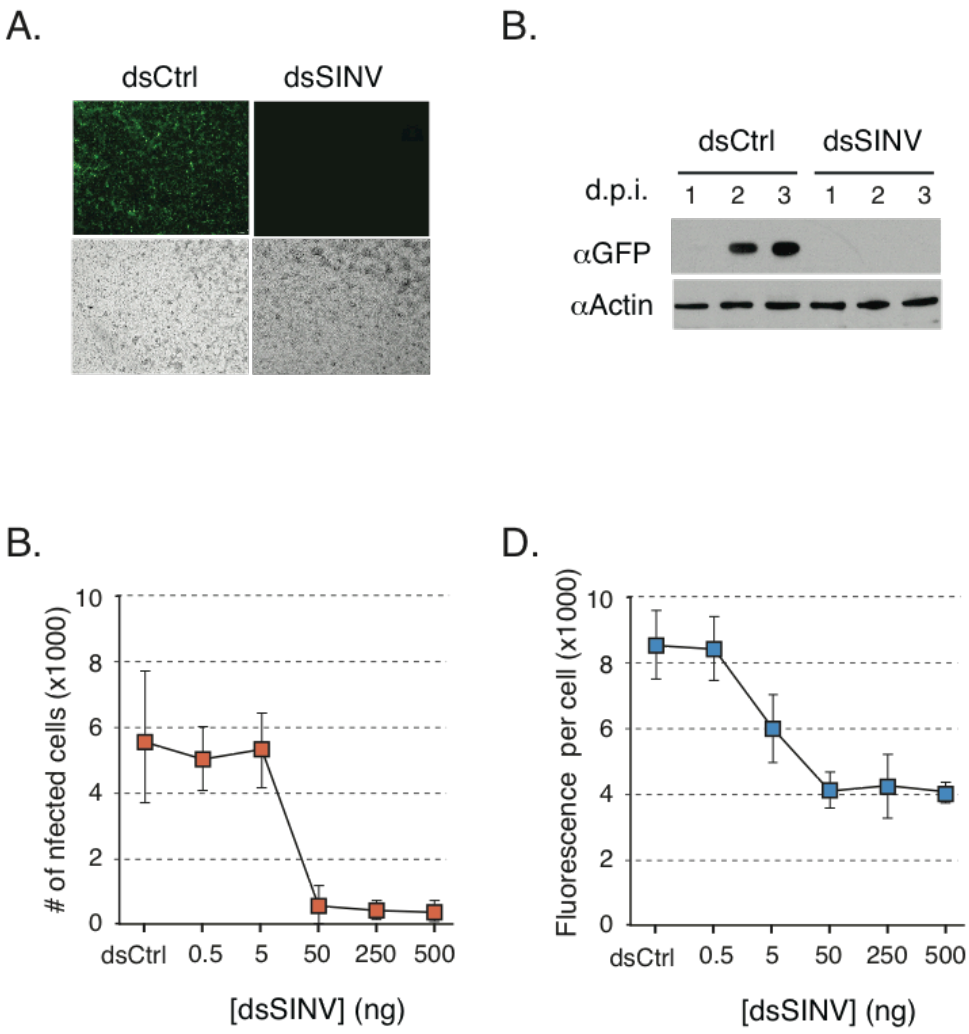

Fig 4. | Vaccination of Aag2 cells by long dsRNA.

Aag2 cells were soaked in 500 ng/ml of dsRNA targeting SINV for 3 days and then infected with SINV-eGFP at an MOI of 0.1. Three days post infection eGFP expression was observed by live cell microscopy (A, i and ii) and total cells by bright field (A, iii and iv). The accumulation of eGFP over time was observed by western blot (B). The effect of dsRNA concentration on SINV-eGFP infection was tested by soaking Aag2 cells in decreasing concentrations of dsRNA targeting

SINV for 3 days and then infected with SINV-eGFP at an MOI of 0.1. Number of infected cells and mean intensity of eGFP / infected cell was measured by CellInsight at 3 days post infection. CellInsight results represent the mean and standard deviation of 6 replicates.

Figure 5

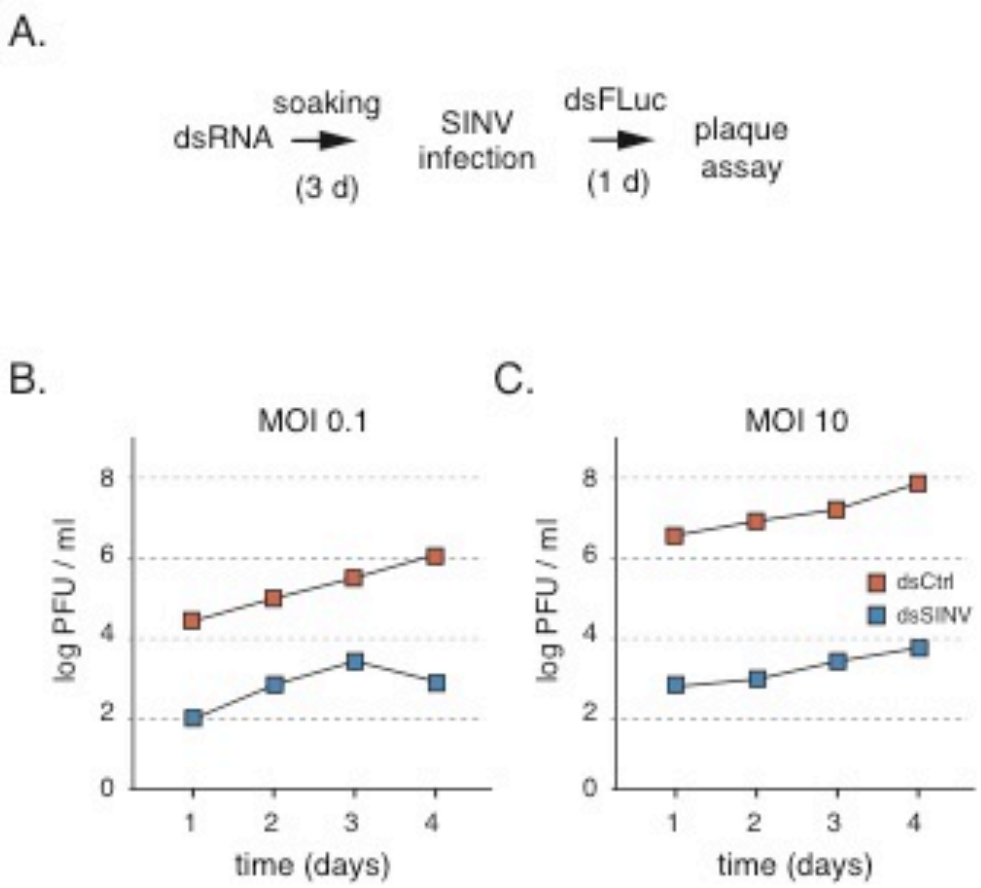

Fig. 5 | Vaccination of Aag2 at multiple MOIs.

