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Identification and Mechanism of Small Molecule Inhibitors of RNA Interference

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ABSTRACT OF THE DISSERTATION

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RNA interference (RNAi) is induced both artificially to knockdown gene expression and naturally during virus infection as a host defense mechanism. Although genetic studies have provided a biochemical framework for RNAi, little is known if key steps in the RNAi pathway can be inhibited by small molecules. This dissertation describes a cell-based small molecule screen to assay for the suppression of naturally occurring RNAi in *Drosophila* Schneider 2 (S2) cells induced by viral RNA replication. This screen resulted in the identification of twenty lead compounds in the primary screen that were narrowed down to five RNAi inhibitors (RINs) in the secondary screen by Northern blot analysis.

Genetic and biochemical approaches were taken subsequently to determine the targets of these RINs in the RNAi pathway. In *Drosophila melanogaster,* RNAi begins with the dicing of long double-stranded RNA (dsRNA) into small interfering RNAs (siRNAs) by the nuclease Dicer-2. This is followed by the assembly of the RNA-induced

silencing complex (RISC) that contains a single-stranded siRNA bound to the RNaseH-like Argonaute-2 (Ago2) and cleaves the target mRNA that is complementary to the siRNA. The first set of experiments in this study divided the five RIN compounds into two groups. Although all of the RIN compounds were highly potent in the suppression of RNAi triggered in S2 cells by long dsRNA, artificial initiation of RNAi by synthetic siRNA was also inhibited by RINs 4 and 5, but not by RINs 1-3. These findings indicate that RINs 1-3 target the upstream steps of the RNAi pathway while RINs 4 and 5 target the downstream steps. Further biochemical experiments demonstrated that RINs 1 and 2 inhibited the dicing of long dsRNA into siRNAs and that RIN5 blocked cleavage (slicing) of target mRNA mediated by an siRNA-programmed RISC. These results demonstrate for the first time that both dicing and slicing in the core RNAi pathway can be targeted for inhibition by small molecules.

The last set of experiments determined if the RIN compounds were able to suppress the RNAi-mediated antiviral immunity triggered by infection initiated by virion inoculation. Both Flock house virus (FHV) and Cricket paralysis virus (CrPV) replicated to much higher levels in S2 cells following treatment with each RIN compound and RINs 3, 4 and 5 dramatically enhanced the cytopathic effect of both positive-strand RNA viruses. The B2 protein encoded by FHV is a virus suppressor of RNAi (VSR) indispensable for infection of S2 cells. In the absence of RNAi suppression, the B2-deficient mutant of FHV (FHVΔB2) is rapidly cleared in S2 cells following challenge inoculation with viral particles unless RNAi is suppressed in S2 cells, for example, by Ago2 depletion. Treatment of S2 cells with each of the five RIN compounds enhanced

the accumulation of FHVΔB2. Notably, FHVΔB2 replicated to similarly high levels in Ago2-depleted S2 cells and S2 cells treated with RIN5. Moreover, both RINs 2 and 5 enhanced the accumulation and virulence of FHV in adult flies and RIN treatment of *Caenorhabditis elegans* also partially inhibited RNAi induced by a B2-deficient replicon of FHV or exogenous dsRNA.

In summary, the experiments detailed in this body of work established a cell-based screen for the identification of small molecule antagonists of RNAi. These experiments also provided a pipeline of experiments to establish where and how the identified compounds affect the RNAi pathway.

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Chapter 1: Background

Abstract

Small RNA gene silencing pathways, or RNA interference (RNAi), have been implicated in a wide array of biological processes, including genome defense, developmental regulation, heterochromatin formation and antiviral defense in a multitude of eukaryotic organisms. In this chapter I will review the available literature on RNAi in *Drosophila melanogaster*. *D. melanogaster* has been an invaluable source for the understanding of both the genetic and biochemical factors that govern RNAi. The fruit fly has also been important in the understanding of the antiviral role RNAi plays in animal systems and has paved the way of its understanding in pathologically important organisms like mosquitoes. This chapter will also review the published literature available on virus suppressors of RNAi (VSRs), a group of molecules that viruses have evolved which can be used to subvert antiviral RNAi. Several viruses have been shown to encode VSRs that are capable of suppressing RNAi at different steps of the pathway, such as dicing or slicing. In this section antiviral RNAi in other organisms, such as *Caenorhabditis elegans*, will be discussed as well.

1.1 Introduction

In 1990 the Jorgensen lab published the first report of the phenomenon that is now known as RNA interference (RNAi). Their finding, which they named cosuppression, involved the silencing of an endogenous gene by the overexpression of a homologous transgene in the same plant (Napoli et al., 1990; van der Krol et al., 1990). Subsequent studies showed that RNA virus replication can be down regulated in plants that carry fragments of the viral genome as transgenes (Lindbo et al., 1993). These two pioneering studies led to the explosion of publications which developed into the field of RNAi. Later work established the presence of RNAi in a multitude of organisms, including the fungi *Neurospora crassa*, the nematode *Caenorhabditis elegans*, arthropods such as *Drosophila melanogaster* and in humans (Billy et al., 2001; Cogoni et al., 1996; Elbashir et al., 2001; Fire et al., 1998; Kennerdell and Carthew, 1998).

The hallmark of RNAi is the production of small RNAs of 21 to 28 nucleotides (nt) in length. These small RNAs are what give specificity to the pathway and serve as guides for several RNAi functions such as RNA cleavage, translational repression, DNA methylation and chromatin methylation (Hannon 2002; Bartel 2004). Currently there are three known classes of small RNAs; small interfering RNAs (siRNAs), microRNAs (miRNAs) and Piwi interacting RNAs (piRNAs). Plants were the first organisms where siRNAs were detected and described as well as the first organism where antiviral RNAi was characterized (Hamilton and Baulcombe, 1999; Ding and Voinnet, 2007; Li and Ding, 2006; Voinnet, 2005). Further research has shown that small RNAs play an

antiviral role or are closely associated with virus infection in other organisms such as in insects, nematodes and mammals (Ding and Voinnet, 2007; Marques and Carthew, 2007; Muller and Imler, 2007; Parameswaran et al., 2010; Sullivan and Ganem, 2005).

Viruses are parasites which rely on their host to complete their replication cycle while at the same time contend with the host's antiviral immunity mechanisms. Plant and invertebrate viruses trigger the RNAi pathway via the generation of double-stranded RNA (dsRNA). This dsRNA can be synthesis in several manners including from the replicative intermediates formed during RNA virus replication and the convergent transcription of genes in both RNA and DNA viruses (Ding, 2010). In the following section the data supporting antiviral RNAi as well as the mechanisms by which viruses counteract this pathway will be addressed.

1.2 Small RNA Pathways in *Drosophila*

1.2.1 The microRNA Pathway

Of the small RNA pathways found in *D. melanogaster*, the miRNAs are the most numerous with current estimates close to 200 distinct species (miRBase). miRNAs are found encoded in endogenous genes that are transcribed by RNA polymerase II and sometimes RNA polymerase III into transcripts that have been dubbed primary miRNAs, pri-miRNAs for short (Borchert et al., 2006; Lee et al., 2004). pri-miRNAs contain imperfect loop structures which are recognized and cleaved by a protein complex containing the RNAase III enzyme Drosha and the dsRNA binding protein Pasha. This process releases a hairpin structure of approximately 70 nt in length termed the pre-miRNA that contains a 2 nt overhang at its 3' end as well as a 3' hydroxyl and 5'

phosphate group (Lee et al., 2003). The first two steps of miRNA biogenesis occur in the nucleus while the maturation phase occurs in the cytoplasm. To achieve the movement of the pre-miRNA from the nucleus to the cytoplasm the pre-miRNA is carrying over via the nuclear transporter Exportin-5. Once moved to the cytoplasm , the pre-miRNA is then cleaved by another RNase III enzyme, Dicer-1 (Dcr-1), and the dsRNA binding protein Loquacious-PB and PD isoforms (Loqs) into an imperfectly base paired 22nt duplex miRNA (Jiang et al., 2005; Miyoshi et al., 2010). The mature miRNA is then unwound and one strand is loaded into the miRNA induced silencing complex (miRISC) where the now single-stranded miRNA serves as a guide to bring miRISC to an mRNA that is complementary with a few mismatches to it inducing either translational inhibition or cleavage of the mRNA (Miyoshi et al., 2010) (Figure 1.1a).

Aside from the canonical miRNA biogenesis pathway previously described, there has been an alternate miRNA biogenesis pathway found in *D. melanogaster* where miRNAs are produced from the hairpin structures located in the introns of genes that have come to be known as mirtrons (Okamura et al., 2007; Ruby et al., 2007a; Ruby et al., 2007b). The hairpins found in mirtrons are processed by the splicing machinery of the cell to produce pre-miRNA-like structures which are then fed into the canonical miRNA pathway to produce mature miRNAs via the activity of Dcr-1 and Loqs (Miyoshi et al., 2010).

The miRNA pathway is found in many eukaryotic organisms are paramount to their survival and growth. These small RNAs control important functions by modulating the gene expression of endogenous genes involved in cell proliferation, differentiation,

growth as well as development (Wu et al., 2008). It should be noted that miRNAs are not limited to eukaryotes as it has been reported that some DNA viruses found in mammals encode miRNAs as well.

