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RNA-Interference Mediated Antiviral Immunity
in *Schizosaccharomyces pombe* and *Drosophila melanogaster*

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Cell, Molecular and Developmental Biology

by

Ying Jun Luo

December 2010

Dissertation Committee:
        Dr. Shou-wei Ding, Chairperson
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The Dissertation of Ying Jun Luo is approved:

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Committee Chairperson

University of California, Riverside

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ABSTRACT OF THE DISSERTATION

RNA-Interference Mediated Antiviral Immunity
in *Schizosaccharomyces pombe* and *Drosophila melanogaster*

by

Ying Jun Luo

Doctor of Philosophy, Graduate Program in Cell, Molecular and Developmental Biology
University of California, Riverside, December 2010
Dr. Shou-wei Ding, Chairperson

Infection of RNA viruses induces RNA interference (RNAi) – directed antiviral immunity in plants and invertebrates. However, it is not known if the RNAi machinery in the fission yeast *Schizosaccharomyces pombe* has an antiviral function. Replication of Flock House Virus (FHV) genomic RNA1 and its mutant deficient in the expression of the viral suppressor of RNAi (VSR) was examined in both wild type and RNA-defective mutant *S. pombe* strains. The results indicated that the primitive RNAi pathway in *S. pombe* was incompetent to inhibit viral RNA replication. Deep sequencing of small RNAs also did not detect production of virus-derived small interfering RNAs (siRNAs) following FHV replication in *S. pombe.*

Since most of the previous studies in *Drosophila* used cell culture and embryos, little is known about the population structure, biogenesis pathway and relative potency of virus-derived siRNAs in adult *Drosophila melanogaster*. Deep sequencing of small RNAs from wild type and RNAi-defective *Drosophila* mutants challenged by a VSR-deficient FHV

mutant showed that virus-derived siRNAs were produced by the type III ribonuclease Dicer-2, were predominantly 21 nucleotides in length with an approximately equal ratio of positive and negative strands, and sufficiently potent to terminate virus infection in adult flies. Importantly, two dsRNA-binding proteins, Loquacious and R2D2, which act sequentially in the biogenesis and loading of siRNAs in the canonical RNAi pathway, appeared to function redundantly in antiviral RNAi, whereas loss of Argonaute-2 resulted in a strong bias for positive-strand viral siRNAs.

Further genetic studies revealed a prominent antiviral role for the *PIWI* gene, which encodes an Argonaute protein required for silencing transposons and repeat elements in the *Drosophila* germline. Notably, enhanced accumulation of FHV proteins in the epithelial sheath and follicle cells surrounding the developing egg chambers in the ovaries was illustrated in the *Drosophila* mutant heterozygous for *PIWI*. Consistently, presence of the *piwi* mutant allele resulted in significantly enhanced levels of FHV and Drosophila C virus in the progenies of virus-infected adult flies. Thus, Piwi may be essential for virus clearance in the developing oocytes, and hence prevent vertical transmission of certain RNA viruses in fruit flies.

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CHAPTER 1. INTRODUCTION

1.1 Discovery of RNAi and antiviral RNAi in plants

RNA interference (RNAi) is a small RNA-guided gene silencing mechanism conserved in a wide variety of eukaryotic organisms including fungi, plants and animals. It was first discovered in plants as co-suppression between introduced transgenes and its homologous endogenous gene, which can occur on both the transcriptional and posttranscriptional levels (Napoli *et al.*, 1990; van der Krol *et al.*, 1990). Later on plant virologists demonstrated transgenic plants expressing either full-length or truncated segment of viral genomic RNA led to resistance and sequence-specific degradation of the infecting viral RNA (Lindbo *et al.*, 1993). In 1998, collaborative work from the groups led by Dr. Fire and Dr. Mello elucidated that RNAi, which has been used by worm geneticists to knock down gene expression for years, was actually sequence-specific gene silencing triggered by double-stranded RNAs (dsRNAs) in *C. elegans* (Fire *et al.*, 1998). It was then found to occur in diverse eukaryotic model organisms in the subsequent years, such as *Drosophila melanogaster*, *Schizosaccharomyces pombe*, *Dictyostelium*, *Neurospora*, mice, human and probably many more eukaryotes (Kennerdell and Carthew, 2000; Paddison *et al.*, 2002; Hall *et al.*, 2003; Baulcombe, 2004), suggesting it is an evolutionarily conserved regulation mechanism involved in a broad spectrum of cellular activities, including as a potent defense against RNA viruses and transposable elements in the genome (Sijen and Plasterk, 2003; Kalmykova *et al.*, 2005; Li and Ding, 2005).

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Consistently diverse viral proteins have been identified as suppressors of RNAi, providing strong validation for the effectiveness of this antiviral immunity (de Vries and Berkhout, 2008; Wu *et al.*, 2010b).

1.2 RNAi-based antiviral immunity in *S. pombe* and adult *Drosophila*

Researchers have been seeking the possibility to use nucleic acids as antiviral therapeutics for almost three decades, for compounds such as antisense oligonucleotides, ribozymes, aptamers and so on can theoretically mediate sequence-specific degradation of particular viral genomes. However, their application in the clinic was hindered by the unsatisfying efficiency, target-specificity, toxicity, delivery difficulty and stability (von Eije and Berkhout, 2009). Since the discovery of RNA interference (RNAi), it has become a promising nucleic acid-based therapeutics as the closely related microRNA (miRNA) and small interference RNA (siRNA) pathways emerged to be important regulators and defense mechanism during virus infection in both plants and animals (Scherr *et al.*, 2003; Aliyari and Ding, 2009; Umbach and Cullen, 2009; Ulvila *et al.*, 2010). Although both pathways are relatively well conserved, there are important differences between these organisms which need to be comprehensively studied for a better understanding of the diverse viral pathogenesis and the host innate immune response to viral infection. For instance, the budding yeast *Saccharomyces cerevisiae* has been an invaluable model for assessing fundamental cellular response, disease states and host factors of relevance to higher eukaryotes during virus infection (Nagy, 2008).

Unfortunately RNAi is lost in the budding yeast, so I determined to use another yeast model, *Schizosaccharomyces pombe* to explore the potential involvement of primitive RNAi in antiviral immunity. Notably, *S. pombe* has only one dicer as found in human (the human genome encodes a Drosha for processing of miRNA precursor) (Shabalina and Koonin, 2008) and the dicing activity has been reported to involve in transcriptional gene silencing in both organisms (Volpe *et al.*, 2003; Kawasaki *et al.*, 2005; Kim *et al.*, 2006). Moreover, insect models for virus-host interaction are of great concern because insect viruses threaten beneficial insects in agriculture and human enterprises, such as honeybees and silkworm respectively. Some of the insect viruses also infect humans. Over 500 known varieties of arthropod-borne viruses can efficiently infect and replicate in cells from both invertebrate and vertebrate hosts, for instance, Dengue viruses, West Nile virus, Sinbid virus and yellow fever virus (Cherry, 2008; Sabin *et al.*, 2010). Therefore, *D. melanogaster* providing the powerful genetic and molecular tools is an excellent model to assess the virus-host interaction in disease-transmitting vectors.

1.3 RNAi mediated heterochromatin silencing in *Schizosaccharomyces pombe*

The discovery that RNAi plays an important role in the formation of heterochromatin at discrete chromosomal loci including centromeres, telomeres, the mating-type locus and ribosomal DNA in the fission yeast *S. pombe* has made it a model of interests in the field (Verdel and Moazed, 2005b; White and Allshire, 2008). Heterochromatin is an epigenetically inherited attribute that is essential for gene regulation, chromosome

segregation and chromosome stability in all eukaryotic cells. The heterochromatin assembly pathway involves posttranslational modifications of histones and a group of DNA-binding proteins including chromo-domain proteins that are conserved in *Drosophila* and mammals (Horn and Peterson, 2006). Distinct mechanisms are responsible for the initial heterochromatic protein assembly in different chromosomal contexts, and those loci often contain repetitive DNA elements which are transcribed and thus lead to recruitment of heterochromatic factors. Methylation of histone H3 at lysine 9 (H3K9me) by Clr4 – a homolog of Drosophila SU(VAR)3-9 and human Suv39 h, provides binding site for the HP1 family protein, Swi6 and chromo-domain protein Chp1 (Jenuwein and Allis, 2001). The pericentromeric repeats surrounding the site of kinetochore formation are transcribed and then processed by the RNAi machinery with the core components Dicer (Dcr1), Argonaute (Ago1) and RNA dependent RNA polymerase (Rdp1) (Hall *et al.*, 2002; Volpe *et al.*, 2002).

Fission yeast possesses a single gene encoding each of the key proteins required for RNAi, and they are not essential for cell viability, which greatly simplify the analyses of the pathway without the impact of other Dicer and Argonaute homologues. Two key complexes, the RNA-induced initiation of transcriptional gene silencing complex (RITS) has been identified to contain Chp1, Ago1, Tas3 and siRNAs derived from the pericentrimeric region by Biochemical assays (Motamedi *et al.*, 2004; Verdel *et al.*, 2004). siRNAs generated by Dcr1 help recruiting the complex to chromatin region presumably by base-pairing with the nascent centromeric transcripts (Motamedi *et al.*,

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2004; Sugiyama *et al.*, 2005). Roles of the other complex, the RNA-directed RNA polymerase complex (RDRC) are poorly understood relative to RITS. Targeting of Rdp1 along with another two components, Hrr1 and Cid12 in RDRC to the heterochromatic domains depends on the chromatin-localization of RITS by binding to H3K9me (Motamedi *et al.*, 2004; Zhang *et al.*, 2008). Chromatin association of Rdp1 also requires another H3K9me-binding protein Swi6 (Sugiyama *et al.*, 2005). Loss of methylation in *clr4Δ* strain leads to defective localization of RITS and its associated factors (Zhang *et al.*, 2008). Therefore, it appears to be a self-enforcing mechanism to ensure the RITS chromatin association and processing of centromeric siRNAs - Clr4 is not only creating the H3K9me binding site for RITS, the recruitment of RITS may facilitate the processing of centromeric transcripts into siRNAs, which in turn brings in Clr4 associating with RITS to mediate further H3K9me at adjacent loci. This may explain why the long-range spreading of heterochromatin requires a unique characteristic of Clr4. Clr4 not only methylate H3K9, but also bind to H3K9me via its chromo-domain (Zhang *et al.*, 2008). The ability of Clr4 to both 'read' and 'write'H3K9me presumably allows it to modify adjacent nuclesomes, and mutations in Clr4 that abolish its binding to H3K9me *in vitro* affect spreading of heterochromatin in vivo (Hall *et al.*, 2002). Taken together, these experiment evidences support a widely accepted model of the heterochromatin formation initiates from the transcription of non-coding RNA derived from the repetitive DNA sequences. The transcripts form a double stranded RNA and/or hairpin-structured template which is then processed by Dicer into siRNAs. These siRNAs are incorporated

into the RITS effector complex, which was then tethered to the nascent transcripts via sequence complementary and facilitate recruitment of histone-modifying activities such as Clr4. In support of this model, artificial tethering of RITS to transcripts has been demonstrated to induce local heterochromatin formation (Buhler *et al.*, 2006). Binding of Chp1 to H3K9 methylated by Clr4 in turn allows RITS to localize stably across heterochromatic domains. RITS engages nascent transcript, activating Rdp1 for generation of double stranded RNAs, which are then processed by Dcr1 into more abundant siRNAs.

1.4 Drosophila Viruses and their <u>V</u>irus-encoded <u>S</u>uppressors of <u>R</u>NA silencing (VSRs)

The fruit fly has emerged to be a powerful model to study innate immunity and host-pathogen interactions, originally on bacteria and fungi. More recently, viruses have come into the spotlight. Yet no DNA viruses have been recovered in *Drosophila melanogaster*, a diverse set of RNA viruses belonging to several families (Rhabdoviridae, Dicistroviridae, Birnaviridae, Reoviridae, Errantiviridae) has been reported as natural viral pathogens of *D. melanogaster*, some small RNA viruses isolated from other insects are also proven to exhibit substantial infectivity and replication in fruit flies and serve as insightful models for understanding antiviral response and important aspects in virology (Huszar and Imler, 2008; Kemp and Imler, 2009; Sabin *et al.*, 2010).

1.4.1   Rhabdoviridae

Sigma virus is widespread in natural populations of fruit flies and belongs to an important

family of RNA viruses, *Rhabdoviridae*, infecting animals and plants (Hogenhout *et al.*, 2003). The genome of Rhabdoviridae is composed of a single 11-15 kb single stranded RNA molecule which tightly associated with the nucleoproteins surrounded by an external membrane in a characteristic bullet shape, and trimers of glycoprotein spikes cover the entire outer surface of the virions (Huszar and Imler, 2008). Little is known about its replication cycle in Drosophila cells yet, and the genome of Sigma virus has not been fully sequenced yet, it has been documented that sigma virus infection is maintained in *Drosophila* populations through vertical transmission (Bregliano, 1973; Fleuriet, 1982). The flies infected with Sigma virus suffer few adverse effects, including reduced viability of infected eggs, and hypersensitivity to exposure to CO_2 (Fleuriet, 1981a, b). It establishes persistent infections in Drosophila cell culture under the control of genes such as *ref(1)H*, *ref(2)P* and *ref(3)D* (Gay, 1978), which have been proposed to function in the Toll pathway and to regulate the activation of NF-κB pathway (Avila *et al.*, 2002).

1.4.2   Dicistroviridae

DCV is one of the best studied Drosophila viruses, and it was first discovered in 1972 in a French laboratory stock (Jousset *et al.*, 1977). It is a non-enveloped RNA virus that exhibits many properties similar to picornaviruses, and hence was once considered as an insect picornavirus until its genome was sequenced in 1998 and from then on became the prototype member of a new family of RNA viruses, the *Dicistroviridae* (Johnson and Christian, 1999). One of the significant differences is that the 9264nt genome of DCV contains two ORFs. ORF1 is located at the 5' end and encodes a 202kDa polyprotein

containing RNA-dependent RNA polymerase, helicase and protease domains. The 3'

ORF2 encodes a 100 kDa capsid protein precursor. No subgenomic RNA is produced and

translation of both ORFs proceeds from the genome-long bicistronic RNA, using two

internal ribosomal entry sites located at the 5' end of the genome for ORF1 and in the

191nt intergenic region for ORF2 (Johnson and Christian, 1998; Sasaki and Nakashima,

2000; Wilson *et al.*, 2000). Using genome-wide RNAi screens, it has been established

that DCV replicates on cellular vesicles derived from the Golgi apparatus after entry of

the drosophila cells by clathrin-mediated endocytosis, and the viral protein synthesis

triggers a remodeling of the Golgi apparatus (Cherry *et al.*, 2005; Cherry *et al.*, 2006).

The outcome of the DCV infection in adult flies is controversy based on the infection

route. Natural infection by the oral route does not result in major pathogenic symptoms,

whereas injection of purified particles can lead to multiplication and lethality in a couple

days, and the virus accumulates in multiple organs such as the fat body, trachea, a subset

of somatic muscles, visceral muscles along the midgut and the epithelial sheath of the

female ovaries (Cherry and Perrimon, 2004). CrPV is also classified as a member of the

Dicistroviridae. It was originally isolated from crickets in 1970 by Carl Reinganum and

turn out to has a wide host range including insects belonging to Diptera, Lepidoptera,

Orthoptera, and Heteroptera species (Wilson *et al.*, 2000). It can replicates efficiently in

Drosophila cells and is pathogenic when injected into flies (Scotti, 1975; Moore *et al.*,

1980). Its genome is of 9185nt and is in a similar arrangement as the genomic RNA of

DCV. A global microarray analysis displayed that DCV infection in Drosophila S2 cells

induced a set of genes that did not include the well-characterized antimicrobial peptide genes, while the Jak-STAT pathway is required but not sufficient for the antiviral response in *D. melanogaster* (Dostert *et al.*, 2005a).

1.4.3 Birnaviridae

DXV was first identified as a contaminant in uninfected fly-controls for experiments with Sigma virus (Dobos *et al.*, 1979). Although this virus is found in many Drosophila cell lines, it has never been found in wild populations of flies. DXV can cause death in the inoculated flies depending on the inoculums concentration and induce hypersensitivity to anoxia: infected flies are killed by exposure to pure CO_2 or nitrogen for several minutes; while the uninfected flies can recover rapidly after returning to normal atmosphere and temperature. DXV belongs to the Birnaviridae family which is characterized by a double stranded RNA genome packaged in a non-enveloped, icosahedral particle (Chung *et al.*, 1996). The two RNA segments are called segments A and B. Segment A is 3360-bp-long, and contains two ORFs. The large ORF encodes a polyprotein of 128 kDa, which is cleaved into outer capsid protein (preVP2), inner capsid protein (VP3), and proteolytic enzyme (VP4) that catalyze the precursor processing. Viral protein preVP2 undergoes further cleavage into pVP2 and then to its final 45 kDa- final form VP2, which mediates the DXV interaction with host-cell membranes for internalization (Galloux *et al.*, 2007). The 3243bp- Segment B encodes the 110-kDa RNA-dependent RNA polymerase (VP1), which can undergo self-guanylation and bind to the 5' end of both genomic fragments to initiate RNA synthesis (Shwed *et al.*, 2002).

1.4.4   Reoviridae

DFV and DSV are members of the *Reoviridae* family, which has dsRNA genomes as birnaviruses. DFV has been identified in flies collected from laboratory stocks and from some wild populations. Its genome is segmented in 10 fragments and packed in a spherical particle with a two-layer capsid comprising eight polypeptides ranged from 40-150 kDa (Chandran and Nibert, 2003). Detail mechanism of the replication of DFV in Drosophila cells remains largely unknown, and the pathogenic effects of this virus in flies upon inoculation of viral suspension seem questionable as well (Huszar and Imler, 2008). Another reovirus DSV, however, is associated with a mild reduction in fitness of the infected flies (Lopez-Ferber *et al.*, 1989).

1.4.5   Unclassified viruses

Some RNA viruses found in laboratory stocks and natural populations have not been classified into any of the known families as the particle feature, genomic sequences and/or replicative mechanism are very poorly understood, such as DPV and Nora virus. DPV mainly replicates in the intestine, malpighian tubules, tracheae, periovarian sheath and follicle cells. It was firstly isolated from wild population of flies from tropical areas, and documented that it can be transmitted vertically through the female germline (Huszar and Imler, 2008). Another recently described virus causing persistent infection in Drosophila is Nora virus, which possesses a polyadenylated single positive stranded RNA genome of 11,879nt (Habayeb *et al.*, 2006). Nora virus is often found in the intestine of infected flies, which exhibit only a mildly adverse effect, and is horizontally transmitted

via feces. Variability in virus clearance has been recently reported in the laboratory stocks of Drosophila upon infection, and surprisingly neither the clearance of low-level Nora virus infections nor the stability of persistent infections caused by higher-titer infection seem to require the RNAi pathways or the Toll and Jak-Stat pathways (Habayeb *et al.*, 2009a; Habayeb *et al.*, 2009b).

1.4.6 Errantiviridae

Retrovirus are transposable elements containing two large ORFs encoding the capsid and nucleocapsid components and the factors required for replication, long terminal repeats (LTRs) and a *env − like* gene which typically encodes a transmembrane glycoprotein (Kaminker *et al.*, 2002). They can form infectious particles, replicate by reverse transcription of an RNA intermediate, followed by integration of the resulting DNA into the host genome. These LTR retrotransposons comprise a large proportion of the Drosophila genome, while only a few of them contain an *env*-like gene. They are classified in the *Errantiviridae* family, in which *gypsy* is the best studied member (Kim *et al.*, 1994; Song *et al.*, 1994). Its 7469bp genome contains the three canonical ORFs *gag*, *pol* and *env*, flanked by two 482nt- LTRs (Marlor *et al.*, 1986). Most drosophila stocks contain a few *gypsy* elements located on chromosome arms and larger number of defective copies in the pericentromeric heterochromatin regions (Marlor *et al.*, 1986; Bucheton, 1995). *Gypsy* and other retroviruses such as *ZAM* and *idefix* are normally repressed by the *flamenco* locus which is located at the pericentromeric heterochromatin on the X chromosome (Prud'homme *et al.*, 1995; Mevel-Ninio *et al.*, 2007). When it is

deregulated, these elements can transpose at high rates leading to induced insertion mutability. How these retroviruses are under the control of piRNA pathway will be discussed in the following section.

1.4.7 Exogenous viruses

Many viruses that infect or are transmitted by other insects including SINV, WNV, FHV and NoV also serve as important models for antiviral response in *Drosophila*. Sindbid virus (SINV) and West Nile virus (WNV) are both viruses transmitted by mosquitos to vertebrate hosts, while they can infect adult Drosophila. SINV is a member of the genus Alphavirus in the family Togaviridae. It was first identified in a pool of *Culex pipiens* and *Cx. univittatus* mosquitoes in Egypt (Taylor *et al.*, 1955), and mosquitoes serve as an effective transmission vector to infect the vertebrate hosts (birds). It is an enveloped virus with a genome of single-stranded, positive-polarity RNA of approximately 11,7000nt (Strauss *et al.*, 1984). Injection of SINV into the thoraces of wild type flies did not lead to significant lethality despite the presence of viral antigen and infectious particles, but injection into *dcr2* mutant flies caused dramatic increase in death (Galiana-Arnoux *et al.*, 2006). Similar to SINV, WNV is a virus also mainly infecting birds, and has been used to infect both adult flies and *Drosophila* cultured-cells. WNV belongs to the family Flaviridae with a single-stranded RNA genome between 11,000 and 12,000nt in length which encodes the capsid (C), envelope (E), and premembrane (prM) proteins, as well as 7 nonstructural proteins that likely contribute to viral replication (Beasley, 2005; Hayes and Gubler, 2006). WNV infection led to higher virus titer in flies containing mutations

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in *spindle-E*, *piwi*, *dcr-2*, and *ago2*, and the latter mutant flies also showed enhanced susceptibility to WNV infection (Chotkowski *et al.*, 2008). Nodaviruses Flock house virus (FHV) and Nodamura virus (NoV) become our favorite virus models in exploring antiviral immunity since the discovery and characterization of their VSRs. FHV is the most extensively studied member of Alphanodavirus genus in the Nodaviridae family (Schneemann *et al.*, 1993). FHV is a natural pathogen of the Coleopteran insect *Costelytra zealandica*, but it has also been demonstrated to replicate robustly in yeasts, plants, *C. elegans*, mammalian cells and *Drosophila*. (Dasgupta *et al.*, 1994; Price *et al.*, 1996; Li *et al.*, 2002; Johnson *et al.*, 2004; Lu *et al.*, 2005) FHV contains a 4.5 kb bipartite (+) RNA genome that is packaged in a single, nonenveloped, icosahedral virion. RNA1 (3107 nt) encodes protein A of 112 kDa, the RNA dependent RNA polymerase (RdRp), whereas RNA2 (1400 nt) encodes the capsid protein precursor that is 43 kDa (CP). Thus, RNA1 can replicate in absence of RNA2 and the RdRp is required for RNA2 replication. The RdRp also transcribes a single subgenomic RNA (sgRNA), RNA3 (387 nt) during RNA1 replication, which is overlapping with the 3' end of RNA1. RNA3 contains two overlapping open reading frames encoding protein B1 and B2, and both can be detected in infected cells (Venter and Schneemann, 2008). The function of B1 has never been revealed, and our unpublished data indicated that it is dispensable for FHV RNA1 replication in Drosophila S2 cells. NoV is another member of the alphanodaviruses (Murphy *et al.*, 1970). Similar as FHV, NoV has bipartite positive-sense RNA genome and ORF arrangement. RNA1 is slightly longer (3204 nt) than FHV, and

RNA2 is 1336 nt in length (Johnson *et al.*, 2003). NoV is unique in that it can lethally infect both insects and mammals.

1.4.8 VSR encoded by Drosophila or insect viruses

The 106-residue B2 protein encoded by FHV is the best-characterized VSR in animal viruses. It is translated from RNA3 within the +1 reading frame relative to protein A, but is not required for RNA1 self-replication (Venter and Schneemann, 2008). Surprisingly, the VSR activity of B2 protein was found to be as potent as the plant viral 2b protein in the Agrobacterium coinfiltration assay (Li *et al.*, 2002). Transient B2 expression inhibited the silencing of GFP transgenic plants, leading to high intensity of green fluorescence in the infiltrated patch on the leave. Consistently, the B2-deletion mutant of FHV RNA1 (FR1ΔB2) could not accumulate to detectable level in wild type Drosophila S2 cells showed much more RNA abundance in the cells when Ago2 were down-regulated by RNAi or B2 protein was expressed in *trans*. Those findings indicated that the B2 depletion does not inhibit FHV RNA replication and the essential role of B2 is to suppress Dcr2/Ago2-dependent RNA silencing pathway. Similar suppression activity was also found in NoV B2 in both Drosophia cells and mosquito cells (Li *et al.*, 2002; Li *et al.*, 2004). As many other VSRs encoded by plant viruses, B2 contains a dsRBD at the N terminus by both NMR and crystallization structural analyses (Chao *et al.*, 2005b; Lingel *et al.*, 2005). In vitro binding-assay has demonstrated that B2 is able to bind to a 21-nt siRNA duplex as well as 100-nt long dsRNA (Lu *et al.*, 2005). A single amino-acid substitution at position 54 (R54Q), which is located in the center of dsRNA binding

surface of B2 protein, affected both dsRNA binding and dicing inhibition of B2 indicating that the dsRNA-binging activity is required for B2 to inhibit Dcr-2-dependent biogenesis of siRNAs, the RNAi suppressor activity may count on this inhibition of dicing in the organism.

VSRs have also been identified in viruses naturally infecting the fruit flies, such as DCV and CrPV. The VSR activity was discovered in CrPV using a screening in S2 cells based on a recombinant RNA1 of FHV, FR1gfp, in which the B2 ORF in RNA1 was replaced by GFP. (Wang, 2006) The N-terminal fragment of 140 amino acids from the CrPV nonstructural polyprotein referred as CrPV-1A later on was then identified as an RNAi suppressor. It does not affect dsRNA processing (van Rij *et al.*, 2006) but turns out to interact with the endonuclease Ago2 and inhibits its activity without affecting the miRNA pathway. (Nayak *et al.*, 2010) Being another member of the Dicistroviridae family, DCV also encodes a suppressor protein DCV-1A at the N terminus of ORF-1 of its genome (van Rij *et al.*, 2006). DCV-1A binds long dsRNAs with high affinity and prevents process by Dcr2, but it does not bind small RNA (21nt) duplex as illustrated in B2 of FHV. Those findings together suggested VSR encoding may be common in RNA viruses in diverse families, and on the other hand, it was also reflecting the predominance of RNAi-directed antiviral immunity in D. melanogaster.

1.5 Small RNA pathways in *Drosophila*

Small RNAs in *Drosophila* can be classified into microRNAs (miRNAs), endogenous

and exogenous small interfering RNAs (endo- and exo-siRNAs) and Piwi-interacting RNAs (piRNAs) based on their biogenesis mechanism and the type of Argonaute (Ago) protein that they are associated with (Figure 1-1). miRNAs are generated from endogenous hairpin-structured transcripts by the catalytic activities of two RNase III-type proteins, Drosha and Dicer 1 (Dcr1). Mature miRNAs of mainly 22nt are then bound by Ago-subfamily proteins, Ago2 and predominantly Ago1. miRNAs function as post-transcriptional regulators by base-pairing with the target mRNAs, usually in the 3' untranslated region (UTR). Binding of a miRNA to its target mRNA typically leads to translational repression and exonucleolytic mRNA decay, whereas highly complementary can also result in endonucleolytical cleavage. Other types of regulation, such as translation activation and transcriptional gene silencing have also been reported in mammalian systems. siRNAs are distinguished from miRNAs in that they are derived from long double-stranded RNAs (dsRNAs) and requires Dicer 2 (Dcr2) but not Drosha. They are slightly shorter than miRNAs (21nt), and are associated with Ago-subfamily proteins, Ago2 predominantly. They can be further classified into endo-siRNAs and exo-siRNAs, based on the sources of the dsRNAs. Endo-siRNAs are generated from transposons and cellular transcripts, while exo-siRNAs refer to those derived from viruses or experimental dsRNAs. piRNAs are the longest of the three small RNA populations in Drosophila (24-31nt), and are defined by their association with Piwi-subfamily proteins, and its biogenesis does not depend on Dicer. piRNAs are highly abundant in germlines and at least some of them are involved in silencing of transposons

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and repetitive elements through heterochromatin formation or RNA destabilization.

1.5.1 miRNA pathway

miRNAs are the most abundant and also best studied small RNA class in animals. Their expression is sophisticated regulated in the cells mainly at the transcription level under developmental and/or tissue-specific signaling, consistently, they are often associated with crucial controlling of cell growth, development and differentiation. Transcription of miRNA genes by RNA polymerase II or RNA polymerase III generates primary-miRNA (pri-miRNA) transcripts containing imperfect intra-molecular stem loop that is cleaved by an RNase III enzyme Drosha in the nucleus (Lee *et al.*, 2004a; Borchert *et al.*, 2006). The cleavage product of ~70nt, pre-miRNAs are exported to the cytoplasm by Exportin-5 and processed by another ribonuclease Dcr1 in the aid of a dsRBP, Loquacious (Loqs), giving rise to miRNA duplex ranged from 21-23nt. One strand of the miRNA duplex is preferentially loaded into miRNA-induced silencing complex (miRISC) with Ago1 as the key components and then mediates translational repression or RNA decay of the target transcripts. For some cases, the other strand termed as miRNA*, will be loaded into Ago2 and functionally active to mediate target silencing (Forstemann *et al.*, 2007; Tomari *et al.*, 2007; Czech *et al.*, 2009; Okamura *et al.*, 2009).

More recent findings have identified a group of miRNAs referred as Mirtron in *Drosophila* that are originated from short intronic hairpins (Okamura *et al.*, 2007; Ruby *et al.*, 2007a; Ruby *et al.*, 2007b). Mirtron hairpins are processed by the splicing machinery and lariat-debranching enzyme, and the resulting pre-miRNA-like products

are also exported to the cytoplasm and then processed by Dcr1/Loqs hetero-dimer as the canonical miRNAs.

Loqs is a *Drosophila* homolog of human TRBP, and it binds to Dcr1 to facilitate its function in small RNA biogenesis. It has been found to play a role in germline stem cell maintenance, and be required for the efficient miRNA-directed silencing of a reporter transgene, complete repression of white by a dsRNA trigger, and silencing of the endogenous Stellate locus by Suppressor of Stellate (Forstemann *et al.*, 2005; Liu *et al.*, 2007; Park *et al.*, 2007; Marques *et al.*, 2009). Four different isoforms of Loqs have been predicted but no significant peptide evidence has been detected for Loqs-PC in cultured S2 cells and flies. Endo-siRNA production seemed to depend on a specific Loqs isoform, Loqs-PD, which is distinct from Loqs-PB that is required for the production of microRNAs, because normal levels of particular endo-siRNA could not be rescued in homozygous mutant *loqs* null-flies carrying a Loqs-PB transgene, while the miRNAs such as *miR-8* was restored (Zhou *et al.*, 2009; Miyoshi *et al.*, 2010). Moreover, all the isoforms of Loqs were shown to be capable of interacting with Dcr2 when they were overexpressed, whereas Dcr1 preferentially bound to Loqs-PB (Hartig *et al.*, 2009). Overall, these Loqs isoforms may have different Dicer-binding specificity and individual roles in miRNA and siRNA pathways, and their possible involvement in Dcr2-dependent antiviral immunity remains to be determined in both cultured Drosophila cells and adult flies.

1.5.2   siRNA pathway

In the past few years, a number of deep-sequencing studies in *D. melanogaster* somatic tissue, culture cells and ovaries have identified a class of ~21nt RNAs derived from transposon, sense-antisense transcript pairing and long stem-loop structures of cellular transcripts (Babiarz *et al.*, 2008; Chung *et al.*, 2008; Czech *et al.*, 2008; Ghildiyal *et al.*, 2008; Kawamura *et al.*, 2008b; Okamura *et al.*, 2008c). They are termed endogenous siRNAs to be distinguished from those derived from exogenous dsRNAs, siRNA duplexes or RNA viruses. Biogenesis of both endo- and exo-siRNAs is dependent on an RNase III enzyme, Dcr2 in *D. melanogaster* (Bernstein *et al.*, 2001). Dcr2 forms a hetero-dimer with a dsRNA-binding protein (dsRBP) R2D2, on which the siRNA duplexes are loaded as an intermediate RNA-protein complex (Liu *et al.*, 2003). Maturation of RNA-induced silencing complex (RISC) containing Ago2 was accomplished following the removal of one strand of the siRNA duplexes. The guide strand of siRNA duplex within the RISC then recruits the complex to target RNAs based on sequence complementary, and thus Ago2 catalyzes endonucleolytic cleavage (Kim *et al.*, 2007). Endogenous siRNA, especially those originated from long stem-loop structures rely on Loqs as described earlier. (Czech *et al.*, 2008; Okamura *et al.*, 2008a; Okamura *et al.*, 2008c)

1.5.3   siRNA-mediated antiviral Defense in *D. melanogaster*

Three lines of evidence could serve as the criteria to establish the antiviral RNA silencing in a given organism: (1) detection of small RNA derived from the viral dsRNA; (2) increased accumulation of viral RNA in RNAi-defective host; and (3) the RNAi-mediated

silencing may be inhibited by the viral silencing suppressor. Experimental evidences provided by several independent groups including ours have established the essential role of Dcr2 in host defense by the enhanced disease susceptibility associated with higher virus load in dcr2 mutant fruit flies during infection of five distinct single-stranded positive-sense RNA viruses: FHV, CrPV, DCV, SINV and WNV (Galiana-Arnoux *et al.*, 2006; Wang, 2006; Chotkowski *et al.*, 2008). We also observed similar hypersensitivity and increased viral accumulation in *r2d2* homozygous flies, which also showed defective exo-siRNA loading into RISC. Interestingly, Dcr-2 seemed not to be essential for the clearance of a dsRNA virus DXV (Zambon *et al.*, 2006) in adult flies suggesting that the genetic requirements for immunity against dsRNA-viruses may differ from that initiated by single-stranded RNAs. In addition, possibility of various VSR activities encoded by different viruses could not be excluded yet.

Ago2 acts downstream of Dcr2 to recruit siRNAs into siRISC and catalyzes slicing of the target transcripts through sequence-specific manner. As a matter of fact, prior to establishing the antiviral role of Dcr2, we have observed a dramatic increase in accumulation of FHV RNAs and restored abundance of the VSR-depleted mutant RNA, FR1ΔB2 in *Drosophila* S2 cells when Ago2 was knocked down by RNAi, or in both *dcr2* and *ago2* mutant embryos (Li *et al.*, 2004; Aliyari *et al.*, 2008a). Consistent with this proposed model, *ago2* mutant flies were also susceptible to the infection of DXV, DCV, CrPV and WNV (van Rij *et al.*, 2006; Zambon *et al.*, 2006; Chotkowski *et al.*, 2008). Taken together, genetic analyses from both cultured cells and adult *Drosophila* have

suggested a model for the antiviral response mediated by Dcr-2/R2D2/Ago-2-dependent siRNA pathway, in which double-stranded replicative intermediates produced during viral replication may be recognized by Dcr2 acting as a host sensor and initiating biogenesis of viral siRNAs, which are then loaded into the silencing effector Ago2.

Detection and analysis of virus-derived siRNAs (viRNAs) have provided convincing evidence supporting the existence and insights on the mechanistic of the antiviral response. viRNAs of both positive and negative strands were first detected in plants infected with positive-strand RNA viruses (Hamilton and Baulcombe, 1999). Subsequently, viRNAs of both polarities were also observed in *Drosophila* S2 cells infected with FHV virions (Li *et al.*, 2002). The existence of viRNAs in adult flies was firstly demonstrated in adult flies infected with FHV (Galiana-Arnoux *et al.*, 2006; Wang, 2006). Although both *dcr2* and *r2d2* mutant flies showed hypersensitivity to FHV infection and similar abundance of virus RNAs in the infected flies, viRNAs were only easily detected in infected *r2d2* flies but not *dcr2* mutant, indicating an essential role of Dcr2 in the viRNA biogenesis. R2D2 is not required for the viRNA production but is essential for the activity of viRNAs, consistent with the previous report on its function in RISC-loading in siRNA pathway (Liu *et al.*, 2003). Apart from FHV, CrPV infection also leads to the accumulation of CrPV-specific siRNAs of 21-nt in S2 cells. In contrast, a population of 25nt- but not 21nt- small RNAs derived from WNV was detected in infected S2 cells (Chotkowski *et al.*, 2008), and even the presence of this population seemed questionable according to a more recent report using the same system. So viRNA

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biogenesis during WNV infection in Drosophila cells remains an unsolved issue. More extensive sequencing of small RNAs in adult flies during FHV and FHVΔB2 infection are presented.

1.5.4 piRNA pathway

piRNAs were originally discovered during profiling studies of small RNAs *D. melanogaster* during development. These germ-cell-specific small RNAs (24-29 nt) were firstly termed as repeat-associated small interfering RNAs (rasiRNAs) as they were mainly mapped to intergenic repetitive elements, including retrotransposons (Aravin *et al.*, 2001; Aravin *et al.*, 2003). Report by Aravin et al. in 2001 suggested that this small RNA species was involved in the silencing of transposable elements. In addition, proceeding studies demonstrated that PIWI protein was essential for self-renewal of germline stem cells (Cox *et al.*, 1998a; Cox *et al.*, 2000; Szakmary *et al.*, 2005) and for transposon mobility control(Sarot *et al.*, 2004; Kalmykova *et al.*, 2005). Aubergine (AUB), another Piwi-subfamily member in *D. melanogaster*, was also shown to act in repression of retrotransposons (Vagin *et al.*, 2004) and be required for pole-cell formation (Harris and Macdonald, 2001). Mutations of these two genes led to a loss of rasiRNA accumulation in ovaries, and detection of rasiRNAs immunopurified with Piwi complexes strengthened the connection between rasiRNAs and Piwi proteins (Saito, 2006; Vagin, 2006; Brennecke *et al.*, 2007a; Gunawardane *et al.*, 2007; Nishida *et al.*, 2007). Later, the Piwi-subfamily proteins in mice (MIWI, MILI and MIWI2) were also found to be associated with small RNAs (24-31 nt) that resemble *D. melanogaster* rasiRNAs

(Aravin *et al.*, 2006; Girard *et al.*, 2006; Grivna *et al.*, 2006; Watanabe *et al.*, 2006). They are now both termed piRNAs based on their common features. Most, if not all of the piRNAs identified so far are generated from intergenic repetitive elements in *D. melanogaster*. They can be mapped to particular loci on chromosomes, which are termed piRNA clusters. piRNAs associating with each of the Piwi proteins differ in size, polarity and nucleotide bias, which might due to the differences in subcellular localization, expression patterns of these proteins, and their roles in piRNA production.