(A) Schematic description of experimental design. Aag2 cells were soaked in 500 ng/ml dsRNA targeting SINV for 3 days and then infected at an MOI of 0.1 (B) or 10 (C). Viral titer was quantified by plaque assay. For all the above experiments, results represent the average and standard deviation of 4 replicates.

Figure 6

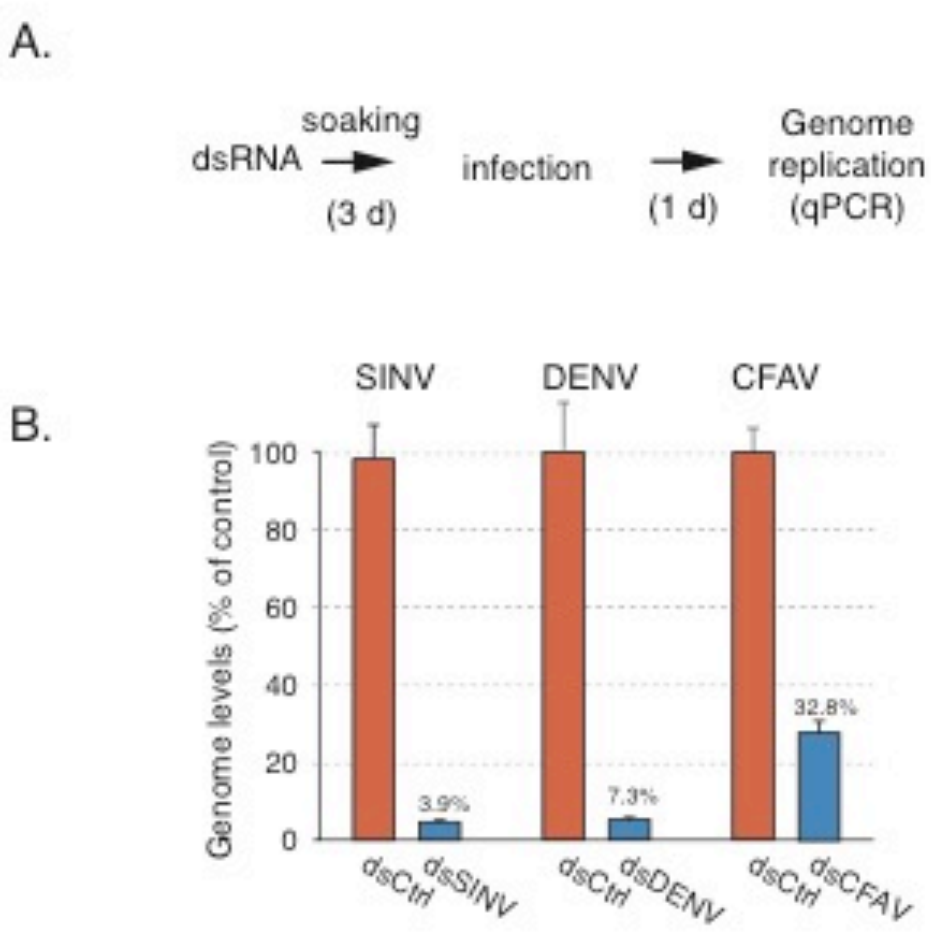

Fig. 6 | dsRNA vaccination of Aag2 cells inhibits a range of Arboviruses. (A) Schematic description of experimental design. (B) Aag2 cells were soaked in 500 ng/ml of dsRNA targeting the indicated virus for 3 days then infected at an MOI of 0.1. Virus was measured by qPCR 3 days after dsRNA treatment. For all the above experiments, results represent the average and standard deviation of 6 replicates.

Figure 7

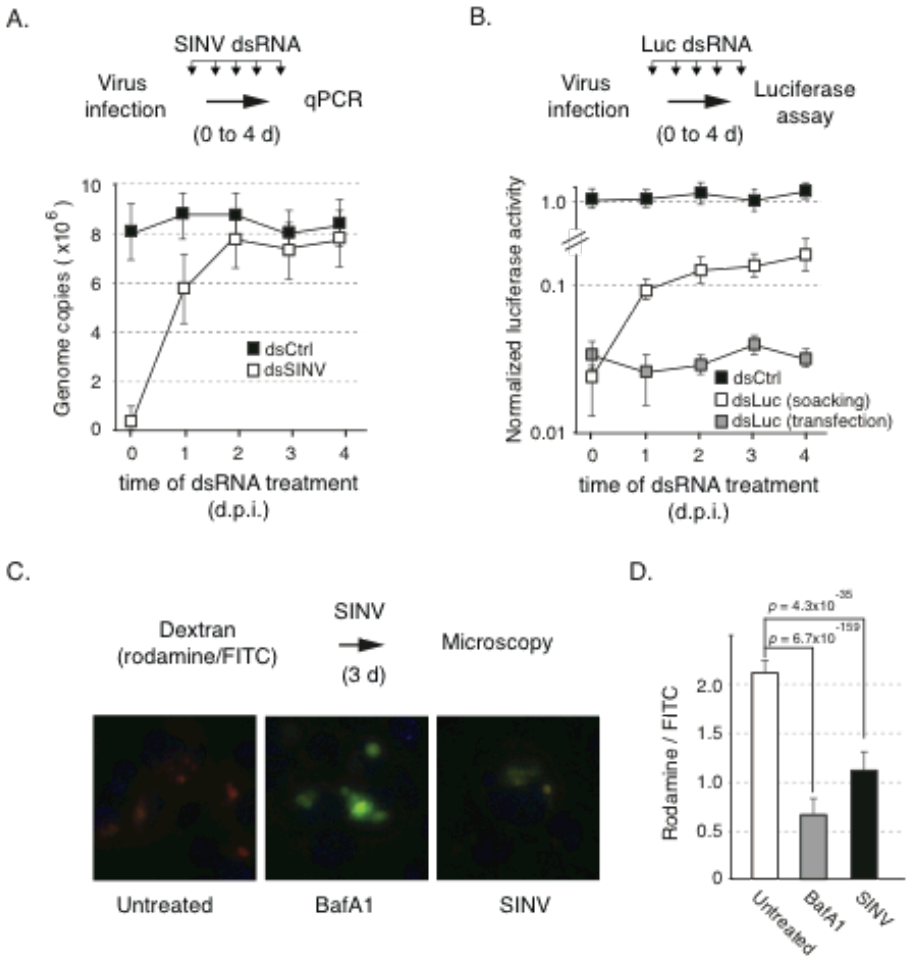

Fig. 7 | Suppression of dsRNA uptake by Sindbis virus infection.