1.2.1 Piwi Interacting RNAs

Of the three small RNA pathways in *Drosophila*, the piRNA pathway is the newest and least understood. What is known however is that piRNAs are endogenous small RNAs, like miRNAs, that are 24 to 30 nt in length which interact with the Piwi subfamily of Argonaute proteins to protect the germline from selfish genetic elements such as transposons (Brennecke et al., 2007; Nishida et al., 2007). It has also been shown that neither of the two Dicer proteins found in *Drosophila* are required for their biogenesis but that two helicases, Spendle-E (Spn-E) and Armitage (Armi), are required for piRNA mediated silencing (Vagin et al., 2006). As piRNA biogenesis is Dicer independent and they occur only as a single-stranded RNA species, a new model for biogenesis has been proposes based of the sequencing data available which is referred to as the “ping pong” cycle. This cycle is started by the transcription of a piRNA cluster encoded in the genome which is subsequently cleaved into piRNAs that have a strong bias for a uridine at the first position at the 5’ end. This primary piRNA is then loaded into Piwi or Aubergine (Aub) which then seeks out its target sense strand transposon transcript and cleaves it at a position ten nucleotides downstream of the 5’ end. This cleavage event results in the production of a secondary piRNA, with an adenine at the tenth position from the 5’ end, which binds to Ago3 and mediates cleavage of a piRNA precursor transcript which in turn gives rise to more piRNAs that have a uridine at the

first position at the 5'end. These piRNAs are then fed back into the piRNA amplification loop to generate more piRNAs (Thomson et al., 2009) (Figure 1.2). It is still unknown how the primary piRNAs are excised from the transcripts transcribed from the piRNA clusters, however according to this model either Piwi or Aub performs these cleavage events.

1.2.3 The siRNA Pathways

In *D. melanogaster* there has been two siRNA pathways that have been characterized, the exogenous siRNA pathway and the endogenous siRNA pathway. The exogenous siRNA pathway is triggered when foreign dsRNA is introduced into the fly while the endogenous siRNA pathway deals with small RNAs encoded in the fly genome that are not miRNAs or piRNAs. The exogenous siRNA pathway starts when dsRNA is recognized by a complex containing the RNaseIII enzyme Dcr-2 and the dsRNA binding protein Loq-PD and cleaved into 21 nt duplex siRNAs (Hannon et al., 2002; Marques et al., 2010). An intermediate complex composed of Dcr-2 and the dsRNA binding protein R2D2 then starts the loading of the siRNA into the RNA induced silencing complex (siRISC) (Liu et al., 2003). The final step of this maturation process occurs when the siRNA is unwound and one of the strands in then loaded into Argonaute 2 (Ago2) which results in the formation of the mature siRISC (Kim et al., 2007). The loaded siRNA serves as a guide to bring siRISC to a perfectly complementary mRNA target that triggers Ago2 to cleave the mRNA via an endonuclease reaction called "slicing" (Okamura et al., 2004) (Figure 1.1b). Studies have shown that this exogenous siRNA pathway can be

triggered in response to viral dsRNA, transgene driven expression of dsRNA and exogenously introduced dsRNA (Aravin and Tuschl, 2005).

The endogenous siRNA pathway is characterized by production of siRNAs from convergent transcription or highly structured genomic segments that are found in both somatic tissue and in gonadal tissue in *D. melanogaster* (Czech et al., 2008; Ghildiyal et al., 2008; Kawamura et al., 2008; Okamura et al., 2008a; Okamura et al., 2008b). As in the exogenous siRNA pathway, the biogenesis of these endo-siRNAs requires the function of the Dcr-2 protein to cleave the dsRNA into siRNA duplexes. One of the main differences in these two pathways is that the endo-siRNA pathway makes use of the Loqs-PB isoform as the dsRNA binding protein partner of Dicer-2 as opposed to Loqs-PD (Czech et al., 2008; Miyoshi et al., 2010; Okamura et al., 2008) (Figure 1.1c).

1.3 RNAi as an Antiviral Pathway in *Drosophila melanogaster*

D. melanogaster has been an ideal model organism for the study of innate immunity against viruses which has produced advances in the fields of the Toll and immune deficiency-mediated signaling pathway as well as the Janus kinase signal transducer and activator of transcription (Jak-STAT) pathway (Dostert et al., 2005; Hoffman, 2003; Zambon et al., 2005). In 2002 it was shown the RNAi plays an antiviral role in the *D. melanogaster* Schneider cell line when challenged with Flock House Virus, an RNA virus with a positive sense genome (Li et al., 2002). Later work has shown that RNAi also plays an antiviral role in adult flies and requires components of the siRNA pathway, but not the miRNA pathway, when challenged with a diverse array of RNA viruses (Galiana-Arnoux et al., 2006; van Rij et al., 2006; Wang et al., 2006).

1.3.1 Viruses that Induce an Antiviral Response

Several viruses have been shown to illicit an antiviral RNAi response when infecting *Drosophila melanogaster*. This section will highlight the viruses that have been used to study antiviral RNAi in *D. melanogaster* and that have been used in the following chapters.

Flock house virus (FHV) is part of the genus *Alphanodavirus* and in the family *Nodaviridae* (Ball and Johnson, 1998). Although an insect virus, FHV has been shown to be able to replicate in yeast, plants, nematodes and in mammalian cell lines in the laboratory setting (Ball et al., 1992; Lu et al., 2005; Price et al., 1996; Selling et al., 1990). The FHV particle carries a bipartite, positive sense RNA genome that is 4.5kb in length. RNA 1 is 3107 nt in length and encodes protein A, which is an RNA-dependent RNA polymerase (RdRp) that the virus uses for both replication of the viral genome as well as the transcription of viral mRNAs. RNA 2 is 1400 nt in length and encodes in precursor to the coat protein. Through the course of replication the subgenomic RNA 3 is produced which is 387 nt is length and is derived from the 3' end of RNA 1 (Ball and Johnson, 1998). RNA 3 encodes the B2 protein which is a potent viral suppressor of RNAi (VSR) (Li et al., 2002). When FHV carries a mutation in the B2 ORF that renders the protein inactive it can no longer replicate to high levels in both cell culture as well as adult flies (Li et al., 2002; Wang et al., 2006). Nodamura virus (NoV) is closely related to FHV and a member of the genus *Alphanodavirus* and the family *Nodaviridae* that has a similar genome structure and protein composition as FHV. This virus has been shown to be targeted by RNAi in S2 cells and that its B2 protein has been shown to be a suppressor

of RNAi as well (Li et al., 2004). A key difference between FHV and NoV is that NoV can be lethal to mammalian cells (Ball and Johnson, 1998).

A second group of viruses that belong to the family *Dicistroviridae* have been used to study the antiviral RNAi response in *D. melanogaster*; Drosophila C virus and Cricket paralysis virus (CrPV) (Wilson et al., 2000). CrPV's genome is composed of one single stranded, positive sense piece RNA of 9185 nt in length. This RNA contains two ORFs (ORF 1 and ORF 2) that code for the nonstructural and structural proteins and are each preceded by an internal ribosomal entry site (IRES). Although this virus was first discovered in crickets it has then been shown to be able to replicate in a wide variety of insects and insect cell lines including *Drosophila* S2 cells (Scotti et al., 1975). CrPV has also been shown to have strong pathogenic effects in adult fruit flies (Wang et al., 2006). DCV is a closely related virus to CrPV that also has a single stranded, positive sense RNA genome that is slightly larger than CrPV's genome at 9264 nt in length that is divided in two ORFs, 1 and 2. ORF 1 is located at the 5' end of the viral genome and encodes the RdRp, helicase and protease. ORF 2 which is on the 3' end encodes the capsid protein. Unlike CrPV, DCV is a true *Drosophila* virus that was discovered in a laboratory strain (Jousset et al., 1977). Also unlike CrPV, DCV does not normally have pathogenic effects on adult flies.

1.3.2 Dicing and Slicing Play Key Roles in Antiviral RNAi

It has demonstrated in both genetic and biochemical studies that the previously described exogenous siRNA pathway has strong antiviral function in *D. melanogaster* (Aliyari et al., 2008; Wang et al., 2006). It has been shown that the dsRNA produced

during viral replication serves as a pathogen-associated molecular pattern (PAMP) that Dcr-2 can recognize and cleave into siRNAs (Ding, 2010).

Dicer serves as the gatekeeper of antiviral RNAi, not only in fruit flies but in all invertebrate animals and plants. In *Drosophila* it has been shown the Dcr-2 has antiviral activity against several RNA viruses included FHV, CrPV, DCV as well as the human pathogens Sindbis virus (SINV) and West nile virus (WNV) (Chotkowski et al., 2008; Galiana-Arnoux et al., 2006; Wang et al., 2006). It has been shown the *dcr-2* mutant flies that have been challenged with FHV show enhanced disease susceptibility (EDS) as compared the wild-type flies infected with FHV in both the number of flies that die and that the flies succumb to the virus at earlier time points (Galiana-Arnoux et al., 2006; Wang et al., 2006). This decrease in survival in *dcr-2* flies also coincides with an increase of viral RNA accumulation as compares to wild-type flies. When flies carrying a mutation in *r2d2*, the dsRNA binding partner of Dcr-2 in the exogenous RNAi pathway, were infected with FHV similar results to the *dcr-2* mutant flies were observed (Wang et al., 2006). *dcr-2* mutants also exhibit the EDS and increased viral replication levels for CrPV, DCV, SINV and WNV, all of which are single stranded RNA viruses (Chotkowski et al., 2008; Galiana-Arnoux et al., 2006; Wang et al., 2006). The requirement of Dcr-2 in antiviral RNAi is not universal however. In flies challenged with Drosophila X Virus (DXV), a dsRNA virus, Dcr-2 does not appear to be necessary for the antiviral response (Zambon et al., 2006). However, genes that are required in the piRNA pathway were shown to be important in the antiviral response to DXV as flies carrying mutation in *piwi, aub,* and *armitage (armi)* all showed EDS when infected with DXV (Zambon et al.,

2006). This requirement for piRNA related genes was also observed in flies infected with WNV as *spn-E* and *piwi* mutants showed EDS (Chotkowski et al., 2008). These pieces of data indicate that there is some cross talk between the RNAi pathways in *Drosophila* that is required for efficient viral immunity.