PIWI- and AUB-associated piRNAs are mainly derived from antisense transcripts of retrotransposons, whereas those associated with AGO3, the third member of the subfamily, arise mostly from sense transcripts (Saito, 2006; Vagin, 2006; Brennecke *et al.*, 2007a; Gunawardane *et al.*, 2007; Nishida *et al.*, 2007). Moreover, PIWI- and AUB-associated piRNAs show strong preferences for uracil at their 5' ends, while AGO3-associated piRNAs do not have this bias but mostly have adenine at nucleotide 10. Interestingly, AUB-associated piRNAs show frequent complementarity to those associated with AGO3 in their first 10 nucleotides. Combined with the evidence that Piwi cleaves target RNA at between positions 10 and 11 relative to the 5' end of the associated small RNAs, a ping-pong model has been proposed to explain the piRNA biogenesis (Brennecke *et al.*, 2007b; Gunawardane *et al.*, 2007). AUB and/or PIWI associated with antisense piRNA cleave(s) sense strands of retrotransposon transcripts, producing the 5' ends of sense piRNAs that in turn binds to AGO3. Newly-assembled AGO3-piRNA complexes subsequently cleave the antisense transcripts and thereby make the 5' ends of

antisense stranded piRNAs that bind to AUB or PIWI. This continuous cycle thereby amplifies the piRNAs to maintain the silencing of retrotransposons. Furthermore, piRNAs, presumably in a form that is associated with Piwi proteins, can be maternally inherited to the embryos to mediate transposon silencing and/or initiate amplification of piRNA biogenesis between generations (Brennecke *et al.*, 2008).

Many processing steps in this model have yet to be determined, including the primary piRNA formation and loading, factors regulating the formation of 3' end of piRNAs. Moreover, the requirement for Drosha has not been formally tested, although that for Dicer can be excluded, based on the fact that piRNAs accumulate in *Dicer* mutant ovaries (Vagin, 2006). In addition, the precise roles of *Squash*, *Zucchini*, *Spindle-E*, *Krimper* and *Maelstrom* are being examined, as mutations of which cause the depletion of piRNAs in fly ovaries (Findley *et al.*, 2003; Vagin, 2006; Lim and Kai, 2007; Pane *et al.*, 2007). Noticeably, not all the piRNA production depends on the ping-pong pathway, such as those derived from *flamenco* (Aravin *et al.*, 2007b) and those identified recently in ovarian somatic sheet (OSS) cells (Lau *et al.*, 2009). Making the entire picture even more complicated, some piRNA-generated loci can produce endo-siRNAs (Czech *et al.*, 2008; Kawamura *et al.*, 2008a). Similar to endo-siRNAs, piRNAs have 2'-O-methyl groups at their 3' ends in flies, which might be added by the methyltransferase Hen1 (Kawamura *et al.*, 2008b). Consequently, the possibility of interaction between two pathways may be another open issue to be addressed.

As mentioned in the earlier section, many endogenous retroviruses are found to be

controlled by piRNAs in the germline, and flamenco locus which is a major piRNA cluster, emerged to be a good example in case. *flamenco* produces large amounts of sense and antisense stranded piRNAs that are associated with Piwi, Aub and Ago3 (Pelisson *et al.*, 2007). The piRNAs amplication via ping-pong cycle may also occur for many, if not all, retroviruses, with Ago3 associating with sense piRNA recognizes and cleave long antisense RNAs, leading to generation of the 5' end of new antisense piRNAs, and the 3' end formation may require the nucleases Squash and/or Zucchini (Pane *et al.*, 2007). Therefore, de-repression of *gypcy* is observed in flamenco or other piRNA-defective mutant flies in which piRNA derived from *flamenco* is lost (Aravin *et al.*, 2007b; O'Donnell and Boeke, 2007). Currently, further genetic, molecular and biochemical studies on the detailed mechanism of this process in different cell type in the germline are of great interest in this field.

1.6 Conclusions and perspectives

The past decade has been a fast-moving period in term of investigating the mechanism of RNAi and its biological significance in antiviral defense. Accumulating evidences have indicated a complicated and dynamic interaction between the insect host and invading viruses. During infection, the host utilizes small RNA pathways to control the disease development and eliminate viral replication. On the other hand, viruses have evolved suppressors or various mechanisms to surpass the immunity defense, and probably use the same pathways to modulate gene expression in the host in order to maintain their

abundance and propagation in the host cells. Mammalian hosts have evolved inducible response such as production of interferons to fight infection, as well as specific antibodies and lymphocytes that are adapted to specific viral antigens, and thus RNAi-mediated antiviral silencing may only an auxiliary part of the host defense against virus infection. However, in plants, fungi and invertebrates, RNAi emerges as a dominant and potent antiviral response protecting the hosts from developing disease symptoms and lethality. So insights derived from researches of the RNAi-mediated antiviral immunity in fungi and invertebrates, which include important pathogens or vectors transmitting diseases to plants and human, will facilitate a better understanding of viral pathogenesis, medical therapy, pathogen control and crop improvement. Many aspects of genetic requirement and molecular mechanism of this immune response remain unclear, some of which will be addressed in this work and future direction. For instance, whether the RNAi-directed immunity is sufficient to ensure virus clearance and elimination of virus infection and VSR activity encoded by the viral genome is necessary for virus infection. Moreover, it is not completely clear whether miRNA and piRNA pathways also involved in resistance to RNA viruses in invertebrates. The current knowledge on mechanistic of the siRNA pathway, including the viral siRNA biogenesis, assorted loading into Argonaute and viral RNA degradation, remains to be proven in the intact organisms.

Figure 1-1 *Drosophila melanogaster* **encodes three small RNA pathways that are highly conserved in mammals.**

(A) miRNA genes are transcribed by RNA polymerase II (Pol II), primary miRNA (pri-miRNA) transcripts are processed sequentially by the type III ribonucleases (RNases) Drosha, in the nucleus, and Dicer1 (DCR1), in the cytoplasm — which form heterodimers with the double-stranded RNA (dsRNA)-binding proteins Pasha and Loquacious-isoform PB (Loqs-PB), respectively. This processing generates precursor miRNAs (pre-miRNAs) and then 22-nucleotide miRNAs. miRNAs bind to Argonaute 1 (AGO1) in an RNA-induced silencing complex (RISC) to mediate translational arrest or the degradation of target mRNAs.

(B) Production of small interfering RNAs (siRNAs) from long dsRNA precursors involves one type III RNase (DCR2) and possibly Loqs-PD, although another dsRNA-binding protein, R2D2, is required for siRNA binding to AGO2 and loading into the RISC, which mediates the cleavage of target mRNAs.

(C) The biogenesis of Piwi-interacting RNAs (piRNAs) is Dicer-independent. piRNAs are mostly antisense (shown in red) to transposon transcripts and bind to Piwi or Aubergine (AUB), but AGO3 binds to the low abundant sense piRNAs (shown in blue) and collaborates with AUB to amplify piRNAs. The methyltransferase HEN1 adds the 2´-O-methyl modification (depicted by a black circle) at the 3´-end of siRNAs and piRNAs after binding to AGO proteins. Yb, Armitage (Armi) and Zucchini (Zucc) are components required for Piwi nuclear-localization and biogenesis of primary piRNA. Additional components that interact with AGO proteins in the RISC and related effector complexes are not shown.

(A) miRNA genes

Pol II

pri-miRNA

Stem-loop region

Drosha / Pasha

pre-miRNA

DCR1 / Loqs-PB

miRNA 22-nt

AGO1 — RISC

Translational arrest or degradation of target mRNAs

(B) Endo-siRNA genes

Pol II

DCR2 / Loqs-PD

siRNAs 21-nt

DCR2 / R2D2

AGO2

HEN1

AGO2 — RISC

3' methylation

Cleavage of target mRNAs

(C) piRNA loci

flamenco   Transposon

Yb / Armi / Zucc

piRNAs 24 to 31-nt

PIWI

HEN1

PIWI

AUB

HEN1

AUB

AGO3

HEN1

AGO3

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1.7 References

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CHAPTER 2. RNA INTERFERENCE AND VIRUS-HOST INTERACTION IN *SCHIZOSACCHAROMYCES POMBE*

2.1 Abstract

Heterochromatic silencing directed by siRNAs was first described in the fission yeast *Schizosaccharomyces pombe* and then plants, *Drosophila melanogaster* and mammalian cells. It has been associated with the induction of epigenetic changes and the formation of complexes containing RNA interference (RNAi) and chromatin-remodelling factors. The cytoplasmic localization, dicing and slicing activity of Dcr1 and Ago1, respectively, suggest that the same components involved in transcriptional gene silencing (TGS) may also mediate post-transcriptional gene silencing (PTGS) in the cells. Here whether the cytoplasmic RNAi in *S. pombe* plays a role in antiviral silencing was examined. Infectious FHV RNA1 or RNA1 mutant (R1ΔB2) was inducibly expressed from cDNA clones either in plasmids or genomic locus generated by homologous recombination. Autonomous replication of RNA1 was observed in both cases, as well as the production of subgenomic RNA3 and RNAi-suppressor B2 protein. By comparing the viral accumulation in various integrant-strains, I found that in the absence of B2, FHV RNA1 and RNA3 accumulated to lower levels in wild type yeast before reaching the stationary phase; and the *dcr1Δ* strain showed more robust viral replication compared to wild type. However, the variation declined dramatically when the cells were entering the stationary phase, indicating RNAi may only eliminate the viral RNAs at low abundance or at the

early stage of replication. In addition, virus-derived small interfering RNAs (siRNAs) following FHV replication were not detected in *S. pombe* either by northern hybridization or deep sequencing. The small RNAs derived from FHV RNA1 represented the degradation intermediates of abundant viral RNAs.

2.2 Introduction

RNAi has been observed in the vast majority of the eukaryotic cellular processes such as the regulation of development, differentiation and apoptosis (Chen, 2005; Wienholds and Plasterk, 2005). It can act in the nucleus and the cytoplasm by mediating TGS and PTGS, respectively. In both cases, double-stranded RNAs are processed into siRNAs by Dicer, a type III ribonuclease. siRNAs are then incorporated onto the Argonaute-containing effector complexes which then associate with targets through a sequence-specific manner (Baulcombe, 1996; Bernstein *et al.*, 2001). The targets are consequently silenced undergoing RNA degradation or chromatin modification. In plants, *Neurospora crassa*, *Caenorhabditis elegans* and *Schizosaccharomyces pombe*, the silencing response can be amplified by RNA-dependent RNA polymerases (RDRs), which synthesize more dsRNAs that fuel the pathway by using the target RNAs as templates. Flies and vertebrates may lack this amplification step or maintain by other functional analogues for they do not encode proteins homologous to RDRs (Shabalina and Koonin, 2008). *Schizosaccharomyces pombe* has become a favorable biological system to study the mechanisms and function of RNAi-directed chromatin modification in the past decade.

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The fission yeast possesses relatively large domains of constitutive heterochromatin at the pericentromeric, telomeric regions and the silent mating type loci, which controls yeast cellular identity (Verdel and Moazed, 2005b). The RNAi proteins have been found associated with every major heterochromatic locus by site-specific and genome-wide chromatin immunoprecipitation experiments (Allshire, 2002). Notably, deletion of the RNAi machinery does not disrupt heterochromatin formation and silencing at the mating-type interval and telomeres, which is due to the existence of parallel pathways involved other transcription factors or histone-binding proteins that are conserved in a wide variety of eukaryotes at these sites (Bailis and Forsburg, 2002; Ekwall, 2004; Martienssen et al., 2005). It is believed that Clr4, homolog of the human and drosophila Suv39h methyltransferases, catalyzes methylation of histone H3 lysine 9 (H3K9me), which serves as a docking site for chromodomain proteins such as Swi6 and Chp2, which are both homologues of the vertebrates heterochromatin proteins1 (HP1) (Jenuwein and Allis, 2001). Another chromodomain protein, Chp1, which also binds to H3K9me and is essential for heterochromatin formation, associates with Argonaute in the RNA-induced transcriptional silencing (RITS) complex (Motamedi et al., 2004; Verdel et al., 2004). It is proposed that once the chromodomain protein bound to an H3K9 methylated nucleosome, it recruits Clr4 and probably other chromatin-modifying enzymes, including histone deacetylases, to initiate another cycle of H3K9 methylation and histone-binding onto the adjacent nucleosome (Grewal and Rice, 2004; Grewal and Jia, 2007). Furthermore, a few more recently reports indicate that heterochromatin formation at

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pericentromeric repeats is cell cycle regulated, and robust transcription by RNA polymerase II at each cell cycle might be an important step for the efficient epigenetic maintenance and recruitment of RITS and Clr4 complexes (Chen *et al.*, 2008; Kloc and Martienssen, 2008; Kloc *et al.*, 2008; Zhang *et al.*, 2008; Kavi and Birchler, 2009). Another benefit of using *S. pombe* to investigate the mechanism of RNAi pathway is the fact that its genome encodes single copy of each of the components required for RNAi with no redundancy among the mains actors (Hall *et al.*, 2002; Volpe *et al.*, 2002). Experiments based on deletion of *dcr1*, *ago1* and *rdp1* genes have shown a direct role of RNAi in the establishment and maintenance of pericentromeric heterochromatin in a siRNA-dependent manner. It is believed that transcription from both strands of the pericentromeric repeat region leads to the synthesis of a low amount of mRNA from both the template and non-template strands. These dsRNA then are processed by the Dicer to produce "trigger" siRNAs which are then loaded on RITS complex and guide the complex to homologous transcripts produced by RNA polymerase II. RNA-directed RNA polymerase complex (RDRC) which contains Rdp1 and required for siRNA loading onto RITS, is recruited by RITS to the nascent transcripts and generates dsRNA probably dependent on the activity of Rdp1. Larger amount of dsRNAs produced by RDRC are then processed by dicer to produce siRNA and the cycle continues to maintain the heterochromatic state (Verdel and Moazed, 2005b). If any of the RNAi machinery or components in RITS or RDRC is removed and hence disrupts the complex assembly, proper heterochromatin silencing fails implicating RNAi-mediated heterochromatin

formation relies on the production of dsRNA by bi-directional transcription of pericentromeric DNA repeats, siRNA, and the integrity and interaction of the RITS and RDRC (Motamedi *et al.*, 2004; Noma *et al.*, 2004; Verdel *et al.*, 2004; Buratowski and Moazed, 2005; Sugiyama *et al.*, 2005).

RNAi machinery in *S. pombe* can initiate another distinct silencing response besides the nuclear TGS and histone modification, without resorting to specialized forms of Dicer, Argonaute or RdRP. Introduction of a dsRNA hairpin corresponding to a green fluorescent protein (GFP) transgene triggers PTGS in the cytoplasm. GFP reflects a change in the steady-state abundance of GFP mRNA, whereas no detectable transcription effect or chromatin modification occurred at the GFP reporter that is artificially integrated into the chromosome. This cytoplasmic RNAi requires *dcr1*, *rdp1* and *ago1*, but does not require *chp1*, *tas3* or *swi6*, genes required for transcriptional silencing. (Sigova, 2004) The evidence of a functional classic RNAi pathway in *S. pombe* suggests it may provide a simplified, genetically tractable model in which I can study the requirement of each step: dicing, slicing and RdRP amplication in RNAi-mediated antiviral response; and whether the response occurs at the transcriptional level.

Although the budding yeast *Saccharomyces cerevisiae* provides all or most of the functions for successful replication of various RNA viruses and has been used as a model to unravel the host-virus interaction in the past two decades, it fails to be used in our investigation for it lacks recognizable homologs of RNAi machinery. Despite this perceived loss, Argonaute genes are present in some budding yeasts, including

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Saccharomyces castellii and *Kluyveromyces polysporus* (both close relatives of *S. cerevisiae*) and *Candida albicans* (the most common yeast pathogen of humans) and found to be associated with 22- or 23-nt RNAs which resemble to those Argonaute-bound guide RNAs of animals, plants, and other fungi. Analogous RNAs were not found in *S. cerevisiae*, and all the essential reads from small RNA sequencing represent degradation fragments of rRNA, tRNA, and mRNA (Drinnenberg *et al.*, 2009). Therefore, *S. cerevisiae* cannot be used directly for our purpose, but it implies very good possibility that RNA viruses can readily replicate in the fission yeast, for the complete replication of FHV in *Saccharomyces cerevisiae* was first demonstrated by Price *et al.* in 1996 (Price *et al.*, 1996). Transfection of yeast with FHV genomic RNA induced viral RNA replication, transcription, and assembly of infectious virions. All replicating FHV RNA species were readily detected in yeast by Northern blot analysis and yields of virions per cell were similar to those from Drosophila cells. Here I report the first instance to our knowledge of autonomous replication of FHV RNA1 in *S. pombe*. Nevertheless, *S. pombe* appears not to be an optimal system to study the RNAi-mediated antiviral response for the reasons presented in the discussion section.

2.3 Materials and methods

2.3.1   Plasmids and genome integration

For transcient expression of viral sequence, a full-length FHV RNA1 cDNA fragment followed by a tobacco ringspot virus satellite RNA ribozyme was cloned into the

pNMT41 expression vector by Dr. Feng Li in our laboratory. The construct for integration was designed based on pFA6a-3HA-hphMX6, which was originally applied as heterologous modules for PCR-based gene targeting. (Bahler *et al.*, 1998) The two stretches of homologous sequences (70-75nt) flanking the *leu1* gene in chromosome 2 were synthesized and annealed before cloning, and they terminated with recognition sequence of *Spe*I enzyme, therefore the entire cassette was released from the plasmid by *Spe*I digestion and transformed into yeast strains. The cDNA of FHV R1ΔB2 is derived from RNA1 and contains the two point mutations (T2739C and C2910A) described previously that abolished the coding potential of ORF B2 but had no effect on the overlapping ORF A in the genomic RNA or ORF B1 in RNA3.

2.3.2   Yeast transformation

Plasmids or PCR products described above were transformed into the yeast cells using the LiOAc-based method (Bahler *et al.*, 1998) with minor modification. Cells from fresh YEA plate were inoculated in 20ml YEA liquid medium and grown at 30℃ overnight before OD_{600} reached up to 1.0. On the next day, the overnight culture was diluted into 20 ml YEA per transformation to an OD600 of about 0.08. Continue growing the cells for 4-6 hours with gentle shaking until an OD600 of 0.2-0.5, which was equivalent to 0.5-1 $\times$ 10^7 cells per milliliter. Spin down the cells in Beckmann tabletop centrifuge at 3000 rpm for 5 min. Wash 1$\times$ with ddH_2O and 1$\times$ with 1 ml LiOAc mix, pellet again and resuspend in 100 μl of LiOAc mix for every 20 ml of culture starting with. For each transformation reaction, mix 100 μl of cells with 2 μl of cells Salmon Sperm DNA (10 mg/ml) and 1-5

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µg of transforming DNA in less than 15 µl volume. For integration of PCR fragments, use about 10-30 µg DNA per reaction. The DNA-cell mixture was incubated at room temperature for at least 10 minutes, and mixed well with 260 µl PEG mix by pipeting up and down. After incubation at 30℃ for another hour, 43 µl DMSO was added to the reaction and heat shocked at 42℃ for 5 minutes. The cells were then briefly spun down and washed once with 1ml deionized-distilled water, plated on YEA plates supplemented with 200 mg/ml hygromycin B. The transformants were incubated at 30℃ until the colonies formed, which usually took 3-4 days.

The hygromycin-resistant transformants are checked by PCR amplifying the 5' junctions of chromosomal and inserted DNA, using appropriate primers. (Table 2-1) The positive colonies revealed by PCR were further confirmed by southern blotting, to detect a 4.5 kb *Spe*I-*Cla*I segment containing B2 coding sequence.

2.3.3   Genomic DNA extraction

Cells were stroked in 5ml of YEA liquid medium containing 200 mg/L hygromycin B (Calbiochem) and 50mM thiamine (Sigma) when the transformant colonies appeared in proper size. Genomic DNA was prepared from culture at stationary phase (OD600 of ~2.0) following the procedures below. The harvested cells were treated with 300 µl of the Zymolase solution and incubated at 37℃ for at least 30 minutes to remove the cell wall. The yeast cells were then pelleted, resuspended in 550 µl 1×TE, 1% SDS, incubate at 65°C for 1 hour, and then kept on ice for at least 30 minutes after adding 175 µl 5M potassium acetate. Spin down the cell debris at 4℃ for 15 minutes, and transfer the

supernatant to a new tube containing equal volume of ice-cold isoproponal. Precipitate at -20 ℃ for 20 minutes and pellet the nucleic acids by centrifugation at full speed at 4 ℃ for 15 minutes. The pellet was then washed once with 70% ethanol, air-dried, dissolved in 350 μl of TE containing 10 μg/ml RNaseA (Sigma) and incubated at 37°C for 1 hour. Genomic DNA was further purified by phenol-chloroform extraction, and then precipitated from the aqueous phase with 1/10 volume of 3M sodium acetate and 2.5 volume of ethanol. The DNA pellet was dissolved in 20 μl 1×TE buffer.

2.3.4 PCR screening and Southern Blotting

The genomic DNA was diluted 10 times and 1 μl was used as template in a 20 μl reaction. Sequences of primers used for PCR screening were listed in Table 2-1. Positive colonies were then proceeding to southern blotting for further confirmation. About 5-10 μg DNA sample was digested with 30-40 units of *Sal*I and *Cla*I for at least 6 hours in a 100 μl reaction, followed by phenol/chloroform extraction and ethanol precipitation. The DNA pellet was resuspended in 10 μl 1×TE buffer, and then loaded on a 1.2% agarose gel. The gel was prepared by boiling 2.4 g agarose in 200 ml 1×TAE buffer, adding ethidium bromide (0.5 μg/ml) after it cooled down to 60°C and pouring into a gel casting tray (BIO-RAD). The gel was run in a DNA SUB CELLTM (BIO-RAD) at 100 V for about 3 hours until the bromophenol blue ran through 2/3 of the gel. Then the gel was photographed using a GEL SYSTEM (BIO-RAD) to assure equal loading and marked the position of DNA ladder. Next the gel was subjected to depurination by incubating in 0.2 M HCl for 10-20 min till the bromophenol blue turned yellow. Then it was washed

briefly in water and denatured by incubating in 0.4 M NaOH for 30 min till the bromophenol blue turned blue again. Finally, it was neutralized by incubating in neutralization buffer for 30 min and preceded to transfer step. Thereafter, every step was the same as described in Northern blotting analysis.

2.3.5   Cell grow and viral RNA induction

Integrants are maintained on rich media (YEA: 5 g/L Yeast extract, 30 g/L Glucose, 225 mg/L Adenine) plus Thiamine (Sigma) and 200mg/L Hygromycin B (Calbiochem). In order to ensure the healthiness of each yeast strain and any potential effects on viral replication during cell proliferation, cells were stroked on proper plates from glycerol stocks prior to each experiment. In order to induce the expression of viral cDNAs, cells are grown in liquid minimum media (EMM) plus all the supplements and Thiamine at the concentration for fully suppression until the mid-log phase is reached. (OD_{600} ~ 0.5) Equal amount of cells for each testing sample are washed two times with double distilled water and once with EMM medium. Inoculate cells to fresh EMM medium plus all the supplements except Thiamine, and grow cells at 30 ℃ by constant shaking. 1% glucose is added to the culture when OD_{600} reaches 0.5 ~ 1 to maintain cells at mid-log phase.

2.3.6   Total RNA extraction and small RNA fractionation

Total RNA are extracted from *S. pombe* using TRIzol reagent (Invitrogen) vortexing with acid-washed glass beads. For better purification, 1ml of TRIzol was applied to approximately 2 × 108 cells. 200 μl of chloroform was added to the TRIzol after removing the glass beads and vortex vigorously for 30 seconds. After chloroform

extraction, precipitate the aqueous phase with equal volume of isopropanol, and the mixture was incubated at -20 ℃ for at least 20 min. Total RNA was then centrifuged at 14,000 rpm for 10 min, washed once with 1ml 70% ethanol, air-dried, dissolved in DEPC-treated water. To purified RNAs subjected to fractionation, hot phenol extraction had been applied in which rather than TRIzol, cells were vortex for 30 min with phenol (pH~5), glass beads and extraction buffer at 65 ℃ as described by Verdel et al.(Verdel *et al.*, 2004; Verdel and Moazed, 2005a). However, no significant difference in RNA yield or quality was observed. The low molecular weight (LMW) RNA was separated from those of high molecular weight (HMW) by 5M LiCl precipitation. Following LiCl precipitation overnight at -20 ℃, the supernatant containing small RNA fraction was precipitated with equal volume of isopropanol, 1/10 volume of 3M sodium acetate and 20 μg of glycogen. *mir*Vana miRNA isolation kit (Ambion) was also applied for the enrichment of LMW RNA following the manufacturer's instructions, and better isolation was obtained compared to the LiCl method.

2.3.7   Northern Blotting analysis (HMW RNA)

RNA samples were quantified by BioPhotometer (Eppendorf), and ~5 μg total RNA in less than volume of 10 μl was mixed with 7.8 μl sample buffer (Table 2-6), heated at 65°C for 10 min and immediately cooled on ice. After adding 2 μl of 6 × loading buffer (Table 2-6), RNA samples were loaded into the 1.2% (v/v) formaldehyde-denaturing gel and run at 100 volts for 2-3 hours using FB600 Electrophoresis System (FisherBiotech) until the bromophenol blue ran through 2/3 of the gel. 1.2% (v/v) formaldehyde-

denaturing gel was prepared by adding 2.4g of agarose into 174 ml RNase-free water and boiled in a microwave oven until the agarsoe was completely dissolved. 20 ml of 10 × MOPS buffer (Table 2-6) and 6 ml of 37% formaldehyde were added and mixed thoroughly after the agarose cooled down to around 60 ℃. The total 200 ml solution was poured into the gel casting tray (Amersham Phamacia Biotech) with proper comb, and the electrophoresis was carried out in a MAX HORIZ SUB system (Amersham Phamacia Biotech) containing 1×MOPS buffer.

After electrophoresis, the gel was rinsed in 10× SSC (Table 2-6) and RNA was transferred for 3 h onto a Hybond-N+ membrane (Amersham Bioscience) using a VacuGene XL transfer unit (Pharmacia Biotech) according to the manufacture's instruction. The gel was discarded. The membrane was briefly washed in 10×SSC and then cross-linked in a XL-1000 UV crosslinker (SPECTROLINKERTM, 180mj/cm2). The membrane was then stained with methylene blue solution (Table 2-6) to estimate the amount of RNA and be used as loading control. The stained membrane was scanned and subjected to pre-hybridization (Table 2-6) at 65 ℃ for at least one hour in a hybridization oven (Amersham Pharmacia Biotech).

Both FHV RNA1 and RNA3 can be detected by α^{32}P-labelled cDNA probe that corresponded to the last 387 nucleotides of FHV RNA1, which is also used in southern blotting to detect the integrated copy of viral cDNAs. The probe was prepared using a RediprimeTMII kit (Amersham). Specifically, a DNA template about 20-50 ng was added into 45 μl 1×TE buffer, boiled for 5 min, chilled on ice for 3 min, and spun down to the

bottom. Then the template was transferred to reaction tube from the kit, 4 μl α-^{32}P-dCTP was added (Amersham), mixed well, and incubated at 37 ℃ for 20 min. Finally the reaction tube was boiled for 5 min, chilled on ice for 3 min, and half or total of the reaction was added to the hybridization tube depending on the expression level of the gene to be detected. Hybridization continued at 65 ℃ usually overnight or for at least 16 hours. The membrane was washed once in $2\times$ SSC/0.1% SDS at 65 ℃ for 20 min; twice in $0.2\times$ SSC/0.1% SDS for 20 min. Then the membrane was dried by filter paper, sandwiched in plastic membranes, and exposed to a phosphoimage screen (Kodak) or HyBlot-CL$^{®}$ Autoradiography Film (Denville Scientific).

If re-probing was needed, the membrane was stripped by incubating in stripping solution (Table 2-6) at 80 ℃ in the hybridization oven. After stripping, the membrane was rinsed in distilled water and re-used for pre-hybridization. Strand specific oligos were used for probing the sense and antisense strands of RNA1 and RNA3. The ^{32}P-ATP-labelled oligo-probes were used for detecting the FHV-specific siRNAs as well, and they are composed of 11 head-to-tail overlapping oligonucleotides that cover the sequence of last 387 nt of RNA1 (Li *et al.*, 2002) Probes detecting the centromeric siRNA control were as previously reported (Reinhart and Bartel, 2002). Probe preparation was described as below. Intensity-measurement of radioactive signal was done by using Quantity One (Discovery Series) software.

2.3.8   LMW RNA detection

Cells have been subjected to small RNA isolation contains FHV RNA1 or R1ΔB2 in the

expression plasmid under a stronger promoter *nmt1*. Cells are collected at 24 hours post induction (thiamine depletion). Small RNA concentrations are normalize and used for blotting following the protocol described by Verdel et al. (Verdel *et al.*, 2004; Verdel and Moazed, 2005a). Briefly, 25 to 100 μg of RNA samples were mixed with equal volume of formamide, heated at 90 °C for 5 minutes, and added 2 μl 6× loading buffer. A 15% polyacrylamide gel containing 8M urea was prepared and run in 0.5×TBE buffer (Table 6) using a Dual Adjustable Slab Gel Unit (C.B.S Scientific Co.)

Once electrophoresis was completed, the resolving gel was removed gently from the glass plate, rinsed in 0.5×TBE buffer, and transferred onto a Hybond-N$^+$ membrane (Amersham Biosciences) using semidry transfer unit (Hoefer SemiPhor, PharmaciaBiotech). Then the membrane was UV cross-linked, hybridized in PerfectHyb™ Plus Hybridization Buffer (Sigma) at 40 °C and the washing was carried out at 50 °C.

The strand-specific oligonucleotide probes (40nt in length) corresponding to nucleotides B2 region were labeled at their 5' end by polynecleotide kinase (PNK) in the following reaction: 2μl of DNA oligo (10 μM), 2μl of 10× PNK buffer, 4 μl of α-^{32}P-ATP, 1 μl of PNK enzyme (NEB), and 11 μl of water. The end-labeling reaction was incubated at 37 °C for 30 minutes, chilled on ice, added for hybridization after denaturing at 95 °C for 3 minutes.

2.3.9   Western blotting

Total proteins were isolated from yeast treated with Zymolase solution for at least 30

minutes by proper volume of cracking buffer. Protein sample was mixed with equal volume of 2×SDS sample buffer and heated at 95-100 ℃ for 5 min prior to loading. 15% SDS-PAGE gel was prepared to detect B2 protein and run at 100-150 volts in 1×TTS buffer (Table 2-5) in a vertical Electrophoresis System (FisherBiotech) until the bromophenol blue tracking dye reached the bottom of the resolving gel. Transfer of proteins from the gel to a Hybond-P+ membrane (Amersham Biosciences) was performed using a semidry transfer unit (Hoefer SemiPhor, PharmaciaBiotech) according to the manufactures instructions. After transfer, the gel was stained with Coomassie Brilliant Blue (Table 2-6) for 2-4 hours, and destained with the destaining solution. The gel was scanned for loading control. The membrane was gently agitated in the block solution (1×TBST with 1% nonfact dry milk) for at least 1 hour, and then incubated with the primary antibody diluted in block solution (1:3000) for 1-2 hours and followed by two washes with 1×TBST 15 min each. Protein A-conjugated alkaline phosphatase (1:5000 diluted in block solution) was added afterwards and incubated for 1 hour followed by washing three times as above. All the blotting and washing steps were carried out at 4 ℃. Immunodetection was carried out using a Lumi-Lightplus Western Blotting Substrate (Roche).

2.3.10 Small RNA library construction

Cloning of small RNAs was carried out as described previously (Langmead *et al.*, 2009; Malone *et al.*, 2009; Wu *et al.*, 2010a) with minor modification. Small RNAs ranged of 18-25nt were isolated from 20-50 μg of LMW RNAs in a urea-denaturing gel as

described in LMW RNA detection. The desired section of gel was excised, eluted in 1mL 0.4 M sodium chloride overnight at 4 ℃, and precipitated in 20 μg of glycogen and equal volume of isopropanol. RNA pellet was washed once with 70% ethanol, air-dried and dissolved in 13μl DEPC-treated water. The small RNAs were then cloned by the procedure that require 5'-mono phosphate and 3'-hydroxyl group. Briefly, small RNAs were ligated to one of the three adaptors at their 3' end. The ligation products were isolated from the non-ligated ones and excessive primers by the same procedure as the previous step. The ligation products were then precipitated by adding 4 μl of 3M sodium acetate (pH 5.2) and 3 volumes (90 μl) of 100% ethanol. The recovered pellets were subjected to the reverse transcription followed by PCR amplification. The PCR products were purified from a 12% polyacrylamide gel through the same procedures as the previous step, quantified and sequenced by Illumina GA Analyzer.

2.3.11 Sequence analysis

Data analysis was carried out by Dr. Qingfa Wu in our laboratory. Sequence of FHV RNA1 (Genbank Accession No NC_004146) was downloaded from NCBI. The adapter sequences were masked with cross_match, only these reads containing both adaptors were kept. After removing the adaptor sequences, the reads (18-25 nt in length) were mapped to RNA1 with BLASTN. Only reads that can be perfectly mapped on the genome sequences were kept for further analyses. All other analysis was carried out with in-house scripts.

2.4 Results

2.4.1 FHV RdRP-dependent replication of RNA1 under the control of thiamine-

repressible promoter

The *nmt1* (no message in thiamine) promoter is the most frequently used promoter for

gene expression in fission yeast, and its transcription can be fully suppressed in the

presence of thiamine at a concentration of 0.5 μM or greater, according to the laboratory

handbook and protocols from Dr. Nurse, P. and Dr. Forsburg, S. Previous colleagues in

our laboratory had been using *nmt1* and *nmt41*, an intermediately attenuated version of

nmt1, to express the cDNA of FHV RNA1 (R1) or R1ΔB2 mutant in yeast cells prior to

the beginning of this project. The major drawback of using the transient transformation

system is impossible to monitor the inheritance of the plasmid in each cell during cell

division, which would be a trivial factor for cellular gene; whereas in the case of viral

RNA that are self-replicable independent of transcription, the plasmid system may cause

dramatic fluctuation in each experiment. Therefore, I decided to replace the *leu1* gene in

the chromosome 2 with the cDNA clone of FHV RNA1, in order to make a reasonable

comparison of the viral accumulation between various RNAi mutant strains.

The PCR-based gene targeting approach has been commonly used for gene deletion and

tagging, and it is indeed more convenient and straightforward to create the fragments for

homologous recombination in most cases (Bahler *et al.*, 1998). However, for our purpose,

the length of FHV RNA1 cDNA driven by the *nmt41* promoter (~1 kb) was over 3 kb,

and a hygromycin selection marker was used for screening at the proceeding step. It was

technically difficult to amplify such a large DNA fragment by PCR with a pair of long

primers due to the problem of secondary-structured primers, and more importantly, point

mutations would be introduced into the PCR product unavoidably. Therefore, I decided to

clone the 5' and 3' UTRs (70nt each) of *leu1* gene into the pFA6a-3HA-hphMX6 plasmid

by stepwise cloning, and FHV RNA1 would be transcribed in the same orientation as the

endogenous *leu1* gene. A tobacco ringspot virus satellite RNA ribozyme (Li *et al.*, 2002)

was cloned immediately following the FHV RNA1 cDNA at its 3'-end, and immediately

upstream of T$_{ADH1}$ sequence (Terminator sequence of the ADH1 gene from *S. cerevisiae*).

As a result, the nascent transcripts were supposed to contain the full-length R1 and

ribozyme sequence. The ribozyme was required to cleave off any non-viral sequence

from the 3' end, producing infectious RNA1, which can serve as both template for

translation of FHV RdRp and RNA replication. The HphMX6 cassette following the

T$_{ADH1}$ sequence consists of the promoter and terminator sequences of the *Ashbya gossypii*

translation elongation factor 1α gene, followed by a hygromycin resistant gene, which is

used as a selectable marker for positive integrants (Bahler *et al.*, 1998). The plasmid does

not contain any replication origin and thus cannot replicate by itself in *S. pombe*, and the

cassette for integration could be easily cleaved from the finished construct (Figure 2-1)

by *Spe*I restriction digestion, and transformed into the yeast cells. The transformants that

were capable to grow on medium containing 200mg/L hygromycin B were subjected to

genomic DNA isolation, and the genotype was further confirmed by PCR amplication of

a 400bp fragment covering the HphMX6 and the 5'UTR of *leu1*, and southern blotting

probing for a 4.5 kb *Sal*I – *Cla*I fragment (Figure 2-2). Only those positive integrants

containing one single copy of FHV RNA1 cDNA sequence at the *leu1* loci were selected. A relatively high recombination rate ranged from 12.5% - 50% was observed among the yeast strains tested (Table 2-3).

In both FHV-integrated strains and transient expression system, autonomous replication of FHV RNA1 was detected. The patterns of RNA1 and subgenomic RNA3 were consistent with those that were demonstrated in other organisms, including *S. cerevisiae*, *Drosophila* and *C. elegans*. Replication of FHV RNA1 was supported by three lines of evidence: (i) detection of antisense strand of RNA1 by strand-specific oligonucleotide probes; (ii) abundant RNA3 of both sense and antisense strands, for RNA3 is a subgenomic RNA transcribed during RNA1 replication from an internal site of the complementary, replicative intermediate of RNA1; (iii) No FHV RNAs accumulated in the cells expressing with R1fs transcripts that contain a frameshift mutation in the FHV RdRp open reading frame, suggesting that the replicating viral RNAs were dependent on the functional FHV RdRp (Figure 2-3B). Similarly, both sense and antisense strands of FHV RNA1 and RNA3 were readily detected in yeast strains expressing either R1 or R1ΔB2 from their genomes. Transcription of the viral sequence was efficiently repressed in the presence of thiamine and hence the viral replication was below detectable level. (Figure 2-3C) The VSR B2 protein could be detected in *S. pombe* strain that was transformed with R1 but not R1ΔB2, by the polyclonal serum recognizing B2 in FHV-infected S2 cells (Figure 2-3D).