(A) Aag2 cells were infected with SINV at an MOI of 0.1 concurrent to or following the addition of 500 ng/ml of dsRNA targeting SINV. Virus was measured by qPCR 1 day after dsRNA treatment. (B) Aag2 cells were infected with SINV at an MOI of 0.1 concurrent to or following the addition of 500 ng/ml of dsRNA targeting Fluc. Following a 6 hr incubation dsRNA was washed off and replaced with fresh complete media, and transfected with Firefly and Renilla expression plasmids. Luciferase activity was measured 1 day after dsRNA treatment. (C) Representative images of Aag2 cells incubated with dextran

doubly conjugated with FITC and TMR 3 days after infection with SINV (MOI 0.1) or 1 hour after Baf-A1. (D) Ratio-metric comparison of Rhodamine/FITC signal in ROIs for the experiment in (C). ROIs were determined using Volocity software based on a minimum Rhodamine intensity. For the qPCR and luciferase experiments above, results represent the average and standard deviation of 6 replicates. At least 200 ROIs for each sample

Chapter 3:

Identification of components of the antiviral piRNA pathway in Aedes aegypti

Abstract

Mosquito transmission of arboviruses such as Dengue Virus (DENV) causes widespread and debilitating disease across the globe[1]. The kinetics of viral replication is of critical importance to the dissemination of virus within the mosquito and ultimately transmission between hosts[4]. In mosquitos the siRNA pathway of RNA interference (RNAi) is an indispensible component of the antiviral immune system and a key repressor of viral replication[38]. However, during infection both virally derived siRNAs (v-siRNAs) and piRNAs (v-piRNAs) are detected signifying additional complexity to antiviral immunity in *Aedes aegypti* mediated by RNAi[23, 39]. Although the piRNA pathway is generally associated with germline defense against selfish genetic elements such as transposons[21], in *Aedes aegypti* the Piwi family of genes has expanded[32]. This observation in conjunction with the detection of v-piRNAs suggests a diversification of function within the Piwi family in *Aedes aegypti*. Here we demonstrate that the piRNA pathway in mosquitos mediates antiviral immunity *in vivo* in somatic tissues and characterize the mechanism of v-piRNA biogenesis. We show that retro-transcription dependent synthesis of viral DNA and that Piwi4 is essential for the biogenesis of virally derived small RNAs and that disruption of either causes an increase in viral replication. We propose that the synthesis of DNA from non-retroviral RNA viruses form loci termed Endogenous Viral Elements (EVEs) that are transcribed as piRNA precursors and feed into the 'Ping-Pong' mechanism of secondary piRNA synthesis using viral RNA as a

target. Our results illustrate a novel somatic function for the piRNA pathway where retro-transcription of viral RNA produces DNA loci that initiate small RNA synthesis to target and degrade RNA viruses.

Introduction

Biogenesis of piRNAs begins in the nucleus with transcription of long single-stranded piRNA precursor RNAs. These precursor transcripts undergo a maturation process that includes the binding of a Piwi protein with a bias for association with uridine at the 5' end (U1), trimming to ~24-30 nt, and 2'-O-methylation of the 3' end. These antisense primary piRNAs target complementary single stranded RNAs for cleavage by the piwi protein and degradation. This cleavage generates a free 5' end where Argonatue-3 (Ago3) binds and initiates the maturation of a new sense piRNA that will have a bias for adenosine at the 10th position (A10). These new piRNAs can then target piRNA precursor transcripts to generate more antisense U1 Piwi bound piRNAs. This 'Ping-Pong' mechanism selectively amplifies piRNAs that have complementary targets. Loaded Piwi proteins also transit to the nucleus where they are thought to define new piRNA clusters in the genome. This complex and poorly understood pathway is primarily utilized to silence selfish genetic elements (primarily transposons) in the germline, but surprisingly, in mosquitos piRNAs have been detected following viral infection[24].

In this work we identify and characterize a key player in v-piRNA biogenesis, Piwi4, and show that it is a viral restriction factor both in cell culture and somatically *in vivo*. Surprisingly, v-piRNA biogenesis is initiated by the formation of viral DNA that serves as a template for transcription. Once primary piRNAs mature, they enter the ping-pong cycle where they target the viral genome. Interestingly, v-piRNA abundance correlates with viral RNA abundance with a bias towards the 5' end of both the sub-genomic and genomic RNAs.

Results

Identification of Piwi4 as a viral restriction factor. On the basis of the observation of virally derived piRNAs both in mosquito cell culture[23, 39] and somatically *in vivo*[24], we hypothesized that the piRNA pathway acts as a somatic antiviral immune system in *A. aegypti*. To determine the ability of the piRNA pathway to restrict viral replication, we screened the 7 *A. aegypti* piwi orthologues in addition to the single Ago3 orthologue as well as orthologues to known piRNA biogenesis factors for antiviral activity by depleting Aag2 cells of these genes and infecting with Sindbis virus encoding eGFP (Fig. 1a). Of the genes screened, several displayed significant increases in eGFP expression including Ago3 and Piwi4. Piwi4 knockdown displayed a phenotype similar to that of Ago2 (Fig. 1a).

Piwi4 and Ago3 are viral restriction factors. In order to confirm that Piwi4 and Ago3 represent bona fide viral restriction factors, we proceeded to individually test the effect of their depletion on viral replication of Sindbis virus by one-step growth curves. Following gene knockdown, cells were infected with WT SINV and viral replication was measured by qPCR (Fig. 1c) and plaque assay (Fig. 1b). Knockdown of Ago2 results in a substantial increase in Sindbis replication, validating its role as major antiviral restriction factor, whereas Piw4 knockdown results in an intermediate increase and Ago3 results in a mild increase of viral replication. Knockdown of Piwi4 and Ago3 showed similar effects on Dengue replication as with Sindbis virus.

Piwi4 and Ago3 are necessary for virally derived piRNAs. Previous studies in *Aedes aegypti* and *Aedes albopictus* have shown that virally derived piRNAs accumulate following infection. In order to determine the relationship between Piwi4, Ago3, and virally derived piRNAs, we depleted Aag2 cells of these genes and infected them with either SINV. Following a four-day infection, we harvested the small RNA (~18-35 nt) for deep sequencing. Analysis of these small RNA libraries shows that, similar to previous results, small RNAs bearing the hallmark signatures of both siRNAs and piRNAs map to the SINV genome (Fig. 2a). The virally derived siRNAs (v-siRNAs) are 21 nt in length and map in proportionately equivalent numbers to both the sense and antisense strand of the genome without any discernable bias for sequence (Fig. 2a,b). The virally derived piRNAs (v-piRNAs) are ~24-30 nt in length. Both v-siRNAs and v-piRNAs are beta-

elimination resistant indicating that they are methylated at 3' end and mature small RNAs.

In contrast to the v-siRNAs, these v-piRNAs are asymmetrically distributed across the genome with many 'hot spots' of high abundance and a strong bias towards the sense strand. The asymetric distribution of v-piRNAs strongly correlates with relative abundance of the viral RNA during SINV infection (+ strand sgRNA > +strand genome > - strand genome). Sequence analysis reveals that the antisense v-piRNAs have a strong bias for a uracil at the 1st position and sense v-piRNAs have a strong bias for an adenosine at the 10th position(Fig. 2g). In addition, read distribution overlap analysis shows that a large proportion of the v-piRNAs between the sense and antisense strands overlap by 10 nt (Fig. 2h). These two pieces of evidence indicate that the majority of v-piRNA biogenesis occurs by the classic ping-pong mechanism.