In fruit flies Ago2 is the last line of defense from invading viruses. Ago2's antiviral activity was first described in 2002 making it the first RNAi gene to be implicated in antiviral RNAi (Li et al., 2002). This study showed that when Ago2 was depleted in S2 cell that was infected with FHV there was an increase in the amount of viral RNA accumulation. The role of Ago2 in antiviral RNAi was further reinforced by the fact that when FHV RNA 1 transcripts containing a mutation in the B2 ORF (FR1ΔB2) were introduced into S2 cells they could not replicate, but the same transcript could replicate in S2 cells depleted for Ago2. Similar results were found in *D. melanogaster* embryos; FR1ΔB2 could not replicate in wild-type embryos but could in *ago2* mutant embryos (Wang et al., 2006). It has also been reported the *ago2* flies infected with DXV, DCV, CrPV, and WNV display EDS as compared to wild-type flies (Chotkowski et al., 2008; van Rij et al., 2006; Zambon et al., 2006). In the case of DCV and WNV there was upwards of a 100 fold increase in the amount of virus production in these flies as compared to wild-type (Chotkowski et al., 2008; van Rij et al., 2006). More recent biochemical evidence has shown that Ago2, and not Ago1, loads virus specific siRNAs (Aliyari et al., 2008). The wealth of genetic and biochemical data available has made it clear that Ago2 plays an irreplaceable and paramount role in the antiviral RNAi pathway.

1.3.3 Viral Small RNAs (viRNAs)

viRNAs were first discovered in *Nicotiana benthamiana* plants infected with the single stranded, positive sense RNA virus Potato Virus X (PVX) (Hamilton and Baulcombe, 1999). The viRNAs detected were 25nt in length and both sense and antisense strands were present in infected plants. The first reported findings of viRNAs in animals were in cultured *Drosophila* S2 cells infected with FHV which produced viRNAs of 22nt in length (Li et al., 2002). These two findings reinforced the importance of antiviral RNAi as a host immune response (Ding, 2010). In *D. melanogaster* it has been shown the Dcr-2 is essential for production of viRNAs. FHV infection of flies carrying mutations in the *dcr-2* gene fails to accumulate FHV specific viRNAs while wild-type and *r2d2* flies can accumulate FHV specific viRNAs (Wang et al., 2006). The role of *r2d2* in viRNA function is not at the level of biogenesis, but rather downstream of it, presumably in loading of viRNAs into RISC as *r2d2* mutant flies show EDS and increased viral mRNA accumulation while still maintaining a high level of FHV specific viRNAs (Wang et al., 2006). This hypothesis is in agreement with the data showing the requirement of R2D2 in loading of siRNAs in siRISC in the exogenous RNAi pathway (Liu et al., 2003). FHV is not the only virus reported to generate viRNAs in *Drosophila*; CrPV infection of S2 cells has shown to generate 21nt CrPV specific siRNAs (Wang et al., 2006). Deep sequencing has provided a new avenue of viRNA detection which has been shown to be more sensitive than Northern blot detection. Deep sequencing provided the first evidence of viRNA biogenesis for dsRNA viruses, including Drosophila

biranvirus (DBV) Drosophila totivirus (DTV) and DXV, in *D. melanogaster* (Wu et al., 2010). The same technique was also used to find viRNAs for more plus strand RNA viruses such as American nodavirus (AMV) and DCV. Not all viral small RNAs detected by Northern blot analysis fit into the siRNA size category. Deep sequencing of *Drosophila* OSS cells, a germline derived cell line, also led to the detection of AMV and DCV viRNAs of 27 to 28nt in length, putting it in the piRNA size range (Wu et al., 2010). These 27 to 28nt length viRNAs share much in common with piRNAs including a 5' uridine bias and only sense and not antisense strand specific RNAs were found (Wu et al., 2010). Northern blot analysis has also yielded the detection of piRNA-like viRNAs. It was found that when S2 cells are infected with WNV, viRNAs of 25nt in length were detected, which fall into the piRNA size range (Chotkowski et al. 2008). This data coincides well with the fact that antiviral RNAi against WNV requires Piwi and Spn-E, which are required in the piRNA pathway.

1.3.4 Viral Suppressors of RNAi (VSRs)

As in most host-pathogen interactions, viruses have evolved of a means of counter acting antiviral RNAi in the form of VSRs. Many plant and invertebrate viruses encode at least one or more VSRs (Li and Ding, 2006; Wu et al., 2010). The first and best studied VSR in invertebrates is the B2 protein of FHV which is encoded on the subgenomic RNA 3. B2 was first discovered in 2002 when it was shown to have VSR activity in both plants and S2 cells (Li et al., 2002). Further studies have demonstrated in importance of the B2 protein in the life-cycle of FHV. FR1ΔB2, a viral RNA 1 transcript containing a B2 deletion, is unable to replicate in S2 cells as well as wild-type fruit fly embryos, while

FR1 can replicate to high levels under the same conditions. However, FR1ΔB2 can replicate in S2 cells in which Ago2 has been depleted or in *ago2* or *dcr-2* mutant embryos (Li et al., 2002; Wang et al., 2006). In wild-type embryos FR1ΔB2 accumulated virtually no viral RNAs whereas in *dcr-2* and *ago2* mutant embryos viral RNAs replicated to very high levels. This data implies that B2 is not required for replication of the virus but rather is required for suppression of the host RNAi machinery. The closely related virus NoV also encodes a B2 protein which has been shown to inhibit RNAi in *Drosophila* as well as mosquito cells (Li et al., 2004). Studies using FHV virions carrying a B2 mutation cannot replicate in S2 cells proving the B2 is essential for the life-cycle of a fully intact virus, not just the FR1ΔB2 transcript (Aliyari et al., 2008). The importance of B2 suppression of RNAi is a key piece of evidence in support of antiviral RNAi as an innate immunity pathway against viruses.

Many VSRs have been shown to be able to bind to dsRNA or have a dsRNA binding domain. Both crystallization and NMR analysis of B2 have revealed the presence of a dsRNA binding domain on the N terminal region of the protein (Chao et al., 2005; Lingel et al., 2005). B2's dsRNA binding activity was first shown *in vitro* to be able to bind to both duplex siRNAs and long dsRNA (Lu et al., 2005). A mutation in the dsRNA binding domain (R54Q) abolished the binding of B2 to both siRNAs and dsRNAs. In the same study it was shown that the B2 protein could inhibit Dicer processing of long dsRNA *in vitro*. The same R54Q mutation of B2 that abolished dsRNA binding also abolished B2's ability to block siRNA biogenesis mediated by Dicer. More recently it has been shown that both FHV and NoV's B2 proteins can bind viral specific dsRNA in

Drosophila S2 cells during virus infection confirming the *in vitro* binding data results (Aliyari et al., 2008).

Both CrPV and DCV have been shown to be able to inhibit RNAi. CrPV was first shown to inhibit RNAi in *Drosophila* S2 cells by being able to rescue the replication of the FR1gfp construct, which is unable to replicate in S2 cells unless Ago2 has been depleted or by expression of B2 in *trans* (Wang et al., 2006). Deletion analysis of the CrPV genome placed the VSR activity in the first 140 amino acids of the nonstructural polyprotein encoded on ORF 1; later named 1A. It was later shown that the CrPV 1A protein does not inhibit dsRNA processing by Dicer nor does it bind to long dsRNA (Nayak et al., 2010; van Rij et al., 2006). However, CrPV 1A can block siRNA mediated cleavage of mRNA *in vitro* as well as being able to block the silencing of a luciferase mRNA by siRNAs in S2 cells (Nayak et al., 2010). The same study reported that CrPV 1A can bind to the Ago2 protein in S2 cells at the level of holo-RISC as RISC formation was not perturbed in CrPV infected cells nor in cells where CrPV 1A was transiently expressed. The closely related DCV has also been shown to encode a 1A protein capable of suppressing antiviral RNAi as well. The function of DCV 1A has been shown to be different than that of CrPV 1A. Firstly, DCV 1A can strongly bind to long dsRNA and showed weaker affinity to duplex 21nt siRNA, which can be abolished with a L28Y mutation (van Rij et al., 2006). Also, like with B2, DCV 1A can block Dicer mediated processing of dsRNA into siRNAs *in vitro* (Nayak et al., 2010). DCV 1A is the first and only VSR which has been shown perturb proper holo-RISC formation in *Drosophila* embryo lysates.

1.4 Antiviral RNAi in other Ecdysozoans

1.4.1 Antiviral RNAi in Mosquitoes

As in *D. melanogaster* it has also been shown that RNAi is a functional antiviral pathway in mosquitoes. Early results from *Aedes* C3/C6 cells infected with a SINV strain carrying a fragment of the Dengue Virus (DENV) genome were resistant to infection by DENV (Olson et al., 1996). This result is similar to phenomenon observed in plants called virus induced gene silencing, or VIGS. Researchers have used engineered SINV strains to infect adult mosquitoes in order to induce VIGS against viral genes as well as endogenous genes (Adelman et al., 2001; Johnson et al., 1999). The same resistance to DENV was found in the C3/C6 cell line when they were transfected with a plasmid coding for an inverted repeat RNA corresponding to DENV (Adelman et al., 2002). Using the same inverted repeat technology that was successful in the C3/C6 cell line, *Aedes aegypti* transgenic mosquitoes were made to express an inverted repeat using a genome fragment from DENV. These mosquitoes exhibited the same resistance to DENV infection that was found in the C3/C6 cell line (Franz et al., 2006).