2.4.2   RNAi defective mutants exhibited higher viral RNA accumulation only at early

phases of viral replication

All the integrant strains were maintained and grown in medium containing 10-50 μM thiamine to achieve full repression of the viral transcription, and cells were washed multiple times with deionzed distilled water before culturing in thiamine-free medium in each experiment. In order to figure out the optimal experimental condition, various set-ups were tested, including culturing the yeast strains under different temperature, inducing viral transcription at various growth stages. Eventually I determined to conduct the experiment at 28 °C, at which the balance of efficient RNAi and cell growth was reached. The cells were either kept diluted in fresh medium or 1% glucose was added to the medium when we need to study the viral accumulation for longer period of time, i.e. 2 days post induction. I decided to induce transcription in the thiamine-free medium for 12 hours according to the discovery (Maundrell, 1990) that removal of thiamine from the medium produced detectable massage RNA after 10 hours and maximal steady-state levels after 16 hours by northern blotting.

Interestingly, when the viral transcription was induced for 12 hours, the cells were at mid-log phase and the viral RNA accumulated to very low level, deletion of *dcr1*, *rdp1* and *ago1* resulted in higher replication of R1 compared to wild type, indicating these key components of RNAi pathway play a role in restricting the virus in the cells. (Figure 2-4A) This was also supported by the more robust replication of R1ΔB2 in *dcr1Δ* and *rdp1Δ* null mutants, whereas *ago1Δ* showed comparable or even lower level than wild type. Thiamine was added to the medium again after 12 hours to shut-off transcription, so

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that the viral RNA levels would only be influenced by FHV RdRp replication and potential effects by RNAi-elimination of the virus. However, the variation among different strains disappeared when the cell culture was harvested after another 36 hours (2 days post induction), even if we kept refreshing the culture so the cells were maintained at healthy condition. Both R1 and R1ΔB2 accumulated to similar levels in wild type and all the other three mutant strains. This was significantly distinct from what we observed in *Drosophila*, in which R1ΔB2 accumulated under detectable level in either wild type embryos or S2 cells, and only managed to replicate robustly in the absence of key RNAi enzymes such as Ago2. Consequently, I suspected that the primitive RNAi machinery in *S. pombe* might only be capable for eliminating the virus efficiently when the viral RNAs were of low abundance, and B2 might have little activity in suppressing RNAi. The impact of RNAi-deficiency on virus clearance was examined before the system was saturated by high levels of viral RNAs, by shorten the time of de-repressing the viral transcription to 6 hours, and shut-off the transcription by the end of 6 hours.

As expected, strains containing R1 and deletion of *dcr1*, *rdp1* and *ago1* genes showed 7.1, 6.0 and 6.4 times higher virus abundance than wild type after 12 hours (6-hour induction and 6-hour replication), respectively. While the ratio of RNA3 between *dcr1Δ*, *rdp1Δ*, *ago1Δ* and wild type decreased to 1.6, 1.8 and 3.0, respectively, at 24 hours post induction (6-hour induction and 18-hour replication), (Figure 2-4B) indicating these three proteins play very weak role in virus clearance during the early stage of viral replication, and the function of Dcr1 may be more dominant among them. The idea was further

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confirmed by the comparison of R1ΔB2 replication in *dcr1Δ* and *ago1Δ* strains. (Figure 2-4C) The subgenomic RNA3 level in *dcr1Δ* was 4.7 times higher than that in the wild type at 12 hours post induction (thiamine depletion), and remained to be around 4 times more abundant at 24 hpi. RNA3 accumulation in *ago1Δ*, however, which was 4.6 times higher level of RNA3 at 12 hpi, turned out to be almost equal to wild type after another 12 hours of replication. This may correspond to the fact that no matter in TGS or PTGS, dicing and primary siRNA production is the most upstream step, and dicing itself can be a process degrading the viral RNAs. Therefore, I observed stronger defects in restricting the viral replication in *dcr1Δ* than *ago1Δ* mutant.

2.4.3 FHV-derived small RNA analysis

Production of virus-specific small RNA is the most important feature in RNAi-mediated antiviral response characterized in plant and invertebrate systems. As a result, small RNAs from R1ΔB2-transformed yeast strains were examined, in order to conclusively determine whether the primitive RNAi in yeast inhibited the viral replication through the canonical pathway. For better yield and quality of small RNAs, yeast cells should be collected at mid-log phase and according to the previous experience in detecting the genomic and subgenomic RNA of FHV, sufficient viral accumulation should be reached at a relatively short time. So a stronger promoter, *nmt1*, was chosen to drive the expression of viral transcripts in the plasmid. R1ΔB2 was used for this study to eliminate any possible effects of B2 on siRNA-dependent pathway or the yeast cells. Total RNAs shorter than 200 nt in length were isolated and probed for antisense strands of either 5' or

3' end of RNA1, because they were supposed to target the sense viral genomic RNAs, which were the predominant species in the infected cells. Most of the FHV-specific siRNAs were derived from the first 400 nucleotides the 5' terminus of FHV RNA1 in FHVΔB2-infected S2 cells, and good amount also targeted the last few hundreds of nucleotides at the 3' end probably due to the existence of RNA3. However, probes targeting these two regions did not revealed any convincing signals at either 21 nt (according to viRNAs in *Drosophila*) or 22-23 nt, which was the size of endogenous siRNAs from *S. pombe*. (Figure 2-5A) The smeary signal at the top portion of the gel may represent the degradation products of viral RNA1, and the corresponding signals appeared at much stronger intensity when the membrane was probed for sense strands of RNA1. Similar hybridization pattern was also detected in a yeast strain expressing a C-terminal TAP-tagged Ago1 for siRNA enrichment (personal communication with Dr. Danesh Moazed). Whereas the viral degradation products was absent in *dcr1Δ* strain implied that dicer in *S. pombe* might process the viral RNAs in a diverse manner from that for dicing the heterochromatic transcripts or antiviral dicers in other species. The involvement of Dcr1 in processing was supported by our observation that in the presence of either R1 (not shown) or R1ΔB2, the endogenous siRNAs significantly decreased no matter in total small RNA population or in those associated with Ago1 (personal communication with Dr. Danesh Moazed).

Considering the limited sensitivity of detection by northern blotting, small RNAs of 18-25 nt were cloned from wild type and *dcr1Δ* mutant for deep sequencing. To our surprise,

the majority of small RNAs that could be mapped perfectly to the RNA1 were of 18nt, and exhibited gradual decline in quantity as the length increased. (Figure 2-5B) The reads corresponding to the genome also revealed similar pattern with enrichment in 18-21nt, which was distinct from the previously reported library constructed from wild type strain free of viruses. (Figure 2-5C) Since the reference library composed of much smaller number of reads in total, further normalization was required to make a reasonable comparison of the two libraries from wild type strains in actual number of a particular size population probably by using a well-characterized endogenous siRNA, as there was no miRNA or piRNA identified in the fission yeast so far. Nevertheless, it was apparent that the endogenous small RNA profile with the major peaks at 22-and 23 nucleotides could be changed by the presence of viral RNA probably due to the competition between the virus-degradation and heterochromatin silencing pathway. Moreover, most virus-specific small RNAs were derived from the sense strand of FHV RNA1 (Figure 2-5D) and the relatively even distribution along the viral genome suggested these small RNAs might represent the products of RNA decay.

2.5 Discussion

Viruses are intracellular parasites that rely on the molecular machinery of the hosts for propagation and replication. To investigate the virus-host interactions, including identification of host factors that initiate the defense response, and factors with which

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viruses interact for their replication and cytopathic effects has always been a challenging subject in virology. A number of filamentous fungi species including *Olpidium bornovanus* and *O. brassicae* in the phylum Chytridiomycota have been identified as transmission vectors of various plant viruses (Rochon *et al.*, 2004; Rochon, 2009). These two filamentous fungi can reproduce asexually through the production of zoospores within a spherical cell. The zoospores, which have a single flagellum, settle upon release and grow into new chytrids (Kassanis and Macfarlane, 1964; Fry and Campbell, 1966; Kakani *et al.*, 2001). These motile zoospores are employed by viruses within five of the eight genera of the family Tombusviridae, which are all icosahedral RNA viruses. (Rochon *et al.*, 2004) Members of Tombusviridae, such as cucumber necrosis virus (CNV) are carried by the fungus externally by attaching to the surface of zoospores prior to zoospore encystment on host roots. (Kakani *et al.*, 2001) Whereas other viruses in unassigned families, including lettuce big vein virus (LBVV) and tobacco stunt virus (TSV) are carried internally in the resting spores as well as the zoospores of *O. brassicae*. (Kakani *et al.*, 2003) Being the transmission vector of viruses, fungi should be capable to support replication of viruses. On the other hand, they have also evolved defense mechanism to restrict the viral replication and prevent the pathogen from causing massive damage. Therefore, it is intriguing to understand the dynamic virus – vector interaction and how the balance described above is reached.

Although RNAi pathways in filamentous fungi are well understood in *Neurospora*, there is no experimental viral system that can infect this organism. Therefore, I meant to

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develop the fission yeast as a system to study the virus-fungi interaction, for it represents eukaryotic life in a single, non-motile cells, while possess a wide variety of biochemical and genetic pathways that are conserved among higher multicellular organisms. Moreover, their genomes are almost completely sequenced, and thus many genetic and cell biological techniques are available for generating mutants of interest, such as genetic crosses, gene deletion, tagging and overexpression; GFP fluorescent microscopy, immunoprecipitation and so forth. For specific aims of our laboratory to prove that RNAi is an evolutionally conserved antiviral defense and understand the mechanism of this pathway, *S. pombe* appeared as a promising model as it possess a very simple and conserved machinery, and no microRNA (miRNA) pathway has yet been discovered. The siRNA-mediated silencing could be studied without the potential involvement of homologs or miRNA components in the antiviral response. In addition, there is also only dicer protein encoded by human genome. And it has been demonstrated that RNAi-mediated heterochromatin assembly exits in mouse cells. In mammalian cells, conditional knock down of a component of RNAi such as dicer led to increased expression of genes in the repeat regions, suggesting that RNAi-mediated heterochromatin assembly is needed to maintain transcriptional silencing in certain repeat regions (Kanellopoulou *et al.*, 2005; Murchison *et al.*, 2005). So I assumed the *S. pombe* could be used as a model to address some important questions in the field: (1) whether a single Dicer protein is capable to recognize and process the dsRNA intermediate during viral replication, even if the same Dicer is required for nuclear transcriptional silencing; (2) if the Dicer protein

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serve as a sensor of viral dsRNA generation, how the host and viral components interact with each other within the cells and (3) whether the RNAi-dependent heterochromatin assembly would be affected by the antiviral silencing pathway.

Disappointedly, I found that FHV replication did not trigger strong RNAi-based antiviral response in *S. pombe*. The viral replication levels were much lower than what we detected in other organisms, such as *Drosophila melanogaster*, under our experimental condition, even if I replaced the ribozyme in our constructs with a more efficient ribozyme from HDV (Data not shown). As described in the introduction section, the RNAi was reported to be regulated by cell cycle, and the transcription-dependent viral replication was also easily affected by the condition of cell culture, temperature and other environmental situation. Therefore, variation of viral RNA abundance was observed occasionally. More importantly, typical virus-derived siRNA of specific lengths failed to be detected by sequencing, indicating that the primitive dicer in *S. pombe* seemed to be not processing the viral transcripts efficiently, if there is any processing at all, and the system was probably overwhelmed by the RNA degradation products in the presence of FHV replication.

Another possibility leading to a weak antiviral response against FHV in *S. pombe* may be the predominant nuclear-localization of the RNAi machinery. Studies in both *S. cerevisiae* and *Drosophila* imply that FHV may be a virus propagating in the cytoplasm. Miller and Ahlquist have demonstrated FHV RNA polymerase is a transmembrane protein with amino-terminal sequences inserted into mitochondrial membrane in *S.*

cerevisiae (Miller and Ahlquist, 2002). In *Drosophila* S2 cells, FHV RdRP is localized on the outer membrane of mitochondria (Miller *et al.*, 2001). However, the preferentially nuclear localization of RITS and RDRC has been shown by insertion of 13 myc epitopes at the C terminus of all the subunits except that Ago1 is tagged with myc3 at the amino terminus (Motamedi *et al.*, 2004). They found that Chp1 and Tas3 are predominantly localized to the nucleus in a speckled pattern, which may represent the different heterochromatic chromosome regions. These results are consistent with previous report and their chromatin immunoprecipitation experiments showing that the RITS complex is specifically localized to heterochromatic DNA region and their involvement in heterchromatin establishment (Volpe *et al.*, 2002; Noma *et al.*, 2004; Verdel *et al.*, 2004; Verdel and Moazed, 2005b; Moazed *et al.*, 2006). Rdp1-myc13 could only be detected under overexpression condition, and it was localized to the nucleus in most cells but also was present in the cytoplasm of mitotic cells. The signal of myc13-Ago1 may be so weak that they did not show in their literature, but they state that it is present in a speckled pattern in the nucleus and throughout the cytoplasm (Sigova, 2004). As a result, Dcr1, Rdp1 and Ago1 may have very limited access to the replicating viral RNAs in the cytoplasm.

Quite recently the antiviral role of RNAi has been shown in the filamentous Ascomycete fungus *C. paracitica*, the chestnut blight fungus. It provides an excellent experimental system for the study of hypoviridae family of mycoviruses that reduces its pathogenicity and can support the viral replication of five different RNA virus families (Pearson *et al.*,

2009). Another group also reported the infection of *Aspergillus nidulans* with three mycoviruses (Coenen *et al.*, 1997; van Diepeningen *et al.*, 1998). While *C. paracitica* appeared to be a better studied fungus, whose genome encodes two dicer-like genes DCL-1 and DCL-2. *dcl-2* and *dcl-1*/*dcl-2* mutant strains are highly susceptible to the infection of hypovirus or reovirus. On the other hand, infection of the *dcl-2* mutant by a hypovirus mutant lacking the RNAi suppressor exhibited elevated viral RNA levels compared to the wild-type (Segers *et al.*, 2007; Zhang and Nuss, 2008; Sun *et al.*, 2009). These results combining with the detection of virus-derived siRNAs dependent on DCL-2 establish that a fungal Dicer can function to regulate virus infection and RNAi plays an important role in antiviral defense in fungi. Alternatively, in order to study the siRNA-mediated silencing pathway in an isolated cellular environment, such as an in-*vitro* system, one may consider expressing the key components, Dicer, RdRP and Argonautes from *Saccharomyces castellii* or *Kluyveromyces polysporus* in *S. cerevisiae*, (Drinnenberg *et al.*, 2009) which may provide better condition for supporting the replication of RNA viruses.

Table 2-1 Sequences of primers used for generation and screening for virus-integrants

YL19	GGCCGCACTAGTAACCAGGCATTTTTGCGGTAGGGTCTGAAACTTTCACGAAACCAT TTCACAACAAAACTTCTTCAACAACGC
YL20	GGCCGCGTTGTTGAAGAAGTTTTGTTGTGAAATGGTTTCGTGAAAGTTTCAGACCCT ACCGCAAAAATGCCTGGTTACTAGTGC
YL21	AAACTAGGGTTTAATGTAGAATAAATTCATATGATTTGATTTGACCTAATTTTTATTGAT TAATTGTGTCGCATA
YL22	CTAGTATGCGACACAATTAATCAATAAAAATTAGGTCAAATCAAATCATATGAATTTAT TCTACATTAAACCCTAGTTT
YL26	CGGATGTCGTAAATCAATTCCATGC
YL27	GCCGATAGTGGAAACCGACGC

Table 2-2 Sequences of linkers (adaptors) for small RNA cloning

3'linker-1	5rApp/CTGTAGGCACCATCAAT/3ddC
3'linker-2	5rApp/CACTCGGGCACCAAGGA/3ddC
3'linker-3	5rApp/TTTAACCGCGAATTCCAG/3ddC
5'linker-1	GUUCAGAGUUCUACAGUCCGACGAUCAUC
5'linker-2	GUUCAGAGUUCUACAGUCCGACGAUCCGU
5'linker-3	GUUCAGAGUUCUACAGUCCGACGAUCUAC
SBS3-1	CAAGCAGAAGACGGCATACGATTGATGGTGCCTACAG
SBS3-2	CAAGCAGAAGACGGCATACGTCCTTGGTGCCCGAGTG
SBS3-3	CAAGCAGAAGACGGCATACGCTGGAATTCGCGGTTAAA
SBS5	AATGATACGGCGACCACCGACAGGTTCAGAGTTCTACAGTCCGA

Table 2-3 Genotype of yeast Strains

Strain	Genotype
SPY28	(SPG1011) h+ *leu1-32 ade6-216 ura4DS/E imr 1*R*(NcoI)::ura4+ oriI*
SPY86	h+ *leu1-32 ade6-216 ura4DS/E imr 1*R*(NcoI)::ura4+ oriI dcr1Δ::TAP-kanR*
SPY87	h+ *leu1-32 ade6-216 ura4DS/E imr 1*R*(NcoI)::ura4+ oriI rdr1Δ::TAP-kanR*
SPY120	h+ *leu1-32 ade6-216 ura4DS/E imr 1*R*(NcoI)::ura4+ oriI ago1Δ::TAP-kanR*

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Table 2-4 Buffers for transformation and nucleic acid extraction from *S. pombe*

LiOAc mix	1 ml 10x TE (pH 7.5), 1 ml 1 M LiOAc (pH 7.0), 8 ml ddH2O
PEG mix	5 ml 10x TE (pH 7.5), 5 ml 1 M LiOAc (pH 7.0), 20 g PEG (3850)
Zymolase solution	α-Sorbitol 16.4g, 0.5M EDTA 20ml, Zymolase (20T) 30mg, 2-mercaptoethanol 200ul, ddH2O up to 100 ml

Table 2-5 Buffers for protein extraction

Cracking buffer	8 M Urea, 5% (w/v) SDS, 40 mM Tris-HCl (pH 6.8), 0.1 mM EDTA, 10 mM beta-mercaptoethanol, 30x diluted protease inhibitor
Protease Inhibitor	One cocktail tablet (Roche) dissolved in 1 ml distilled water
2×SDS sample	0.125 M Tris-HCl (pH 6.8), 4% (w/v) SDS, 20% (v/v) glycerol, 0.3 M β-mercaptoethanol, 0.05% (w/v) bromophenol blue

Table 2-6 Buffers for Northern, Southern, and Small RNA Blot analyses

Northern Blot	
10x MOPS	0.5 M MOPS, 0.01 M EDTA, pH 7.0
Sample buffer	100 μl 10x MOPS buffer, 180 μl formaldehyde, 500 μl formamide, 220 μl H2O
6x loading buffer	0.25% (w/v) Bromophenol Blue, 0.25% (w/v) Xylene cyanol FF, 40% (w/v) Sucrose in water
20x SSC	3 M NaCl, 0.3 M Trisodium citrate
Methylene blue solution	0.04% (w/v) Methylene blue, 0.5 M Sodium acetate, pH 5.2
1x TE	10 mM Tris-HCl, 1 mM EDTA, pH 8.0
Pre-hybridization mix	1 ml 10% (w/v) BSA; 4 ml 1M NaPO4 pH 7.0; 1.5 ml Formamide; 20 μl 0.5 M EDTA; 3.5 ml 20% (w/v) SDS
Stripping buffer	2% (w/v) SDS, 10 mM Tris, pH 7.4
Southern Blot	
50x TAE	2 M Tris-acetate, 1 M Sodium acetate, 50 mM EDTA, pH 8.2
Neutralization buffer	1.5 M NaCl, 0.5 M Tris-HCl, 1 mM EDTA, pH 7.2
Western Blot	
1x TTS buffer	100 mM Tris, 100 mM Tricine, 0.1% (w/v) SDS, pH 8.3
Transfer buffer	1x lammli buffer plus 20% (v/v) methanol
Staining buffer	0.1% (w/v) Coomassie brilliant blue R-250, 45% (v/v) methanol, 10% (v/v) glacial acetic acid
Destaining solution	45% (v/v) Methanol, 10% (v/v) glacial acetic acid
1x TBST	50 mM Tris, 150 mM NaCl, 0.05% (v/v) Tween 20, pH 7.5
10x TBS	0.5 M Tris, 1.5 M NaCl, pH 7.5
Small RNA Blot	

Stacking gel	1 ml Polyacrylamide:Bis (19:1 40% w/v); 1 ml 5x TBE; 8 ml water; 100 μl 10% (w/v) APS; 10 μl 0.5 M TEMED, pH 8.0.
Resolving gel	12 ml Polyacrylamide:Bis (19:1 40% w/v); 3 ml 5x TBE; 14.4 g urea; add ddH2O to 30 ml; 300 μl 10% APS, 15 μl TEMED added when everything dissovled
5x TBE	0.45 M Tris-borate, 10 mM EDTA, pH 8.0

Figure 2-1 Schematic map of pFA6A-R1-leu-hphMX6.

Two recognition sites of *Spe* I were fonted in black, *Bsi*WI, *Sal*I and *Pme*I having single cutting-site were in dark red, and the following numbers in parenthesis represent the nucleotide position on the plasmid. Leu1 3'UTR was shown in purple arrow starting from *Spe*I on top. Promoter pNMT41 (Pnmt41) was shown as the aqua arrow following by the dark blue arrow representing the full length cDNA clone of FHV RNA1 (RNA1). The transcription start site, Tobacco ringspot virus satellite RNA ribozyme sequence were displayed as black lines as stated, so were the ORFs of FHV B2 (B2) and B1 (B1) proteins, HA-tag sequence (3HA), Terminator sequence of the *ADH1* gene from *S. cerevisiae* (TADH1), Promoter (PTEF) and Terminator (T TEF) of the *Ashbya gossypii* translation elongation factor 1 á gene represented in purple arrows. ORF of Hygromycin resistant (hph) gene and ampicillin resistant genes (amp r) were shown in organge arrows.
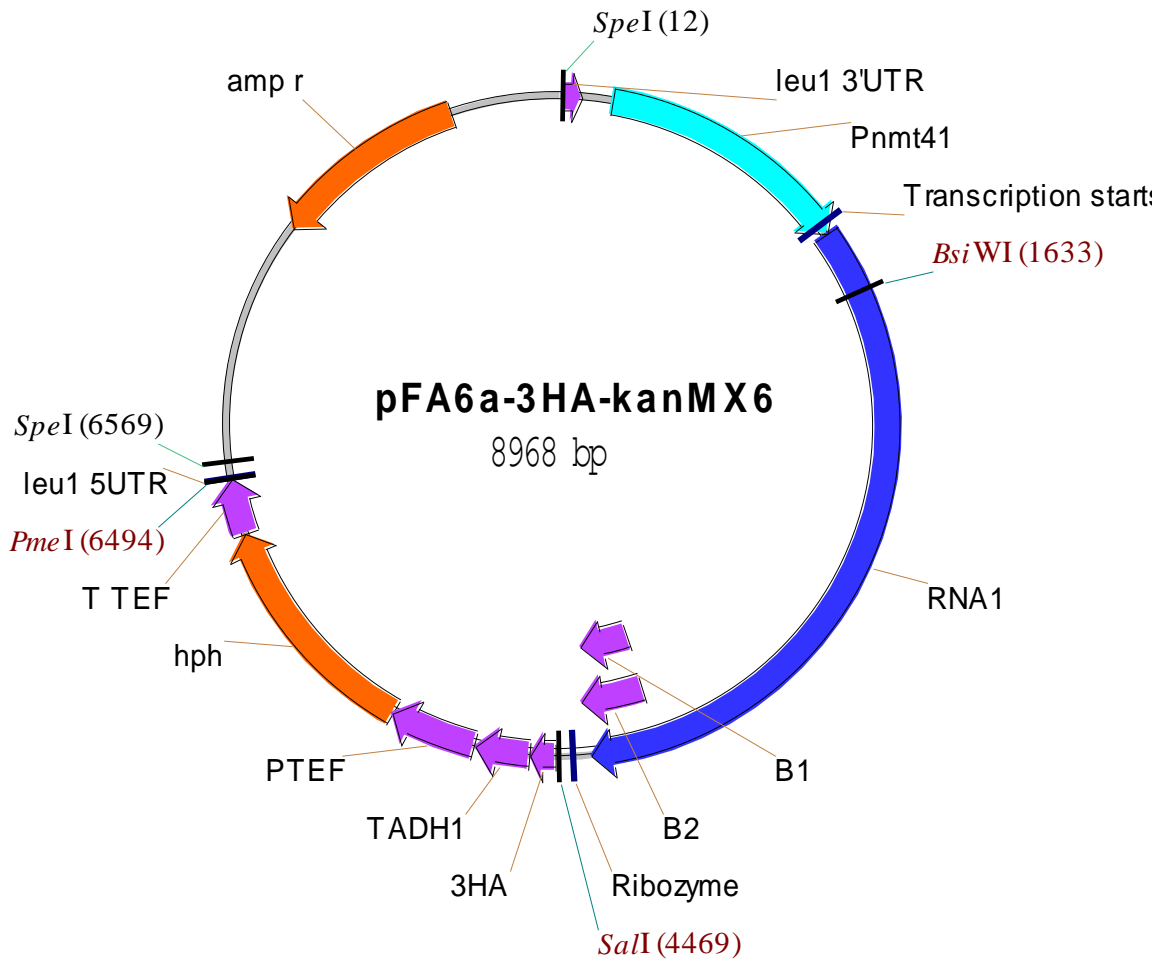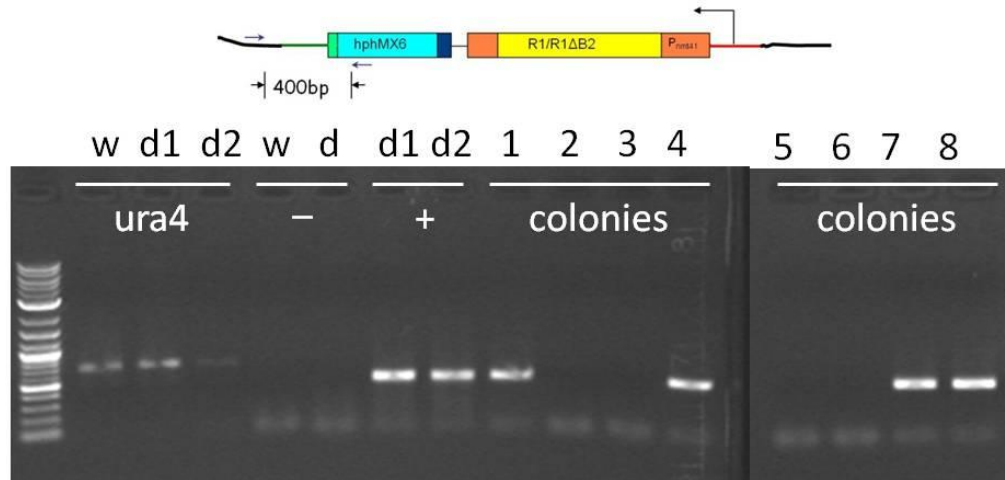
Figure 2-2 Screening for strains containing single copy of either R1 or R1ΔB2 in the leu1 locus.

(A) Diagram (top) and result examples (bottom) of PCR screening strategy for integrants. The integrated cassette was shown in colors and the black lines flanking represented the chromosomal loci. The transcription orientation of the FHV-cDNA clone was shown in a blended arrow initiated from the 5'UTR of leu1 gene (red line), and the pair of primers used to amplify the specific region only existed in integration-positive colonies in expected orientation were illustrated as two small black arrows and the size of positive-PCR product (400bp) was shown on the schematic map. The PCR fragments were visualized on 1% agarose gel along with 2-log DNA ladder (first lane on the gel). ura4 gene was shown as positive control for PCR reaction. Genomic DNAs from wild type strain (w) and *dcr1Δ* (d) without integration, two colonies (d1, d2) proven to be positive by previous round of PCR screening were used as negative- (-) and positive- control (+) for ampligying the viral sequence, respectively. Four out of eight colonies being tested turned out to be positive with expected fragment in the genome as shown.

(B) Southern blotting confirmation of single copy insertion of FHV cDNAs in selective strains. Diagram comparison of gene arrangement (top) in the yeast chromosome represented in the rod-shape yeast cell and the isolated plasmid pFA6a-hphMX6-R1/R1ΔB2 was shown to illustrate the expected size of *Sal*I-*Cla*I fragment from the chromosome (4.5kb) or the plasmid (~8.9kb). Southern blotting analysis of positive colonies from PCR screening was conducted using probes hybridized to FHV B2 ORF (bottom). Description of samples loaded from lane 1 to 12 was shown on the left side of Marker lane.

A

B

B2 from plasmid – 8.9kb

pFA6a-hphMX6-R1/R1ΔB2

Lane 1. WT
2. dcr1Δ
3. WT-R1
4-6. WT-R1ΔB2
7,8. dcr1Δ-R1
9-12. dcr1Δ-R1ΔB2

Marker 1 2 3 4 5 6 7 8 9 10 11 12

◄ 4.5kb
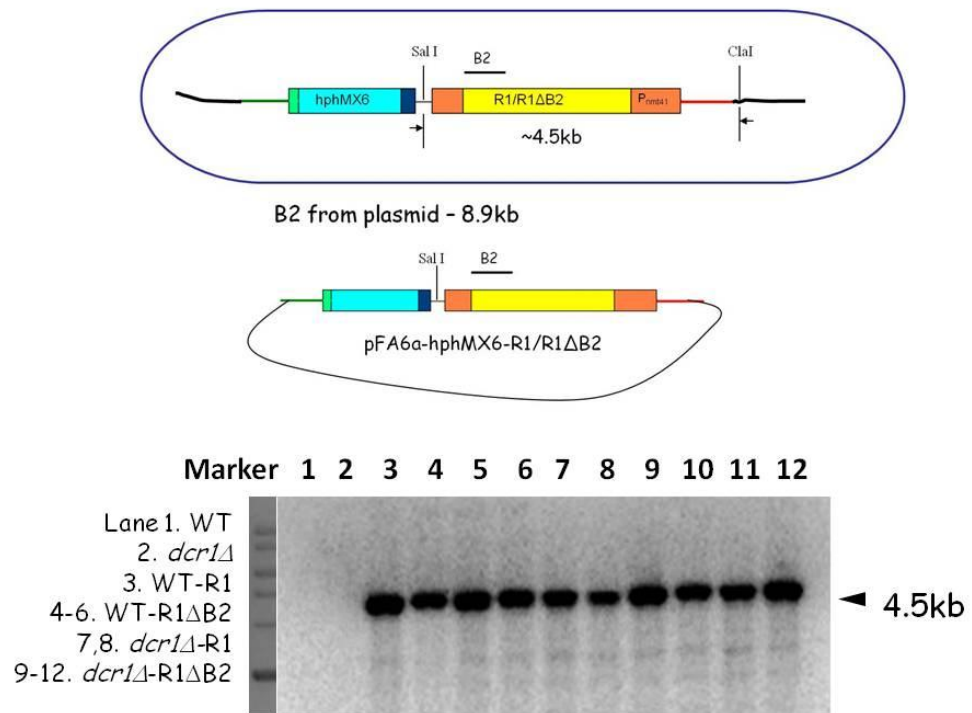
Figure 2-3 Fission yeast supports autonomous replication of FHV RNA1.

(A) The schematic diagram of homologous recombination between our constructed gene segment and endogenous target locus. cDNA of FHV RNA1 could be transcribed into mRNA when cells are grown in medium depleted of thiamine, and the nacent transcripts contain the full-length R1 and ribozyme sequence. The ribozyme then cleave any non-viral sequence from the 3' end, producing RNA1, which can serve as both template for viral replicase translation and RNA replication.

(B) Viral RdRp-dependent RNA1 replication in the wild type yeast cells. Northern blotting analysis of positive (sense) and negative (antisense) strands of RNA1 and RNA3 in wild type yeast strain transformed with empty vector, FR1, FR1fs or FR1ΔB2 from the pNMT41-plasmid.

(C) Thiamine-repressible detection of positive (sense) and negative (antisense) strands of FHV RNA1 and RNA3 in wild type yeast cells expressing FR1 (R1) or FR1ΔB2 (ΔB2) from the genome. The third lane (-) represented the yeast containing cDNA of FR1 that was grown in medium with 50 μM thiamine under similar temperature and period of time.

(D) Detection of B2 protein in the wild type strain. Total protein extracted from S2 cells (S2) was used as positive control. (M: protein Marker; V: empty vector; FR1; R1fs: FR1fs; ΔB2: FR1ΔB2)
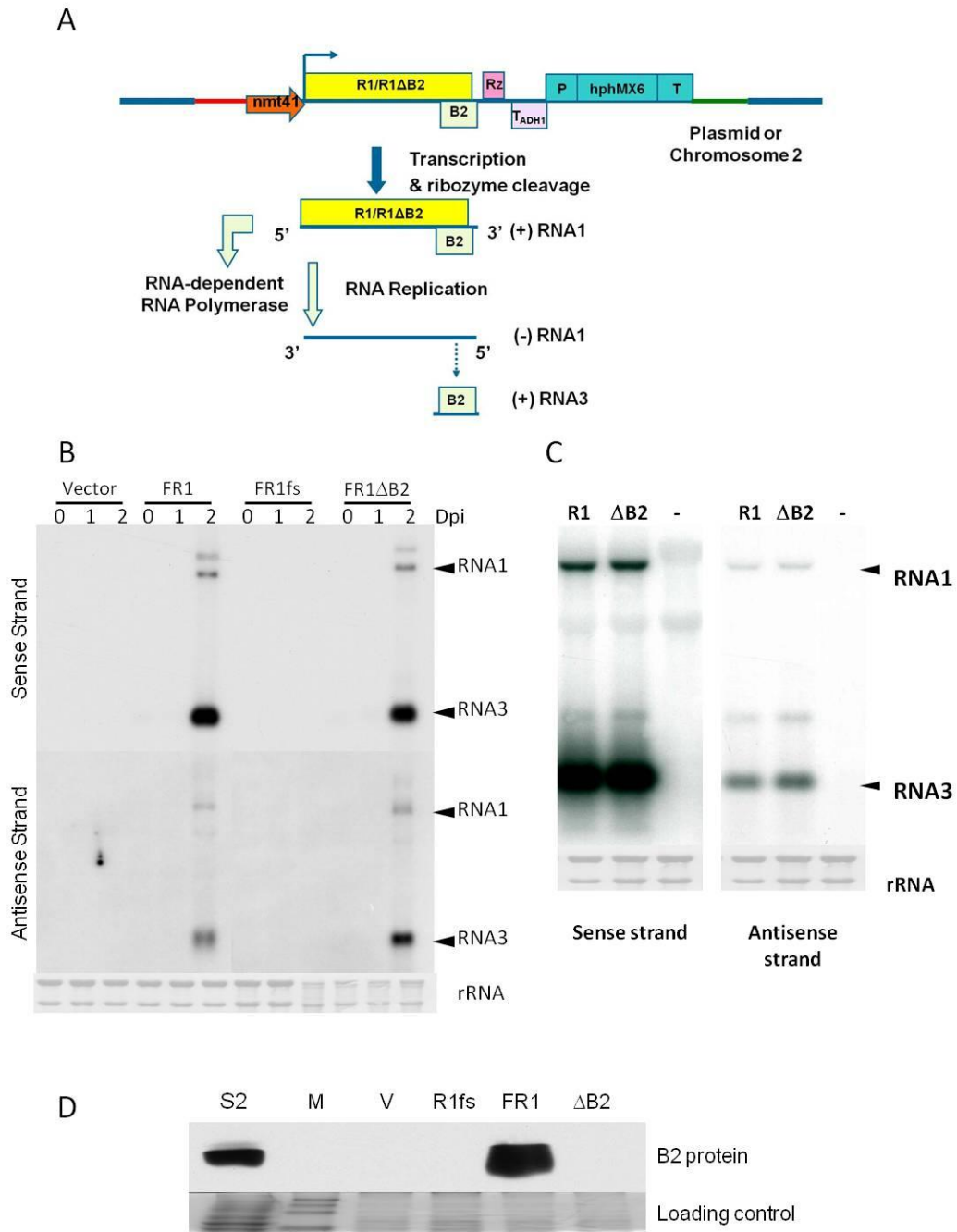
Figure 2-4 RNAi-defective strains exhibit enhanced viral RNA accumulation only at early phase of viral replication.

(A) Comparison of RNA1 and RNA3 accumulation in wild type (*wt*), *dcr1*Δ, *rdp1*Δ and *ago1*Δ mutant strains expressing either FHV RNA1 (1) or FHV R1ΔB2 (2). 12 hours induction: cells were harvested 12 hours after transferring to thiamine-free medium. 2 dpi: 2 days post induction. Cells were cultured for two days in thiamine-free medium.

(B) Comparison of RNA1 and RNA3 accumulation in wild type (*wt*), *dcr1*Δ, *rdp1*Δ and *ago1*Δ mutant strains expressing FHV RNA1. 12hpi: 12 hours post induction by depleting thiamine. The cells were cultured in thiamine-free medium for 6 hours before 50mM thiamine was re-added to the cell culture to repress the viral transcription for another 6 hours. 24hpi: 24 hours post thiamine depletion. The yeast culture were treated as those shown in 12hpi, and harvested at 18 hours after thiamine addition. The overexposure image for 12hpi was to show the presence of FHV RNA1 and RNA3 in the wild type strain.

(C) Comparison of RNA1 and RNA3 accumulation in wild type (*wt*), *dcr1*Δ, *rdp1*Δ and *ago1*Δ mutant strains expressing FHV R1ΔB2. 12hpi: 12 hours post induction by depleting thiamine. The cells were cultured in thiamine-free medium for 6 hours before 50mM thiamine was re-added to the cell culture to repress the viral transcription for another 6 hours. 24hpi: 24 hours post thiamine depletion. The yeast culture were treated as those shown in 12hpi, and harvested at 18 hours after thiamine addition. The overexposure image for 12hpi was to show the presence of FHV RNA1 and RNA3 in the wild type strain.