Both abundance and diversity of mature v-piRNAs is significantly decreased when either Ago3 or Piwi4 are depleted by dsRNA knockdown (Fig. 2a compare Fig. 2b,c,d). Thus piRNAs are derived from Sindbis virus during infection and these v-piRNAs are dependent upon both Ago3 and Piwi4 for their biogenesis. Knockdown of Piwi4 also causes a depletion of mature siRNA. This indicates that the siRNA and piRNA pathway are in some way intertwined in *Aedes aegypti* and that Piwi4 acts as a hub to coordinate this relationship.

To confirm that the dependence of v-piRNA biogenesis and stability is Piwi4 is due to their association, we purified a Piwi4-FLAG construct and

sequenced the associated small RNAs. Indeed, we find enrichment for v-piRNAs associated with Piwi4 (Fig. 2e,f).

Piwi4 and AZT. Although it is clear that v-piRNA biogenesis involves the ping-pong pathway, the initiation of this pathway classically begins in the nucleus with the transcription of primary piRNA precursors from DNA loci defined as piRNA clusters. Coincidentally, integration of DNA derived from non-retroviral RNA viruses has been identified in a wide variety of animal genomes. In *Drosophila melanogaster* S2 cells treated with AZT, viral DNA synthesis was inhibited and the majority of v-siRNAs were depleted. These observations led us to hypothesize that the biogenesis of these small RNAs was dependent on the formation of loci of virally derived DNA.

To test this hypothesis we first identified viral DNA formation in Aag2 cells infected with SINV. In agreement with previous studies, reverse-transcriptase (RT) inhibitor AZT counteracts the synthesis of viral DNA (Fig. 3a). In addition, the RT-inhibitor Stavudine (d4T) inhibits the synthesis of viral DNA (Fig. 3a). Treatment with AZT not only inhibits the formation, but it also causes an increase in viral replication of SINV (Fig. 3b). Analysis of virally derived small RNAs shows that both AZT and d4T inhibit biogenesis of v-piRNAs, but not effect on v-siRNAs (Fig. 3c). Neither RT-inhibitor has an effect on either the siRNA or piRNA pathway, as transposon-derived small RNAs (which come from DNA loci formed long before drug treatment) are unaffected. These results suggest that initiation

of the bulk of primary v-piRNAs are synthesized from DNA loci as opposed to being appropriated from viral RNA.

Piwi4 expression is up-regulated by blood meal. On the basis of our cell culture experiments, we proceeded to investigate the function of Piwi4 *in vivo* (Fig. 4). Analysis of several studies examining transcriptional changes in response to blood feeding reveal that Piwi4 expression is up-regulated under these conditions in multiple stains of *Aedes aegypti*[40-42]. However, generally the piRNA pathway is active exclusively in the germline and in *Aedes aegypti* the ovaries are transcriptionally activated following blood meal.

To determine if Piwi4 is expressed and/or induced in tissues that are relevant to arboviral infection and dissemination, we extracted RNA and performed RT-qPCR on dissected ovaries, midguts, and remaining carcasses of Chetmul strain female mosquitos fed a blood or sugar meal (Fig. 4).

Piwi4 mRNA is increased in both the midgut and carcass 2 days after blood meal (Fig. 5a). In contrast to previous organism-wide analysis, we observe no change in Piwi4 transcript abundance in the ovary post blood meal (Fig. 5a). This confirmation of Piwi4 induction outside of the ovary is suggestive of additional functions of Piwi4 beyond germ line defense *in vivo*.

Piwi4 is antiviral *in vivo*. Previous studies have shown that injection of dsRNA into adult female mosquitos can initiate RNAi and that knockdown of known antiviral restriction factors results in increased viral replication[4, 11, 12].

In order to examine the ability of injected dsRNA to knock down Piwi4 mRNA, we extracted RNA and performed RT-qPCR on dissected ovaries, midguts, and remaining carcasses of female mosquitos injected with dsRNA targeting either Piwi4 or control dsRNA and fed a blood meal. Treatment with dsRNA effectively knocks down Piwi4 mRNA levels in both the midgut and the remaining carcass from 3 days post injection until 14 days post injection.

To determine if the antiviral activity of Piwi4 in cell culture is recapitulated *in vivo*, *Aedes aegypti* females were depleted of Piwi4 by dsRNA injection and then infected with Dengue virus by blood meal. Viral replication was quantified by plaque assays of virus from whole mosquitos and in a tissue specific manner by qPCR (Fig. 4).

Dengue genomic RNA is significantly increased in both the midgut and carcass at days 7 and 10 days post infection (Fig. 5b), whereas we detect little viral RNA in the ovaries with no effect of dsRNA treatment on Piwi4 expression or genomic viral RNA. Additionally, injection of Piwi4 dsRNA causes an increase in infectious virus titers in whole females at 7 and 10 days post infection (Fig. 5b). Overall these results confirm our initial observations in cell culture that Piwi4 is an antiviral restriction factor. In addition to Piwi4's induction by blood meal

Discussion

These finding represent the first observations that piRNA pathway can restrict viral replication in somatic tissues *in vivo*, a function that is a major

departure from previously identified functions of this pathway. We show that piRNA biogenesis is dependent upon reverse-transcription of viral RNA and identify the core components of the piRNA pathway that are required for this process.

Surprisingly, the piRNA pathway acts antivirally in *Aedes aegypti* without any significant alteration as compared to *Drosophila melanogaster* or even *Mus musculus*. Biogenesis of primary v-piRNAs begins at DNA loci and are processed in a typical fashion into ~24-30 nt single stranded, 2'-O-methylated, and U1 biased small RNAs. These piRNAs enter the 'Ping-Pong' cycle when they encounter single stranded viral RNA genomes producing complementary A10 piRNAs.

Several species of mosquito, including *Aedes aegypti*, have an expanded set of piwi genes compared to many of the model species studied. It is tempting to speculate that this expansion has led to additional roles for the piRNA pathway in these species. Indeed, virally derived piRNAs are also observed in *Aedes albopictus* and *Culex quinquefasciatus*.

MATERIALS AND METHODS

Cells culture and viral propagation. *Aedes aegypti* Aag2 cells were cultured at 28 °C without CO_2 in Schneider's *Drosophila* medium (GIBCO-Invitrogen), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% non-essential amino acids (NEAA, UCSF Cell Culture Facility, 0.1 µM filtered, 10 mM each of Glycine, L-Alanine, L-Asparagine, L-Aspartic acid, L-Glutamic Acid, L-Proline, and L-Serine in de-ionized water), and 1% Penicillin-Streptomycin-Glutamine (Pen/Strep/G, 10,000 units of penicillin, 10,000 µg of streptomycin, and 29.2 mg/ml of L-glutamine, Gibco).

Human Huh7 and African green monkey Vero cells were cultured at 37 °C with 5% CO_2 in Minimum Essential Media with 10% heat-inactivated FBS, 1X NEAA, 1% sodium pyruvate (Gibco,100 mM), 1% Pen/Strep/G.