Although the above results were promising, they still did not prove that RNAi was antiviral in mosquitoes. Some of the first evidence came in NoV infection of *Anopheles gambiae* cell lines. It was found that NoV replication in *A. gambiae* cells required the expression of the B2 protein in that the NR1ΔB2 mutant could not replicate to detectable levels. However, in *A. gambiae* cells that were depleted for Ago2 the NR1ΔB2 construct could accumulate to high levels (Li et al., 2004). This was the first demonstration that an RNAi gene plays an antiviral role in mosquitoes. Further work has shown that antiviral

RNAi also functions in adult mosquitoes. *Ae. Aegypti* that were depleted for Dcr-2 by transgenic hairpin RNA and infected with SINV showed increased virus titers as well as lower rates of survival as compared to non-transgenic flies (Khoo et al., 2010). It has also been shown that long dsRNA injection into adult mosquitoes has been a successful means of mRNA depletion. This technique has been used to demonstrated that mosquitoes depleted for Tudor staphylococcal nuclease (TSN), Ago2, R2D2 and Dcr-2 by dsRNA show an increase in both viral mRNA levels and virus particle production for both DENV and SINV (Campbell et al., 2008; Sanchez-Vargas et al., 2009).

viRNAs have also been shown to be a key component of antiviral RNAi is mosquitoes providing another line of evidence that Dicer is an important component of this pathway. The first reported viRNAs in mosquitoes were found in SINV infected adult *Ae. Aegypti* (Campbell et al., 2008). Both plus strand and minus strand viRNAs were detected in these infected animals with the plus strand viRNA being the more abundant species. Later studies in the Aag2 cell line infected with SINV confirmed the results found in the adult mosquitoes (Cirimotich et al., 2009). This study also showed that B2 expression in conjunction with SINV decreased the level of SINV viRNAs. *Aedes Albopictus* and *Culex tritaeniorhynchus* either injected with SINV expressing the B2 protein or fed on blood meals loaded with the same virus had a decrease in the survival of the infected population, as compared to infection with a non-B2 expressing SINV strain, highlighting the importance of the viRNAs in controlling virus infection (Cirimotich et al., 2009). viRNAs have also been found by Northern blotting in DENV infected Aag2 cells and by deep sequencing of WNV infected *Culex pipiens quinquefasciatus* adults

(Brackney et al., 2009; Sanchez-Vargas et al., 2009). In both these cases, as in the case of SINV, both plus and minus strand viRNAs were detected with the vast majority of the viRNAs being plus stranded. Although a bounty of work has been done to elucidate the antiviral RNAi pathway in mosquitoes more work need to be done to better understand this pathway as well as to investigate whether arthropod born viruses that mosquitoes traffic encode VSRs.

1.4.2 Antiviral RNAi in Ticks and Shrimp

Similar antiviral research that was done in mosquitoes has also been conducted in cultured tick cells. Using a VIGs approach researchers we able to use a genetically modified Semliki forest virus (SFV) replicon carrying a segment of Hazara virus (HAZV) to silence HAZV in Tick cell culture (Garcia et al., 2005). These infected Tick cell also accumulated SFV and HAZV specific viRNAs indicating that these two viruses induced an antiviral RNAi response. Also as in mosquitoes, the RNAi response in ticks can also be suppressed by VSRs. A study has shown that the NS1 protein of influenza and two plant VSRs, NSs of Tospovirus and HC-Pro of Zucchini yellow mosaic virus, can suppress the antiviral response in ticks resulting in a higher accumulation of viral mRNAs (Garcia et al., 2006).

Antiviral research has also been going on in shrimp as yield loss due to viruses is a source of great annual financial loss in the shrimp aquaculture industry. Functional knockdown of hemocyanin and CDP mRNA, both endogenous genes, by dsRNA injection has been shown in *Litopenaeus vannamei*, however when siRNAs targeting the same genes are used no knockdown could be detected (Robalino et al., 2006). This data

indicated that exogenous RNAi, which has been shown to be antiviral in other arthropods, is functional in shrimp. Furthermore, when dsRNA targeting viral sequences are injected into shrimp a reduction in the mortality rate is observed in virus infected shrimp. The mortality rate was unaffected in infected shrimp that were injected with non-viral dsRNAs. This was observed for three different viruses; White soot syndromes virus (WSSV), Taura syndrome virus (TSV), and yellow head virus (THV) (Robalino et al., 2005; Tirasophon et al., 2005). As was the case with endogenous gene silencing, siRNAs targeting viral sequences could not reduce mortality rates in infected shrimp (Robalino et al., 2005). As in other organisms, viruses in shrimp have been shown to be able to block exogenous RNAi. When shrimp that are infected with WSSV are injected with hemocyanin dsRNA, no hemocyanin mRNA knockdown could be detected, indicated that WSSV has the capacity to suppress RNAi (Robalino et al., 2007). The same study showed that TSV could not suppress dsRNA knockdown of hemocyanin. RNAi components have also been characterized in shrimp. A Dicer protein has been identified in the shrimp species *Penaeus monodon* and was named Pm Dcr-1 (Su et al., 2008). When this gene is knocked down in shrimp and then subsequently infected with Gill associated virus (GAV), there is an observable increase in viral mRNA levels which is associated with an increase in the mortality rate. Also of interest, q-PCR analysis of infected shrimp showed an increase in Pm Dcr-1 mRNA levels as compared to uninfected shrimp (Su et al., 2008). This is the first evidence of an RNAi component being transcriptional induced by a virus.

dsRNA seems to trigger a non-sequence specific immune response in shrimp which can lead to reduced levels of mortality rates in infected shrimp (Robalino et al., 2004). This was observed in *L. vannamei* shrimp infected with WSSV as well as shrimp infected with TSV. The reduced mortality rates that were observed were much lower than those found when dsRNA with viral specific sequences were used. These results were also recapitulated in *P. monodon* adults as well as primary cell culture infected with Yellow head virus (YHV) (Tirasophon et al., 2005; Yodmuang et al., 2006). These findings are akin to those found in mammalian systems where dsRNA serves as a PAMP to trigger IFN responses, RIG-I as well as Toll-like receptor mediated immunity.

1.4.3 Antiviral RNAi in *C. elegans*

C. elegans was the first invertebrate in which dsRNA induced RNAi was discovered (Fire et al., 1998). Since then it was revealed that *C. elegans* encoded a very complex yet elegant RNAi pathway. Firstly, it only encodes one Dicer protein, which like in mammals is responsible for all of the Dicer dependent siRNAs and miRNAs. *C. elegans* encodes the most expansive family of Argonaute proteins found in one organism, comprised of 27 family members. As there is only one Dicer protein, it is the Argonaute family of proteins that determine the specificity and function of the small RNAs (Yigit et al., 2007). And lastly like in plants, nematodes encode RNA dependent RNA polymerase (RDRP) proteins that are required for both systemic and transitive RNAi (Voinnet, 2005). The function of the RDRPs in *C. elegans* is tissue specific, with RRF-1 required for RNAi in the soma and EGO-1 in the germline. A third RDRP, RRF-3, was first shown to be a negative regulator of RRF-1 and EGO-1. Subsequent experiments have shown that

this RDRP acts in a parallel pathway which competes for access to Dicer in order to generate a 26nt endogenous siRNA required for spermatogenesis and zygotic development (Han et al., 2009).

Several strategies were taken to study antiviral RNAi in *C. elegans*. One of these methods was using a primary cell culture system infected with Vesicular stomatitis virus (VSV). Not only did VSV replicate in these cells, but cell cultures derived from *rde-4, rde-1, rrf-1,* and *rde-3* mutant worms showed enhanced VSV replication (Wilkins et al., 2005; Schott et al., 2005). Also of note was that cell cultures derived from *rrf-3* and *eri-1* mutant lines had lower levels of viral replication as compared to N2 cell cultures which is in line with results describing the exogenous RNAi pathway in worms. A second strategy used to test antiviral RNAi in worms was creating transgenic lines carrying the FHV genome as a replicon (Lu et al., 2005). N2 worms with the transgene were able to accumulate viral RNAs, furthermore when put into the *rde-1* background (an Argonaute mutant) the viral RNAs accumulated to much higher levels than in N2 worms. This study also showed that the FR1ΔB2 mutant could not replicate in N2 worms but could be rescued in the *rde-1* mutant. This is very similar to FR1ΔB2 being able to replicate in *Drosophila* S2 cells in which Ago2 has been knocked down. Further studies in transgenic worms carrying the FHV replicon showed that *drh-1*, and not *drh-2*, is required for antiviral RNAi (Lu et al., 2009). This is of note as previous reports in exogenous RNAi studies placed these genes as being redundant to each other.

1.5 Small Molecules and RNAi

Several small molecules that interact with the RNAi pathway have been identified in mammalian systems. One group used a HeLa cell line carrying a modified luciferase mRNA that had a miR-21 target site inserted in the coding region of the mRNA (Gumireddy et al., 2008). The system led to the identification of the small molecule diazobenzene, which led to a 5-fold increase in luciferase activity. However, upon further analysis, this small molecule not only reduced mature miR-21 levels but also reduced the levels of the pri-miRNA transcript. This indicates that this small molecule affects the transcription of pri-miRNA 21 and does not target the core RNAi machinery. In two separate studies using HEK293 cell, the small molecule enoxacin was found to be able to enhance siRNA guided mRNA degradation as well as enhance miRNA processing (Shan et al., 2008; Zhang et al., 2008). Furthermore, enoxacin was found to be able to promote the binding of RNA to TRBP, which could explain the enhancement of miRNA production (Shan et al., 2008). A third small molecule isolated from a screen using a chemical library of ATP analogues in HeLa cells was found to be inhibitory to RNAi (Chiu et al., 2005). This small molecule was found to be able to block the unwinding of siRNAs which prevented the loading of siRNAs into the RISC complex.