A

wt   dcr1Δ   rdp1Δ   ago1Δ   wt   dcr1Δ   rdp1Δ   ago1Δ

1 2   1 2   1 2   1 2   1 2   1 2   1 2   1 2

◄ RNA1

◄ RNA3

12 hour induction   2 dpi

1: wt FHV RNA1; 2: FHV R1ΔB2

B   C

Overexposure   Overexposure

12hpi   24hpi   12hpi   24hpi
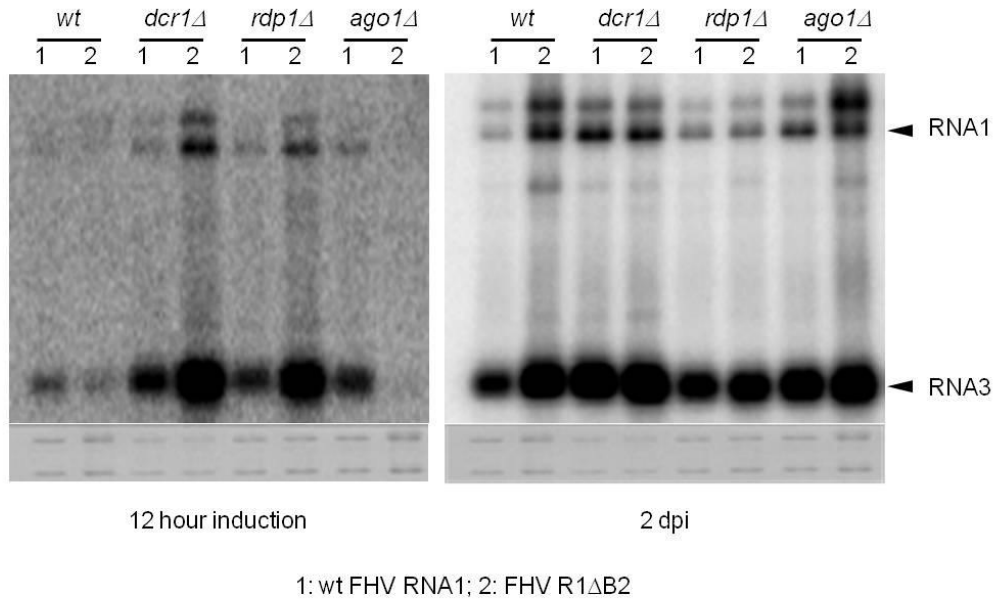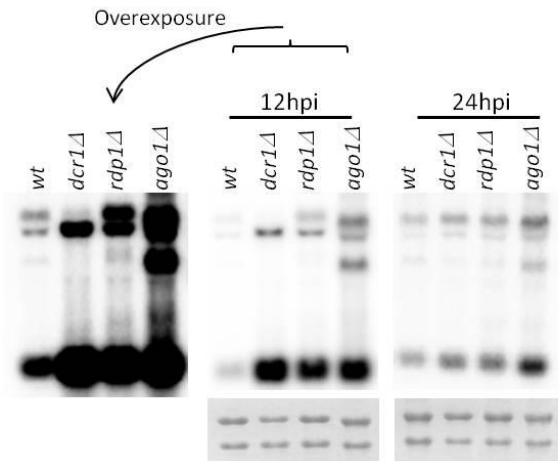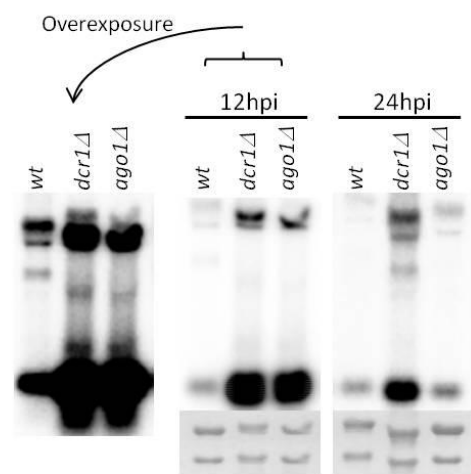
Figure 2-5 Detection of FHV RNA degradation products in the yeast cells expressing FHV R1ΔB2.

(D) Northern blotting analysis of FHV specific small RNAs in wild type strains transformed with either empty vector (Vector) or FR1ΔB2 (ΔB2) and mutant strains with FR1ΔB2. The LMW fraction of RNA from different conditions was probed subsequentially for negative (-) strand of FHV RNA3 region, centromeric siRNAs targeting the dg-dh outer repeats of pericentromeric region and U6 as loading control.

(E) Size distribution of FHV RNA1-specific small RNAs in wild type and *dcr1Δ* strains expressing FR1ΔB2 from pNMT1 plasmid (FHV). Small RNAs from wild type were illustrated in blue bar and those from *dcr1Δ* were in dark red.

(F) Size distribution of small RNAs mapped to the yeast genome in wild type and *dcr1Δ* strains expressing FR1ΔB2 from pNMT1 plasmid (FHV). Previous reported small RNA library from non-infected yeast (GSM311596) was shown as profile reference and positive control.

(G) Sequence alignment of FHV specific 21nt-small RNAs to the genome of FHV RNA1. X axis represented each nucleotide on RNA1, and the y axis represented the reads per million.
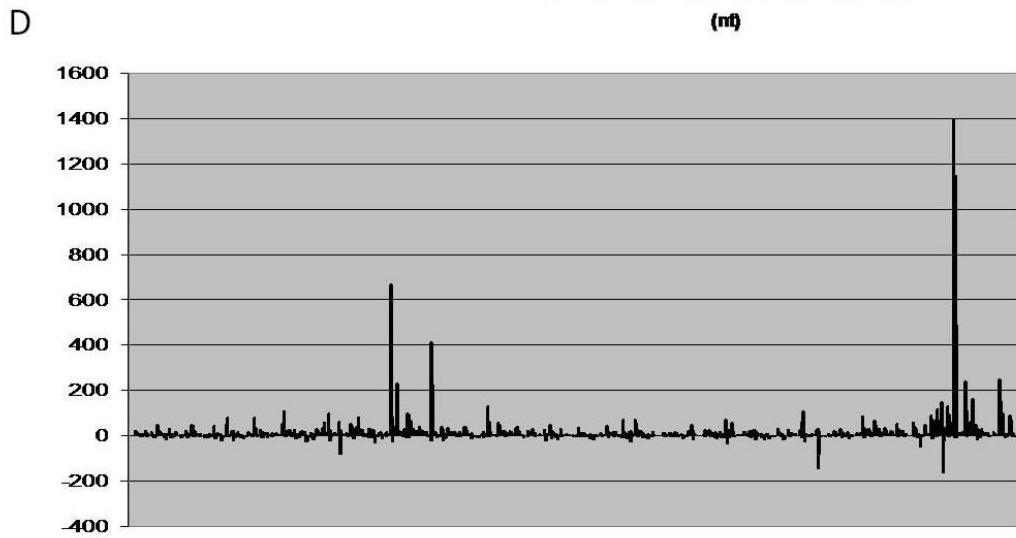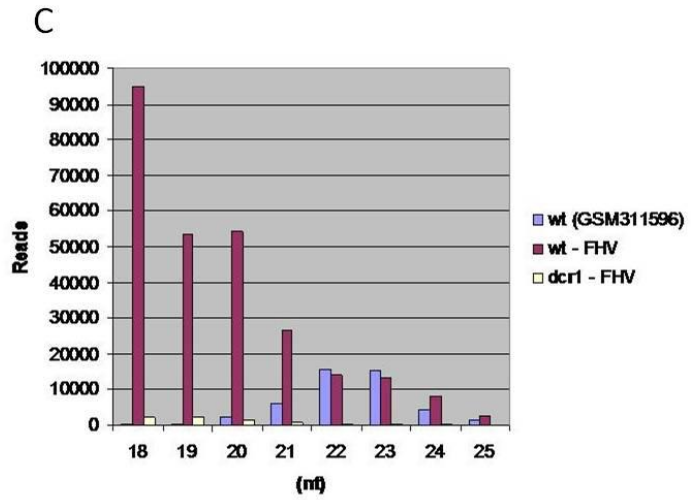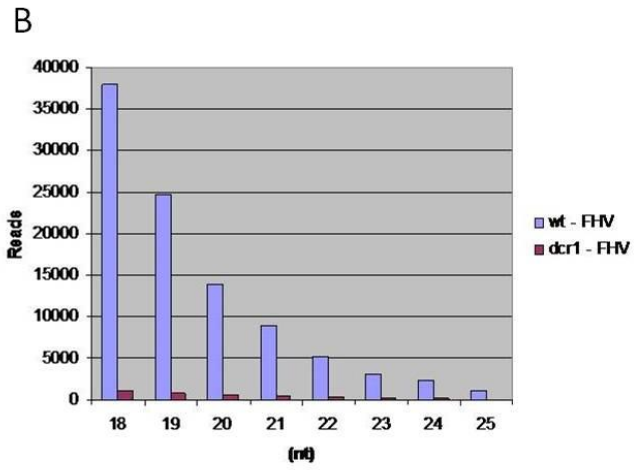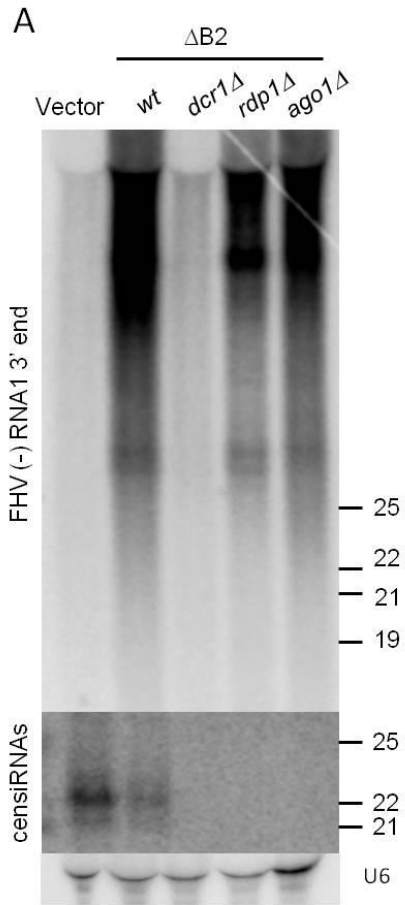
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2.7 Appendices - Protocol for small RNA cloning

3'end ligation (25 μl):

RNA	12 μl
10X ligase buffer (NEB B0242S)	2.5 μl
DMSO	2.5 μl
3' adaptor Modban 50 μM	2 μl
T4RLN2(NEB M0242L)	2 μl
H_2O	4 μl

Incubate at room temp for 1 to 2 hours.

5' end ligation (25 μl)

Ligation product	13 μl
10×T4 RNA ligase buffer (NEB B0204S)	2.5 μl
DMSO	2.5 μl
Solexa linker 100μM	0.7μl
T4 RNA ligase I (NEB B0204L)	2 μl
H_2O	4.3 μl

Incubate at 37 ℃ for 2 hour.

Reverse transcription (annealing reaction)

10 μl RNA ligation product

6.7μl 5 μM SBS3

Incubate at 72 ℃ for 2 min.

Spin at RT for 1 min.

Cool on ice for 2 min.

Add the following solution up to final volume of 30 μl:

13.3 μl "RT Mix" Stock of RT Mix(30 μl 5× first strand buffer ,3μl 100 mM DTT ,15 μl

dNTPs (10 mM each) ,12μl MQ)

Split the sample into two tubes:

+ RT (15 μl): add 2.5 μl Superscript III (Invitrogen Superscript™).

- RT (5 μl):   add 0.5 μl water.

Incubate at 50 ℃ for 1 hour. Heat at 70 ℃ for 15 minutes.

PCR amplification of cDNA (100 μl)

16 μl first-strand cDNA, or "minus RT control" (from above)

51 μl water

20 μl 5× Phusion HF Buffer

4 μl dNTP Mix (10 mM each)

4 μl 10 μM 5' PCR primer,

4 μl 10 μM 3' PCR primer

1.5 μl Phusion polymerase (NEB F-530L)

 PCR program

98 ℃ 30 sec

15 cycles of

98 ℃ 10 sec.

60 ℃ 30 sec

72 ℃ 15 sec

72 ℃ 10 minutes

CHAPTER 3. GENETIC REQUIREMENTS FOR VIRAL SIRNA BIOGENESIS IN THE ADULT FRUIT FLIES

3.1 Abstract

Extensive studies on antiviral immunity at molecular level have demonstrated the importance of siRNA-based silencing in virus clearance in *Drosophila* cell culture, but whether the conclusion could be applied to the adult fruit flies and the detailed mechanism, including the relative potency, population structure and biogenesis pathway of the virus-derived siRNA (viRNA) remain to be determined. In this study by using a mutant Flock house virus FHVΔB2 combined with Illumina deep sequencing, it provided clear evidence supporting that viRNAs were produced by the type III ribonuclease, Dicer-2, predominantly 21 nucleotides long with an approximately equal ratio of positive and negative strands, and sufficiently potent to eliminate virus infection in adult *Drosophila*. Moreover, another dsRNA-binding protein, Loquacious (Loqs) was found to play redundant role with R2D2 to eliminate the virus, probably by enhancing both biogenesis and RISC-loading of viRNAs. In addition, FHVΔB2 which was incapable to suppress dicing of long dsRNA into siRNAs and activity of viral siRNAs, caused increased mortality only in RNAi-defective flies such as *Dicer-2* and *Argonaute-2* mutants rather than wild type adult flies, indicating the VSR encoded by FHV is essential for symptom development by inhibition of the RNAi-mediated antiviral response in the insect host.

3.2 Introduction

Antiviral immunity directed by virus-derived siRNAs provides a major host defense against various RNA viruses in fungi, invertebrates and plants (Aliyari and Ding, 2009; Ding, 2010). It is supported by three lines of evidence which include the detection of viRNAs in virus-infected hosts, enhanced disease susceptibility of mutants defective in mediating RNAi, and the existence of virus-encoding suppressor of RNAi (VSR) in a great variety of RNA viruses. Based on the current knowledge of its mechanism, it is believed that upon viral RNA replication, the dsRNA intermediates are processed by a host Dicer ribonuclease and the viRNAs are loaded into an Argonaute protein to guide the clearance of cognate viral RNAs. The siRNA pathway can be triggered by double-stranded RNA (dsRNA) either generated from either transposons or exogenous transgenes or upon viral infection in *Drosophila*. However, it is not completely clear whether the genetic requirements for silencing various targets differ from one another. The recognition and processing of exogenous dsRNAs require Dicer-2 (Dcr-2) and a dsRNA-binding protein (dsRBP), R2D2 (Galiana-Arnoux *et al.*, 2006; Wang, 2006). These two components have been documented to participate in the innate immunity pathway that protects adult flies from FHV infection. Genetic mutants containing loss-of-function mutations in *dcr-2* and *r2d2* mutant flies exhibited enhanced disease susceptibility to two evolutionarily diverse viruses: Flock House Virus (FHV) and Cricket Paralysis Virus (CrPV), and support higher viral load relative to wild type. However, only Dcr-2 but not R2D2 seems to be essential for production of virus-derived siRNAs (viRNAs) based on small RNA blotting detection (Wang, 2006). Overall, our findings

established that the *dcr-2*-dependent siRNA pathway play a key role in limiting the viral infection in adult *D. melanogaster*, and further experimental evidence was required to determine the contribution of R2D2 protein in the virus clearance process. On the other hand, the endogenous siRNA pathway, which is activated by dsRNA derived from transposons, base-paring transcripts and repetitive elements (Babiarz *et al.*, 2008; Chung *et al.*, 2008; Czech *et al.*, 2008; Ghildiyal *et al.*, 2008; Kawamura *et al.*, 2008b; Okamura *et al.*, 2008c), requires Dcr-2 interacting with a different dsRBP, Loquacious (Loqs) (Czech *et al.*, 2008; Okamura *et al.*, 2008a; Okamura *et al.*, 2008c), although more recent findings showed that Loqs and R2D2 interact with Dcr-2 sequentially to regulate the biogenesis and RISC loading of siRNAs, respectively for dsRNAs of various sources (Marques *et al.*, 2010). Therefore, I was interested in verifying the potential involvement of Loqs and the specific role of R2D2 in the RNA-based antiviral response in the adult flies.

The biological significance of antiviral silencing mediated by RNAi in insects has also been well illustrated by the discoveries of VSRs encoded by viruses in a number of evolutionally diverse families (Li and Ding, 2006; de Vries and Berkhout, 2008; Diaz-Pendon and Ding, 2008). Nonstructural protein B2 encoded by FHV subgenomic RNA3 may be one of the best studied VSRs, and in-*vitro* activity assay and studies in *Drosophila* cell culture have indicated that B2 suppresses dicing of long dsRNA into siRNAs by binding the dsRNAs and is recruited to the viral replication complex by association with the viral RNA-dependent RNA polymerase (Lu *et al.*, 2005). Prior to our

studies presented in this Chapter, the small RNA profile has only been comprehensively analyzed in FHV or FHVΔB2-infected S2 cells (Aliyari *et al.*, 2008b; van Rij and Berezikov, 2009). Comparison of these two conditions indicated that in the absence of B2, 21 nucleotides small RNA revealed to be dominant species among the virus specific small RNAs cloned from cultured Drosophila cells infected for all of the positive stranded and double-stranded RNA viruses, and they were mapped to the sense- and antisense- strands of viral genomes in approximately equal percentages. Additionally, the majority of viRNAs were specifically located in the 5' -terminal approximate 400 nucleotide region of FHV RNAs (Aliyari *et al.*, 2008b). Therefore, I proposed that the 5' nascent replication intermediates formed during initiation of viral progeny RNA synthesis may be the trigger of the silencing response. Those 5' dsRNAs may be efficiently processed into viRNAs, which will be loaded into Ago2 preferentially to mediate cleavage of the viral genomes. Dcr2-dependent processing of dsRNA was mainly supported by our finding that production of viral siRNAs induced by autonomous replication of FHV RNA1 in *Drosophila* embryos seemed to be abolished in *dcr2* but not *ago2* mutants (Aliyari *et al.*, 2008b), no direct evidence yet is available to prove that such role of Dcr2 exists in the adult flies infected with FHV. Here these unsolved issues were addressed by infectivity assays and deep sequencing in wild type and RNAi-defective mutant flies challenged by FHVΔB2.

3.3 Materials and methods

3.3.1   Fly strains, virion preparation and inoculation

All the virions used in this study were propagated in *Drosophila* S2 cells. Plaque forming

units per ml (pfu/ml) of the purified FHV and FHVΔB2 suspensions were determined by

a standard plaque assay by staining with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-

tetrazolium bromide (MTT) before fly injections. Purified virions were diluted in 1 $\times$

PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO4, 1.76 mM KH_2PO4; pH7.4)

for microinjection.

Canton S *white* flies were used as wild type control, *dcr-2*^{L811fsX} (*dcr-2*), *ago2*

homozygous mutants (Lee *et al.*, 2004b; Okamura *et al.*, 2004) and *r2d2*^{S165fsX} were

described as previously (Wang *et al.*, 2006). *loqs*^{f00791} (*loqs*) used in our experiments is a

partial loss-of-function allele, while *R2D2*^{S165fsX} (*R2D2*) is a null allele. *loqs-r2d2*

double-mutant was generated by crossing *loqs*^{KO}- *r2d2*^{S165fsX} and *loqs*^{f00791}- *r2d2*^{1} as

reported previously (Marques *et al.*, 2010). 30-50nl of a viral suspension (FHV: 1.47×10^6

pful/ml; FHVΔB2: 7×10^6 pfu/ml) was injected into the thorax of adult flies of 3-4 days

old with a microinjector (FemtoJET 5247, Eppendorf, Germany). Equal volume of blank

buffer was injected as mock controls. All the flies were anaesthetized by CO_2 exposure,

except that *ago-2*^{414} was anaesthetized on ice. For survival test, over 50 adults of each

genotype were divided into three groups and each experiment was repeated at least three

times. Flies were transferred to fresh medium every 2-3 days after injection, and

monitored for survival on a daily basis. All the fly stocks and inoculated flies were reared

on standard medium at room temperature.

3.3.2 Quantitative real-time PCR

For viral accumulation analysis, total RNA was extracted from flies at 7 days post injection (dpi) using TRIzol (Invitrogen) following the procedures described in Chapter 2 with minor modification. Briefly, 20-30 adult flies were homogenized in 500 μl of TRIzol reagent with a motor pestle. Volume of TRIzol used for extraction should be adjusted based on the number of flies. Insoluble material was removed by centrifugation at 13,500 rpm for 10 minutes after vortexing for at least 10 minutes at room temperature. 100 μl chloroform was added to the supernatant followed by vigorous vortex for 20 seconds and the mixture was centrifuged at no more than 13,500 rpm for 15 minutes at 4°C. Following centrifugation, RNA in the colorless upper aqueous phase was precipitated with equal volume of isopropanol at -20°C for at least 20 minutes and pelleted by spinning at 13,500 rpm for 10 minutes. The RNA pellets were washed by 500 μl of 70% ethanol, air-dried and resuspended in DEPC-H2O, and the concentration and purity were monitored by a NanoDrop 2000 (Thermo Scientific).

500ng of RNA was used for cDNA synthesis using Superscript III reverse transcriptase (Invitrogen) and gene-specific primers, and real time qPCR were carried out in the presence of iQ SYBR Green Supermix (Bio-rad). The relative abundance of sense- and antisense-strands of FHV genomic RNAs was normalized to an internal control (rp49) using the ΔCt method. Primers used for cDNA synthesis and qPCR were listed in Table 1, and we used a two-tailed student's *t*-test to statistically analyze the differences in vRNA replication level.

3.3.3 Northern blotting

Total RNA extraction was performed as described above. For detecting the viral genomic RNAs, 5 μg of total RNA in less than 10 μl volume were mixed with 7.8 μl sample buffer and 2 μl 6× loading buffer (Table 2-6), denatured at 65°C for 10 minutes and placed immediately on ice. RNAs were separated on 1.2% (m/v) agarose gel containing 3% (v/v) formaldehyde and 1 × MOPS buffer in a MAX HORIZ SUB system (Amersham Phamacia Biotech), with 1 × MOPS buffer as running buffer. After electrophoresis RNAs were transferred on Hybond-N+ membranes (Amersham Bioscience) using a VacuGene XL transfer unit (Pharmacia Biotech) for at least 3 hours according to manufacturer's instruction. 5×SSC buffer was used as transferring buffer. The membrane was briefly washed in distilled water and cross-linked in a XL-1000 UV crosslinker (SPECTROLINKERTM, 180mj/cm^2). Ribosomal RNA could be visualized by staining the membrane with methylene blue solution and used as loading control. The membrane was then hybridized to ribonucleotide probes labeled with α-^{32}P-UTP (Perkin Elmer) in a hybridization oven (Amersham Pharmacia Biotech).

Fragments containing T7 promoter at one strand were amplified from cDNA clones of FHV RNA1 and RNA2. The primers were listed in Table 3-1. The in-*vitro* transcription reaction was assembled as listed in Table 3-2, and incubated at 37°C for at least 4 hours. 1 μl Turbo DNase (Ambion T7 SuperScript kit) was added to the reaction and kept at 37°C for another 30 minutes. Therefore, the ribonucleotide probes were corresponding to either the sense or antisense strand of FHV RNA3 or the last 400 nucleotides of RNA2.

Probes detecting Rp49 was prepared using a Rediprime^{TM} II kit (Amersham). Briefly, PCR product targeting the 114-264 nucleotide of Rp49 was synthesized by RT-PCR using total RNA from adult flies or S2 cells, and purified from 1% agarose gel. 20-50ng of the PCR fragment was diluted in 46 µl DNase-free water, denatured and mixed with 4µl α-^{32}P-dCTP. The reaction was incubated at 37°C for at least 30 minutes, denatured and added to the membrane in the hybridization tube. The membrane was incubated with the probes for at least 16 hours and washed twice in 2×SSC, 0.1% SDS for at least 20 minutes each, and twice in 0.2×SSC, 0.1% SDS for at least 20 minutes each. The hybridization and washing process for Rp49 and ribo-probes were carried out in hybridization buffer described in Table 2-6 at 65°C, and the membrane was exposed to either the phosphoimage screens (Kodak) or X-ray films (Hyblot CL$^{®}$, DENVILLE Scientific).

For enrichment of low-molecular-weight (LMW) RNA, total RNA was resuspended in 4M LiCl and incubated in -20°C for at least 2 hours; the supernatant after LiCl precipitation was further incubated with two volumes of isopropanol in -20°C overnight. 10 µg of LMW RNA was electrophoresed in denaturing gel containing 0.5×TBE, 8M urea, 15% polyacylamide in 0.5×TBE buffer. For easier loading and better resolution of the small RNAs, a 4% polyacylamide stacking gel containing 0.5×TBE was used. The top part of the gel in proper width was stained with ethium bromide to visualize the RNAs and used as loading control in certain cases, and the rest of the RNA was transferred onto Hybond-NX membrane using a semi-dry apparatus (Hoefer SemiPhor, PharmaciaBiotech)

followed by EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) cross-linking. 0.5×TBE buffer was applied as transfer buffer and for soaking the gel. Immediately prior to the termination of transferring, prepare a solution of 1-methylimidazole (Sigma) by diluting 245 μl of 12.5M stock in 24ml RNase-free water, and then adjusting the pH to 8.0 with 1M HCl approximately 300 μl. 0.753g of EDC powder (Sigma) was added to the solution, mixed well, and then soak one sheet of Whatman 3MM filter paper in a plastic container. The nylon membrane was dissembled from the electroblot and placed on the solution-saturated filter paper with the RNA-side up, and incubated at 60°C for at least 2 hours. The membrane was then washed with excess RNase-free water and preceded to prehybridization.

Mixture of 13 oligonucleotide-probes targeting the first 400 nucleotides of FHV RNA1 was end-labeled with γ-^{32}P-ATP (Perkin Elmer) using T4 polynucleotide kinase (Aliyari *et al.*, 2008b). End-labeling reaction was set up as listed in Chapter 2, incubated at 37°C for at least 30 minutes, denatured at 95°C for 3 minutes and added to the membrane. Hybridization was carried out in PerfecHyb hybridization buffer (Sigma) at 35°C for at least 16 hours, washed at 40-45°C following the same procedure for the HMW RNA, and exposed to X-ray films.

3.3.4 Western blotting

The interphase and phenol-chloroform phase from TRIzol-RNA isolation was used for protein analysis. For 1mL of TRIzol used in the initial homogenization, 300 μl of 100% ethanol was added to the mixture of interphase and lower phase, mixed well and spun

down the genomic DNA pellet at no more than $2,000 \times g$ for 5 minutes at 4°C. The supernatant was then precipitate with 1.5mL isopropanol per 1mL of TRIzol used for the initial step. Store samples for 10 minutes at room temperature, and centrifuge the protein precipitate at $12,000 \times g$ for 10 minutes at 4 °C. Protein pellets were then washed 3 times in 95% ethanol containing 0.3M guanidine hydrochloride, air-dried, dissolved in $1 \times$ SDS loading buffer (80 mM Tris-HCl, pH 6.8; 2% SDS; 20% glycerol; 100 mM Dithiothreitol and 0.01% bromophenol blue) and electrophoresed in 12.5% SDS-PAGE. The proteins were then transferred to nitrocellulose membranes (GE Healthcare). Membranes were blocked overnight at 4 °C in TBST buffer (10 mmol Tris-HCl, pH8.0; 150 mmol NaCl; 0.05 % Tween-20) containing 5% non-fat milk. After washing briefly in TBST buffer, the membrane was incubated with polyclonal anti–FHV CP, –FHV B2 or –tubulin antibodies (Santa Cruz) diluted in TBST buffer containing 1% non-fat milk for at least one hour at 4 °C followed by three washes with TBST for 15 minutes each. Secondary goat anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (Thermo scientific) was applied to the membrane in the same way as for the primary antibodies, and immunodetection was carried out using a Lumi-Light Western Blotting Substrate (Roche).

3.3.5   Deep sequencing and bioinformatics

Total small RNAs in the range of 18-30nt were cloned by the procedure that require 5'-mono phosphate and 3'-hydroxyl group as described in Chapter 2. Cloning and sequencing of small RNAs by Illumina 2G Analyzer and mapping onto target reference

sequences by Bowtie were carried out as described(Langmead *et al.*, 2009; Wu *et al.*, 2010a). FHV RNA1 and RNA2 sequences (GenBank Accession NC_004146 and NC_004144) were retrieved from NCBI. Sequences of fruit fly ribosomal RNA and endogenous siRNAs and miRNAs were downloaded from FlyBase (http://flybase.org/). Genome sequences of fruit fly and other annotation data set were downloaded from UCSC genome browser (http://genome.ucsc.edu/) as described previously (Ref as Chapter 2). Prior to any analysis, small RNAs with up to 2 mismatches to rRNA, tRNA and snoRNA of fruit fly were removed with the strategy described previously (Ref as Chapter 2). Only those reads that matched perfectly to the references (such as FHV viral sequences) were used for comparison and downstream analysis. Virus derived small RNAs were normalized to 1 million sequenced small RNAs. The normalization factors for each library is determined by the overall counts of small RNAs in the length of 18-28nt after subtraction of RNA degradation fragments from tRNAs, rRNAs and snoRNAs.

3.4 Results

3.4.1   VSR-deficient mutant FHV showed virulence to RNAi-defective mutants but not
        wild type adult flies

Distinct from those injected with FHV virions (50 pfu per fly), which resulted in 70% mortality by 16 dpi, wild type flies that were inoculated with even higher dose of FHVΔB2 (280 pfu per fly) exhibited little defects over the same period of observation. (Figure 1) However, FHVΔB2 was highly pathogenic to fly mutants carrying loss-of-

functions in *Dcr2*. Same inoculation of FHVΔB2 in *dcr2* mutant flies started causing gradual death from 6dpi, and resulted in 95% mortality by 16 dpi, indicating that the virulence of FHVΔB2 is indeed lower than FHV, which causing rapid death from 7dpi and 100% mortality by 12-13dpi in *dcr2*. Ago2 was required for slicing of the single-stranded RNA target in the canonical RNAi pathway. Approximately 40% of *ago2* flies on average survived the FHVΔB2 injection up to 16 days post infection, indicating that FHVΔB2 was less virulent in the *ago2* mutant than in the *dcr2* mutant (Figure 1). Wild type, *dcr2* and *ago2* flies were collected at 7dpi for detection of FHVΔB2 replication abundance, when their survival rates are 100%, 84% and 61%, respectively. Consistent with the survival rates, northern blot analyses revealed that while FHV RNAs were readily detected in wild type flies, FHVΔB2 RNAs only accumulated to high levels in *dcr2* and *ago2* mutants, but not wild type flies (Figure 2A). Quantitative real-time PCR (Figure 3A) revealed that the level of sense-stranded RNA1 in *ago2* was over 5000 times higher than that in wild type strain, and over 10000 times more abundant in *dcr2* relative to wild type. The abundance of sense-stranded RNA2 also appeared in similar pattern, which corresponded well with the coat protein (CP) expression in these strains. Western blot analyses demonstrated that FHVΔB2 CP only accumulated to high levels in *dcr2* and *ago2* mutants but not the wild type at 7dpi. B2 protein was not detected in all the flies infected with FHVΔB2, confirming that FHVΔB2 virion used in our studies was truly defective in B2 expression (Figure 3B). These findings suggest that VSR B2 supports effective viral infection and accumulation in the hosts, and this activity of B2 depends on

the intactness of RNAi-mediated antiviral defense in the infected animals. Although the mortality of *ago2* mutant flies increased rapidly after FHVΔB2 inoculation, which I believed might be due to some undetermined defects in this mutant rather than the viral replication, as the survival rate of *ago2* that were injected with blank buffer was also slightly lower than that of *dcr2*. The fact that *ago2* mutant flies exhibited less severe susceptibility to FHVΔB2 infection in general and slightly lower viral RNA accumulation compared to *dcr2* supports our model that dicing is more upstream than slicing in the antiviral pathway. It is possible that dicing itself may also contribute to the viral clearance and/or other components with slicing activity such as Ago1 may also aid in this process in the *ago2* mutant flies.

3.4.2   Loqs act synergistically with R2D2 to mediate the virus clearance

To investigate whether Loquacious (Loqs) is involved in the antiviral defense, and if it is, whether there is any interaction with R2D2, the other Dcr2-binding partner, I injected *loqs- r2d2* single- and double mutant flies with purified FHVΔB2. I found that accumulation of FHV ΔB2 in *loqs* was low compared to *r2d2*, quantitative real-time PCR revealed that the level of sense-stranded RNA1 in *loqs* mutant was approximately 5 times higher than the wild type (Figure 3A), this also reflected on the fact that the viral genomic and subgenomic RNAs in the *loqs* mutant were only visible by Northern blotting hybridization after at least 60 times longer exposure (Figure 2A). FHVΔB2 replication was rescued to stronger extent in *r2d2* single mutant relative to *loqs* mutant strain, indicating that R2D2 may play a more significant role in antiviral silencing and/or

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the dominant binding preference to Dcr2. Strikingly, FHVΔB2 accumulated to a much higher level in the *loqs-r2d2* double mutant, which was over 60 times stronger than *r2d2* and comparable to *ago2* and *dcr2* flies (Figure 3A), as well as an enhanced expression of CP but not B2 protein relative to wild type or any of the single mutants (Figure 3B). These data together showed that Loqs and R2D2 have a redundant role in this RNAi-based antiviral pathway.

I further measured the ratio of the sense (+) and antisense (-) strand RNA1 of FHVΔB2 in the inoculated flies (Figure 3A). FHVΔB2 produced approximately 15 times more (+) RNA1 than (-) RNA1 in wild type and mutant flies (Figure 3B). A similar ratio of (+) RNA1/ (-) RNA1 was also detected in FHV-infected wild type flies as expected for (+) RNA viruses, which produce low levels of (-) RNA as the template for the synthesis of the abundant (+) RNA to be packaged into progeny virions. Overall the comparison of (+) RNA1/ (-) RNA1 ratios in wild type and all the mutant-strains being examined indicated that the enhanced viral RNA abundance observed in the mutant flies was due to defective antiviral silencing by RNAi, which occurred independent of viral RNA replication.

3.4.3   DCR-2 is required for the biogenesis of virus-derived siRNAs in adult flies

To gain an insight of the molecular mechanism of the viRNA biogenesis, the small RNA profiles of the wild type and mutant flies infected with FHVΔB2 at 7dpi were examined by Illumina sequencing. Although FHVΔB2 accumulation was hardly detectable in the infected wild type flies, 1,198 small RNAs per million of total 18- to 28-nucleotide small RNAs were 100% identical or complementary to the genomic RNAs 1 and 2 of FHVΔB2

(Figure 4A). These virus-derived small RNAs were approximately equally divided into positive (46%) and negative (54%) strands (Figure 5A) and the predominant species was 21 nucleotides in length for siRNAs derived from both RNA1 (66%) and RNA2 (56%). (Figure 4B) Moreover, the 21nt- viral siRNAs in the libraries contained the point mutations introduced into the FHV genome for depletion of B2 translation, indicating that these virus-derived small RNAs were products of Dcr2 with the dsRNAs formed during viral replication as substrates. Notably, the density of the 21nt- viral siRNAs targeting the 5'-terminal region of RNA1 and RNA2 were much higher than the rest of viral genome (Figure 6), which was observed previously in cultured *Drosophila* cells challenged by FHVΔB2. Three pools of small RNA samples isolated from three independent biological replicas were sequenced, and the abundance of FHV specific small RNAs in all the libraries was very low relative to other mutants or wild type flies infected with FHV. The 21nt-viRNAs constituted only 0.65% of total 21nt- RNA reads and were 43 times less abundant than the endogenous siRNAs encoded by the fly genome (data not shown), probably reflecting the instability of small RNAs after mediating target cleavage in the hosts.

In contrast, over 50% of the small RNAs (18-28nt) sequenced from the infected *dcr-2* mutant flies were derived from FHVΔB2. However, these highly abundant virus-derived small RNAs from *dcr-2* flies did not have a predominant length (Figure 4B) expected for siRNAs, miRNAs or piRNAs, and 98% of them corresponded to the viral positive-strands (Figure 5A). Considering the hypersensitivity and higher viral accumulation in *dcr2*

mutant flies, I believed these virus-derived small RNAs were inactive to virus RNA clearance by RNAi and hence terminate the viral infection. They may represent non-specific degradation products of the abundant viral genomic and subgenomic RNAs in the infected cells. In addition, the consensus sequence of 21-siRNAs in *dcr-2* flies seemed to be different from those from wild type and other genotypes (data not shown). Further experiments and analysis is required to determine the physiological significance of these small RNAs and accordingly to determine how they are produced. Nevertheless, these findings provide the first experimental evidence indicating that virus-derived siRNAs in adult flies are processed by Dcr2 from dsRNA replicative intermediates. Detection of viral siRNAs is consistent with the idea that lack of undetectable accumulation of FHVΔB2 in the inoculated wild type flies resulted from virus clearance by the RNAi-mediated immunity, but not due to defective replication and infectivity of FHVΔB2.

3.4.4   Roles of Loqs and R2D2 in the viRNA biogenesis and activity in adult flies

In *loqs* and *r2d2* single mutants infected with FHVΔB2, the most dominant small RNA species mapped to the viral genome was 21 nucleotides (Figure 4B and 5A) and these 21nt virus-derived small RNAs were approximately equally divided into positive and negative strands as those in the wild type flies (Figure 5A). Moreover, the 21nt- viRNAs derived from the 5' terminus of FHVΔB2 RNA1 and RNA2 accumulated to high density in both of *r2d2-* and *loqs-* single mutants (Figure 6). Thus, the population feature of viRNAs observed in wild type strain was basically preserved in *loqs* and *r2d2* flies,

indicating that neither Loqs nor R2D2 was essential for the biogenesis of viral siRNAs. I did however notice an interesting comparison between these two single mutants. The percentage of 21nt-viRNAs in the total small RNAs decreased by nearly 15% in *r2d2* single mutant, while the *loqs* mutant displayed no significant change relative to wild type flies. In the same *loqs* mutant allele, I observed a roughly 20% reduction of the 7 known endogenous siRNAs, which was as abundant as the wild type flies in *r2d2* mutant (Figure 5B). In addition, I estimated the efficacy of viRNA production in these mutants by calculating the ratio of negative-stranded viRNA molecules over the copy number of the antisense RNA genome, as the negative-strand RNA1 serves as both the template for RNA replication and precursor of viral siRNA duplex. I found that the ratio of viral negative-stranded siRNA/ (-) RNA1 decreased in *loqs* and *r2d2* mutants to 89% and 22.6% respectively of that in the wild type (Figure 4C), these data all together suggested a more dominant impact of Loqs in endogenous siRNA pathway, and R2D2 may be more important in antiviral silencing pathway by enhancing the relative abundance of viRNAs in the virus-infected flies.