Sindbis viral stocks were produced by infecting Vero cells at low MOI (below 1) in MEM with 2% heat-inactivated FBS. After CPE was observed (~72 hours post infection), the supernatant centrifuged at 3,000 g for 10 min, passed through a .45 µm filter, supplemented with 10% glycerol, flash frozen, and stored at -80 °C.

Dengue viral stocks were produced as described above except that Huh7 cells were used for propagation and the viral stock was supplemented with 20% FBS instead of glycerol.

Virus titration. Virus was titrated by plaque assay by infecting confluent monolayers of Vero cells with serial dilutions of virus.

Cells were incubated under an agarose layer for 2 to 3 days at 37°C before being fixed in 2% formaldehyde and stained with crystal violet solution (0.2% crystal violet, and 20% ethanol).

Viral titers were calculated, taking into account plaque numbers and the dilution factor.

dsRNA preparation. PCR utilizing primers including the T7 RNA polymerase promoter were used to amplify *in vitro* templates for RNA synthesis using Phusion polymerase (NEB). Manufacturer's recommendations were used for the concentrations of all reagents in the PCR. Primers were synthesized by *Integrated DNA Technologies*, Inc. (IDT). The thermocycling protocol is as follows: 98°C 2:00, (98°C 0:15, 65°C 0:15, 72°C 0:45, these three cycles were repeated 10X with a lowering of the anneal temperature by 1°C per cycle), (98°C 0:15, 60°C 0:15, 72°C 0:45, these three steps were repeated 30X), 72°C 2:00. For a 100 µl IVT reaction the composition was as follows: 30 µl of PCR product, 20 µl 5X IVT buffer (400 mM Hepes, 120 mM $MgCl_2$, 10 mM Spermidine, 200 mM DTT), 16 µl 25 mM rNTPs, and 1 unit of T7 RNA polymerase. IVT reaction was incubated @ 37°C for 3-6 hours and then 1 µl of DNase-I (NEB) was added and the reaction was further incubated at 37°C for 30 min. The RNA was purified by phenol-chloroform-isoamyl alcohol followed by isopropanol precipitation.

Products were quantified using a Nanodrop (Thermo Scientific) and analyzed by agarose gel electrophoresis to ensure integrity and correct size.

dsRNA soaking. Monolayers of Aag2 cells were seeded into 96-well plates (40,000 cells per well) in 100 µl of complete media (Schneider's *Drosophila* medium, 10% FBS, 1X NEAA, and 1X Pen/Strep/G) and allowed to attach overnight. Prior to dsRNA soaking, cells were washed once with Phosphate Buffered Solution w/o calcium or magnesium (dPBS, 0.1 µM filtered, 0.2g/L KH_2PO_4, 2.16g/L Na_2HPO_4, 0.2g/L KCl, 8.0g/L NaCl). Cells were soaked in dsRNA in minimal media (Schneider's *Drosophila* medium, 0.5% FBS, 1% NEAA, and 1% Pen/Strep/G) for the time indicated by the experiment. All incubations were performed at 28 °C without CO_2.

qPCR Analysis. Total RNA was extracted using TRIzol (Life Technologies). cDNA synthesis was performed using the iScript cDNA synthesis kit (Bio-Rad). Primers for qRT-PCR were obtained from IDT and are listed in the supplementary material.

Specific genes or viral genomes were analyzed using SYBR green methods on a CFX-Connect (Bio-Rad). All genes / viruses tested for relative quantitation were normalized to RP49 expression. Relative quantitation was calculated by the $2^{-\Delta\Delta CT}$ method. Absolute quantitation was calculated using a standard of known quantity.

Statistical analysis. Values were expressed as the means +/- standard deviation or Standard Error of the Mean as indicated, and statistical analysis was performed with Microsoft Excel using an unpaired, one-tailed Student's t-test or

one-tailed Mann–Whitney U test to determine significance.

Mosquito rearing and infection with arboviruses. Ae. aegypti Chetmul mosquito eggs were hatched from an egg liner (containing 10,000–100,000 eggs) in 150ml of deionized, autoclaved water. Larvae were transferred to a large rearing pan, collected as pupae 7–9 days later, transferred to an emergence container within a cage and maintained in the insectary at 28uC, 82% relative humidity until adult mosquitoes were harvested. Groups of 200 one-week-old adult females were placed in 2.5 liter cartons, deprived of sugar source overnight and allowed to feed on artificial blood meals consisting of virus-infected C6/36 cell suspension (60% vol/vol), 40% (vol/vol) defibrinated sheep blood (Colorado Serum Co., Boulder, CO) and 1 mM ATP. Virus titers were usually 106–107 pfu/ml for DENV2 [48]. The artificial blood meal was prewarmed to 37uC and then pipetted into water-jacketed glass feeders covered with a hog gut membrane and maintained at a constant temperature of 37uC. Feeders were placed onto the net covering the cartons to allow females to feed through the hog gut membrane for 1 h. Fed females were selected, put into new cartons, provided with water and sugar and maintained in the insectary for analysis.

Analysis of infected mosquitos. Following blood meal at selected time points mosquitos were dissected and RNA was extracted from tissues with TRIzol for RT-qPCR analysis. For plaques assays mosquitos were homogenized in PBS to extract virus.

Affinity purification 5 x 10^6 Aag2 cells were seeded in 10 cm dishes and allowed to attach overnight. Cells were transfected with expression plasmids using Transit2020 (Mirius Bio) using the manufacturer's instructions. 24 hours post transfection cells were washed with dPBS three times, scrapped off of the dish in IP-buffer (pH 7.5 @ 4°C, 10 mM tris, 2.5 mM EDTA, 250 mM NaCl, and cOmplete protease inhibitor, Roche), and spun down @ 2000 rcf for 5 min @ 4°C. Cell pellets were resuspended in 300 µl lysis buffer (IP-buffer + 0.5% NP-40) and incubated @ 4°C for 30 min and then spun down @ 12000 rcf for 10 min @ 4°C. The supernatant was added to 50 µl of Protein A conjugated beads (Sigma) and rotated for 1 hr @ 4°C. Lysate was adjusted to 1% NP40 (by adding 4 volumes IP-buffer) and then transferred to 50 µl of anti-FLAG conjugated beads (Sigma) and rotated for 6-16 hr @ 4°C. Beads were then washed 6 times with wash-buffer (IP-buffer + 0.05% NP-40). Following the final wash 30 µl of elution buffer was added (IP-buffer + 100 µg/ml 3x Flag peptide, Sigma) and rotated for 1 hr @ 4°C.

Mass Spectrometry To ensure samples were appropriate for mass spectrometry, eluates from affinity purification were analyzed by western blot and silver stain (Pierce). Samples were run on an Orbitrap LC-MS and analyzed with MaxQuant software.

AP for deep sequencing Elution was omitted. After washes, 1 ml of TRIzol was added to Flag beads and RNA extraction was performed according to the

manufacturer's protocol. Cloning of small RNAs was performed as described previously.