1.6 Conclusion

Put together, the available data in invertebrate animals have demonstrated that RNAi plays an important role in controlling virus infection. At the same time however viruses exert control of the RNAi pathway through the use or VSRs. The resulting picture is a

genetic arms race in which host and pathogen are battling each other to insure their own survival.

Invertebrates have evolved a complex antiviral RNAi pathway is order to control and stem the tide of invading viral pathogens. In insects specifically, antiviral RNAi appears to be the major antiviral pathway present as loss of it is detrimental to the survival of the organism. In more complex arthropods like shrimp there appears to also be an IFN-like pathway to fight virus infection in addition to RNAi, although RNAi is the more potent antiviral pathway in this system.

The body of work in this field has increase exponentially since the first reports of antiviral RNAi in S2 cells came out in 2002. Even with this large body of work published there are still many questions that have yet to be answered. There has been evidence that the piRNA pathway may play an antiviral role. However more work needs to be done to test whether or not this pathway is truly antiviral or if the data observed is an artifact of having a deficiency in an important transposon defense pathway. Another area that requires more work is in mosquitoes. Do mosquito vectored viruses encode VSR? Does RNAi reduce transmission of mosquito born viruses? And if so would mosquitoes overexpressing key RNAi components reduce transmission of these viruses even further?

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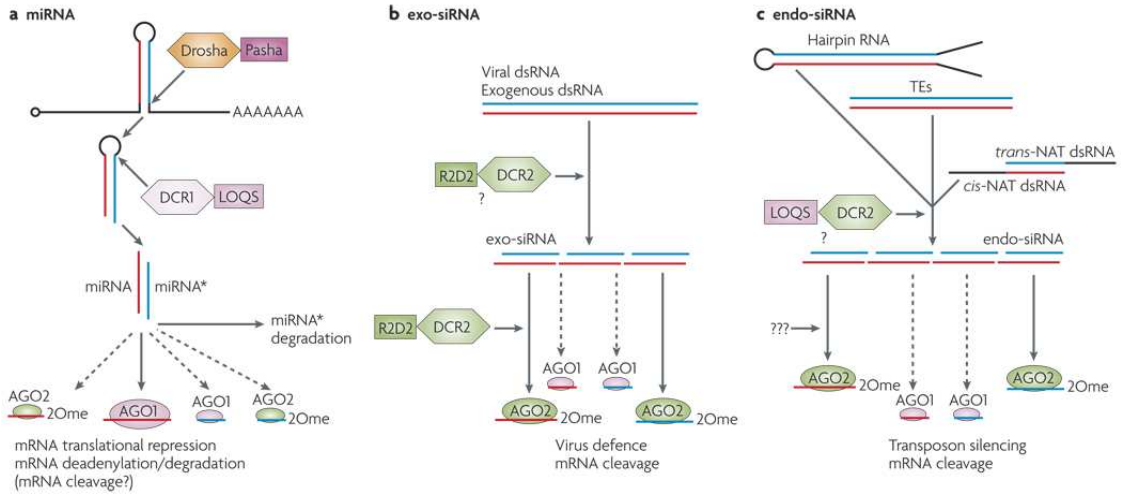
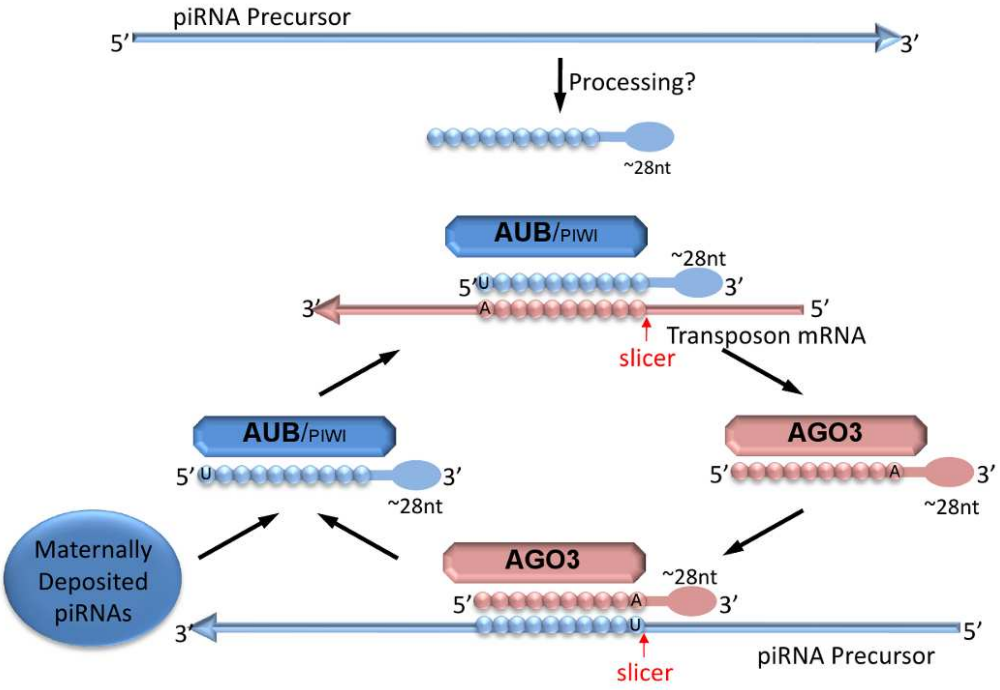


From Okamura and Lai, 2005

Figure 1.1 Dicer dependent small RNA pathways in *Drosophila*

(A) The miRNA pathway requires the activity of Drosha and Pasha to release the hairpin pre-miRNA precursor form the pri-miRNA. Dicer-1 and Loqs then cut the mature miRNA out of the pre-miRNA which is then unwound and loading into miRISC, which Argonaut 1 is a main component. (B) The exogenous RNAi pathway is triggered by exogenously introduced dsRNA which is recognized and cleaved by Dicer-2 and R2D2. The siRNAs that are generated by this process are preferentially loading into the Argonaut 2 siRISC complex which them mediates slicing of the target mRNA. (C) Much like the exogenous RNAi pathway, the endogenous RNAi pathway employs Dicer-2 to cleave endogenously produces dsRNA. However Loqs, and not R2D2, serves as the binding partner to Dicer-2 in this process. The siRNAs that are produced here are the loaded into Argonaut 2 which mediates slicing of the target mRNA.



From Thomson and Lin, 2009

Figure 1.2 The "Ping-Pong" model for piRNA biogenesis

The Ping-Pong model involves successive rounds of slicing by Aub and Ago3 to generate piRNAs. As Dicer is not involved in this pathway it is believed the slicing in responsible to generate the primary piRNA from the piRNA precursor, however there has been no evidence to support this hypothesis thus far.

Chapter 2: Small Molecule Screening Reveals Inhibitors of Antiviral Activity in *Drosophila* cell culture

Abstract

RNA interference, or RNAi, has been a well-documented means in which plants and invertebrate animals can fend off invading viral pathogens. Our laboratory has previously established RNAi as an effective means of antiviral defense against viruses such as Flock house virus and Cricket paralysis virus in *Drosophila melanogaster* S2 cells. Here we describe a small molecule screen performed in S2 cells looking for inhibitors of RNAi. We used a modified Flock house virus amplicon derived from FHV RNA 1 made to express GFP from the RNA 3 open reading frame, called R1gfp. Approximately twenty small molecules were identified in the primary screen with five molecules confirmed via secondary screening. These molecules were named RNAi inhibitors, RIN for short.

2.1 Introduction

Flock house virus (FHV) is in the genus *Alphanodavirus* and a member of the family *Nodaviridae* (Ball and Johnson, 1998). FHV has a wide host range and has been shown to be able to infect insect cells, mammalian cells and adult fruit flies while viral RNAs are able to replicate in yeast, plants and nematodes (Ball et al., 1992; Lu et al., 2005; Price et al., 1996; Selling et al., 1990). The FHV particle encapsulated a bipartite, positive sense RNA genome that is 4.5kb in length (Schneeman et al., 1998). RNA 1 encodes protein A and is 3107 nt in length. Protein A is an RNA-dependent RNA polymerase (RdRp) that the virus uses for both replication of the viral genome as well as the transcription of viral mRNAs. RNA 2 is 1400 nt in length and encodes in precursor to the coat protein. As the virus replicates in a host cell a third viral RNA, called RNA 3, is produced through a subgenomic promoter located on the 3' end of RNA 1 (Ball and Johnson, 1998). RNA 3is 387 nt in length and encodes the B2 protein which is a potent viral suppressor of RNAi (VSR) (Li et al., 2002). Two point mutation introduced in the B2 open reading frame (T2739C and C2910A) can deactivate the protein making it unable to replicate to high levels in both cell culture as well as adult flies (Li et al., 2002; Wang et al., 2006).

As antiviral RNAi is a potent means of clearing virus infections, viruses have adapted by evolving proteins that can inhibit this pathway called VSRs. Many plant and invertebrate viruses encode at least one or more VSRs (Li and Ding, 2006; Wu et al., 2010). As mentioned above, FHV encodes a single VSR named B2. B2 was discovered in 2002 and was shown to be active in *Nicotiana benthamiana* and *Drosophila* S2 cells (Li

et al., 2002). Transcripts generated from FHV RNA 1containing a B2 deletion mutant, called FR1ΔB2, are unable to replicate in *Drosophila* cell culture and embryos, while the wild-type FR1 can replicate to high levels under the same conditions. FR1ΔB2 viral RNAs can accumulate in S2 cells in which Ago2 has been depleted or in *ago2* or *dcr-2* mutant embryos (Li et al., 2002; Wang et al., 2006). From this data it can be inferred that B2 is not required for virus replication but is required for suppression of the antiviral RNAi. Work performed using FHV virions carrying the ΔB2 mutation showed that these particles cannot successfully infect S2 cells proving that B2 is important for completion of the virus' life-cycle (Aliyari et al., 2008).