Interestingly the deficiency in viRNA biogenesis that was observed in the *loqs* and *r2d2* single mutants seemed to be more severe in the *loqs-r2d2* double mutant strain. The ratio of viral siRNA/genomic RNA decreased to 2.3% in *loqs-r2d2* mutant flies (Figure 4C). Although both the positive and negative strands of 21nt siRNAs derived from RNA1 of FHVΔB2 still remained to be the dominant species, they were 25% less abundant than that in the wild type flies, and 21nt siRNAs no longer the dominant species for the sense-

strands of RNA2 in the double mutant (Figure 4B, 5A). Similar to the viRNAs, the relative abundance of 21-nt siRNAs from 7 known endogenous siRNA loci was reduced in *loqs-r2d2* mutant strain as exhibited in *loqs* single mutant (Figure 5B). It was noticeable that despite the high density of 5' terminal viRNAs, relative abundance of siRNAs targeting the 3'terminal region of RNA1 greatly increased in the double mutant (Figure 6), further supporting that R2D2 and Loqs have redundant roles in Dcr2-dependent biogenesis of viral siRNAs in adult flies.

Unexpectedly, over 80% of small RNAs (18-28nt) derived from RNA2 in the *loqs-r2d2* mutant were positive strands, which differed from the approximately equal distribution of positive and negative strands for those derived from RNA1 in the same mutant. I could not explain yet why two segments of viral genomic RNAs showed different pattern of viRNA production in the same mutant flies without additional data support, and this inconsistency was also observed in the FHV-infected wild type flies as described in the following section (Figure 5A).

3.4.5   Strong positive-strand bias of viRNAs in *ago-2* flies

Prior to this study, evidence indicating a prominent role of Ago2 in antiviral immunity was based on the enhanced disease susceptibility and increased virus titers in *ago2* mutant flies upon virus infection. Previous works from our laboratory have showed the detection of FHV-specific siRNAs in the *ago2* mutant embryos injected with in-*vitro* transcript FR1ΔB2 at a remarkably higher level than that in the wild type, but an explanation how Ago2 mediated virus clearance was lacking as it seemed to have no

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negative influence on viRNAs. Here I found that viral siRNAs accumulated to the highest level in the *ago-2* mutant flies among the wild type and all other mutant flies tested infected with FHVB2 virions (Figure 4A). Unlike *dcr-2* flies, 21-nt RNAs were the predominant species for small RNAs derived from both RNAs 1 and 2 of FHVΔB2 in *ago-2* flies (Figure 4B), confirming that Ago2 is not essential for the production of viral siRNAs by Dcr2, but probably for executing the activity of the viral siRNAs instead because the highly accumulated viral siRNAs in the *ago-2* mutant flies failed to inhibit the infection and pathogenicity of FHVΔB2. Analysis of viral siRNAs in the infected *ago-2* flies showed that although in total, 17.7% of the 18-28nt small RNA reads sequenced from the infected *ago-2* flies were derived from FHVΔB2, the ratio of viral siRNAs over viral genomic RNA was dramatically reduced to 0.4% in *ago-2* flies as compared to the wild type flies (Figure 4C), and the higher density of 5'-terminal siRNAs of RNA1 was lost in *ago-2* mutant (Figure 5A) More importantly, 92.4% of the total FHVΔB2-specific small RNAs were positive-strands in contrast to the approximately equal ratios of positive over negative strand viral siRNAs in the wild type, *r2d2* and *loqs* flies. This strong bias for the positive-strands was also observed for the 21-nt small RNAs derived from both RNA1 (87%) and RNA2 (91%) of FHVΔB2 in *ago-2* mutant flies (Figure 5A and data not shown). These results indicate that Ago2-loading may enhance the stability of both total and 5'terminal viRNAs, as well as alter the ratio of positive and negative strands of viRNAs. As strand-bias of viral siRNAs were also observed in those derived from RNA2 in loqs-r2d2 double mutant flies infected with

FHVΔB2, I proposed that Loqs and R2D2 may also regulate the Ago2 loading of viRNAs in the adult flies.

3.4.6 Properties of viral siRNA population in wild type flies infected by FHV

I also determined the population structure of viral siRNAs produced in the wild type flies inoculated with FHV to understand the impact of VSR on the viRNA biogenesis. As shown in Figure 1 and 2, FHV RNAs and proteins accumulated to high levels in the wild type flies, but only 1.52% of the total small RNAs sequenced from these flies were derived from FHV. The 21-nt small RNAs were the most dominant species for both the positive and negative strands among those small RNAs derived from RNA1, whereas the percentage decreased to 28% in contrast to 66% in FHVΔB2-infected wild type flies (Figure 4B, 5A). However, multiple defects were observed in the presence of B2 expression in the infected adult flies. Firstly, the efficacy of viral siRNA production was very low as the ratio of viral siRNAs versus viral genomic RNA in FHV-infected wild type flies was 0.4%, lower than all the fly strains infected with FHVΔB2 (Figure 4C). Production of the abundant 5'-terminal siRNAs of RNA1 was also largely suppressed (Figure 6). Surprisingly, strong positive-strand bias for small RNAs derived from both RNAs 1 (88%) and 2 (91%) of FHV was detected in the infected flies (Figure 5A), even the 21nt viRNAs derived from RNA1 as the dominant species were strongly biased for positive strands, which was also defects observed in *ago-2* and *loqs-r2d2* flies infected with FHVΔB2 to a lesser extent. These results indicated that expression of an RNAi suppressor not only inhibited the Dcr2-production of viral siRNAs in adult flies, but also

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led to alteration in strand bias of the 21nt viRNAs as found in *ago2* mutant flies. This finding provides the first evidence that B2 interferes with both dicing and RISC-loading in adult flies.

3.5 Discussion

Valuable insights into the mechanism of RNA-based antiviral immunity were obtained in adult *Drosophila* by deep sequencing the 18-28nt small RNAs from various genetic mutants infected with a VSR-deficient FHVΔB2. The findings presented in this chapter showed that potent infection of adult flies required a specific VSR activity as the infectivity and virulence of FHVΔB2 that were abolished in wild type could be probably fully restored in *dcr2* and *ago2* mutant flies and develop disease symptoms. The virus abundance in *dcr2* and *ago2* infected with FHVΔB2 was even slightly higher than that in wild type flies inoculated with FHV as shown in Figure 2 may be due to a higher FHVΔB2 virion concentration for microinjection. However, increased mortality caused by FHVΔB2 in *dcr-2* and *ago-2* flies further supports the idea that induction of viral diseases does not depend on the disruption of endogenous small RNA pathways in the insect hosts and this RNAi-mediated antiviral immunity in adult fruit flies is sufficiently potent to rapidly terminate virus infection before the onset of disease.

Biogenesis and population structure of viral siRNAs in adult flies have not been investigated in depth since our first demonstration of virus-derived small RNAs by Northern blot hybridization in FHV-infected wild type and r*2d2* but not in *dcr2* mutant

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flies (Wang *et al.*, 2006). Later on small RNAs from FHV and FHVB2-infected S2 cells were sequenced and analyzed (Aliyari *et al.*, 2008b; van Rij and Berezikov, 2009). Studying the RNAi-directed antiviral immunity using *Drosophila* cell culture is beneficial with more straight forward and convenient procedures for molecular or biochemical assay and representing most aspects of cellular activities in the entire organism with less noise or interference from other pathways. Nevertheless I encountered numerous problems in mapping the genetic requirement for the antiviral activity and biogenesis of viral siRNAs in the *Drosophila* S2 cells, as the efficiency of knockdown RNAi components by RNAi itself is relatively low and inconsistent between experimental replicas compared to that for RNAi-independent genes. By using genetic mutant adult flies, I provide evidence clearly illustrating that virus-derived small RNAs produced in the infected flies are Dcr2-dependent siRNAs, which are predominantly 21 nucleotides in length and divided approximately equally into positive and negative strands. Moreover, higher density of siRNAs targeting the 5'-terminal region of FHV RNA1 than those mapped to the rest of the viral genome were detected, similar to the previous observation in cell culture by 454 sequencing. Recent Illumina sequencing of approximately 45,500 RNA1-derived small RNAs from cultured *Drosophila* cells challenged by FHVΔB2 revealed an essentially identical pattern of RNA1-derived siRNAs (data not shown). These results indicate that in both cell culture and adult flies viral siRNAs are processed by Dcr-2 from viral dsRNA replicative intermediates and that initiation of the progeny positive-strand RNA synthesis from the anti-genomic RNA

template yields dsRNA molecules of about 400 nucleotides in length to serve as the major precursor of viral siRNAs. It is still not clear why in certain cases the pattern of viRNAs derived from RNA2 was slightly different from those from RNA1, which does not generated any defective interfering (DI) RNA as RNA2 does (Li and Ball, 1993; Schneemann *et al.*, 1993; Venter and Schneemann, 2008). Direct evidence supporting a biological significance of DI-RNAs in viRNA biogenesis and RNAi-mediated virus clearance in adult flies remains to be determined in the future.

Furthermore, the sequencing analysis confirmed that Ago2 was not essential for the Dcr2-dependent biogenesis of siRNAs because 21nt- siRNAs remained the most dominant species for both FHVΔB2 and endogenous siRNAs in the *ago-2* mutant. However, the ratio of viral siRNA reads over the copy number of viral replication template decreased in the *ago-2* mutant to 0.4% of that in wild type flies and approximately 90% of viral siRNAs detected in *ago-2* flies were positive-strands, in contrast to an approximately equal ratio of positive and negative strands in the wild type. The reduction in relative abundance of viral siRNAs sequenced from *ago-2* flies indicated loss of viral siRNA loading into Ago2 may result in an enhanced degradation as preferential loading of viral siRNAs into Ago-2 has been documented in Drosophila S2 cells, and the endogenous and exogenous siRNAs in *ago-2* and *r2d2* mutant flies also displayed reduced stability in the absence of loading(Czech *et al.*, 2008; Okamura *et al.*, 2008a; Okamura *et al.*, 2008c; Zhou *et al.*, 2009; Marques *et al.*, 2010). However, it does not fully explain why viral siRNAs detected in *ago-2* flies are strongly biased for positive-strands and how the

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negative-stranded viral siRNAs are eliminated preferentially. Degradation of the endogenous small RNAs loaded in Ago1, but not those loaded in Ago2, by perfect complementary target RNAs was recently detected in cultured *Drosophila* cells (Ameres *et al.*, 2010). Co-immunoprecipitation combine with deep sequencing has shown previously that loading of FHVΔB2 siRNAs into Ago-1 occurs at very low levels in the infected S2 cells [our unpublished data and (Aliyari *et al.*, 2008b; Czech *et al.*, 2008)]. Therefore, I hypothesized that in the absence of Ago2-loading, higher abundance of viral siRNAs may be loaded into Ago1, leading to a more robust decay of viral siRNAs following the cleavage of target. Since the target RNA of the negative-strand viral siRNAs is inherently much more abundant in cells infected with a positive-strand RNA virus and the target RNA of the positive-strand viral siRNAs serves as the viral replication template, which is often inaccessible to the RISC activity in infected cells (Bitko and Barik, 2001; Gitlin *et al.*, 2002; Ge *et al.*, 2003; Pantaleo *et al.*, 2007; Schubert *et al.*, 2007), higher abundance of negative-strand viral siRNAs were degraded in this process and hence yielded the strong bias of positive-stranded viRNAs in *ago2* mutant flies. The complicated involvement of Ago1 in antiviral immunity and future works to test our hypothesis will be discussed in Chapter5.

In addition, I provided the first evidence supporting a redundant role of two dsRBPs in the Dcr2-dependent viRNA biogenesis and antiviral immunity in adult flies. Analysis of genetic mutants showed that RNA silencing by siRNAs from endogenous loci and exogenous dsRNA was often inhibited in *loqs* and *r2d2* single mutants as efficiently as in

the double mutant, suggesting they may function sequentially but not redundantly in the pathway. Loqs is required for dicing of the dsRNA, while R2D2 is essential for the subsequent RISC-loading of siRNAs (Czech *et al.*, 2008; Okamura *et al.*, 2008a; Okamura *et al.*, 2008c; Zhou *et al.*, 2009; Marques *et al.*, 2010). Consistently the relative abundance of 21nt- siRNAs from endogenous siRNA loci was dramatically reduced in *loqs* and *loqs- r2d2* mutants, but not in the *r2d2* mutant (Figure 5B). In contrast, RNAi-directed repression of FHVΔB2 was aborted only in the *loqs- r2d2* mutant and presence of either the *loqs* or *r2d2* allele alone had little effect on the silencing of FHVΔB2, indicating largely redundant roles for Loqs and R2D2 genes in the viral siRNA pathway. Comparison of small RNA populations in these mutants showed that the relative abundance of 21nt- viral siRNAs, particularly those targeting RNA2 of FHVΔB2, was markedly inhibited in the *loqs- r2d2* double mutant, but not significantly in *loqs* and *r2d2* mutants. Interestingly, the *loqs- r2d2* mutant exhibited strong positive-strand bias for RNA2-derived siRNAs, a pattern that was also observed in the *ago2* flies, but not in *loqs* or *r2d2* mutants. The efficacy of viral siRNAs comparison was based on the calculation of viral siRNAs over copy number of replication template RNA. In *r2d2* mutant the ratio reduced to only 22.6% of that in the wild type, but only modestly decreased in the *loqs* mutant (89%), suggesting that R2D2 may be dominant over Loqs in the antiviral silencing pathway. The viral siRNAs over viral genomic RNA in *loqs- r2d2* flies became much lower, being only 2.3% of that in wild type flies. Taken together the data indicated that Loqs and R2D2 proteins have redundant roles both in the biogenesis and Ago-2

loading of viral siRNAs. It was also the first instance to show that the genetic requirement for dsRBP as binding partners of Dcr2 in silencing virus RNAs differed from that silenced the transposons or transgenes by endogenous and exogenous siRNAs respectively.

Analysis of virus-specific small RNAs in wild type flies infected with FHV also revealed multiple changes associated with the expression of the B2 protein. The relative abundance of 21nt species viRNAs among the total small RNA reads was markedly decreased in FHV-infected flies, clearly suggested that B2 inhibits Dcr2-dependent production of viral siRNAs in adult fruit flies, which is consistent with previous studies *in vitro* and in cell culture illustrating that B2 protected the long dsRNA from being diced into siRNA duplexes by binding to dsRNA and FHV RdRP inside the viral RNA replication complex (Chao *et al.*, 2005a; Lu *et al.*, 2005; Aliyari *et al.*, 2008b). It was out of expect to find that B2 expression also led to a dramatically reduced ratio of viral siRNAs versus replication template RNA and strong positive-strand bias for siRNAs derived from both RNAs 1 and 2 in the wild type fruit flies. Since both of these changes were found in *ago-2* mutant flies and *loqs-r2d2* double mutant to a lesser extent, I concluded that B2 also inhibits loading of viral siRNAs into Ago2, given that the *in vitro* activity and assays in cell culture have demonstrated its activity to bind and sequester siRNA duplex. Additionally, unlike viRNAs loaded in Ago2 that were methylated at their 3' ends, a portion of viRNAs were found to be unmethylated in S2 cells inoculated with FHVΔB2 virion. Thus, it would be interesting to test whether the abundance of

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unmethylated viRNA population was altered in the presence of B2 expression in wild

type flies.

**Table 3-1 Primers used for qPCR or making templates for ribo-probes**

qPCR	
R1-F	AACTGCTGGTTTCCATCGG
R1-R	GGTCCTTGTGTCCCATACCG
rp49F	ATGACCATCCGCCCAGCATAC
rp49R	CTGCATGAGCAGGACCTCCAG
Amplifying templates for riboprobes and rp49 cDNA-probe	
YL105	CCAATGTTAAACATGCCAAGCAAACTCG
YL130	TAATACGACTCACTATAGGGCGTTACCAATGTTAAACG
YL131	TAATACGACTCACTATAGGGACCTCTGCCCTTTCGG
CR95	ACCTCTGCCCTTTCGGGCTAGAACGG
YL136	TAATACGACTCACTATAGGGTTATACCAATTCGG
YL137	TAATACGACTCACTATAGGGACCTTAGTCTGTTG
R2-F	CCATGATTCGCCTCCTCTCG
R2-R	ACCTTAGTCTGTTGACTTAAACTGG
Rp49F	GCTAAGCTGTCGCACAAATGGCGCA
Rp49R	CCAGGAACTTCTTGAATCCGGTGG

Table 3-2 *In vitro* transcription for ribo-probe synthesis

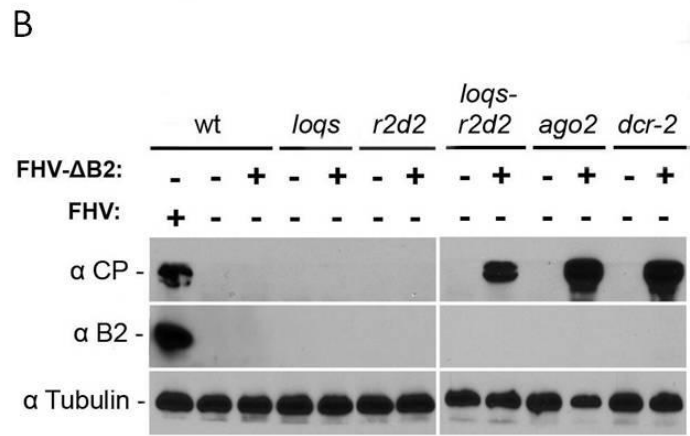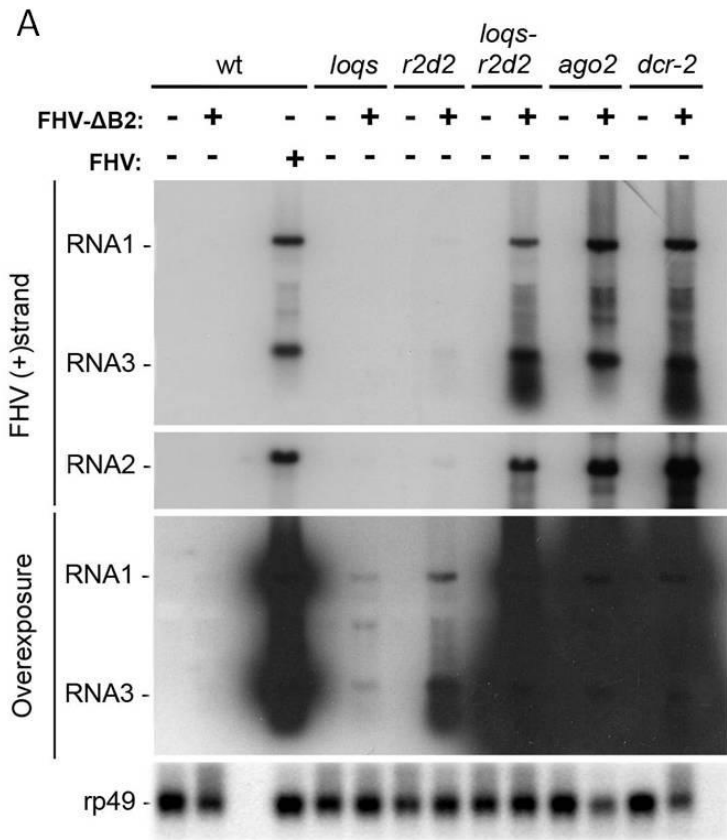| 0.1 M DTT	2 μl
3.3 mM ATP, GTP, CTP	3 μl
0.3 mM UTP	0.8 μl
RNase inhibitor	0.5 μl
10x Buffer	2 μl
DNA template (~200 ng/μl)	5.7 μl
[α-^{32}P] UTP	4 μl
T7 RNA Polymerase 50 U/μl	2 μl

Figure 3-1 VSR-depleted mutant FHVΔB2 exhibits high virulence to adult fruit flies carrying loss-of-functions in Dcr2 or Ago2.

Survival of wild type (wt), *dcr-2*[L811fsX] (*dcr2*) and *ago2*[414] (*ago2*) adult flies after 1×PBS buffer (Mock) or FHVΔB2 (ΔB2) inoculation. Over 60 flies of each genotype were injected and monitored daily. Data shown represented mean value of triplicates, and the error bars indicated standard deviation.
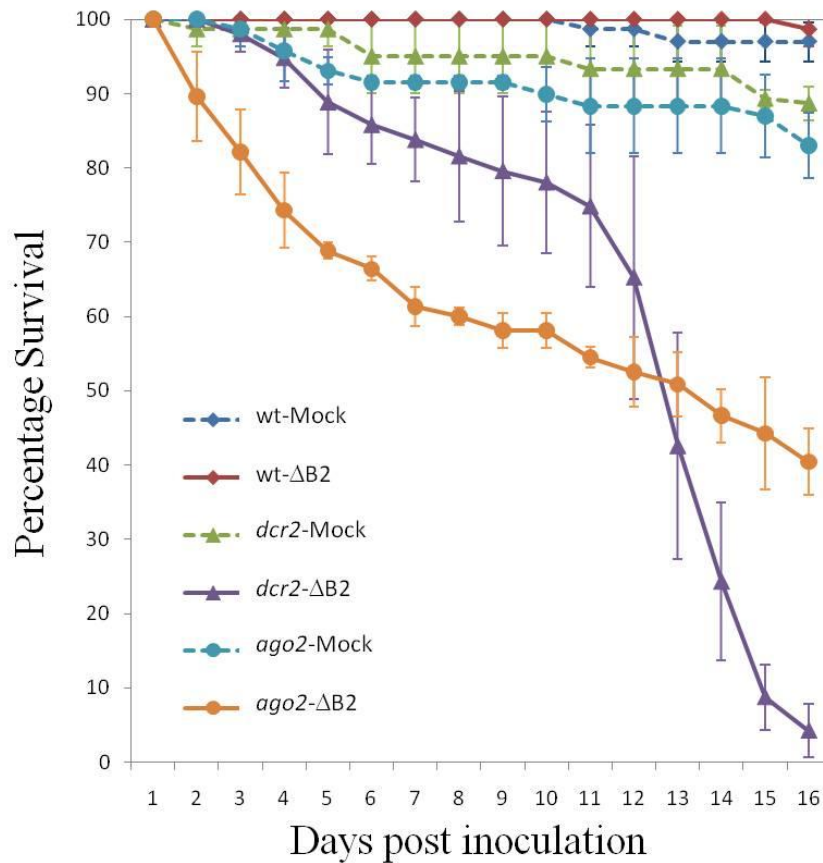
Figure 3-2 Detection of RNA and protein levels of FHV in adult fruit flies

(A) Accumulation of FHV plus strand RNAs in different mutants 7 days after microinjection with FHV or FHVΔB2 virions, loading was monitored by probing rp49.

(B) FHV coat protein and B2 levels in the corresponding mutants, tubulin was detected as loading control.

A

FHV-ΔB2:
FHV:

FHV (+)strand

RNA1 -
RNA3 -
RNA2 -

Overexposure

RNA1 -
RNA3 -

rp49 -

B

FHV-ΔB2:
FHV:

α CP -
α B2 -
α Tubulin -

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Figure 3-3 FHV (+) RNA1 and (-) RNA1 expression levels relative to rp49 (A) and ratio (B) as determined by qPCR of to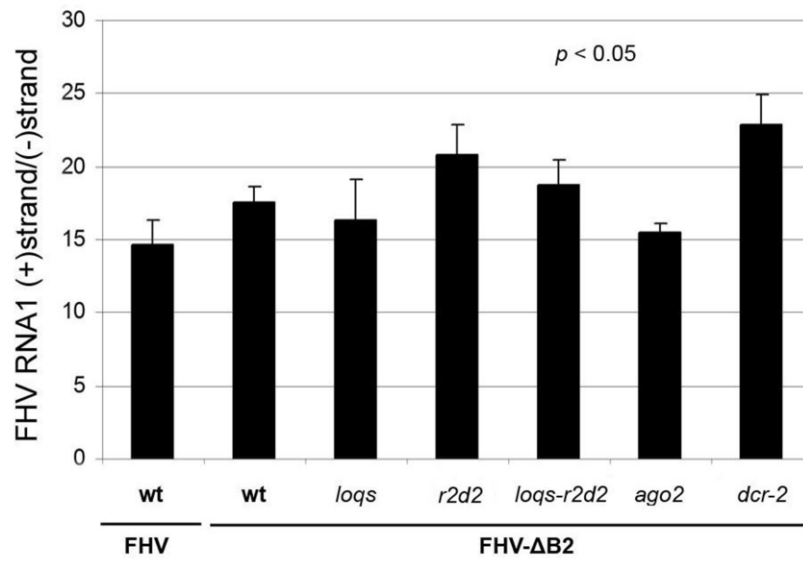tal RNA from FHV or FHVΔB2 microinjected adult fruit flies. The graphs show the mean and s.d.(n=6). Y axis is logarithmic scale in (A). Statistically significant differences are less than 0.02 in (A) and less than 0.05 in (B) (Student's *t*-test, compared to FHVΔB2 injected wild type).

A
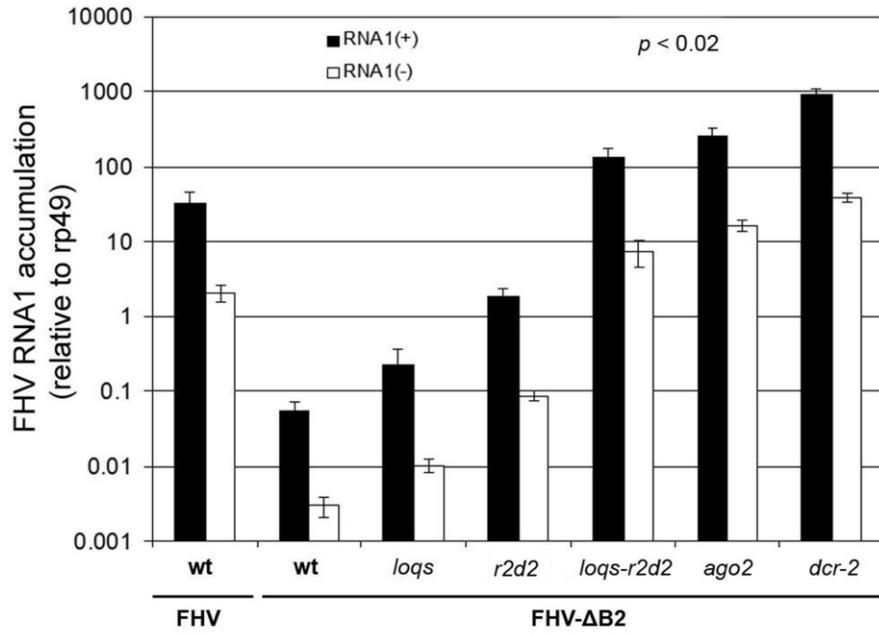
B

Figure 3-4 Virus derived small RNAs of either FHV or FHVΔB2 in adult fruit flies.

Except wild type fruit fly inoculated with either FHV or FHVΔB2 virions, all mutants fruit flies were inoculated with FHVΔB2 virions only.

(A) The abundance of virus derived siRNAs (18-28nt) in wild type fruit fly and mutants. The number of viral siRNAs was normalized to 1 million sequenced small RNAs (18-28nt) of corresponding library.

(B) Percentages of distinct sizes of viral siRNAs derived from either RNA1 or RNA2 of FHV in wild type fruit fly and mutants.

(C) Relative efficiency of 21-nt viral siRNA biogenesis processed by Dcr-2 endonuclease from viral dsRNA replicative intermediates in wild type fruit fly and mutants.
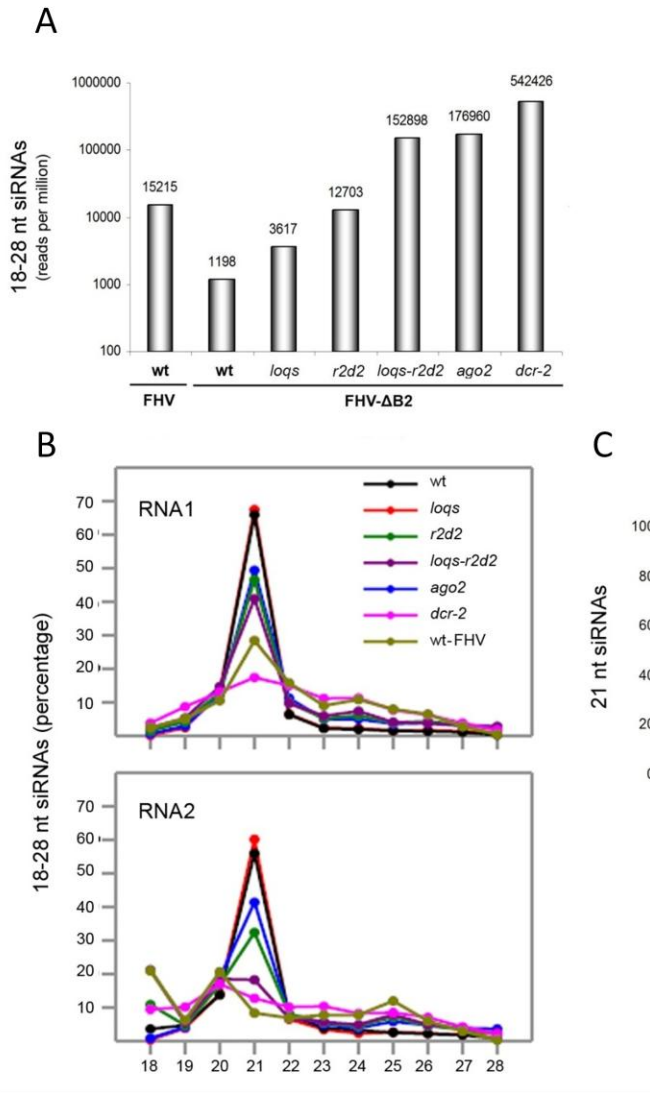
Figure 4

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Figure 3-5 Profiles of virus derived small RNAs in wild type fruit flies and mutants.

(A) Density of 21-nt viral small RNAs derived from FHV RNA1 and RNA2 in wild type fruit flies and mutants. 10-nt size of windows (step size: 10nt) were used to count 21-nt viral small RNAs staring in the region from 5' to 3' end of (+) genomic RNA1/2. (+/-) 21-nt viral small RNAs were shown in red line (top) and blue line (bottom) respectively. It is worthy to note that the scales are different each other. Number on each graph show the "zoom-out" times of viral small RNA density.

(B) Profile of viral small RNAs of FHV RNA1/2 in the wild type fruit flies and mutants. The percentages of (+/-) 18-28nt viral small RNAs were represented by orange bar (top) and blue bar (bottom).
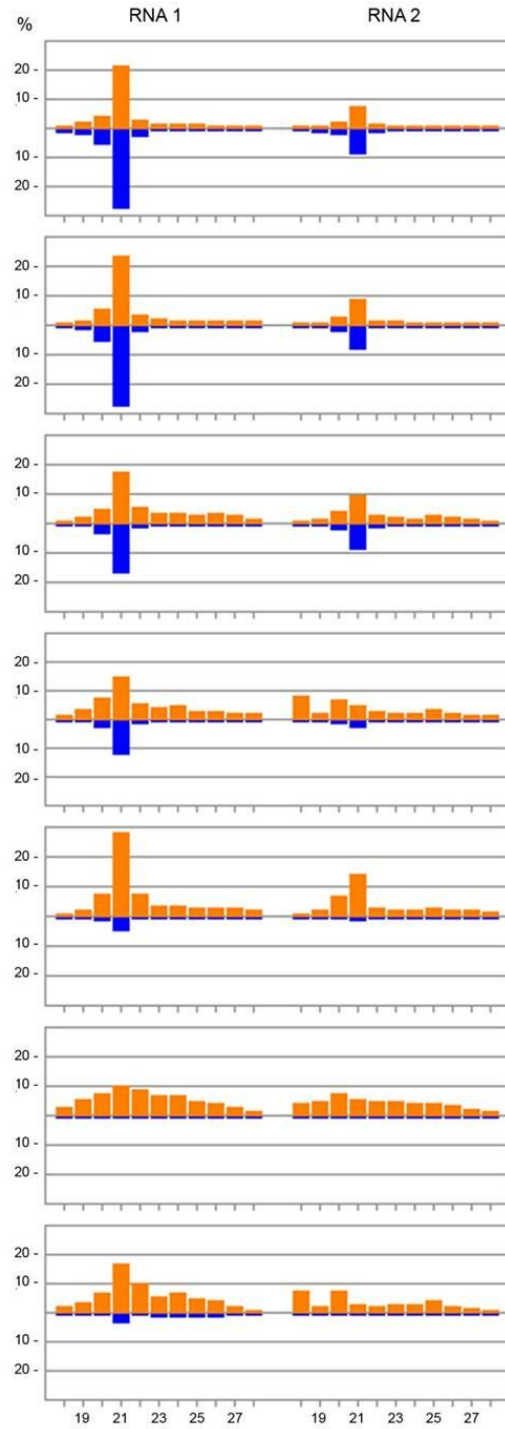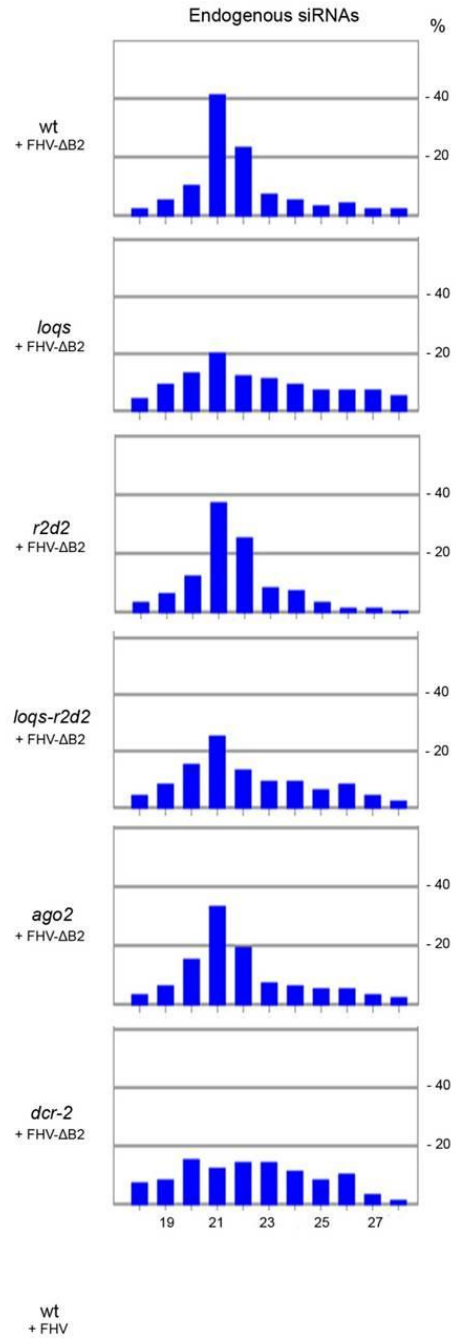
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CHAPTER 4. VIRAL REPLICATION IN GERMLINE AND TRANSMISSION IN ADULT *DROSOPHILA*

4.1 Abstract

In *Drosophila melanogaster*, Piwi-interacting (pi)RNAs, which are 24-30 nt long, are Dicer-independent small RNAs and their biogenesis require proteins in the Piwi subfamily of Argonaute proteins; namely Ago3, Aubergine (Aub), and Piwi. Most *Drosophila* piRNAs are derived from discrete heterochromatic loci in the genome, which are highly enriched in transposons and other repetitive elements. Considerable evidence shows that mutations of piRNA pathway, such as *aub* and *piwi* show increased transposon activity in the germline, indicating their important roles in maintaining genome integrity during germline development. Our recent identification of piRNAs targeting six different viruses in a Drosophila ovary somatic sheet (OSS) cell line indicated the piRNA pathway may protect the oocytes from viral infections. Here I demonstrated that *piwi* mutants exhibited enhanced disease susceptibility to inoculation of FHV, DCV and CrPV. Strikingly, enhanced accumulation of FHV RNAs and proteins was detected in the epithelial sheath and follicle cells surrounding egg chambers in the ovaries of *piwi* mutant flies. Consistent with the viral abundance in ovaries, presence of the *piwi* mutant allele resulted in significantly enhanced levels of FHV and DCV in the progenies of virus-infected adult flies. Although no obvious signature of piRNAs could be detected by deep sequencing of ovarian or Piwi-bound small RNAs from wild type or

dcr-2 mutant flies, I found that negative-stranded 21nt- small RNAs were more abundant in ovaries of *piwi* heterozygous mutants. A preliminary model that integrates our current knowledge of the piRNA pathway into the Drosophila antiviral immunity framework is discussed.

4.2 Introduction

PIWI-interacting RNAs (piRNAs) have been studied extensively for their biogenesis, characteristics, and functions in fruit flies, fish, nematodes and mice since last decade (Cox *et al.*, 1998b; Aravin *et al.*, 2006; Houwing *et al.*, 2007; Batista *et al.*, 2008; Das *et al.*, 2008; Houwing *et al.*, 2008; Kawaoka *et al.*, 2008a; Kawaoka *et al.*, 2008b). piRNAs are longer than miRNAs and endo-siRNAs by several bases; for example, in *Drosophila*, piRNAs range between 24 and 30nt, while miRNAs and siRNAs are 20-23nt long (Aravin *et al.*, 2001). As introduced in the previous chapters, exogenous and endogenous siRNA pathways in *Drosophila* are Dicer 2 – dependent, and reports from various laboratories including ours have established that it is important for antiviral immunity, which is characterized by the recognition and processing of viral dsRNAs by Dicer into siRNAs of discrete sizes. These virus-derived siRNAs are then loaded into an RNA-induced silencing complex to guide the slicing of the target viral RNAs by Ago2. In contrast to siRNAs, piRNAs are Dicer-independent and the genetic requirements for their biogenesis seem to be cell-type specific or even target-specific (Brennecke *et al.*, 2007a; Chung *et al.*, 2008; Lau *et al.*, 2009; Senti and Brennecke, 2010). Most details of the

piRNA production and function remained to be determined, while it has been well established that they are associated with a specialized sub-class of Argonaute proteins termed as PIWI proteins, after the founding member of the sub-family, the *P*-element-*I*nduced *Wi*mpy Testes or Piwi that was originally classified by Dr. Lin, Haifan's laboratory (Cox *et al.*, 1998a; Cox *et al.*, 2000). Members of the PIWI subfamily including AGO3, Aubergine (AUB), and Piwi may be important for both biogenesis and activity of piRNAs in the animal germline based on the currently available evidence (Harris and Macdonald, 2001; Megosh *et al.*, 2006; Brennecke *et al.*, 2007a; Yin and Lin, 2007).