Deep sequencing 7 x 10^6 Aag2 cells were seeded in T-75 flasks in complete media and allowed to attach overnight. Cells were washed with dPBS three times, scrapped off of the dish in dPBS, and spun down @ 2000 rcf for 5 min @ 4°C. RNAs were isolated using the miRvana kit (Life technologies). The large RNA fraction was used for RT-qPCR. The small RNA fraction was precipitated by adding 1/10^{th} the volume 3M NaOaC pH 3.0, 1 µl gylcoblue (Life technologies), and 2.5 volumes 100% EtOH and incubated @ -80°C at least 4 hours and then spun down @ 12000 rcf for 10 min @ 4°C. The pellet was washed with 80% EtOH and then resuspended in Gel Loading Buffer II (Life Technologies) and run on a 20% polyacrylamide gel containing 8M urea. Small RNAs were cut and eluted from the gel overnight @ 4°C and precipitated by adding 1/10^{th} the volume 3M NaOaC pH 3.0, 1 µl glycoblue (Life technologies), and 2.5 volumes 100% EtOH and incubated @ -80°C at least 4 hours and then spun down @ 12000 rcf for 10 min @ 4°C. Small RNAs were cloned using the TruSeq small RNA library preparation kit according to the manufacturer's specifications (Illumina) and run on a HiSeq 1500 using the Rapid run protocol.

Adaptors were trimmed using FastX toolkit and mapped using bowtie[43]. Plots and read distance overlaps were generated by viROME[44]. Sequence bias were determined by Weblogo[45].

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Figure 1

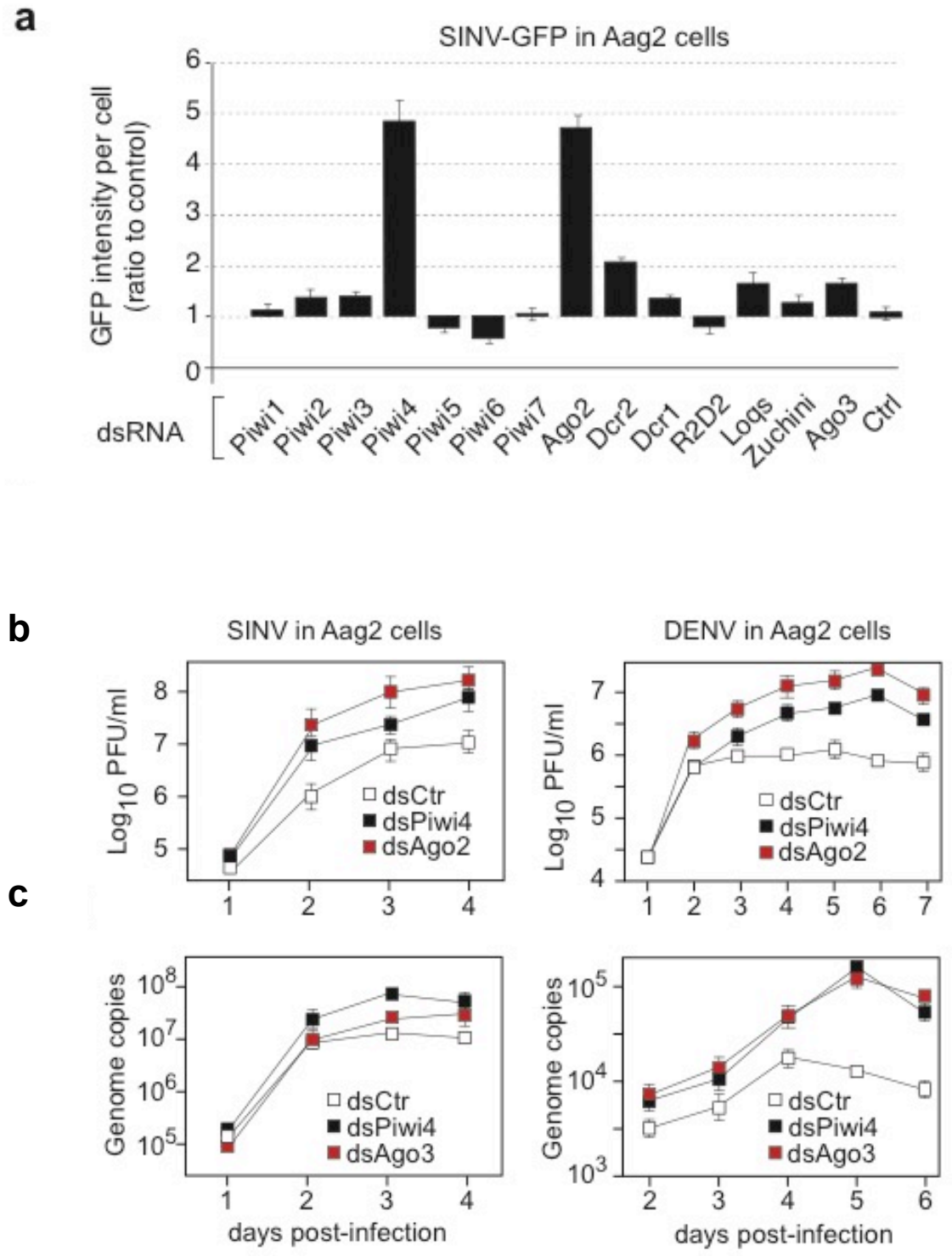
a
SINV-GFP in Aag2 cells
GFP intensity per cell (ratio to control)
dsRNA
Piwi1
Piwi2
Piwi3
Piwi4
Piwi5
Piwi6
Piwi7
Ago2
Dcr2
Dcr1
R2D2
Loqs
Zuchini
Ago3
Ctrl
b
SINV in Aag2 cells
DENV in Aag2 cells
Log10 PFU/ml
dsCtr
dsPiwi4
dsAgo2
c
Genome copies
dsAgo3
days post-infection

Fig. 1 | Identification of Ago3 and Piwi4 as viral restriction factors.

a, Effect of knockdown *A. aegypti* homologues of siRNA and piRNA effector and biogenesis factors on eGFP expression from transgenic reporter SINV.

b, c Multi step growth curve of SINV and DENV measured by plaque assay **b** or RT-qPCR **c** in infected Aag2 cells (MOI 0.1) treated with control, Piwi4, or Ago3 dsRNA. RP49 was used as a normalization control. The error bars depict standard deviation of 4 biological replicates.