Several VSRs have been shown to be able to bind to dsRNA or have a dsRNA binding domain. B2 was shown to be able to bind to both duplex siRNAs and long dsRNA *in vitro* (Lu et al., 2005). A R54Q mutation in B2 can prevent the binding of both these RNA species. This dsRNA binding activity can inhibit Dicer processing of long dsRNA *in vitro* as the same R54Q mutation of that abolished dsRNA binding also abolished B2's ability to block siRNA biogenesis mediated by *Drosophila* Dcr-2 (Lu et al., 2005). Furthermore, structural analysis of the B2 protein via crystallization and NMR analysis have revealed a dsRNA binding domain on the N terminal region of the protein (Chao et al., 2005; Lingel et al., 2005). Recently it has been shown that the FHV and Nodamura virus (NoV) B2 proteins can bind viral specific dsRNA *in vivo* (Aliyari et al., 2008).

There have been several small molecules that have been shown to both inhibit and enhance RNAi. One of these inhibitors was diazobenzene which was identified in a

screen of approximately 1000 compounds found in a library of pharmacologically active molecules screened against a HeLa cell line carrying a modified luciferase mRNA that had a miR-21 target site inserted in the coding region (Gumireddy et al., 2008). When these HeLa cells were treated with diazobenzene there was a 5-fold increase in the luciferase activity levels. Diazobenzene not only reduced the levels of mature miR-21 but also reduced the levels of the pri-miRNA transcript, indicating that diazobenzene effects the transcription of pri-miRNA 21 and does not inhibit the core RNAi pathway. In two separate studies using a HEK293 cell line expressing a short hairpin RNA targeting GFP mRNA, the small molecule enoxacin was found to be able to enhance mRNA degradation as well as mature miRNA levels (Shan et al., 2008; Zhang et al., 2008). The miRNA phenotype that was observed was reported to be due to enoxacin being able to promote the binding of RNA to TRBP, which could explain this observation (Shan et al., 2008). Another screen using a private library of ATP analogues was used to identify the small molecule ATPA18 (Chiu et al., 2005). This screen used HeLa cells co-transfected with both a plasmid that expressed eGFP mRNA and eGFP specific siRNAs. ATPA18 was found to be able to inhibit the unwinding of siRNAs which in turn prevented siRNAs to be loaded into the RNA induced silencing complex (RISC).

Using a S2 cell line stably transfected with the R1gfp amplicon we screened a commercially available library of 10,000 compounds looking for chemicals that can allow for the replication of the amplicon, visualized by GFP florescence. Approximately twenty chemicals were isolated from the primary screen with five of those chemicals

being confirmed through secondary screening and Northern blot analysis. The chemicals that were identified from this screen were and named RNAi inhibitors or RIN for short.

2.2 Materials and Methods

2.2.1 Small Molecule Screen

The stable R1gfp cell line used in the screen was generated as follows; *Drosophila* S2 cells were co-transfected with the pR1gfp and pCoHygro (Invitrogen, CA) plasmids at a ratio of 19:1. Two days after co-transfection of the plasmids, hygromycin B was added into the medium to a final concentration of 500 µg/ml to select for cells that have taken-up pCoHygro, as this plasmid confers resistance to hygromycin B which is normally lethal to S2 cells. The cells were passed onto a new plate with the hygromycin B selective medium every 3-4 days. The stably transformed R1gfp cells were collected after 3 weeks of selection, and maintained in the selective medium. R1gfp cells were then seeded in a tissue culture treated 96 well plate (Product No. 3603 Corning) at a concentration of 1.0-3.5 x 10^4 cells per well in Schneider's insect medium (Product No. S9895 Sigma-Aldrich, Inc. St. Louis, MO, USA) using a Biomek 3000 fluid handling robot (Beckman Coulter). DMSO was added to the cells at a final concentration of 1% to increase cell permeability. Compounds from the ChemBridge Diverset library were then added to the plates using the Biomeck robot's pintool at a final concentration of 20-40 µM. Plates were then incubated in a 27°C incubator for 24 hours. Following the incubation period 0.5µl of $CuSO_4$ was added to each well to induce the transcription of the R1gfp plasmid. Plates were then put back into the 27°C incubator for 2 days to allow for viral replication. Following the incubation period wells were screened for presence of fluorescence via

microscopy. Once lead molecules have been identified secondary screening was conducted in 12 well plates using the same S2 cell line and protocol as described for the primary screen with chemical concentrations at 30µM. Following visual confirmation of GFP fluorescence, RNA was extracted and run out on gels for Northern blot analysis (as described below).

2.2.2 Structure verification of RIN compounds

Electrospray ionization-mass spectrometry experiments were carried out on an LCQ Deca XP ion-trap mass spectrometer (ThermoFinnigan, San Jose, CA). Dimethyl sulfoxide was used as solvent for electrospray, and a 2µL aliquot of a ~5µM sample solution was injected in each run. The spray voltages were 4.5 kV and 3.4 kV in positive- and negative-ion modes, respectively, and the temperature for the ion transport tube was maintained at 200ºC. ^{1}H-NMR spectra were recorded on a Varian Inova 300MHz instrument (Palo Alto, CA). The residual proton signal of the solvent serves as an internal reference.

2.2.3 RNA extraction from S2 cells

Total RNA was extracted by using TRIzol (Invitrogen Life Technologies, Burlington, Ontario, Canada) according to the TRIzol protocol with some modifications. 1mL of S2 were collected in a microcentrifuge tube and spun down to separate the cells from the media. The cell pellet was then washed with 500µl of PBS. 500 µl of TRIzol reagent was added to each tube and the tubes were vortexed of 20 seconds and incubated for at least 5 minutes at room temperature. 200 µl of chloroform was added into each tube and the samples were mixed by vortexing for 20 seconds. After incubation at room temperature

for 2 to 3 minutes, the mixture was then spun at 14,000 rpm for 15 minutes at room temperature. The upper aqueous layer was then transferred to a new tube and 300µl of isopropyl alcohol was added to each tube. After incubation for at least 30 minutes at -20°C, the samples were spun at 14,000 rpm for 15 minutes at 4°C to pellet the RNA. The supernatant was carefully removed and the RNA pellets were washed with 1ml of 70% ethanol, air dried for 10-15 minutes and then resuspended in 30µl of RNase-free water. The RNA quality and concentration was checked by using a Biophotometer.

2.2.4 Northern blot analysis

Total RNA was run on a 1.2% (v/v) formaldehyde-denaturing gel. For a 15 cm x 20 cm gel-casting tray (Amersham Phamacia Biotech), 2.4 g of agarose was added into 174 ml of RNase-free water and heated in a microwave oven until the agarose was dissolved. 20 ml of 10 x MOPS buffer (Table 2-1) and 6 ml of formaldehyde were added and mixed thoroughly after the agarose cooled down to 60°C. The total 200 ml solution was poured into the gel-casting tray and allowed to cool down. After the gel solidified, the gel tray was put in a MAX HORIZ SUB system (Amersham Phamacia Biotech) containing 1x MOPS buffer. While the gel was cooling down, 5 µg of each RNA sample was mixed with 7.8 µl sample buffer (Table 2-1), heated at 65°C for 10 min and cooled on ice. After adding 3 µl of 6x loading buffer (Table 2-1), the RNA sample was loaded into the 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 way down the gel. After electrophoresis, gels were washed in 10x SSC (Table 2-1) and the RNA was transferred onto a Hybond-N+ membrane (Amersham Bioscience) using a VacuGene XL transfer unit (Pharmacia Biotech) for 3 hours according to

manufacturer's instructions. After the transfer was complete the membrane was briefly washed in 10x SSC and then cross-linked in a XL-1000 UV crosslinker (SPECTROLINKERTM, 180mj/cm2). To certify equal loading of RNA samples, the membrane was stained with methylene blue solution (Table 2-1) to visualize the ribosomal RNAs. The stained membrane subjected to prehybridization (Table 2-2) at 65 °C for at least one hour in a hybridization oven (Amersham Pharmacia Biotech). During pre-hybridization, α-32P-dCTP-labeled probe was prepared using a RediprimeTMII kit (Amersham). Specifically, 25 ng of a DNA template was added into 45 µl 1xTE buffer (final volume, Table 2-1) in a 1.5 ml microcentrifuge tube, boiled for 5 minutes, chilled on ice for 5 min, and spun down. The template was then transferred to reaction tube and 4 µl of α-32P-dCTP (10 mCi/ml, PerkinElmer) was added and mixed. The reaction was incubated at 37°C for 20 min. Finally, the reaction tube was boiled for 5 minutes, chilled on ice for 5 minutes, and half of the reaction was added to the hybridization tube. Hybridization continued at 65°C overnight. The membrane was washed once in 2x SSC/0.1% SDS at 65°C for 20 min, and then twice in 0.2x SSC/0.1% SDS for 20 min. The membrane was then sandwiched in plastic membranes, and exposed to X-ray film (HyBlot ClTM, DENVILLE Scientific Inc.) for an amount of time depending on the signal intensity then developed by a Medical film processor QX-70 (KONICA). If re-probing was needed, the membrane was stripped by incubating in stripping solution (Table 2-1) at 80 °C for 30 minutes with gentle shaking in the hybridization oven. After stripping, the membrane was rinsed in distilled water and re-used for prehybridization.