Most *Drosophila* piRNAs are derived from discrete heterochromatic loci in the genome, which are highly enriched in transposons and other repetitive elements. Considerable evidence shows that mutations or RNA silencing of the components in piRNA pathway, such as *aub* and *piwi* resulted in de-repression of certain transposons in the germline, indicating one of the essential roles of piRNAs is to maintain the genome integrity during germline development (Kalmykova *et al.*, 2005; Saito, 2006; Aravin *et al.*, 2007a; Klenov *et al.*, 2007; van Rij and Berezikov, 2009; Olivieri *et al.*, 2010).

We have recently reported the identification of piRNAs targeting six different RNA viruses in a Drosophila ovary somatic sheet (OSS) cell line (including Drosophila X Virus (DXV), Drosophila C Virus (DCV), Drosophila birnavirus (DBV), American nodavirus (ANV), Drosophila tetravirus (DTrV), and Nora virus (Wu *et al.*, 2010a). It is a cell line derived from the follicle cells that line the oocyte in the germarium (Lau *et al.*,

2009; Saito *et al.*, 2009). Interestingly, two of these viruses, DCV and ANV generate more abundant viral piRNAs than the other four. More strikingly, ANV-specific piRNAs are more than twice as abundant as viral siRNAs in the OSS cells. In addition, these viral piRNAs shared several features with the endogenous primary piRNAs found in the same cell line, including being 24-30 nucleotides in length with two peaks at 27 and 28nt, strong 5' – uridine bias but no preference for adenine at the 10th position, and 95% of them are mapped to the sense strand of viral genomic RNAs (Wu *et al.*, 2010a). Based on the sequencing analysis of small RNAs in OSS cell line, I proposed that a robust antiviral immunity mediated by piRNA pathway, which is led by Piwi protein as it is the only PIWI-subfamily member expressed in the OSS cell line, may occur in the follicle cells to protect the developing oocyte during virus infection.

This is also related to a fundamental aspect of virus infections - transmission of the viruses. Transmission process determines the spreading and the persistence of viruses within a host population, which provides crucial information for designing the disease control strategies against the pathogens. Generally transmission can occur horizontally or vertically. In horizontal transmission, viruses are transmitted among individuals of the same generation through either direct or indirect mode. Horizontal transmission by direct routes includes air-borne infection, food-borne infection, feces-borne infection and sexual infection, whereas an indirect mode involves an intermediate host or vector to carry the viruses from one host to another. For vertical transmission, viruses are passed from infected mother to offspring either on the surface of the egg (transovum transmission) or

within the egg (transovarian transmission) (Roxstrom-Lindquist *et al.*, 2004). The identification of virus-derived piRNAs in the cell line related to follicle cells implies that piRNA pathway may be involved in suppressing the vertical transmission of certain viruses.

In this study I examined the viral accumulation in ovaries of adult flies infected with FHV, instead of ANV, as it is a better-characterized member of the alphanodaviruses in the Nodaviridae family, and it shares 83% and 81% sequence-identity to the RNA1 and RNA2 of ANV, respectively (Wu *et al.*, 2010a). I also examined the response of adult flies to two other evolutionarily diverse viruses: DCV and Cricket paralysis virus (CrPV), which are members of the family Dicistroviridae (Plus *et al.*, 1978; Wilson *et al.*, 2000; Huszar and Imler, 2008). Dicistroviruses have a single genomic RNA of positive strand, which comprised of two open reading frames (ORFs). ORF 1 typically encodes the non-structural proteins necessary for virus replication such as protease, helicase and RdRP domains, and ORF2 encodes the structural proteins such as capsid proteins (Johnson and Christian, 1999). DCV was first discovered from a French strain of *D. melanogaster* (Jousset *et al.*, 1977). The pathogenic effects of DCV on adult flies have been documented since then. Persistent infection normally does not produce remarkable disease symptom; when infected by injection, DCV causes mortality typically within a few days (Scotti, 1975; Moore *et al.*, 1980). DCV replicates in a subset of drosophila tissues, including the fat body and the outer sheath of the egg chamber and sheaths surrounding individual ovarioles, visceral muscles along the midgut, and a subset of

somatic muscles (Cherry and Perrimon, 2004). Its transmission can occur both transovumlly and horizontally from females to males (Gomariz-Zilber and Thomas-Orillard, 1993; Gomariz-Zilber *et al.*, 1995; Thomas-Orillard *et al.*, 1995). Unlike DCV as a natural pathogen to Drosophila, CrPV has a wide host range, infecting insects which belong to *Diptera, Lepidoptera, Orthoptera*, and *Heteroptera* species (Wilson *et al.*, 2000). Importantly, CrPV also replicates in cultured cells from various insect species including *Drosophila* S2 cells (Scotti, 1975).

Both viruses have provided intriguing insights of RNAi-mediated antiviral immunity in *Drosophila* in the past few years. A global microarray analysis revealed that DCV infection induced a set of genes independent of the well-characterized antimicrobial peptide genes in adult flies (Dostert *et al.*, 2005b). The same report indicated Janus kinasesignal transducer and activator of transcription (Jak-STAT) pathway is required but not sufficient for the antiviral response in *Drosophila*. Later on Van Rij RP et al. demonstrated that Ago-2-defective flies were hypersensitive to infection with DCV and CrPV, and the increased mortality was associated with a dramatic increase in viral RNA accumulation and virus titers (van Rij *et al.*, 2006; Saleh *et al.*, 2009; Nayak *et al.*, 2010). Furthermore they found that DCV encoded a potent VSR, DCV-1A, which bound specifically to long dsRNAs and prevented Dicer-2-mediated processing of siRNA. The same research group also reported three years later that mutant flies defective in dsRNA-uptake pathway exhibited increased susceptibility to Drosophila C virus, accompanied by dramatic increase in viral titers. In contrast, the VSR encoded by CrPV interacted with

Ago2 and inhibited its activity without affecting the miRNA pathway. Expression of CrPV VSR but not that of DCV increased the pathogenesis of Sindbis virus in *Drosophila*. Here I showed that *piwi* mutants also displayed hypersensitivity to DCV and CrPV correlated to higher viral RNA abundance in both the ovaries and somatic tissues. Dramatic induction of virus accumulation was also observed in the offspring of DCV-infected flies.

4.3 Materials and methods

4.3.1 Fly strains, virion preparation and inoculation

FHV, FHVΔB2, DCV and CrPV particles used in this study were propagated and purified from *Drosophila* S2 cells. Plaque forming units per ml (pfu/ml) were determined by a standard plaque assay as described in the previous Chapter. Virions were diluted in $1 \times$ PBS buffer for injection. Canton S (*csw*) or Oregon R (*OrR*) obtained from Dr. Peter Atkinson's laboratory was used as wild type. $dcr\text{-}2^{L811fsX}$ (*dcr-2*) was described as previously (Lee *et al.*, 2004b; Okamura *et al.*, 2004). $piwi^{1/2}$ homozygous mutant was generated by crossing $piwi^{1}/Cyo$ females and $piwi^{2}/Cyo$ males. $aub^{QC42/HN}$ homozygote was generated by cross of aub^{QC42}/Cyo females and aub^{HN}/Cyo males. $ago3^{t2/t3}$ was generated by cross of $ago3^{t2}/TM6$ females and $ago3^{t3}/TM6$ males. $armi^{1/72.1}$ was generated by crossing $armi^{1}/Sb$ females and $armi^{72.1}/Sb$ males. For virus susceptibility studies, adult flies of 2-4 days old were anaesthetized by exposure to CO_2. Approximate 40 nl of a viral suspension (FHV: 7×10^7pfu/ml; FHVΔB2: $0.7\text{-}2\times10^8$pfu/ml; DCV:

5×10^8 pfu/ml; CrPV: 1×10^6 pfu/ml) was microinjected into the thoraces of adult flies with a microinjector (FemtoJET 5247, Eppendorf, Germany). Injection of the same volume of $1 \times$ PBS buffer was used as blank control. Flies were transferred to fresh medium every 2-3 days after injection. Fly stocks and infected flies were reared on standard cornmeal-agar medium at 25 ℃. *piwi¹/Cyo*, *piwi²/Cyo* and *armi⁷².¹/Sb* mutant strains were generous gifts from Dr. Richard Carthew's laboratory. All the remaining mutants were bought from Bloomington Drosophila Stock Center at Indiana University. Description of these mutants can be found in Flybase (http://flybase.org/).

4.3.2   Immunostaining and Confocal Microscopy

Antibody staining of ovaries from FHV – infected flies at 7dpi was conducted based on protocols from Dr. Xie, Ting's laboratory with minor modification. Ovaries were dissected in ice-cold 1 x PBS buffer in a spotted glass plate and teased apart the epithelial sheath between each ovariole as gentle as possible to allow better penetration of antibodies. Transfer the dissected ovaries to 1.5mL tubes containing 1mL of 4% paraformaldehyde in $1 \times$ PBS (Table 4-2) for fixation, which takes 15-20 minutes in room temperature. The ovaries were then washed briefly three times in PBT (Table 4-2), followed by twice in PBT for 15 minutes on a rocking nutator, and incubated in PBT-NGS (Table 4-2) for at least 1 hour. Piwi monoclonal antibody, FHV-CP or B2 polyclonal serum which were diluted in PBT-NGS were incubated with the ovaries overnight, and washed in $1 \times$ PBT for at least 4 times for 15 minutes each. Fluorescently-labeled secondary antibody was diluted in PBT-NGS and incubated with the ovaries for 4-6 hours.

After removing the secondary antibody, 1 µg/ml DAPI diluted in PBT was added to the ovaries for 6 minutes to stain the DNAs. The ovaries were subjected to $1 \times$ PBT washing for 5 times with 15 minutes each, and then mounted in Vector mounting medium for confocal microscopy. Leica SP2 confocal microscopy in Core facility was employed. The beam path setting was adjusted based on the mock *piwi²/Cyo* –sample stained with FHV-CP, and as following: ArUV 351nm – 91%, 364nm – 91%; Ar/HeNe/GrNe/HeCd 442nm – 0%, 458nm – 0%, 476nm – 0%, 488nm – 20%, 514nm – 0%, 543nm – 0%, 633nm 51 (for FHV-CP and B2 protein) - 71% (for Piwi).

4.3.3 Transmission test

Female and male virgin-flies of different genetic backgrounds were separated within 10 hours after they hatched. Approximately 50pfu of purified FHV or DCV was injected into each fly of 2-4 days old, and immediately after inoculation, the parent-flies were kept in the same vials for mating for 7 days, and collected for viral RNA and protein analysis. Adult progenies were harvested from the day the first fly hatched from pupa for approximately 5 days. 5 females and 5 males were randomly selected from each group as one sample in each biological replica and applied for quantitative RT-PCR.

For DCV transmission study, virgin parents injected with DCV suspension (50pfu/fly) were kept in the same vial for mating for 5 days. Dead flies were immediately removed or transferred the survivors to fresh medium every two days. All the living parents were replaced into fresh vials of medium at 5 days post inoculation, and the newly laid eggs were collected with brushes, transferred into 1.5ml centrifuge tubes and treated with 60%

bleach for less than 2 minutes. After washing with distilled water briefly twice, the embryos were placed into fresh medium for full development to adult flies. Each sample of total RNA for quantitative real time PCR in all the experimental replicas was extracted from 5 female and 5 male newly-hatched progenies.

Horizontal control was conducted by collecting embryos laid by untreated flies or flies that were injected with blank buffer and the embryos were reared in fresh medium with about 20 male flies of corresponding genetic strains inoculated with FHV (50pfu/fly) for 7 days. The male flies were then removed from the vials and the larvae were left developing in the same vials until they hatched. Same numbers of female and male progenies were harvested from each group, and the FHV-injected flies were used as the positive control for one step RT-PCR using the Titan One Tube RT-PCR kit (Roche).

4.3.4   Plaque assay

Drosophila (DL1) cells were collected in a 15 ml conical tube after determining the cell density and volume of cell suspension. The cells were then centrifuged at 580 x g for 5 minutes at room temperature, resuspended in fresh complete growth medium to a density of 4.2 x 10^7 cells per ml. Dispense aliquots of 95 μl into 15 ml conical tubes and add 5 μl virions or fly extract which were diluted in CGM. The mixture was vortexed lightly, and then shaken gently every 5-10 minutes for 1 hour at room temperature. 5 ml of NHA (125 mM NaCl, 25 mM Hepes pH7, 0.2% (wt/v) BSA, 10 mg/ml trypan blue) was added to the virus/cell mixture and mixed thoroughly by gentel vortexing. The cells were then left attached to 60 mm tissue culture dishes for 1 hour. Gently aspirate buffer and add 3 ml of

1% low gelling temperature agarose overlay. The agarose is dissolved in CGM by heating in 65 ℃ water bath for 10 min; cool to 37-40 ℃ before applying to cells. After the agarose solidified, 2 ml of CGM was added to the mixture. The plates with agarose overlay were incubated at 27 ℃. The plaques were developed after 50 hours by adding 0.5 ml of MTT solution (3mg/ml in distilled water, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma) to each dish as several well-scattered drops. The plates were kept at 27 ℃ for additional few hours for staining. Triplicates were set up for each virus dilution in each experiment in order to obtain more accurate titers.

4.3.5   RNA and protein analysis

Northern blotting, western blotting and quantitative real time-PCR were performed following the same procedure as in Chapter 3, except that the reverse transcription for quantitative real time PCR detecting viral RNAs from the progenies was done by using the iScript cDNA synthesis kit (Bio-rad) according to the manufacture's protocol, in which random primers instead of gene-specific primers were used for first-strand synthesis. Ribonucleotide probes for detection of FHV genomic and subgenomic RNAs, antiserums to detect the FHV capsid protein and B2 were   identical as described in Chapter 3. Piwi monoclonal antibody used for western blotting, co-immunoprecipitation (data not shown) and immunostaining was generous gift from Dr. Mikiko Siomi. Antibodies for –actin and tubulin were purchased from Santa Cruz.

4.4 Results

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4.4.1 Piwi-dependent silencing provided potent protection of the ovaries from FHV infection

Our identification of virus-derived piRNAs in OSS cells, which exclusively expressed Piwi in the PIWI family, suggests that piRNA pathway may be a prominent component of the barrier protecting the germline from virus invasion. However, whether this is the case in adult flies during virus infection need to be tested. The antiviral role of Piwi protein was tested by intrathoracically injecting the *piwi^1* and *piwi^2* mutant flies with purified FHV (50pfu/fly). The *piwi^1* contains a single PZ insertional mutation in the first exon of piwi transcript, whereas the *piwi^2* mutation is a single P-ry11 transposable insertion located in the fourth exon. Homozygous *piwi^{1/1}* and *piwi^{2/2}* mutant flies were viable but in which the development of germline was disrupted and hence sterile. Trans-heterozygous *piwi^{1/2}* mutant was generated by a combination of these two alleles to ensure the stability of mutations. Virus susceptibility and viral RNA abundance in both homozygous mutants and trans-heterozygous mutant were examined in at least two biological replicas using either Oregon R (data not shown) or canton S white (*csw*) as wild type controls. The survival rate of only trans-heterozygous and *csw* strains were presented as no significant difference was observed among these fruit flies. Interestingly the number of *piwi* homozygous (not shown) or trans-heterozygous mutant survivors declined rapidly from 10 days post infection, by dropping approximately 10% each day. Similar enhanced mortality was also observed in *dcr2* mutant flies except that the fly death occurs from 7dpi. In contrast to wild type, which resulted in over 70% survival over the 16 days of

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observation, FHV inoculation resulted in 86% mortality of *piwi*[1/2] mutant and none of the *dcr2* flies was able to survive with FHV infection at the dosage we used. Flies for our detection of viral genomic and subgenomic RNAs and proteins presented in the following sections were harvested at 7dpi, when the survival percentage of wild type, *dcr2* and *piwi*[1/2] flies were 92.5%, 86.3% and 91.3%, respectively (Figure 4-1A).

The abundance of FHV genomic RNA 1, RNA2 and subgenomic RNA3 in the somatic tissues of infected flies was assessed by Northern blot and real time PCR. Both females and males supported accumulation of similar levels of viral RNA (Figure 4-1B) excluding the possibility that a sex would be more susceptible to FHV infection. When quantifying the intensity of virus-derived signals with a phosphorimager system, FHV accumulated to higher levels (ranged to 3.5 – 4.0 times) in *dcr2* mutant compared to the wild type flies as previously reported by our colleague in 2006. Strikingly, FHV RNA levels in *piwi*[1/1] and *piwi*[2/2] homozygous mutants were up to 3.6-8.1 times higher than in wild type flies (Figure 4-1B, female panel), and even the heterozygous mutants exhibited viral accumulation similar to *dcr2* mutant flies. This implied that the Piwi protein may also play an important role in virus clearance outside the germline. Nevertheless, to address our main concern on the antiviral immunity in the germline, I closely analyzed the FHV replication in the ovaries. Consistent with our hypothesis, replication of either FHV RNA1 or 2 could hardly be detected in the ovaries of wild type flies infected with FHV at 7dpi, as demonstrated by the levels of antisense strands of FHV RNAs (Figure 4-2A, bottom panel). Same amount (2 μg) of total RNAs extracted from the heads of FHV-

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infected flies were hybridized under identical experimental condition and exposure time in order to exclude the possibility that the undetectable level of FHV was solely due to defective virus spreading within the host (Figure 2A, upper panel). Although slightly higher than in wild type flies, the levels of FHV RNAs in *dcr2* mutant ovaries were considerably low, despite of the high abundance readily detected by Northern blotting in the heads. Homozygous *piwi$^{1/1}$* and *piwi$^{2/2}$* (data not shown) and trans-heterozygous *piwi$^{1/2}$* mutant flies, on the other hand, allowed robust replication of all three species of FHV RNAs in the ovaries and viral RNA was easily detected by hybridization under the same conditions, even when only half of the amount of total RNAs was loaded (Figure 4-2A). For more accurate comparisons of viral abundance in different tissues among different genotypes, quantitative real-time PCR was performed using rp49 as an endogenous control. In the heads and ovaries of wild type flies, the genomic RNAs of FHV were ~100 and one times more abundant than the ribosomal gene, respectively. More importantly, FHV accumulation in the heads of *dcr-2* and *piwi$^{1/2}$* mutants was approximately 16 and 32 times higher than that in the wild type heads, respectively, while in the ovaries such increase was over 50 and 1400-fold. This strongly suggests that a germline-specific antiviral response dependent on both Dcr-2 and Piwi restricts virus accumulation in the ovaries of FHV-infected flies.

4.4.2   *piwi* mutants exhibited enhanced disease susceptibility to DCV and CrPV

In order to prove that our above-described observation represents a conserved mechanism mediated by Piwi, I challenged the *piwi* mutants with two other evolutionarily diverse

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viruses, DCV and CrPV. According to the small RNA sequencing analysis from OSS cells, DCV also generated abundant siRNAs and piRNAs as ANV, while no small RNAs were corresponding to CrPV. I examined the susceptibility of *piwi* mutant flies to these two viruses by inoculating *piwi^1/Cyo*, *piwi2/Cyo* heterozygous and *piwi$^{1/2}$* trans-heterozygous strains with purified DCV or CrPV virions at 50pfu/fly. Interestingly, CrPV-injected adult flies did not exhibit strong disease symptoms, whereas all the flies including wild type strain displayed slower reactivity and eventually paralysis even at low dosage (50pfu/fly). In contrast to 95% survival achieved by the wild type flies 8 days after infection, only 40% of *dcr2* and 58.2% of *piwi$^{1/2}$* mutant flies survived at the same time point. (Figure 4-3B) When I increased the inoculation dosage to 5000pfu/fly, the flies showed more sever paralysis disease symptoms, and mortality of *dcr2* mutant flies dramatically increased up to less than 50% at 4dpi, and all the *dcr2* flies in each replica of experiments died by the end of 5 or 6 dpi, (Figure 4-3A) which was consistent with the idea that DCV can be efficiently eliminated by Dcr2-dependent siRNA silencing pathway. Similar to *piwi^1/Cyo* and *piwi2/Cyo* heterozygous mutants (data not shown) approximately 70% of *piwi$^{1/2}$* mutant survived by 4 dpi, whereas the average survival percentage dropped to 16.6% by 5 dpi and none could survive by the end of 7 or 8dpi. Higher virulence of DCV in *piwi$^{1/2}$* mutant flies compared to wild type suggests that Piwi also provides some protection against DCV. Although generally CrPV did not cause much symptoms and mortality in all the strains tested, inoculation to *dcr2* and *piwi$^{1/2}$* trans-heterozygous strains resulted in 14% and 18.3% death at 8 dpi, respectively, (Figure 4-3D)

whereas no significant difference in survival was observed in wild type flies injected with CrPV compared to mock (Figure 4-1) over these 8 days. Both *piwi^1/Cyo* and *piwi2/Cyo* heterozygous mutant flies (data not shown) showed similar survival rate as *dcr2* and the trans-heterozygous mutants. Whether the hypersensitivity of *dcr2* and *piwi$^{1/2}$* mutants correlated to increased viral RNA accumulation in the flies was further accessed by northern blotting analysis. An RNA targeting the last 400nt of 3' end of DCV (Figure 4-3C) and oligonucleotides targeting 5' ORF of CrPV (Figure 4-3E) were used to probe for the selected genomic RNAs in the ovaries and somatic tissues. In general, Northern blot results indicated that impairment of Piwi function was associated with higher levels of DCV RNA in both carcasses and ovaries.

4.4.3   Expression of FHV CP and B2 proteins in the cytoplasm of follicle cells in the
    developing egg chamber

The capsid protein (CP) and B2 protein of FHV had been investigated in cultured Drosophila cells and yeast cells, but little was known for their localization in FHV-infected adult flies so far. Our previous investigation in *Drosophila* S2 cells suggested a proportion of B2 protein interacted with the viral RdRP, so the signals of B2 may partly reflect where the FHV replication complexes are localized in the ovaries. Moreover, sub-cellular localization of CP may represent the site of virion assembly. Therefore, in order to prove there is indeed FHV invasion to the fly ovaries, I examined the expression of these two viral proteins in the ovary immunostaining and confocal laser scanning microscopy. This also provides another way to evaluate the susceptibility of our mutants

to allow FHV invasion into the ovaries. Meanwhile, immunofluorescence localized in certain subcellular compartments could exclude the possibility that detection of FHV RNAs in dissected ovaries were actually contamination effects from tissue dissection. Ovaries dissected from the infected wild type, *dcr2* and *piwi²/Cyo* mutant flies were stained for either FHV B2 (Figure 4-4B) or CP (Figure 4-5) and observed under the confocal microscope using the same wavelength and contrast setting. Piwi was used as a positive control for proper sample handling and antibody penetration. Consequently ovaries from homozygous *piwi* mutants were not included in this study. I found that both B2 and CP were greatly induced in *dcr-2* and *piwi* heterozygous mutants, which was consistent with the RNA2 and RNA3 abundance (Figure 4-2A) and was further confirmed by western blotting (Figure 4-4A) using the same antiserum for immunostaining. As previously reported, Piwi protein reduction was observed in the ovaries of *piwi*-heterozygous mutants, while the same antibody could not detect any specific signals from the trans-heterozygous *piwi¹/²* mutant. The CP expression in the wild type and all mutant ovaries was generally correlated to the RNA2 abundance. Unexpectedly distinct from the CP, which was hardly detectable in the wild type ovaries, B2 protein appeared as a faint band in the wild type strain but was visibly more abundant in the *dcr-2* and *piwi²/Cyo* mutant ovaries. Considering the dramatic induction of sense-stranded RNA3s in *piwi¹/²* mutant ovaries, the relatively lower ratio of B2 protein in *piwi¹/²* strain compared to wild type should not simply be explained by cross-interaction of the B2 antibodies. Similar to the observation in the heads of FHV-infected flies, I

noted that CP and B2 expressed in *dcr2* and *piwi* mutants was only a few folds higher than the wild type flies, although the RNA2 and RNA3 levels in those mutants on average were at least 10 folds more abundant relative to those in wild type. I reasoned that may reflect the balanced function of viral RNAs as templates for both protein translation and RNA replication. An overwhelming proportion of RNA2 in this case may be assembled into virions as well. However, possibility of translational inhibition against FHV RNA2 and RNA3 which may involve miRNA pathway could not be excluded so far. I further explored the cellular localization of CP and B2 proteins in the ovarioles of *dcr2* and *piwi²/Cyo* mutants. Viral proteins in wild type ovaries were only detectable when I used higher energy to excite the fluorescence, but the localization of these viral proteins was not altered in *dcr2* and *piwi²/Cyo* mutants (data not shown). I observed strong fluorescent staining in the germarium, the anteriomost structure of each ovariole, probably because it was arranged closest to the thorax and more interaction with the virus spreading from the injection site (Figure 4-5A). In the germarium, germline stem cells (GSCs) divide asymmetrically into GSCs and differentiating cystoblasts. I found that B2 protein in *dcr2* and *piwi²/Cyo* mutant was localized in the cytoplasm of various cell types in the germarium. (Figure 4-5A) As the development proceeds from germarium to the posterial end, four mitotic cystoblast cell divisions produce 15 nurse cells and an oocyte that remain connected by cytoplasmic bridges. Each of these germline cell clusteres is surrounded by an epithelium of somatic follicle cells to form an egg chamber that continuously grows until the oocyte matures into an egg. In those developing egg

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chambers proximal to the germarium, intensive fluorescent signals of B2 and CP aggregated in the cytoplasm of follicle cells (Figure 4-5A, Right panel). Accumulation of both B2 (data not shown) and CP gradually declined in the egg chambers at later development stages (Figure 4-5A, Left panel), and became undetectable in those adjacent to mature eggs as follicle cells eventually undergo apoptosis after depositing the eggshell, suggesting that expression of FHV proteins may only occur in somatic cells but not any germ cells such as nurse cells or oocyte. To confirm that CP protein was exclusively expressed in the follicle cells, I compared the images taken at two different sections of a mid stage egg chamber (Figure 4-5B). Image of the cross section (upper panel) and another one closer to the surface of the egg chamber (bottom panel) showed that the CP signals appeared in the cytoplasm of the cells surrounding the circular hallow cavity where the germ cells were but absent from the center. Besides the follicle cells, both B2 and CP were also detected in the ovariolar wall, which is epithelial tissues that envelop and protect the ovariole. Overall, the immunostaining data supported our idea that in the absence of Dcr2 and Piwi, the virus may replicate and assemble more efficiently in follicle cells.

It should be noted that although Piwi is enriched in the nucleus of both germ cells and somatic supporting cells, recently emerging evidence indicates that primary piRNA biogenesis takes place in the cytoplasm. An N-terminally truncated Piwi protein that failed in localizing to the nucleus is loaded with piRNAs as efficiently as wild type. In addition, a significant proportion of Piwi was co-localized with Yb body, a newly-defined

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organelle localized as discrete cytoplasmic spots and frequently associated with mitochondria exclusively in the somatic cells (mostly follicle cells) of the ovary and testes. Hence there is a good opportunity that Piwi may have direct interaction with the FHV replication machinery in the cytoplasm of follicle cells.

4.4.4 Evidence indicating that piwi may be involved in controlling deposit of viral RNAs into the developing oocyte

Robust replication of two RNA viruses, FHV and DCV, in the ovaries of *dcr2* and mutant strains led us to inspect the virus accumulation in the progenies of flies infected with these two viruses. Ovary is the reproductive organ where occytes developes to be mature eggs, and it is likely that viral RNAs may be deposit into the oocyte and hence be transmitted to the next generation despite the somatic-exclusive expression of FHV proteins. Therefore, I examined the FHV and DCV RNA abundance in F1 generation after the virus inoculation. As neither the *piwi* homozygous nor trans-heterozygous mutant flies were fertile, both female- and male- virgin flies of the wild type, *dcr2*, *piwi^1/Cyo* and *piwi^2/Cyo* background were injected with either FHV suspension or equal volume of blank buffer as mock control. Immediately after microinjection, flies were allowed to mate for seven days, arranged as follows: ♂mock x ♀mock, ♀FHV x ♂mock, ♀mock x ♂FHV and ♀FHV x ♂FHV. No significant changes in mating behaviors or progeny survival rate were observed within the same genetic group. All the parents were harvested at 7dpi and the eggs and larvae were reared in the same vials until they developed into adult flies, in order to distinguish the heterozygous *piwi* - offsprings from

homozygous ones according to their wing-phenotype caused by the *Cyo* balancer.

Quantitative RT-PCR showed that overall extremely low FHV RNA abundance was present in the progenies. FHV RNA1 levels in even the groups demonstrated to harbor the highest viral accumulation were only equal to 7% of the abundance of the ribosomal gene product rp49 (Figure 4-6A), whereas the viral RNAs were at least over 20 folds higher than the rp49 transcripts in the FHV-inoculated parents at 7dpi (data not shown). However, it is noticeable that among the progenies generated by the both-infected parents, *piwi* heterozygous mutant progenies contained over 50 folds higher viral RNA1 relative to wild type and *dcr2*, and the viral RNA1s in homozygous *piwi* progenies were approximately 650 folds higher compared to wild type and *dcr2*. It indicated that firstly, there were indeed FHV RNAs entering the oocyte of *piwi/Cyo* mutants, otherwise it would be impossible to detect viral RNAs in the progenies. The *piwi* mutant progenies produced in the both mock-parents group were just as clear-of-virus as the wild type or *dcr2* mutant flies. The difference observed among the *piwi* mutants and progenies of other genotypes may reflect two layers of variation, the amount of viral RNA deposit in the oocyte and the antiviral immunity during development, as the adult flies were formed after series of cell differentiation and metamorphosis process. The antiviral silencing response during the fly development may also explain the extremely low viral RNA abundance in the F1 progenies compared to the parents.

Supporting the impact of Piwi on transovarian transmission, significantly enhanced viral RNA levels were also observed in *piwi* homozygous mutant offsprings generated by the

mother-infected groups, which were over 800 folds higher compared to wild type on average. Even the heterozygous *piwi* mutant offsprings were about 40 and 10 folds higher than wild type and *dcr2* mutant, respectively. In addition, higher viral RNA abundance was detected in progenies hatched from the both infected groups relative to those from only mother-infected or father-infected groups, indicating virus from the parental side may also be transmitted to the progenies.

In order to exclude the possibility that FHV RNAs detected in the progenies came from physical contact with the infected parents during embryonic or larval stage, I set up a control-experiment to test the occurrence of horizontal transmission during FHV infection. 20 male flies were either injected with equal volume of blank buffer or same dosage of purified FHV used for the vertical transmission experiment, and then reared with the embryos and larvae that were laid by mock or untreated flies and less than 2 days old in the same vial for 7 days. Only male adult flies were used in this experiment to eliminate the chance of producing new eggs in the vials after FHV injection. The embryos and larvae were cultured with flies of the same genotype, and were collected after they differentiated to adult flies to imitate the experiment system for vertical transmission. Results from two independent biological replica were presented for each condition (Figure 4-6B), and no positive band representation RNA1 of FHV (FR1) could be amplified after 37 PCR-cycles in any of the mock progenies as the FHV-infected flies (Figure 4-6B, P lane).

Transmission of DCV was originally investigated in the same system as described above

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for the FHV study. After the microinjection, flies of different genetic backgrounds were cultured in fresh medium for mating, and any dead flies were removed from the medium as soon as possible in order to avoid the influence of horizontal transmission. However, high levels of DCV genomic RNA were still be detected by quantitative real time PCR. The abundance was even comparable to that in the ovaries of injected parents (Figure 4-7A and data not shown). I did observe that flies killed by DCV often underwent tissue lysis soon after death, so the virions may be released from the damaged tissues and thus contaminated the food. In addition, I observed extremely high DCV RNA abundance in the mock flies that were reared with flies injected with purified DCV (50pfu/fly) for 4-5 days (Figure 4-7B), consistent with the previous reports showing that DCV can horizontally transmitted among contaminated adults and larvae. Therefore, I attempted to eliminate the influence of food/dead flies contamination by collecting the eggs that were laid by the DCV-infected parents at 5dpi, removing and/or disinfecting the eggshell by treatment of 60% bleach diluted in distilled water. The progeny-flies developed under this condition contained much lower abundance of DCV RNAs relative to the parents or those generated under the original condition (Figure 4-7C). The viral RNA levels in *dcr2*, *piwi^{1}/Cyo* mutant progenies were so low that no meaningful value could be read by the qPCR machine. DCV RNA levels in the offsprings of wild type and *piwi^{2}/Cyo* mutant strain were less than 5% as abundant as the reference gene, rp49. Interestingly, the *piwi$^{1/1}$* and *piwi$^{2/2}$* homozygous mutant-progenies carried DCV genomic RNAs that were 2 and 24 times higher than rp49 transcripts, respectively. This implied that transovarian

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transmission of DCV may not be detected by other researchers due to the protection by Piwi gene in the wild type ovaries. More comprehensive and extensive studies are required to fully understand the DCV transmission, including monitoring the interaction between infection time and viral titer of the parents and the transmission rate, maternal and parental contribution to the viral accumulation in the progenies.

As shown above, Piwi protein plays prominent roles in protecting the ovaries most likely in the somatic cells. I also examined whether Aub and Ago3, the other two members of PIWI subfamily which are expressed predominantly in the cytoplasm of germ cells and required for secondary piRNA biogenesis in a ping-pong amplification cycle, were also involved in the viral clearance. Moreover, flies containing mutations in an RNA helicase Armi were also tested for antiviral response upon FHV inoculation. It has been recently demonstrated that Armi is one of the main factors in biogenesis of primary piRNA in somatic cells of the ovary, Strikingly, all of the trans-heterozygous mutants, $aub^{QC42/HN}$, $ago3^{t2/t3}$ and $armi^{1/72.1}$ that were generated by crossing of the corresponding mutant alleles showed mild susceptibility to the FHV infection (Figure 4-8A) relative to wild type and $piwi^{1/2}$ mutant flies. Although over 90% of all of these three mutant strains survived up to 7dpi, FHV (50pfu/fly) caused 83%, 49% and 63% mortality in $aub^{QC42/HN}$, $ago3^{t2/t3}$ and $armi^{1/72.1}$ flies at the end of 16dpi, respectively. Real time RT-PCR analysis of the ovarian RNAs in these piRNA-mutants revealed that FHV genomic RNA1 and RNA2 accumulated to highest level in $piwi^{1/2}$ mutant flies, over 1400 folds more abundant than the wild type ovaries. $aub^{QC42/HN}$ trans-heterozygous mutant also

supported relatively high levels (over 500 times compared to wild type) of viral accumulation in the ovaries, whereas FHV RNAs in the ovaries of aub^{QC42} or aub^{HN} heterozygous flies were 10 times lower than $aub^{QC42/HN}$, interestingly such variation was also observed between *piwi* heterozygous and $piwi^{1/2}$ flies. The difference between *ago3* heterozygous and trans-heterozygous flies decreased to around 3-5 folds, while the viral RNA1 and RNA2 levels in $ago3^{t2/t3}$ mutant ovaries were still 135.6 and 158.6 times more abundant respectively than the wild type strain. Furthermore, FHV RNA1 and RNA2 in *armi* mutants were on average 30-50 times higher than the wild type, and I did not see significant difference between the heterozygous mutant ovaries and those in trans-heterozygotes (Figure 4-8B) probably as previously reported none of these mutants eliminated Armi protein expression completely. These results implied that Aub and Ago3, probably Armi proteins may also act in virus clearance in the ovary, and this could be considered as an indirect line of evidence that FHV genomic RNAs may also be present in the germ cells as Aub and Ago3 were undetectable in the OSS cells, and especially Aub has been reported only in germ cells so far by a number of laboratories. It was not completely clear yet whether the antiviral silencing mediated by these proteins involved piRNA biogenesis via a mechanism similar to that against transposons, more small RNA analysis in these mutant ovaries should be helpful to address this question.

4.4.5   Analysis of small RNAs in the somatic tissues and ovaries of FHV-infected flies

In order to explore the molecular mechanisms underlying the genetic and cell biology phynotypes, I exmamined the population structure of virus-derived small RNAs in the

ovaries of wild type, *dcr2* and *piwi* heterozygous mutant flies after FHV inoculation by several means. Firstly, ovary lysates and immune-precipitated Piwi-piRNA complexes were prepared from mock-, FHV- and FHVΔB2-infected wild type and *dcr2* mutant flies, and Piwi-bound piRNAs were sequenced and analysed with the guidance of Dr. Qingfa Wu. We found that very limited numbers of small RNAs were derived from FHV in the wild type ovaries, which may be associated with the low level of viral replication in the wild type ovaries. Piwi-bound small RNAs isolated from the *dcr2* mutant ovaries were in greater abundance relative to wild type, whereas overall the virus-specific reads were still very low in the library. In summary we could not detect any significant population of small RNA with predominant sizes or sharing features with the endogenous piRNAs in either FHV- or FHVΔB2- infected flies, despite the abundant viral accumulation in the *dcr2* ovaries shown in figure 4-2A and the following sections (data not shown).