Figure 2

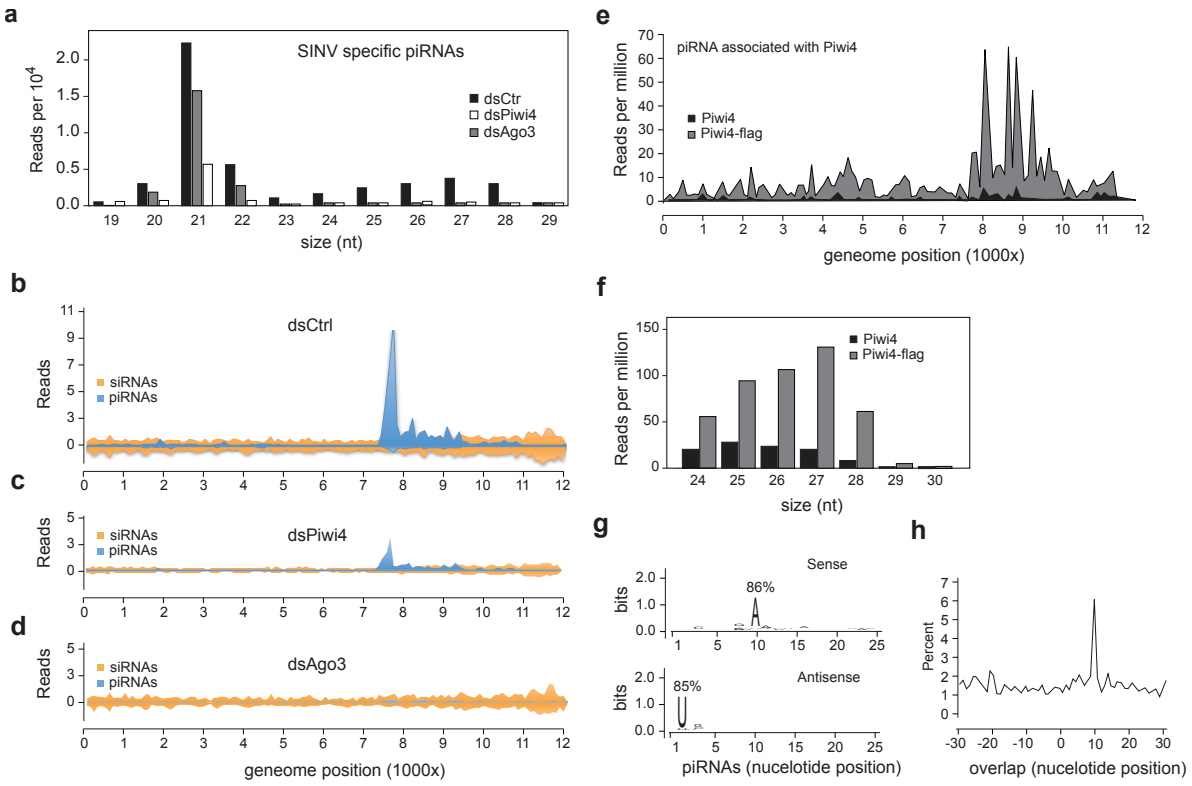

Fig. 2 | Characterization of SINV derived small RNAs.

a, Size distribution plot of small RNAs mapping to the SINV genome from infected Aag2 cells treated with control, Piwi4, or Ago3 dsRNA.

b, c, d, Distribution of 24-30 nt piRNAs mapping to the SINV genome from infected Aag2 cells treated with control **d**, Piwi4 **e**, or Ago3 **f** dsRNA. Positive values represent reads mapping to the sense strand and negative values represent those mapping to the antisense genome.

e, Frequency distribution of 24-30 nt piRNAs that mapped to the negative strand of the SINV genome from Piwi4 purified from infected Aag2 cells.

f, Size distribution plot of of 24-30 nt piRNAs mapping to the negative strand of the SINV genome from Piwi4 purified from infected Aag2 cells.

g, Sequence bias of the piRNAs mapping to the SINV genome.

h, Overlap frequency of the 24–30 nt piRNAs that mapped to opposite strands of the SINV genome.

Figure 3

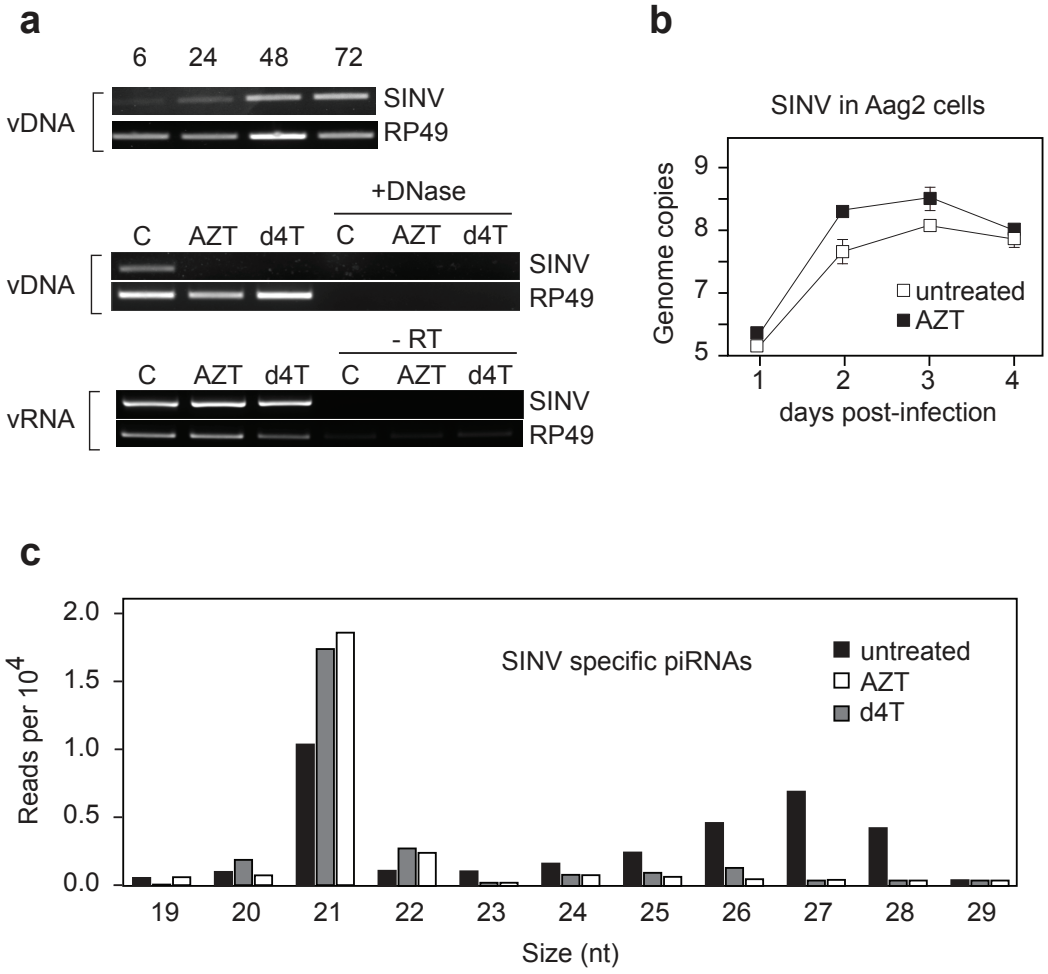

Fig. 3 | v-piRNAs are dependent on the synthesis of viral DNA.

a, Detection by PCR of SINV DNA in genomic DNA extracted from infected Aag2 cells with or without treatment of DNA with DNase I. RP49 was used as control.

b, Detection by RT-PCR of SINV RNA in total RNA extracted from infected Aag2 cells. RP49 was used as control.

c, Size distribution plot of small RNAs mapping to the SINV genome from infected Aag2 cells treated with the RT-inhibitors AZT, d4T, or control.

d, Multi step growth curve of SINV measured by RT-qPCR for infected Aag2 cells (MOI 0.1) with or without AZT treatment. RP49 was used as a normalization control. The error bars depict standard deviation of 4 biological replicates.