2.3 Results

2.3.1 Primary Screen in R1gfp Cells Identifies Twenty Hit Compounds

In order to identify small molecule inhibitors of RNAi we conducted a chemical screen using Flock house virus (FHV) replication as a read-out for silencing. The FHV genome encodes two RNAs; RNA 1 encodes the viral replicase while RNA 2 encodes the coat protein (Ball and Johnson, 1998). Through the course of replication RNA 1 produces the subgenomic RNA 3 that encodes the B2 protein, a viral suppressor of RNAi. Previous studies have shown that FHV carrying a mutation in the B2 ORF is no longer able to suppress RNAi and that said virus is no longer capable of replicating in *Drosophila* S2 cells as well as adult flies (Aliyari et al., 2008; Wang et al., 2006). However viral replication can be rescued with co-transfection of a plasmid encoding the B2 protein or by dsRNA targeting the Ago2 gene (dsAgo2), a key component of the antiviral RNAi pathway (Li et al., 2002). In a previous study conducted in our laboratory, the B2 mutant was simulated by insertion of the GFP coding region in place of B2, called FR1gfp (Li et al., 2004). This substitution not only generates a B2 null mutant but also provides a visual read-out for successful virus replication via RNAi inhibition. This construct is unable to replicate in wild-type S2 cells, however when introduced in cells where RNAi has been rendered defective, this construct can replicate which will give rise to subgenomic RNA 3 which will in turn lead to GFP fluorescence (Lu et al., 2009; Wang et al., 2006). This system has been used to identify VSRs including CrPV's 1A protein as well as the NS1 protein from influenza virus (Li et al., 2004; Wang et al., 2006).

With slight modification, this construct was made amenable to screening by creating a stable cell line carrying the viral amplicon, called R1gfp (Figure 2.1a). R1gfp cells were seeded in 96-well plate format and 10,000 commercially available compounds from the ChemBridge Diverset library were added to each well using the Biomek 3000 fluid handling robot at a concentration of 20-40µM. Cells were allowed to grow for 24 hours at 27ºC and then the R1gfp amplicon was induced with the addition of 0.5M copper sulfate. After a second incubation period at 27ºC, this time for 48 hours, wells were examined for GFP florescence with an IMT-5 inverted microscope (Olympus). Cells treated with only DMSO, the carrier that the compounds were diluted in, did not show any GFP florescence (Figure 2.1b, left panel). As expected, in dsAgo2 transfected cells there was abundant GFP florescence, indicating that viral amplicon replication had occurred (Figure 2.1b, right panel). The primary screen resulted in the identification of twenty compounds that were able to allow for GFP florescence to emerge (Figure 2.1b, middle panel). The twenty compounds varied in both structure and molecular weight (Figure 2.1c).

2.3.2 Secondary Screening Results the Identification of the RIN compounds

The lead compounds isolated in the primary screen were then used to conduct a secondary screen, this time in a 12-well plate format. Using the same R1gfp cell line as in the primary screen, cells were seeded in 12-well plate format at a chemical concentration of 30µM with the twenty lead compounds that were shown in the primary screen to rescue GFP florescence, the carrier DMSO was used as a control. After chemical treatment, cells were incubated for 24 hours at 27ºC, and then the R1gfp amplicon was

induced with the addition of 1µl of 0.5M copper sulfate. After a 48 hour incubation, cells were collected and total RNA was extracted for Northern blot hybridization analysis. Northern blotting revealed that not all the compounds from the primary screen could rescue R1gfp replication (Figure 2.2a). However, five of the compounds allowed for the accumulation of viral RNAs to much higher levels as compared to cells only treated with DMSO (Figure 2.2a; compare lane 2 to lanes 1, 7, 8, 10 and 12). These five compounds hits and were named RINs 1-5, short for RNAi inhibitors (Figure 2.2b). RIN1 corresponds to 1-[3-(benzyloxy)-4-methoxybenzyl]-4-[(4-methylphenyl)sulfonyl]piperazine and has a molecular weight of 467. RIN2 is 1-(3-chloro-4-methylphenyl)-4-[4-(diethylamino)-2-hydroxybenzylidene]-3,5-pyrazolidinedione and has a molecular weight of 400. RIN3 is 3-(2-methyl-1H-benzimidazol-1-yl)-1-(3-methylphenyl)-2,5-pyrrolidinedione and has a molecular weight of 319. RIN4 corresponds to 3-(2-furylmethyl)-2-(9-methyl-9H-carbazol-3-yl)-2,3-dihydro-4(1H)-quinazolinone and has a molecular weight of 407. Finally, RIN5 is 1-(4-bromophenyl)-3-(2-ethoxyphenyl)-2-propen-1-one and has a molecular weight of 331. Their structures of the RIN compounds were validated using NMR and electrospray ionization-mass spectrometry.

Following identification of the RIN compounds, we tested whether any of them exhibited cytotoxic effects in S2 cells. To do this, S2 cells were treated with each RIN compound at a concentration of 30µM each and then the cells were allowed to grow for two days at 27ºC. Following the two day incubation cells were stained with an equal volume of 1% Evans blue dye. Evans blue selectively stains dead cells has healthy cells

can actively pump this dye out, making it an ideal candidate cytotoxic effect assays. Compared to the non-treated and DMSO controls, RINs 1, 2, 4 and 5 showed no significant difference in cell survival (Figure 2.2c). However, RIN3 showed a significant decrease in cell survival with survival rates dropping to around 35% in RIN3 treated S2 cells. With RINs 1 through 5 in hand and the cytotoxic effects known, they were again used to treat R1gfp cells to reconfirm their effects of viral RNA replication. R1gfp cells were seeded and then treated with 30μM of each RIN compound and then incubated for 24 hours. R1gfp was then induced with copper sulfate and allowed to replicate for 48 hours when cells were then collected and total RNA extracted for Northern blot hybridization. In non-treated or DMSO treated cells, only very low levels of viral RNAs were detected (Figure 2.2d, lanes 2 and 3). RINs 1-5 were confirmed to be able to rescue viral RNA replication, but each RIN was able to do so at different levels, with RINs 4 and 5 being the most effective (Figure 2.2d, lanes 1 and 4-7).

2.4 Discussion

Previous work from our laboratory has shown that GFP expressing FHV constructs can be a powerful tool for detection of novel VSRs and well as a screening tool to find new genes involved in antiviral RNAi in both *D. melanogaster* and *C. elegans* (Li et al., 2004; Lu et al., 2009; Wang et al., 2006). In fruit flies FR1gfp was used to show that the 1A protein of CrPV had VSR activity while in nematodes it was used to identify DRH-1 as a component of antiviral RNAi in worms. In this study R1gfp cells were used to screen a commercially available chemical library and led to the identification of twenty compounds in the primary screen. Secondary screening via Northern blot analysis narrowed down the twenty lead compounds that were identified in the primary screen to

identify five chemicals which actually rescued viral RNA levels in S2 cells. These five compounds were named RNAi inhibitors, or RIN for short. RINs 1, 2, 4 and 5 showed no cytotoxic effect in S2 cells as visualized by Evans blue staining, but cells had a strong cytotoxic response to RIN3 which dropped cell survival to ~35%. Finally, the five RIN compounds were able to rescue R1gfp RNA levels to a different degree, indicating that they may be targeting different components of antiviral pathways in *Drosophila*.

As compared to previous small molecule screens that used artificially engineered RNAi systems, such as shRNAs and siRNAs, this screen used a viral derived amplicon system that was modified to visually represent virus replication (Chiu et al., 2005; Shan et al., 2008; Zhang et al., 2008). The benefit of using this system is that all steps of RNAi can be assayed in one screen, as opposed to using siRNA induced RNAi which has a very narrow window. Compared to shRNA induced RNAi, the R1gfp system has the potential of finding small molecules that act upstream of small RNA biogenesis, where a shRNA first triggers the RNAi pathway.

In conclusion, the work presented here provides strong evidence that the R1gfp cells can be used as a screening platform to identify small molecule inhibitors of antiviral pathways in *Drosophila,* being able to use this system from primary screening all the way the lead compound validation. As there is much overlap between antiviral RNAi and the exogenous RNAi pathway in fruit flies there is a high probability that the RIN compounds that were isolated in this screen can in fact inhibit the RNAi pathway. Further work will need to be done to prove if that is this case or not using the many assays that *Drosophila* offers. Potential applications of the compounds that were pulled out of viral

amplicon based screens are as pesticides to deal with pathologically important pests to both plants and animals. As chemicals that are isolated from this screen are able to increase viral RNA levels, it is possible to envision that when applied to insects they would increase viral RNA levels to lethal levels as has been shown in RNAi mutant flies (Chotkowski et al., 2008; Galina-Arnoux et al., 2006; van Rij et al., 2006; Wang et al., 2006; Zambon et al., 2006). Again, more work will need to be done to determine if RIN treated flies can phenocopy RNAi mutant flies.