Meanwhile, I also examined the general profile of small RNAs in the ovaries upon FHV infection by probing the ovarian total RNAs from various mutants with oligonucleotides targeting the 5'terminus of sense-stranded RNA1, as the majority of virus-specific piRNAs identified in OSS cells were of positive stranded. The blotting revealed overwhelming signals that were larger than 30nt, which may represent the large amount of degradation products of viral RNAs in the host (data not shown), thus I analyzed the ovarian small RNAs by Illumina deep sequencing, with small RNAs extracted from carcasses (somatic tissues) as a comparison. Surprisingly, albeit the absence of dominant species ranged from 24-30nt was still observed in all the ovaries or somatic samples of

different genetic backgrounds, the size distribution pattern and strand bias of 21-nt

siRNAs displayed significant changes in the *piwi/Cyo* heterozygous ovaries relative to

wild type and *dcr2* mutant. I found that over 95% of the FHV-derived small RNAs in

wild type and *dcr2* ovaries were positive strands, similar to the observation in the somatic

tissues of all the strains tested. (Figure 4-9A and B) I reasoned that may be caused by the

expression of B2, which interfered with the DCR2-dependent processing of dsRNA in

adult flies and altered the ratio of positive and negative strands of viral siRNAs as

discussed in Chapter 3. However, this inhibition effect of B2 seemed to be released or

eliminated in *piwi[1]/Cyo* and *piwi[2]/Cyo* ovaries, which contained reduced expression of

Piwi protein. A dramatic enhanced accumulation of negative stranded FHV siRNAs was

detected in these mutant ovaries, and the increase of negative strands to a much less

extent was also observed in the carcasses of *piwi[1/1]* and *piwi[2/2]* homozygous mutants.

(Figure 4-9B) Moreover, the 21nt-small RNAs became the most dominant species in the

18-28nt small RNAs derived from both FHV RNA1 and RNA2 in both carcasses and

ovaries of *piwi* mutants. These results could not be explained simply by the B2 protein

levels in the *piwi* heterozygous ovaries, in which B2 expression was comparable to that in

dcr2 mutant ovaries (Figure 4-4A). I also tested the expression of Ago2, a component in

the antiviral RNAi pathway that is not involved in dicing but dramatically altered the

ratio of positive and negative strands of FHV siRNAs in adult flies infected with

FHVΔB2, in the *piwi* heterozygous and *dcr2* mutant ovaries, and no significant

difference could be detected by western blotting analysis (data not shown). It has been

established that both piRNA- and endo-siRNA pathways play a role in regulating a wide variety of transposons, repeat elements and even a small number of protein-coding genes, such as *traffic jam*. Whereas no crosstalk between these two pathways has been reported so far, and it is possible that piRNA pathway member, Piwi may have certain influence on the Dcr2-dependent siRNA pathway besides its involvement in the biogenesis of primary piRNAs. More comprehensive analysis on the ovarian small RNAs and other evidence are required to fully understand how Piwi mediated virus clearance at molecular mechanism.

4.4.6 Partial rescue of FHVΔB2 accumulation in the *piwi* trans-heterozygous mutant

It was surprising to find that Piwi, a protein predominantly expressed and functional in the germline, seemed to also act in the RNAi-mediated antiviral immunity in the somatic tissues as shown in Figure 1 and 2. In order to further confirm this notion, I injected *piwi* mutant flies with the VSR-deficient mutant FHVΔB2. In contrast to FHV, which resulted in over 80% mortality of *piwi*$^{1/2}$ trans-heterozygous mutant flies, FHVΔB2 inoculation only caused mild increased death of *piwi*$^{1/2}$ flies with survival rate of 86.5% over a time course of 16 days, compared to 93% survival rate of wild type (Figure 4-10A). The viral RNA accumulation levels of FHVΔB2 in *piwi*$^{1/2}$ mutant were consistent with the survival change according to the real-time PCR analysis of total RNA extracted from the entire insect in each genetic strain. The abundance of sense-stranded RNA1 in *piwi* mutant was 62.4 folds higher than that in the wild type flies, and over 100 times induction of the antisense-strands was detected (Figure 4-10B). Additionally, FHVΔB2 failed to

accumulate to detectable levels in the ovaries of wild type flies, although same quantity of total RNA extracted from the heads showed extremely weak signal for sense-stranded genomic RNAs by northern blotting under the same experimental condition. Notably, FHVΔB2 accumulated to much higher levels in *dcr2* mutant ovaries than either wild type or *piwi*$^{1/2}$ mutant, indicating in the absence of VSR, siRNA-dependent silencing pathway may provide the most potent virus-clearance in the host. The partial rescue of FHVΔB2 by depletion of Piwi protein in adult flies further confirmed that it has a specific role for antiviral immunity against RNA viruses apart from its role in suppression of transposons in germline. It will be intriguing to investigate the interaction of Piwi and Dcr2-dependent siRNA pathway that was defined in Chapter 3.

4.5 Discussion

In this study I demonstrated genetic, cell biological and molecular evidence that shed light on the role of Piwi in the germline to protect the reproduction organs and thus the offspring of the host during infection of two distinct families of sense-stranded RNA viruses.

Studies in the past few years has been focused on mapping the genetic requirement for RNAi-mediated antiviral immunity, and they provided evidence, including that *piwi*^{06843} mutant line exhibited increased anoxia sensitivity induced death caused by infection with a dsRNA virus, Drosophila X Virus (Zambon *et al.*, 2006) and the enhanced viral titer of West Nile Virus in *piwi*$^{1/2}$ mutant flies (Chotkowski *et al.*, 2008), suggesting that this key

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component of piRNA pathway is involved in the antiviral response. Here I examined in greater depth on the infectivity and pathogenic effects of FHV and DCV in *piwi* mutant flies, not only in specific tissues of the infected insects but also in their progenies produced during viral infection.

Surprisingly, unlike the *gypsy* endogenous retrovirus silenced by piRNAs (Sarot *et al.*, 2004; Pelisson *et al.*, 2006; Mevel-Ninio *et al.*, 2007), the piwi-dependent virus clearance in adult fruit flies did not seem likely to be explained by virus-derived piRNAs generated in the germline, at least in the wild type fly strain I used in this study, there was no FHV-specific piRNA detectable by deep sequencing, and Piwi did no associate with any dominant species of 24-30nt small RNAs that was perfectly matched to the FHV or DCV (data not shown) genome in either wild type or *dcr2* ovaries. Alternative hypothesis need to be develop, and analysis of total small RNA populations in the *piwi* heterozygous ovaries indicates a potential influence on the Dcr2-directed siRNA pathway when the expression of functional Piwi was reduced. The influence may be specific to the processing of virus only, as the size distribution pattern of small RNAs (18-30nt) that were mapped to the genome did not alter in the *piwi* heterozygous ovaries no matter in the absence or presence of FHV infection. Future experiment and directions to investigate the mechanism at molecular level will be discussed in Chapter 5.

Previous reports on the hypersensitivity and viral RNA accumulation in *dcr2* mutant flies upon FHV infection have established the importance of Dcr2-directed RNAi pathway against FHV infection. In this study, although high FHV abundance was detected in the

both the soma and ovaries of *dcr2* mutant, the levels of FHV RNAs in their F1 progenies were as low as those produced by the wild type flies, which implies antiviral silencing may occur during the egg development to prevent the transovarian transmission, and Piwi may be an important factor in this process. I have attempted to performed plaque assay with the guidance of Dr. Juan Jovel to determine if infectious FHV virions were produced in the F1 progenies as that occurred in the parents, but no plaque could be formed even when I infected the cells with fly extracts purified from over 15 flies under our experimental condition. Therefore, infectious virions may not be generated to a detectable quantity in the progenies of FHV-infected flies, as shown by relative abundance by quantitative RT-PCR, the FHV RNA levels in the wild type parents were at least 300 folds greater than the *piwi* homozygous mutant progenies. The viral RNA may instead exist as persistent infection in the newly-born host.

Previous studies on vertical transmission of viruses in insects mainly focused on identification of viral RNAs mostly by RT-PCR in hosts collected in different sources and/or progenies produced in different circumstances (Khurad, 2004; Chen *et al.*, 2006; Carpenter *et al.*, 2007; uuml *et al.*, 2007; Yue *et al.*, 2007). Moreover, none transmission of nodaviruses has been reported in insects so far, except for one member, Macrobrachium rosenbergii nodavirus (MrNV), was found to be vertical transmitted from brooders to progeny in Macrobrachium rosenbergii and Artemia (Sahul Hameed *et al.*, 2004; Sudhakaran *et al.*, 2007; Sudhakaran *et al.*, 2008). Here I demonstrated that Piwi gene had significant influence on transovarian transmission of two viruses, FHV and

DCV. The latter one even accumulated in the *piwi* mutant progenies to the level comparable to the parents, probably reflecting that it naturally infects the fruit flies. As there are conflicting reports as to whether ingestion of DCV leads to mortality (Plus *et al.*, 1978; Cherry and Perrimon, 2003; Costa *et al.*, 2009), many of the studies, including our findings discussed in this chapter, on antiviral immunity against DCV in *Drosophila* deliver virus directly into the haemocoel by injection rather than oral intake, the more natural route of infection for these viruses, to ensure the reproducibility of infection and lethality. (Cherry and Perrimon, 2004) In addition, experimental evidence from either *Drosophila* or honey bees indicated that natural oral infection of DCV resulted in mild symptoms relative to the haemocoel injection (Roxstrom-Lindquist *et al.*, 2004), suggesting that the key or potent antiviral defense may be activated in the gut (Sparks *et al.*, 2008); or DCV entering the hosts through injection and more natural route may spread within the insects via two distinct modes. Hence it would be worthwhile to explore whether the immune response elicited by DCV and higher virus transmission in *piwi* mutants occur during the natural infection process.

It should be also noted that flies isolated in different parts of the world have varying susceptibility to DCV; strains of flies from Japan are highly resistant to DCV (Thomas-Orillard *et al.*, 1995). DCV isolated in Charolles, France kills flies in 3 days, whereas DCV isolated from the French Antilles kills adults in 10 days (Plus *et al.*, 1978). This variation may be due to either different genetic backgrounds or varies in persistent infection of DCV or other viruses. An earlier report was cited to claim that DCV was not

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transovarially transmitted but was horizontally transmitted by adult or larval contamination (Jousset and Plus, 1975). Therefore, it would be interesting to examine whether our observation of transovarially-transmission of DCV occur in strains from other resources.

Table 4-1. Primers for DCV detection by qPCR and Northern hybridization

YL145	GCAGCAAAGAAACAGCGTGA
YL146	ACAATGCGCTTCCGGAGA
P086	TAATACGACTCACTATACATTTTGTAGTTGCATTTCG
P087	CATTCCACAACCTTTGCATC

Table 4-2. Buffers for ovary-immunostaining

4% Paraformaldehyde in 1×PBS (1ml)	250 μl 16% paraformaldehyde, 100 μl 10 ×PBS, 650 μl distilled H2O
1 ×PBT (50ml)	5 ml 10 ×PBS, 45 ml dH2O, 50 μl Triton-X-100
PBT-NGS (1 ml)	1μl Normal Goat Serum, 1 ml 1 ×PBS

Figure 4-1. *piwi* **mutant adult flies exhibited enhanced disease susceptibility to FHV infection.**

A. Survival of wild type (*csw*), *dcr-2*^{L811fsX} (*dcr2*) and *piwi^1/piwi^2* (*piwi$^{1/2}$*) adult flies after $1\times$PBS buffer (Mock) or FHV inoculation. Survival curves of the Mock-injected groups were displayed in dashed lines, and FHV-infected ones were shown in solid lines. Over 60 flies of each genotype were injected and monitored daily. Data shown represented mean value of triplicates, and the error bars indicated standard deviation.

B. Accumulation of FHV sense strand RNAs in the carcasses of adult flies 7 days after microinjection with $1\times$PBS buffer (-) or FHV (+). $5\,\mu$g of total RNAs extracted from female ($\female$) or male ($\male$) flies of *csw*, *dcr-2*^{L811fsX} (*dcr2*), heterozygous *piwi^1/Cyo* (*piwi^1/+*), *piwi^2/Cyo* (*piwi^2/+*) and homolozygous *piwi^1/piwi^1* (*piwi^1*), *piwi^2/piwi^2* (*piwi^2*) mutant flies were probed with mixture of in-*vitro* synthesized RNAs corresponding to antisense-strands of RNA3 and the 3' end of RNA2. Loading was monitored by methylene-blue staining of ribosomal RNA.
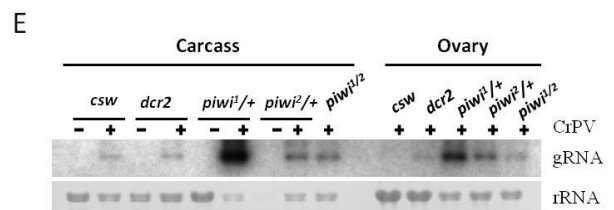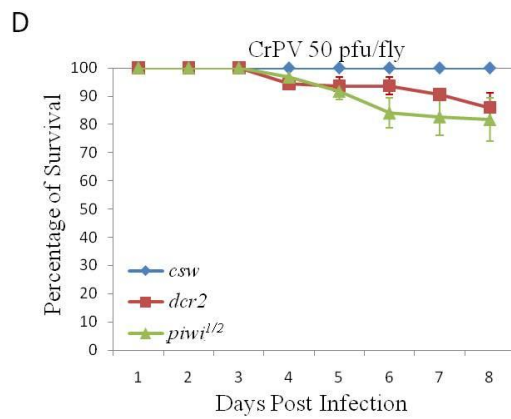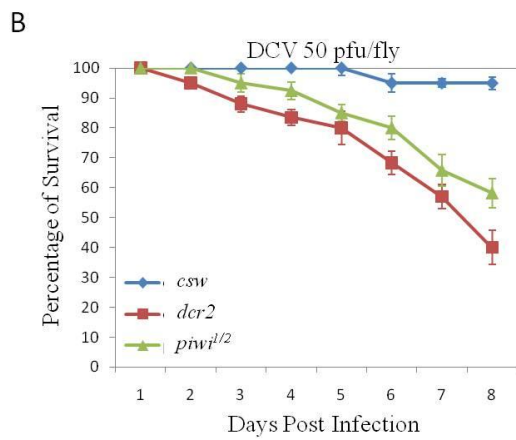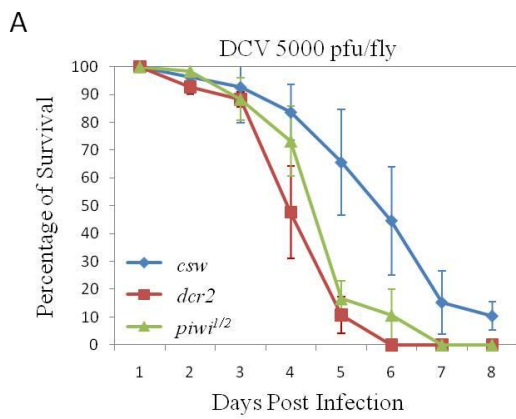
A

B

161
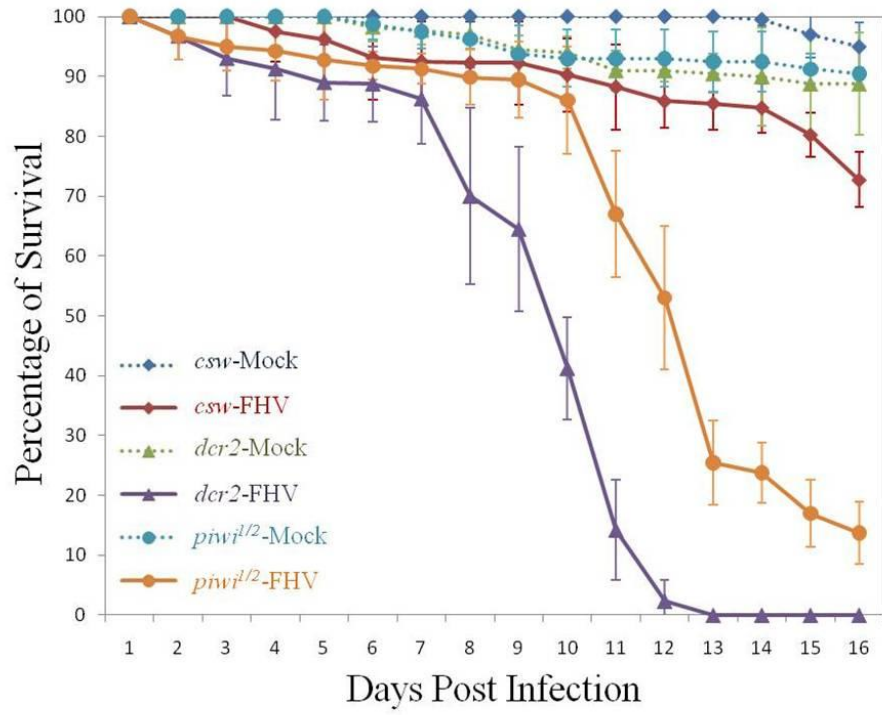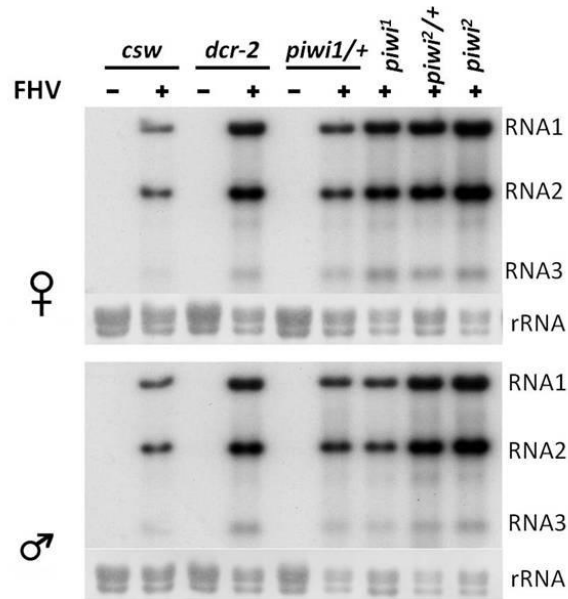
Figure 4-2. Potent antiviral response ensured the viral clearance in the wild type ovaries.

A. Detection of FHV sense and antisense strand RNAs in the heads and ovaries of infected adult flies. 2.5 µg of total RNAs extracted from dissected heads or ovaries of *csw*, *dcr-2*[L811fsX] (*dcr2*), heterozygous *piwi¹/Cyo* (*piwi¹/+*), *piwi²/Cyo* (*piwi²/+*) and *piwi¹/piwi²* (*piwi¹/²*) mutant flies were probed with mixture of in-*vitro* synthesized RNAs corresponding to antisense-strands of RNA3 and the 3' end of RNA2. Only 1.25 µg of ovarian total RNA from *piwi¹/piwi²* mutant was loaded in the lower panel. Loading was monitored by methylene-blue staining of ribosomal RNA.

B. Expression levels of sense-stranded FHV RNA1 (FR1+) and RNA2 (FR2+) relative to rp49 as determined by qPCR of total RNA from the heads of FHV- inoculated adult flies. The graphs show the mean and standard deviation (n≥4). Statistically significant differences are less than 0.055. (Student's *t*-test, compared to heads of FHV- injected wild type strain)

C. Expression levels of sense-stranded FHV RNA1 (FR1+) and RNA2 (FR2+) relative to rp49 as determined by qPCR of total RNA from the ovaries of FHV- inoculated adult flies. The graphs show the mean and standard deviation (n≥4). Statistically significant differences are less than 0.002. (Student's *t*-test, compared to ovaries of FHV- injected wild type strain)

A
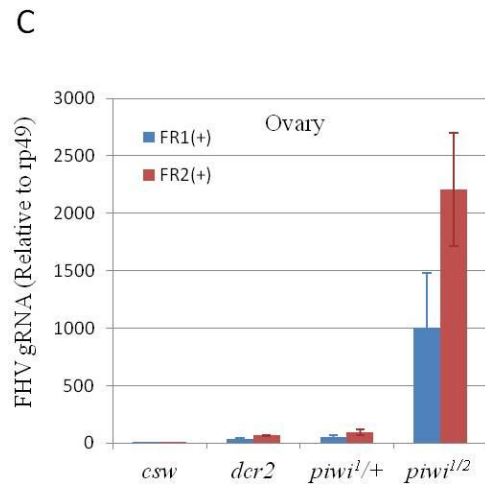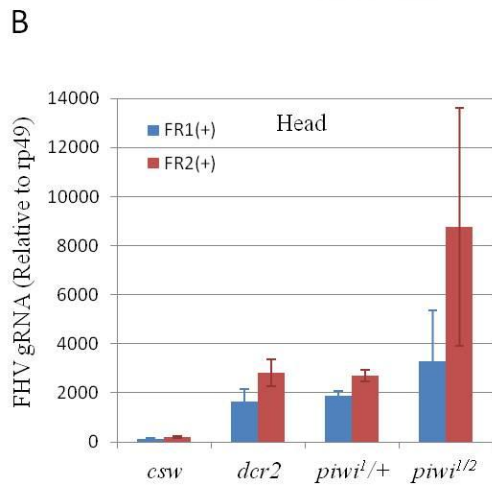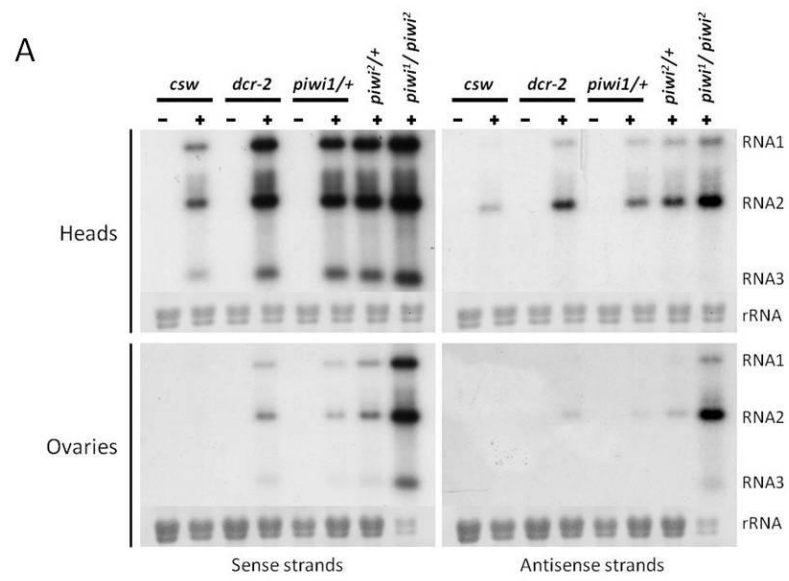
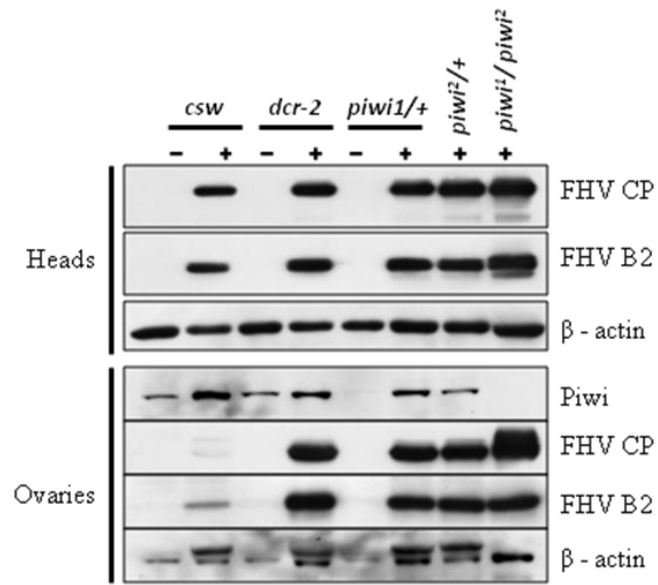**Figure 4-3. Sensitivity of *piwi* mutant strains to DCV and CrPV infection.**

A. Survival of wild type (*csw*), *dcr-2*^{L811fsX} (*dcr2*) and *piwi^1/piwi^2* (*piwi$^{1/2}$*) adult flies after DCV inoculation by 5000pfu/fly. At least 60 flies of each genotype were injected and monitored daily. Data shown represented mean value of triplicates, and the error bars indicated standard deviation.

B. Survival of wild type (*csw*), *dcr-2*^{L811fsX} (*dcr2*) and *piwi^1/piwi^2* (*piwi$^{1/2}$*) adult flies after DCV inoculation by 50pfu/fly.

C. Accumulation of DCV sense strand RNAs in the carcasses and ovaries of adult flies 5 days after microinjection with 1×PBS buffer (-) or DCV (+). 5 μg total RNAs extracted from the selected tissues of *csw*, *dcr-2*^{L811fsX} (*dcr2*), heterozygous *piwi^1/Cyo* (*piwi^1/+*), *piwi^2/Cyo* (*piwi^2/+*) and *piwi^1/piwi^2* (*piwi$^{1/2}$*) mutant flies were probed with mixture of in-*vitro* synthesized RNAs corresponding to antisense-strands of the 3' end of DCV genomic RNA. Loading was monitored by methylene-blue staining of ribosomal RNA.

D. Survival of wild type (*csw*), *dcr-2*^{L811fsX} (*dcr2*) and *piwi^1/piwi^2* (*piwi$^{1/2}$*) adult flies after CrPV inoculation by 50pfu/fly. At least 60 flies of each genotype were injected and monitored daily. Data shown represented mean value of triplicates, and the error bars indicated standard deviation.

E. Accumulation of sense-stranded CrPV genomic RNA in the carcasses and ovaries of adult flies 7 days after microinjection with 1×PBS buffer (-) or CrPV (+). 5 μg total RNAs extracted from the selected tissues of *csw*, *dcr-2*^{L811fsX} (*dcr2*), heterozygous *piwi^1/Cyo* (*piwi^1/+*), *piwi^2/Cyo* (*piwi^2/+*) and *piwi^1/piwi^2* (*piwi$^{1/2}$*) mutant flies were probed with mixture of oligonucleotides targeting the 5' exon of CrPV genome. Loading was monitored by methylene-blue staining of ribosomal RNA.
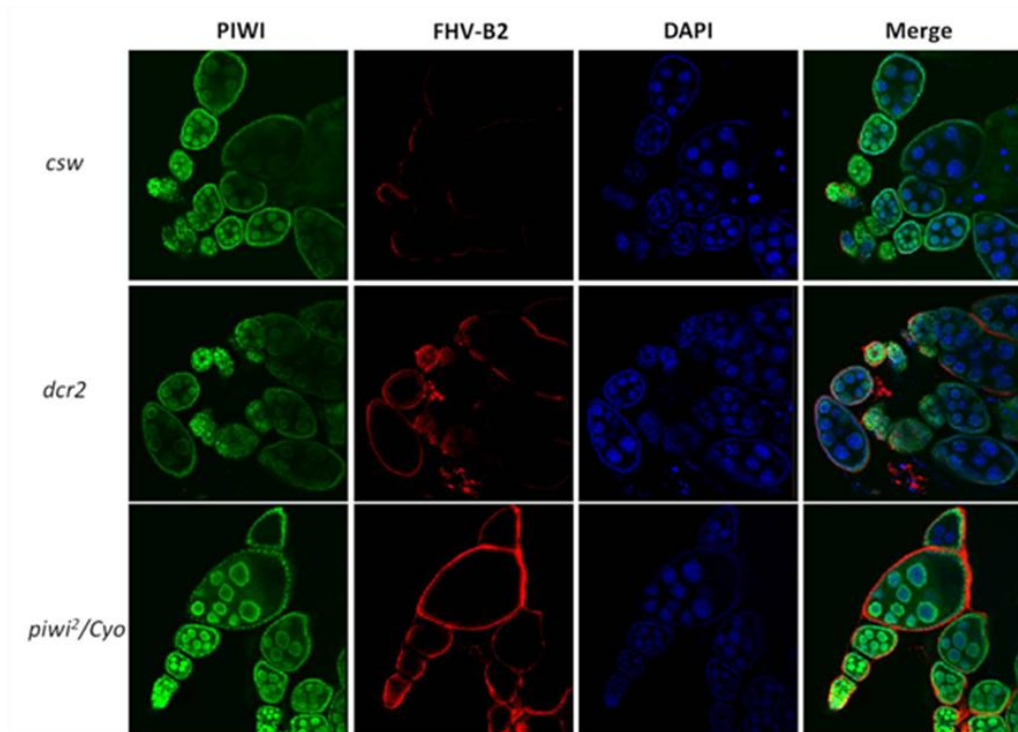
A — DCV 5000 pfu/fly

B — DCV 50 pfu/fly

C — Carcass / Ovary; DCV; gRNA; rRNA

D — CrPV 50 pfu/fly

E — Carcass / Ovary; CrPV; gRNA; rRNA

Figure 4-4. Enhanced accumulation of FHV proteins in the ovaries of *dcr2* and *piwi* mutants.

A. Western blotting detection of the viral capsid protein (FHV CP) and B2 protein (FHV B2) in the dissected heads (upper panel) and ovaries (lower panel) of inoculated wild type and mutant *Drosophila*, using fly β-actin and/or α–tubulin as loading control. Piwi proteins were only readily detectable in the ovaries but not the somatic tissues (data not shown).

B. Immune-staining of the Piwi (green) and FHV B2 proteins (red) in ovaries of *csw*, *dcr-2*^{L811fsX} (*dcr2*), heterozygous *piwi*^{2}/*Cyo* mutants. DAPI marked the DNA in the nucleus. Merged image was generated by Leica SP2 imaging software.

A

Heads

FHV CP
FHV B2
β - actin

Ovaries

Piwi
FHV CP
FHV B2
β - actin

csw   *dcr-2*   *piwi1/+*   *piwi²/+*   *piwi²/piwi²*

B

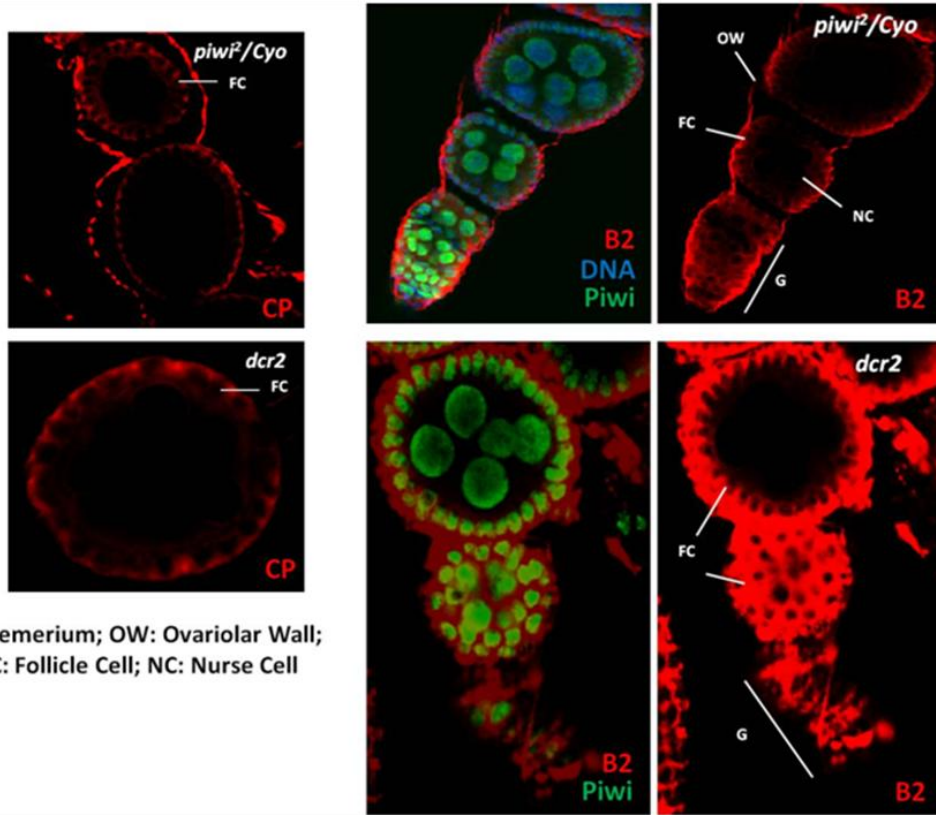| | PIWI | FHV-B2 | DAPI | Merge |

csw

dcr2

piwi²/Cyo

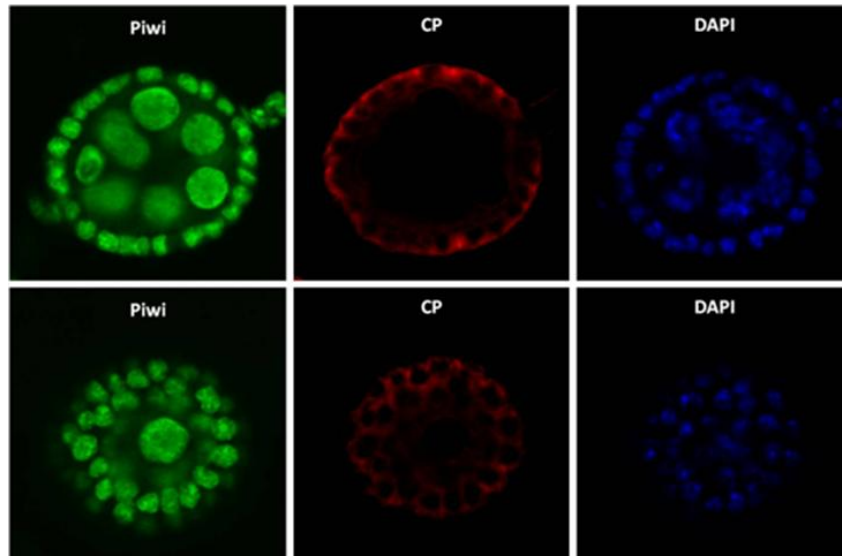**Figure 4-5. Cellular localization of FHV capsid protein and B2 protein in the ovarioles.**

A. Optical sections through mid stage egg chambers (left panel) and gemerium (right panel) of indicated genotypes (top right corner) stained for DNA (blue), Piwi (green) and either CP or B2 (red).

B. Two distinct optical sections through a single mid stage egg chamber stained for Piwi (green), CP (red) and DAPI (blue).

A

**piwi²/Cyo** — CP — FC

B2 / DNA / Piwi

piwi²/Cyo — B2 — OW, FC, NC, G

dcr2 — CP — FC

B2 / Piwi

dcr2 — B2 — FC, G

G: Gemerium; OW: Ovariolar Wall;
FC: Follicle Cell; NC: Nurse Cell

B — Piwi — CP — DAPI

Piwi — CP — DAPI

Figure 4-6. FHV RNA accumulation increased in the progenies of FHV-infected *piwi¹/Cyo* and *piwi²/Cyo* mutants flies.

A. Expression levels of FHV RNA1 relative to rp49 as determined by qPCR of total RNA from the F1 progenies of buffer- (Mock) and/or FHV- (Both FHV) inoculated adult flies of indicated genotypes. Mother- and Father- FHV represent mating groups in which FHV-infected females and males, respectively, were crossed to buffer-inoculated flies of the opposite sexes. The graphs show the mean and standard deviation (n=6). Statistically significant differences are less than 0.04 in all samples except the mother-FHV groups in *csw*, *dcr2* and *piwi¹/Cyo* mutants compared to progenies of buffer- injected (Mock) parents of selected genotype. (Student's *t*-test)

B. Absence of FHV RNA1 (FR1) in progenies developed from eggs or larvae reared with either buffer- (mock) or FHV-infected (FHV) male flies of indicated genotypes for 7 days detected by one-step RT-PCR run on 1.5% agarose gel stained with ethidium bromide. *piwi/+* represents a mixture of heterozygous *piwi¹/Cyo* and *piwi²/Cyo* mutants. Positive control (P) is PCR from total RNAs extracted from FHV-infected males, and buffer-infected male is used as negative control (N). Two lanes of DNA ladders (ladder) indicate the PCR product is of expected size. RT-PCR of ribosomal Rp49 is shown as loading control.

A
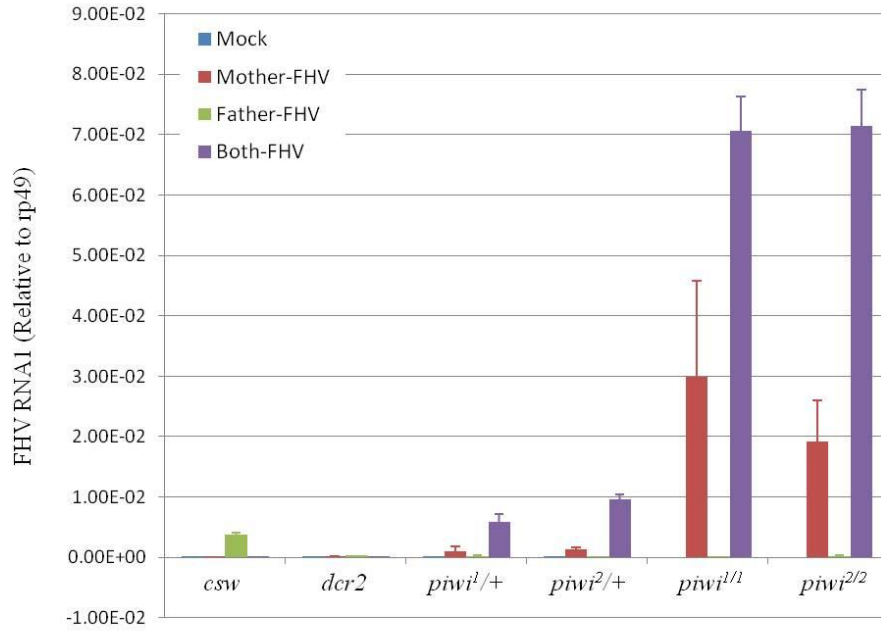
B
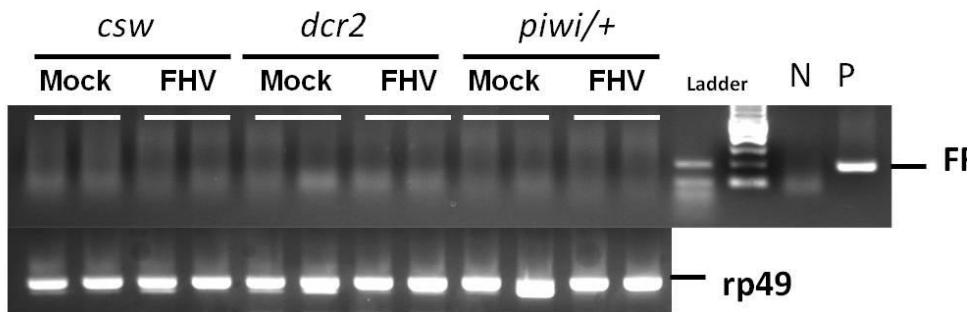
Figure 4-7. DCV RNA abundance in the progenies of DCV-inoculated flies.