Figure 4

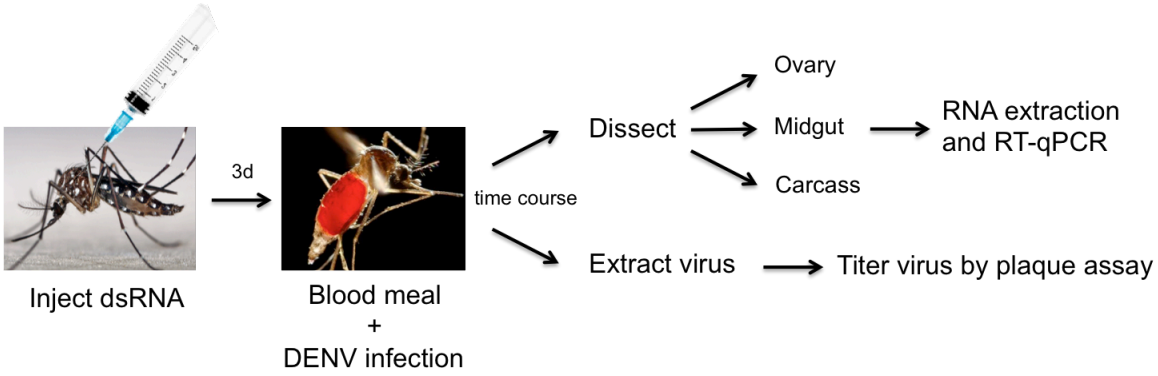

Fig. 4 | Strategy for Piwi4 knockdown and viral infection *in vivo*.

Female *A. aegypti* mosquitoes were injected with dsRNA in order to deplete genes of interest. 3 days after dsRNA injection mosquitoes were infected with DENV by blood meal. Infected mosquitoes were split into two pools. Virus was extracted from one pool of whole mosquitos and tittered by plaque assay. In the other pool, RNA was extracted from the dissected ovaries, midguts, and carcasses of infected mosquitoes and viral replication and gene of interest expression were measured by RT-qPCR.

Figure 5

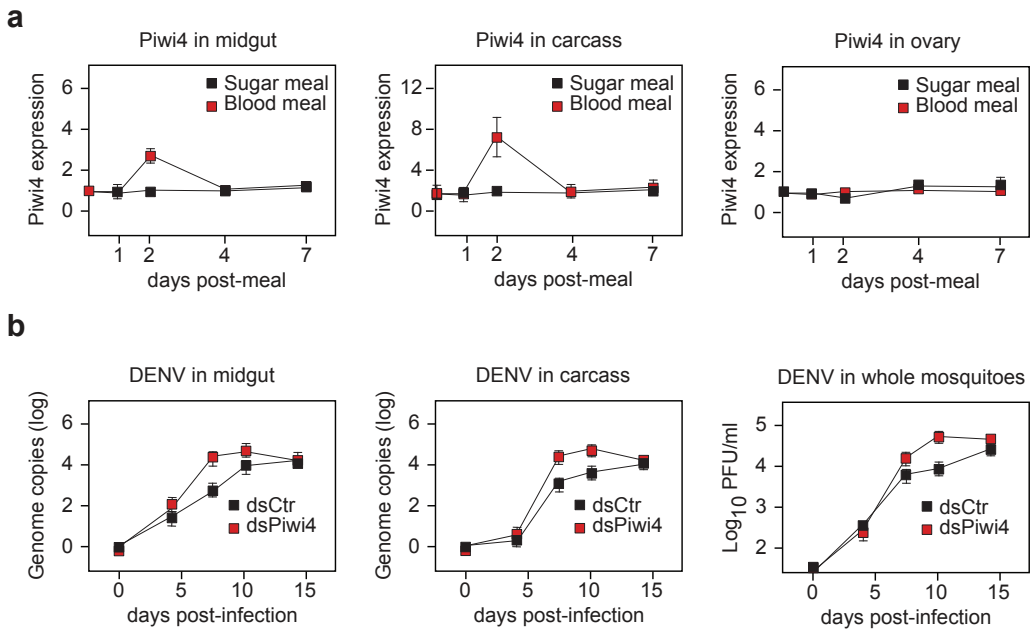

Fig. 5 | Piwi4 restricts DENV *in vivo*.

a, Expression of Piwi4 in dissected midguts, ovaries and carcasses from blood or sugar fed female mosquitos. The error bars depict standard deviation of 4 biological replicates of pools of 5 of the respective tissue. Significant changes over controls are marked with asterisks ($p \leq 0.05$, t-test).

b, Replication of DENV measured by RT-qPCR in infected midguts of female mosquitos injected with either Piwi4 or control dsRNA.

c, Replication of DENV measured by RT-qPCR in infected carcasses of female mosquitos injected with either Piwi4 or control dsRNA. The error bars for **c** and **d** depict standard error of 20 biological replicates of individual midgut or carcasses.

d, Replication of DENV measured by plaque assay in whole infected female mosquitos injected with either Piwi4 or control dsRNA. The error bars depict standard error of 30 biological replicates of individual mosquitos. Significant changes over controls are marked with asterisks ($p \leq 0.05$, Mann-Whitney U test).

Chapter 4:

Future Directions

Future directions

Our study on dsRNA uptake and antiviral immunity in Aag2 cells in conjunction with the observation that injection of naked dsRNA can inhibit viral replication *in vivo* in *Aedes aegypti* point to a similar form of systemic RNAi as in *Drosophila melanogaster*. Formal proof that dsRNA uptake is necessary for antiviral immunity in adult *Aedes aegypti* and identification of the tissues that participate in this immunity is a logical next step in the progression of our understanding of antiviral immunity in *Aedes aegypti*.

Our finding that the piRNA pathway can restrict viral replication in somatic tissues *in vivo*, represents the first observations that piRNA pathway functions antivirally, a major departure from previously identified functions of this pathway. We show that piRNA biogenesis is dependent upon reverse-transcription of viral RNA and identify the core components of the piRNA pathway that are required for this process. Several species of mosquito, including *A. aegypti*, have an expanded set of piwi genes compared to many of the model species studied. It is tempting to speculate that this expansion has led to additional roles for the piRNA pathway in these species. Indeed, virally derived piRNAs are also observed in *A. albopictus* and *Culex quinquefasciatus*. It will be interesting to observe if the antiviral function of the piRNA pathway is conserved amongst mosquitos. In addition, there are many arthropods that have expanded sets of piwi genes, it is possible that antiviral piRNAs is conserved functionally amongst many species.

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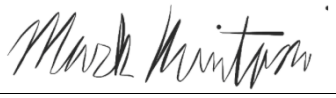
Author Signature  Date 6/11/12015 .