A. Expression levels of DCV RNA1 relative to rp49 as determined by qPCR of total RNA from the F1 progenies of buffer- (Mock) and/or DCV- (Both DCV) inoculated adult flies of indicated genotypes. Mother- and Father- FHV represent mating groups in which FHV-infected females and males, respectively, were crossed to buffer-inoculated flies of the opposite sexes. The graphs show the mean and standard deviation (n=3). Statistically significant differences are less than 0.01. (Student's *t*-test, compared to progenies of wild type strain in selected parents-group)

B. Detection of DCV genomic RNA in mock-infected adult flies of indicated genotypes (females in the first lane and male in the second) reared with either buffer- (mock) or DCV-infected (DCV) flies of the opposite sex for 7 days detected by one-step RT-PCR. PCR products were run on 1.5% agarose gel and stained with ethidium bromide. *piwi/+* represents a mixture of heterozygous *piwi¹/Cyo* and *piwi²/Cyo* mutants. Positive control (P) is PCR from total RNAs extracted from DCV-infected males, and buffer-infected male is used as negative control (N). RT-PCR of ribosomal Rp49 is shown as loading control.

C. Expression levels of DCV genomic RNA relative to rp49 as determined by qPCR of total RNA from the F1 progenies generated by DCV- inoculated adult flies of indicated genotypes at 5dpi followed by bleach treatment. The graphs show the mean and standard deviation (n=6). Statistically significant differences were observed in both alleles of homozygous *piwi* mutant progenies compared to progenies of wild type flies. (Student's *t*-test)

A

B

C
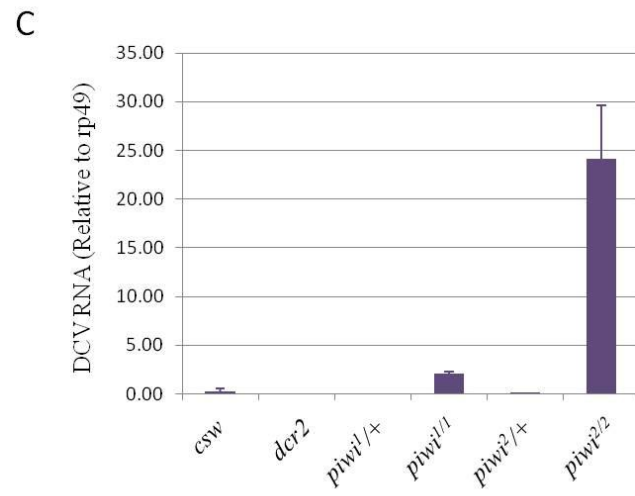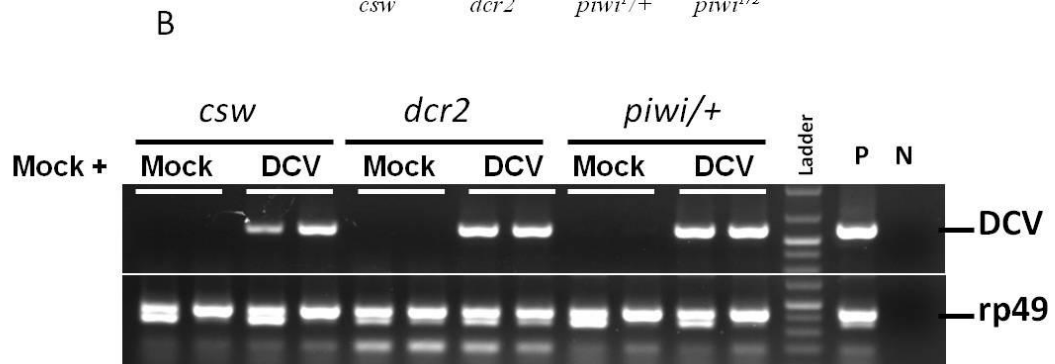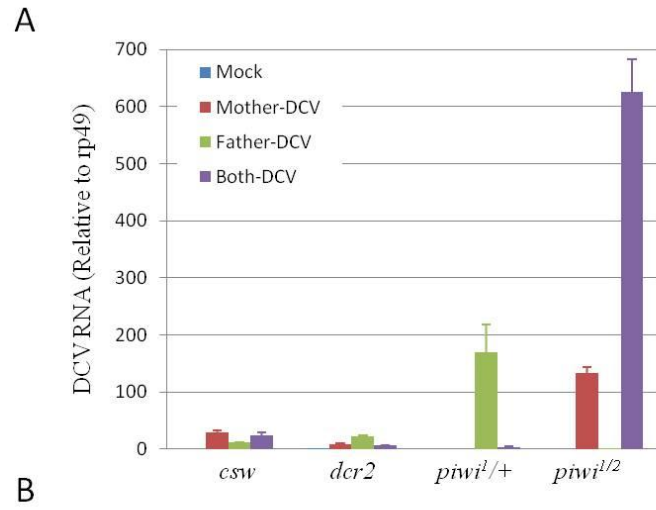
Figure 4-8. Mutations in piRNA pathway members led to enhanced susceptibility to FHV infection in varied extent.

A. Survival rate of wild type (*csw*), *piwi^1^/piwi^2^* (*piwi^1/2^*), *aub^QC42^/aub^HN^* (*aub^QC42/HN^*), *ago3^t2^/ago3^t3^* (*ago3^t2/t3^*) and *armi^1^/armi^72.1^* (*armi^1/72.1^*) adult flies after FHV inoculation. Mock-injected groups were tested but not presented for the figure clarification. Over 60 flies of each genotype were injected and monitored daily. Data shown represented mean value of triplicates, and the error bars indicated standard deviation.

B. Expression levels of sense-stranded FHV RNA1 (FR1+) and RNA2 (FR2+) relative to rp49 as determined by qPCR of total RNA from the ovaries of FHV- inoculated adult flies of indicated genotypes. The graphs show the mean and standard deviation ($n \geq 3$). Statistically significant differences are less than 0.02. (Student's *t*-test, compared to ovaries of FHV- injected wild type strain)

A
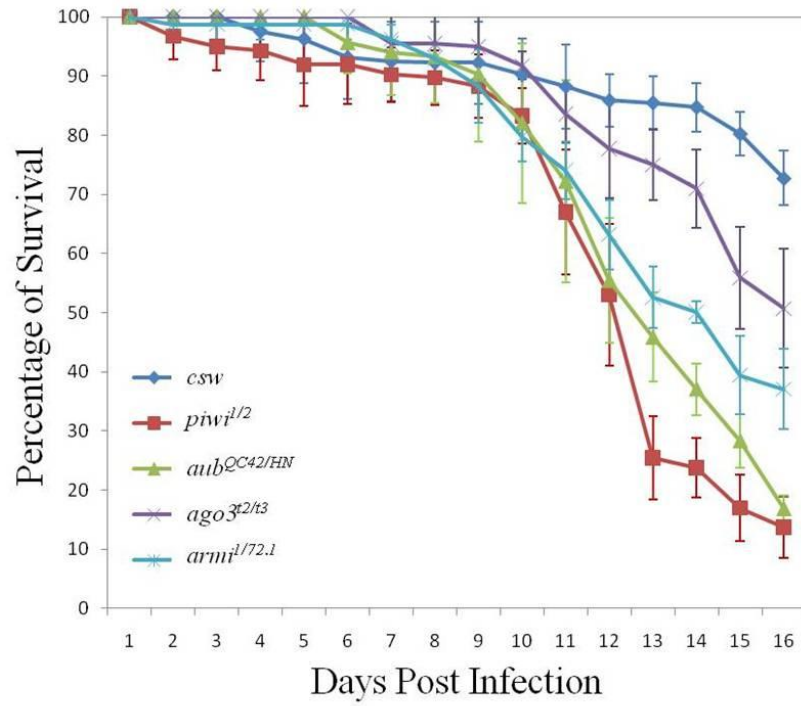
B
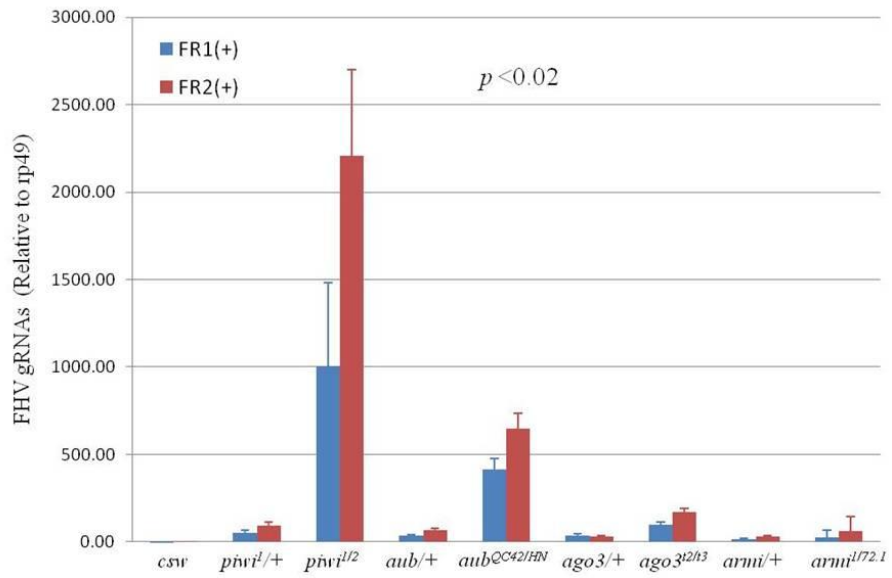
Figure 4-9. Size distribution of virus derived small RNAs in soma and ovaries of adult flies.

A. The abundance of siRNAs (18-28nt) derived from FHV-RNA1 or RNA2, DAV, DCV and Nora virus in the carcasses of wild type (*csw*), *dcr-2*^L811fsX (*dcr2*), homolozygous *piwi¹/piwi¹* (*piwi¹ᐟ¹*), *piwi²/piwi²* (*piwi²ᐟ²*) mutants inoculated with FHV at 8dpi. The number of viral siRNAs was normalized to 1 million sequenced small RNAs (18-28nt) of corresponding library. All the siRNAs were shown in black bars, except that 21-nt positive strands of FHV-derived siRNAs were represented in red bar, negative strands as blue bar.

B. The abundance of siRNAs (18-28nt) derived from FHV-RNA1 or RNA2, DAV, DCV and Nora virus in the ovaries of wild type (*csw*), *dcr-2*^L811fsX (*dcr2*), heterozygous *piwi¹/Cyo* and *piwi²/Cyo* mutants inoculated with FHV at 8dpi. All the siRNAs were shown in black bars, except that 21-nt positive strands of FHV-derived siRNAs were represented in red bars, negative strands as blue bars.

A

FHV-RNA1	FHV-RNA2	DAV	DCV	Nora	
					csw
					dcr2
					piwi¹/¹
					piwi²/²

B

FHV-RNA1	FHV-RNA2	DAV	DCV	Nora	
					csw
					dcr2
					piwi¹/Cyo
					piwi²/Cyo
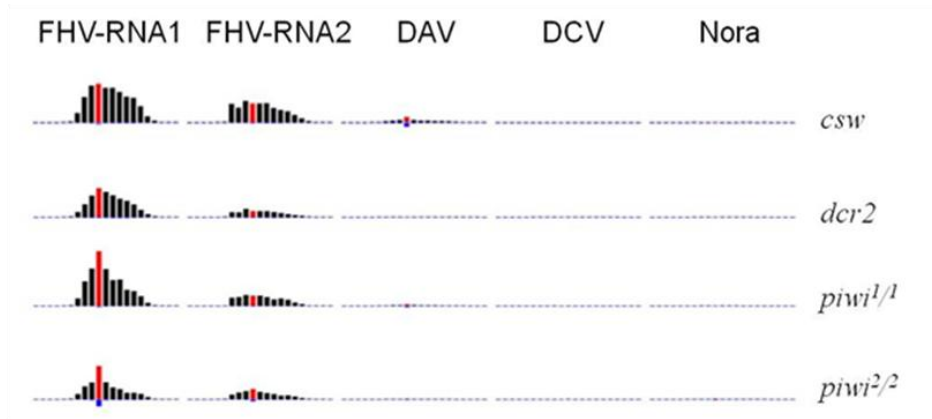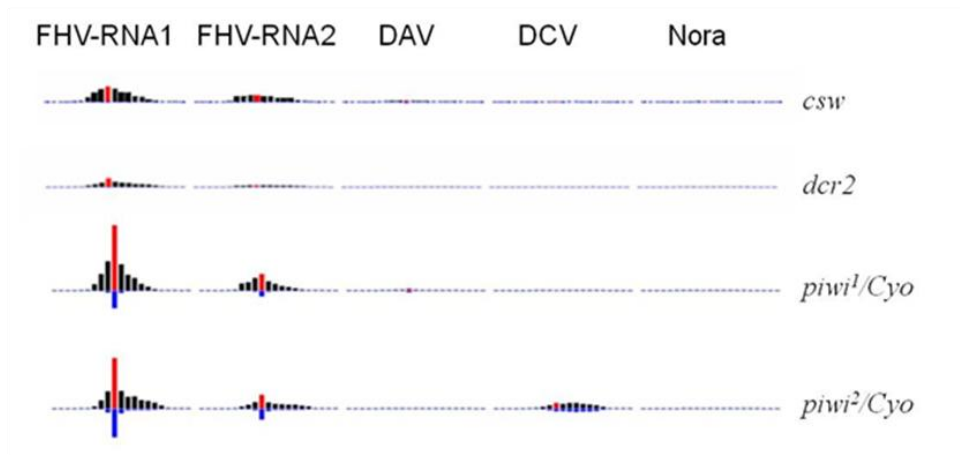
Figure 4-10. Infectivity and accumulation of FHVΔB2 was partially rescued in *piwi* mutant flies.

A. Survival of wild type (*csw*), *dcr-2*[L811fsX] (*dcr2*) and *piwi*[1]/*piwi*[2] (*piwi*[1/2]) adult flies after FHVΔB2 inoculation. Data shown represented mean value of triplicates, and the error bars indicated standard deviation.

B. Expression levels of sense-stranded (+) and antisense-stranded (-) FHV RNA1 (FR1) relative to rp49 as determined by qPCR of total RNA from the FHVΔB2- inoculated adult flies of indicated genotypes. The graphs show the mean and standard deviation (n=6). Y axis is in logarithmic scale. Statistically significant differences are less than 0.001. (Student's *t*-test, compared to wild type strain challenged by FHVΔB2)

C. Detection of FHV sense-stranded RNAs in the ovaries (upper panel) and heads (lower panel) of infected adult flies. 5 μg of total RNAs extracted from dissected heads or ovaries of *csw*, *dcr-2*[L811fsX] (*dcr2*) and *piwi*[1]/*piwi*[2] (*piwi*[1/2]) mutant flies were probed with mixture of in-*vitro* synthesized RNAs corresponding to antisense-strands of RNA3 and the 3' end of RNA2. Loading was monitored by methylene-blue staining of ribosomal RNA.

A
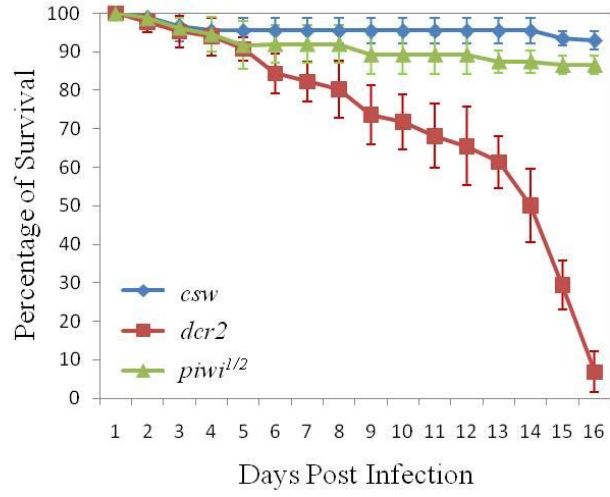
B
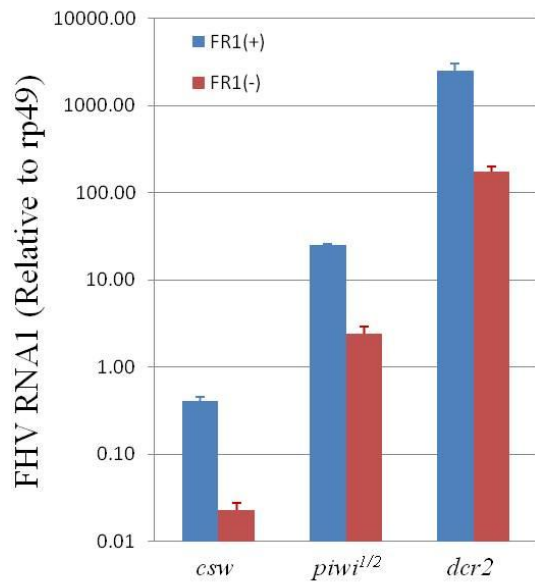
C

4.6 Reference

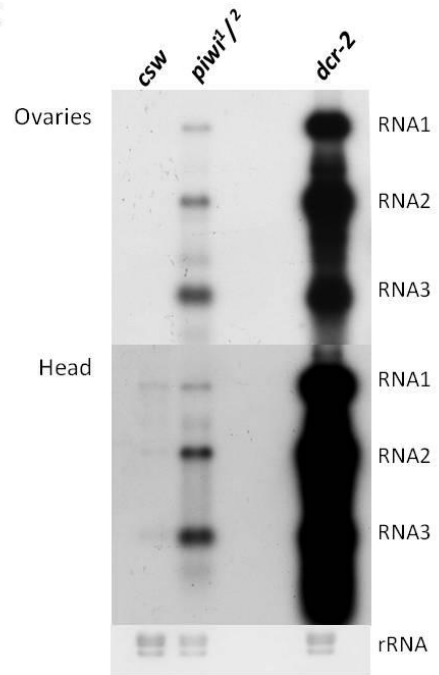Aravin, A., Gaidatzis, D., Pfeffer, S., Lagos-Quintana, M., Landgraf, P., Iovino, N., Morris, P., Brownstein, M.J., Kuramochi-Miyagawa, S., Nakano, T., Chien, M., Russo, J.J., Ju, J., Sheridan, R., Sander, C., Zavolan, M., Tuschl, T., 2006. A novel class of small RNAs bind to MILI protein in mouse testes. Nature 442, 203-207.

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CHAPTER 5. CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Conclusions

Ineffective replication and pathogenicity of FHVΔB2 could be rescued in adult flies by loss-of-function mutations in the antiviral silencing pathway genes, making it a straight-forward and reliable system to map the genetic requirements for RNAi-based antiviral response. Besides Dcr2-R2D2-Ago2 pathway characterized by our previous colleagues and researchers in other laboratories, here I demonstrated that Loqs and Piwi, two components in miRNA and piRNA pathways, respectively, were also important in mediating virus clearance in the adult flies. Notably, Piwi may play a major role in keeping viruses off the germline of the insect host, and hence significantly limiting the virus accumulation in the progenies of infected host. Moreover, Illumina deep sequencing of 18-28 nucleotides small RNAs in wild type and genetic mutant flies challenged by FHV or FHVΔB2 provided an extraordinary progress in understanding the biogenesis of viRNAs, the initial step in antiviral RNAi. Approximately equal ratios of (+) and (-) strand viRNAs were identified from wild type adult flies infected with FHVΔB2, and the density of 21nt-viRNAs located in the 5'-terminal region of FHV RNA1 and RNA2 were much higher than the rest of the genome. These features disappeared in *dcr2* mutant flies, suggesting that the dominant 21-nt species were generated by Dcr2-processing of viral dsRNA replicative intermediates, and the dsRNA precursor formed at the 5' terminus of FHV genomic RNAs were of higher concentration in the replication

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compartment which Dcr2 may get access to. Biogenesis of viRNAs targeting the 5'end of viral genome was also dramatically inhibited in the presence of B2 expression, as observed in wild type flies inoculated with FHV, suggesting the 5' nascent dsRNA replicative intermediates formed during initiation of viral progeny RNA synthesis was actively suppressed by B2 protein. Our sequence analysis in *dcr2* mutant flies also indicated that Dcr1, another dicer in *Drosophila* that is responsible for miRNA production, may play neglectable or very minor role in biogenesis of viRNAs, as the highly abundant virus-derived small RNAs did not have a predominant species with a length of 22 nucleotides as the majority of mature miRNAs defined in *Drosophila*. These Dcr2-independent viral small RNAs may represent non-specific degradation products of the viral genomic and subgenomic RNAs and are non-functional in RNAi considering the robust viral replication and hypersensitivity of *dcr2* mutant flies to FHVΔB2 infection.

Loqs has firstly been established to be required for miRNA maturation and more recently for both processing and RISC-loading of endogenous siRNAs in adult flies. Here I showed that it was not essential for the Dcr2-production of viRNAs, but acted with R2D2 in a redundant manner to facilitate the accumulation of viRNA following viral replication in the adult flies. Virus-derived small RNAs in loqs mutant allele that was used in this study resembled those in the wild type flies except a slight decreased ratio of viRNAs over viral genomic RNAs to 89%, while a more severe disrupted pattern of endogenous siRNA was observed in the same mutant strain. Interestingly, the ratio of viRNAs over genomic RNA of FHV decreased to 22.6% in *r2d2* single mutant flies, in which the

profile of 7 known endogenous siRNAs were similar to that in wild type, suggesting the R2D2 may play a more dominant role on processing of dsRNAs derived from the exogenous source such as RNA viruses, whereas function of Loqs was crucial the endogenous siRNA pathway. Strikingly, I found that FHVΔB2 accumulated to high levels in the *loqs-r2d2* double mutant, in which the viral sense- and antisense-stranded RNA1 was over 1000-time more abundant relative to wild type flies, and comparable to that in the *ago2* flies. Consequently, the ratio of viRNAs over viral genomic RNA decreased to 2.3% in the double mutant flies. More importantly, the percentage of 21nt-viRNAs derived from both sense and antisense strands of FHV RNA1 decreased by 25% and those from RNA2 was even not the dominant species any more. Therefore, our findings indicate that Loqs and R2D2 have redundant roles in Dcr2-dependent biogenesis of viral siRNAs in adult flies. It is notable that component(s) in the piRNA pathway seemed to add another layer of complication to the RNAi-based antiviral immunity. FHVΔB2 accumulation was rescued in *piwi* homozygous or trans-heterozygous mutants to levels similar or even higher than *loqs* or *r2d2* single mutant flies, similar viral RNA abundance was observed in *aub* trans-heterozygous mutant but not *ago3* or *armi* flies (data not shown). Northern blotting analysis and deep sequencing of the small RNAs in FHVΔB2-infected *piwi* and *aub* mutants are in progress.

In addition, an enhanced abundance of positive stranded FHV-derived small RNAs and a decreased ratio of viral siRNAs over FHV genomic RNA were observed in both *loqs-r2d2* double mutant and *ago2* mutant lines, implying that these two components may be

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also redundant in facilitating the viRNA loading into Ago2-containing RISC or other processes more downstream. Ago2 is required for slicing of the single-stranded RNA target in exogenous RNAi and mediate antiviral immunity against a wide variety of viruses in adult fruit flies. In this work, it was proved for the first instance that Ago2 was not required for the biogenesis of 21nt-viRNA by Dcr2 in the adult flies, given our observation that both the positive and negative stranded 21nt- small RNAs were the most dominant species and no significant changes in the relative abundance of 21nt-siRNAs derived from the 7 known endogenous loci for endogenous siRNA were observed. However, over 90% of the FHVΔB2-specific small RNAs in *ago2* mutant flies were positive strands, and the percentage of 21nt-viRNAs derived from RNA1 and RNA2 were up to 87% and 91%, respectively. Overall, Ago2 enhanced the accumulation of both the total and 5'terminal viral siRNAs, and dramatically altered the ratio of positive and negative strands of viral siRNAs.

Another highlight of this study was the germline-specific antiviral response against FHV in the adult *Drosophila* which required Dcr2 and Piwi. I found that albeit the robust replication of both viruses in the somatic tissue of wild type flies, both injection and distal sites, due to the suppressor activity of B2 protein, viral RNAs accumulated to extremely low levels in the ovaries and testes (data not shown). However, both genomic RNAs and subgenomic RNAs of FHV were dramatically increased in the germline of *piwi* heterozygous and homozygous mutant flies, as well as the *dcr2* mutant to a lesser extent. The robust viral accumulation was revealed consistently by northern blotting and

quantitative real-time RT-PCR detecting both sense- and antisense-strands of all the RNA species, western blotting and immunostaining of the capsid protein and B2 encoded by RNA2 and RNA3 of FHV, respectively. Intriguingly, the immunostaining revealed that both viral proteins were specifically localized to the ovariolar walls and cytoplasm of the follicle cells surrounding the developing egg chambers, confirming that the viral RNAs and proteins detected by other experimental strategies were not solely FHV contamination from sample-preparation and dissection. Enhanced viral accumulation was also observed in the ovaries of DCV-infected *piwi* mutant flies, suggesting that the antiviral immunity mounted by Piwi may target viruses in more than one family. Furthermore, I examined the progenies which differentiated from embryos laid by the FHV or DCV-infected flies, and the *piwi* mutant progenies displayed much higher viral RNA levels than those reproduced by wild type or *dcr2* mutant parents. These results suggested a prominent role of Piwi in protecting the germline and progenies of virus-infected hosts, and its function may be more dominant than Dcr2 in the germline. Surprisingly, I have not found any convincing evidence for virus-derived piRNAs or Piwi-bound viral small RNAs yet, but a change in the virus-specific small RNAs and ratio of positive and negative strands of 21nt-viRNAs was observed in the *piwi* heterozygous ovaries during FHV replication, and this will be an intriguing question need to be addressed in the future work.

5.2 Future directions

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5.2.1   Explore the contribution of each component in the antiviral RNAi pathway by

analysis of viRNA profiles in genetic mutants

Sequence-specific small RNAs targeting the viral genome ensure the specificity of RNAi-based antiviral response. Information such as their size distribution, chemical properties, nucleotide bias and alignment to their targets greatly facilitate how the immunity is triggered and proceed in the infected host, and how each components cooperating in the pathway. Therefore, it will be an important follow-up task to analyze small RNA profiles in *Drosophila* mutants defective in specific RNAi genes, including the three members of PIWI subfamily *piwi*, *aub* and *ago3* heterozygous and trans-heterozygous mutants that displayed partial restoration of viral RNA accumulation of FHVΔB2 as mentioned in the previous section.

According to the size distribution and strand bias of viral specific small RNAs in *dcr2* mutant flies infected with FHVΔB2, I believed the possibility of Dcr1-dependent viral siRNA production could be excluded. However, it is still likely that they play a role downstream of the pathway, such as viRNA-loading, viRNA activity and/or target cleavage, as the viral RNA accumulation in *dcr1* and *ago1* embryos was remarkably higher than that in the wild type injected with viral transcript R1ΔB2 (unpublished data), suggesting these two components in miRNA pathway may also involved in virus clearance in the host.

To make the entire picture even more complicated, the findings described in Chapter 3 have demonstrated that Loqs and R2D2 have redundant roles in Dcr2-dependent viRNA

biogenesis and the subsequent loading into Ago2. This may be a delicately regulated process involves timely interaction of these two factors to Dcr2 and/or other factors, for unpublished data from our previous works indicated that both deletion and overexpression of Loqs led to restored infectivity and virus accumulation of FHVΔB2. It has also been documented that Loqs physically interacts with both Dcr1 and Dcr2 and regulate their functions (Ye *et al.*, 2007; Czech *et al.*, 2008; Okamura *et al.*, 2008b). Moreover, although Dcr1 is the key enzyme to process pre-miRNAs into mature miRNAs, it is also required for siRNAs- and Ago2-dependent siRISC assembly (Lee *et al.*, 2004a). These components may as well contribute to the RNAi-mediated antiviral immunity in analogous mechanisms. Unfortunately available mutant alleles of either *dcr1* or *ago1* can only survive as heterozygous mutants, so examining the virus abundance and small RNA population in the null-mutant flies seems not practical. It may be worthwhile to test the protein-protein or protein-RNA interaction in the wild type flies, to test whether the Dcr1-Loqs or Ago1-viRNA occurs in the flies during FHV or FHVΔB2 infection.

In addition, I have performed microarray to examine the transcription profiles in the presence of FHVΔB2 and FHV in S2 cells. Although all the known RNAi genes did not exhibited significant change in expression profile upon replication of the two viruses, I did notice a set of novel genes and those involved in anti-microbial peptides were found to be either up- or down-regulated over 10 folds in S2 cells (data not shown). Further analysis of those lines of information may help identifying more host factors assisting the Dcr2-siRNA pathway during the viral infection.

5.2.2   Characterization of virus-derived small RNAs

Endogenous siRNAs produced by Dcr2 are 21 nucleotide duplex and contain 5'
phosphate and 2', 3' hydroxyl termini (Elbashir *et al.*, 2001a; Elbashir *et al.*, 2001b), and
are modified by S-adenosyl methionine-dependent methyltransferase, *Drosophila*
homolog of Hen1 which was firstly identified in plants (Yang *et al.*, 2006; Yu *et al.*,
2010). Whether the viral siRNAs are also methylated in adult flies remains to be
determined, while our previous works have demonstrated that viRNAs loaded in Ago2
are methylated at their 3' end in the *Drosophila* S2 cells infected with FHVΔB2 virion. I
do not have any evidence proving the biological significance of methylation on the
viRNA activity yet, because a portion of viRNAs are unmethylated in those cells. Those
viral siRNAs may be free-floating in the cytoplasm, as viral siRNAs associated with
Ago1 were undetectable at that time (Aliyari *et al.*, 2008). Moreover, *hen1* mutant flies
available in our laboratory did not show any obvious disease symptoms or susceptibility
upon FHVΔB2 inoculation and any enhanced viral accumulation relative to the wild type
flies (unpublished data). Therefore, whether methylation of viral siRNAs is essential for
their activity/loading remains an open question.

Another important issue need to be verified is the existence and characterization of
piRNAs in adult flies upon virus infection. piRNAs identified to date derived from the
transposons in the germline are 24-30nt in length, contain 5' monophosphate and 2'-O-
methyl, 3'hydroxy termini (Vagin, 2006; Saito *et al.*, 2007). In the ovaries of wild
type flies infected with either FHV or DCV, I could not detect any dominant peaks

representing the small RNA species of 24-30nt derived from the virus genome (Figure 4-9 and data not shown). There is no implication that piRNA biogenesis may be inhibited by the VSR activity of FHV, as B2 suppressed viRNA biogenesis by binding to long dsRNAs, while no existence of dsRNA has been reported during primary or secondary piRNA production in the germline. Supporting this notion, I did not observe any apparent changes in the size distribution of 18-30nt small RNAs cloned from the ovaries of FHV- or FHVΔB2-infected wild type or *dcr2* mutant flies (data not shown). It is noticeable that in the ovaries of *piwi¹/Cyo* and *piwi²/Cyo* heterozygous mutants infected with FHV, the abundance of 21nt- viral siRNAs derived from RNA1 in both positive and negative strands remarkably increased compared to wild type or *dcr2* ovaries. The size distribution and strand bias pattern surprisingly resembled those in wild type flies infected with FHVΔB2. As Dcr2 is not genetically altered in the *piwi* mutant, these dominant 21nt- species should be produced by Dcr2 in the *piwi* mutant ovaries if our undergoing analysis of these 21nt- viral siRNAs were mapped to the 5' terminus of the viral genomic RNA. Furthermore, based on our hypothesis explaining the strong strand bias observed in *ago2* mutant flies infected with FHVΔB2, I assumed that preferential loading of viRNAs into Ago1 in the absence of Ago2 or in the presence of B2 protein might lead to preferential degradation of the negative stranded viRNAs. Similar Ago1-loading may also occur when Piwi is present in the ovaries, thus when the Piwi expression reduced in the heterozygous mutant flies, larger amount of viRNAs produced by Dcr2 were able to associate with Ago2, resulting in enhanced accumulation of negative stranded viRNAs in the ovaries.

This proposed model definitely requires further supporting evidence, but I believe a comparison of the Ago2- and Ago1-immunoprepitated viRNAs in the wild type and *piwi* mutant ovaries would be a reasonable start.

In addition, it would be intriguing to examine whether the population structure, strand bias and genome alignment pattern of the viral siRNAs that were showed in FHV- and FHVΔB2- infected flies is conserved to other positive-stranded RNA viruses. Deep sequencing of small RNAs from DCV- and CrPV- inoculated adult flies has never been documented yet. It would be inspiring to study these two viruses encoding VSRs that inhibit dicing and Ago2-loading, respectively (Nayak *et al.*, 2010), and confirm our idea on the VSR activity of B2 by comparing their impacts on viRNA biogenesis with FHV infection.

5.2.3   Understanding the mechanism of viRNA activity and slicing

Previous studies have clearly proven the crucial role of Ago2 in antiviral immunity in adult flies and cell culture. Little is known about how viRNAs are loaded into Ago2, how the loading process generates the strand bias of viRNAs that was observed in *ago2* and *loqs-r2d2* double mutant flies inoculated with FHVΔB2, the percentage of positive viRNAs loaded into RISC over negative strands in the adult flies. In *Drosophila*, predominant sorting of mature miRNAs and exogenous siRNAs to Ago1 and Ago2 complexes, respectively, was reported earlier (Miyoshi *et al.*, 2005). A byproduct of microRNA biogenesis, miR*s however, was often loaded as functional species into Ago2 (Forstemann *et al.*, 2007; Tomari *et al.*, 2007; Okamura *et al.*, 2009). Accordingly, an

extensive analysis of Ago2- and Ago1- loaded small RNAs in virus-infected wild type, *ago2* and *loqs-r2d2* mutant flies may be necessary to address the issues above. By now, available evidence from S2 cells inoculated with FHVΔB2 virion suggested a preferential loading of viral siRNAs in Ago2 over Ago1 by northern blotting hybridization (Aliyari *et al.*, 2008). It is unclear yet whether this is applicable to the adult flies. By northern hybridization, I detected more abundant positive stranded viRNAs in contrast to the equal ratio in adult flies injected with the same virion. After improving the sensitivity of small blotting and crosslinking, I was able to detect substantial amount of virus specific siRNAs indeed co-immunoprepitated with Ago1 (data not shown). Moreover, I have attempted to examine the Ago1 loading of viral siRNAs when Ago2 was knockdown in S2 cells, but the detection of viral siRNA in cells transfected with double-stranded RNA targeting Ago2 was inconsistent, and the abundance seemed to be distinct from that in the *ago2* mutant flies, which was probably caused by the varied transfection efficiency. Better procedures for Ago2 knockdown is necessary if we need to continue using the cell culture system, meanwhile it would be worthwhile to test our Ago1-immunoprepitation in *ago2* mutant embryos.

5.2.4   Interaction between Dcr2- and Piwi-mediated virus clearances in the germline

A strong antiviral response was observed in the germline, and it may be dependent on both Dcr2 and Piwi. The latter seemed to have a dominant influence as the *piwi* homozygous progenies but not *dcr2* mutants displayed much stronger viral RNA and capsid protein accumulation. Double mutants are being generated by genetic crossing at

the process of dissertation writing, and synergistic effect is expected in the double mutants if these two components or related pathways have redundant roles in keeping viruses out of the germline.

Both endo-siRNA and piRNA pathways are required for specific loci and probably in specific cell types for silencing of transposons and repetitive elements in the germline (Aravin *et al.*, 2007; Brennecke *et al.*, 2007; Nishida *et al.*, 2007; Brennecke *et al.*, 2008; Saito *et al.*, 2009; Rozhkov *et al.*, 2010). So far, it has only been shown that piRNA biogenesis is Dcr2-independent, the possibility of sharing common machinery or target loci by two pathways has not been examined yet. In fact, I did observed a change in viral siRNA strand bias in the *piwi* heterozygous and homozygous mutant, in the ovaries and somatic tissues, respectively. Hence it is intriguing to test the influence of either of the two pathways in the absence of the other. Deep sequencing data not shown in Chapter 4 illustrated that the size distribution of endogenous small RNAs (18-30nt) in *piwi* heterozygous ovaries, both in the presence and absence of FHV infection, displayed similar pattern as the wild type ovaries. The majority of small RNAs were of the size of piRNAs with a peak at 25-26nt, and a less dominant population of 21nt representing the Dcr2-dependent endogenous siRNAs, as this 21nt-population dramatically reduced in *dcr2* mutant ovaries. This result indicates that Piwi may have no influence on biogenesis of endogenous siRNAs. More comprehensive analysis is required to compare the strand bias and Ago2-loading of these siRNAs in the *piwi* mutant ovaries as discussed in the previous section.

5.2.5   Further analysis of progenies of virus-infected flies and paternal effects on virus

transmission

Detection and comparison of viral RNAs in the ovaries and progenies of the infected

flies clearly suggested that vertical transmission of many RNA viruses, including FHV

and DCV, may not occur in the wild type fruit flies due to the astonishing protection of

Piwi. Transmission of FHV has never been reported to our knowledge, and DCV has

been implicated to transmit among adult flies and from infected adults to larvae.

However, I detected high abundance of DCV genomic RNAs by quantitative real time

RT-PCR in the homozygous F1 progenies of DCV-infected *piwi* heterozygous mutants,

and the virus genomic RNA accumulation was comparable to that observed in the

ovaries of inoculated flies.   Plaque assay need to be conducted to verify whether

infectious DCV particles formed in those progenies, and it would also be interesting to

examine if infection in the germline of maternal and paternal side contribute equally to

the viral transmission, as I examined the progenies produced by FHV-infected parents. In

contrast to DCV, FHV RNA abundance in the F1 generation of wild type and all the

mutants was extremely low, probably because it is not a virus naturally infecting

Drosophila as DCV does. Moreover, in the wild type ovaries of DCV or FHV infected

flies, I have identified 21nt-small RNAs with characteristics of viral siRNAs that were

derived from a small RNA virus, Drosophila A virus (data not shown). Whether the

germline protection and transmission resistance function of Piwi also apply to other

natural pathogens of *Drosophila melanogaster* remains to be determined.

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