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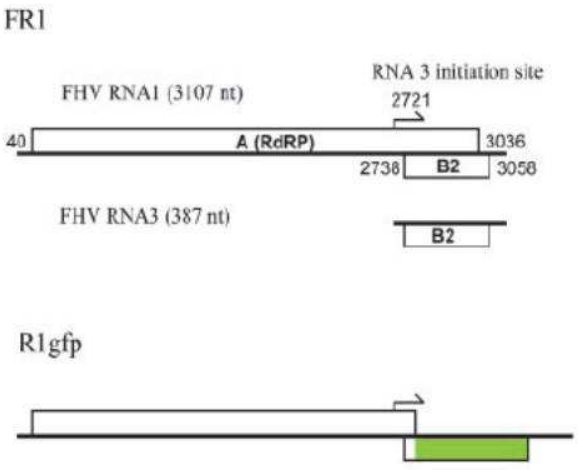
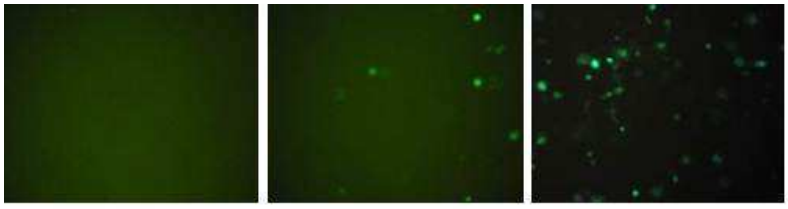
Figure 2.1 Primary Chemical Screen Using the R1gfp Amplicon

(A) Genome organization of FR1 as compared to R1gfp. The B2 open reading frame has been replaced with that of eGFP, which allows for the visualization of virus replication in S2 cells. (B) Detection of green fluorescent S2 cells hits were scored as compared to S2 cells in which no replication has occurred (DMSO) or dsAgo2 transfected cells. (C) Chemical structures of the twenty lead compounds isolated in the primary screen.

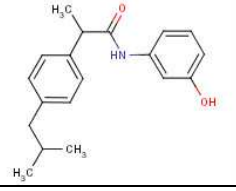
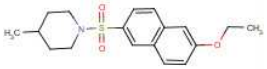
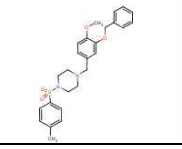
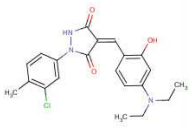
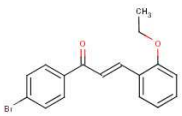
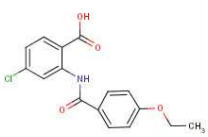
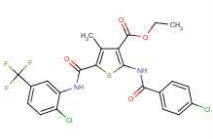
A.


FR1
RNA 3 initiation site
FHV RNA1 (3107 nt)
2721
40
A (RdRP)
3036
2738
B2
3058
FHV RNA3 (387 nt)
B2
R1gfp

B.


DMSO
Hit
dsAgo2

C.

Chemical Structure	Molecular Weight	Chemical ID	Name
	224	5112021	N-1-adamantyl-N'-methylthiourea
	297	5567157	N-(3-hydroxyphenyl)-2-(4-isobutylphenyl)propanamide
	333	5792670	1-[(6-ethoxy-2-naphthyl)sulfonyl]-4-methylpiperidine
	202	5930490	6-methyl-4-phenyl-2(1H)-pyrimidinethione
	467	5956517	1-[3-(benzyloxy)-4-methoxybenzyl]-4-[(4-methylphenyl)sulfonyl]piperazine
	400	5990167	1-(3-chloro-4-methylphenyl)-4-[4-(diethylamino)-2-hydroxybenzylidene]-3,5-pyrazolidinedione
	331	6032376	1-(4-bromophenyl)-3-(2-ethoxyphenyl)-2-propen-1-one
	320	6038111	4-chloro-2-[(4-ethoxybenzoyl)amino]benzoic acid
	545	6065887	ethyl 2-[(4-chlorobenzoyl)amino]-5-({[2-chloro-5-(trifluoromethyl)phenyl]amino}carbonyl)-4-methyl-3-thiophenecarboxylate

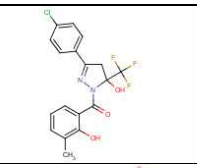
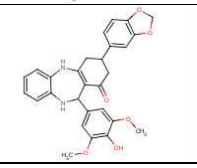
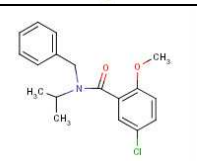
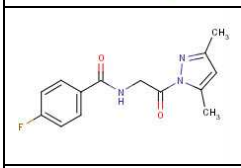
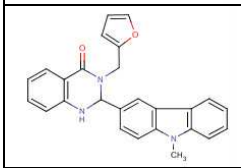
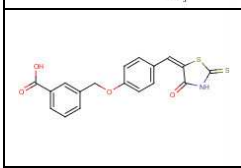
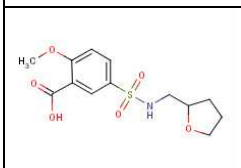
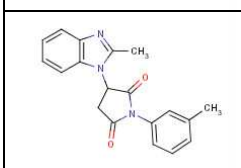
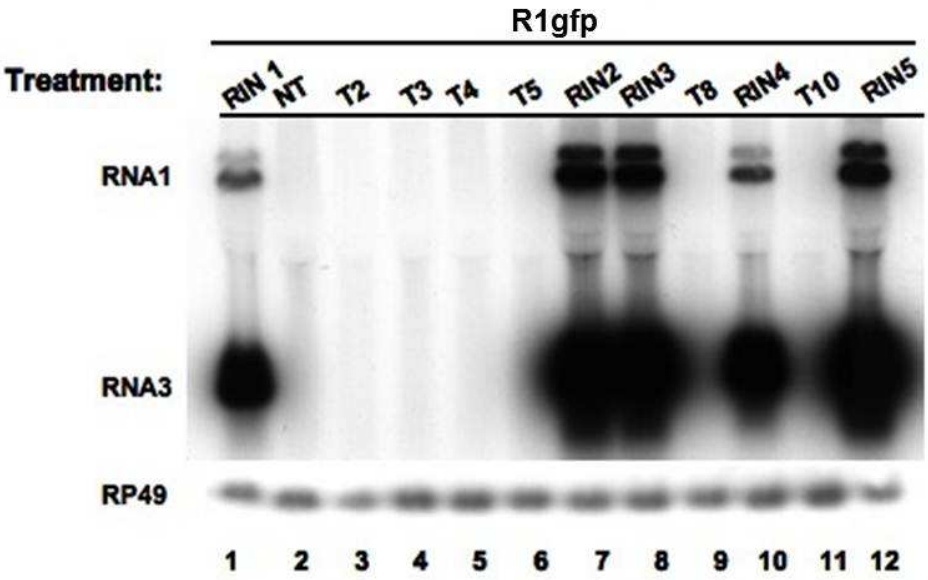
	399	6069394	3-(4-chlorophenyl)-1-(2-hydroxy-3-methylbenzoyl)-5-(trifluoromethyl)-4,5-dihydro-1H-pyrazol-5-ol
	487	6073825	3-(1,3-benzodioxol-5-yl)-11-(4-hydroxy-3,5-dimethoxyphenyl)-2,3,4,5,10,11-hexahydro-1H-dibenzo[b,e][1,4]diazepin-1-one
	462	6128740	N-(4-bromophenyl)-4-(2,3-dimethoxyphenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydro-5-pyrimidinecarboxamide
	318	6129509	N-benzyl-5-chloro-N-isopropyl-2-methoxybenzamide
	275	6130400	N-[2-(3,5-dimethyl-1H-pyrazol-1-yl)-2-oxoethyl]-4-fluorobenzamide
	407	6157070	3-(2-furylmethyl)-2-(9-methyl-9H-carbazol-3-yl)-2,3-dihydro-4(1H)-quinazolinone
	371	6230372	3-({4-[(4-oxo-2-thioxo-1,3-thiazolidin-5-ylidene)methyl]phenoxy}methyl)benzoic acid
	315	6240118	2-methoxy-5-{[(tetrahydro-2-furanylmethyl)amino]sulfonyl}benzoic acid
	319	6240967	3-(2-methyl-1H-benzimidazol-1-yl)-1-(3-methylphenyl)-2,5-pyrrolidinedione

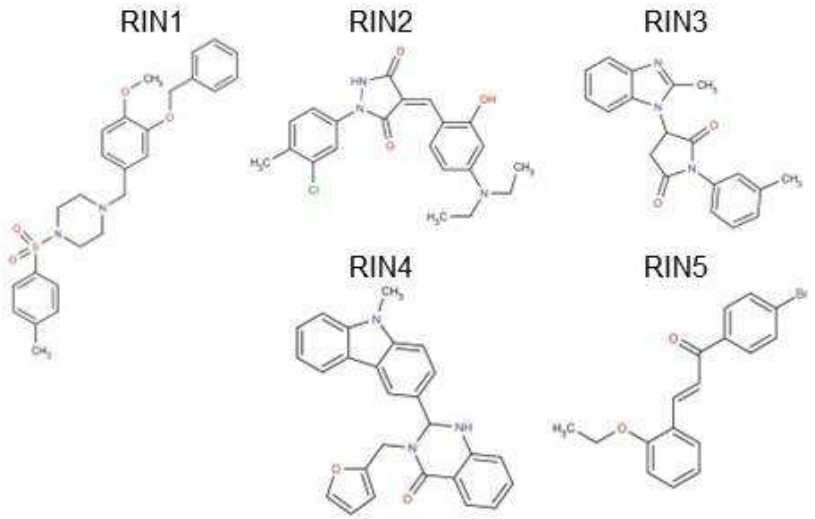
Figure 2.2 Secondary Screen and Northern Blot Analysis Identifies the RINs

(A) Secondary screening in R1gfp cells of the twenty lead compounds at 30µM followed by Northern blot analysis using a GFP specific probe. (B) Chemical structures of RINs 1-5, left to right and top to bottom. (C) RIN compounds at 30µM show no cytotoxic effects in S2 cells except for RIN3 as revealed by staining with 1% Evans blue dye. (D) A 24 hour pretreatment of the R1gfp stable cells with 30µM of each RIN compound enhanced accumulation of RNA1 and RNA3 from the R1gfp replicon revealed by Northern blot hybridization.

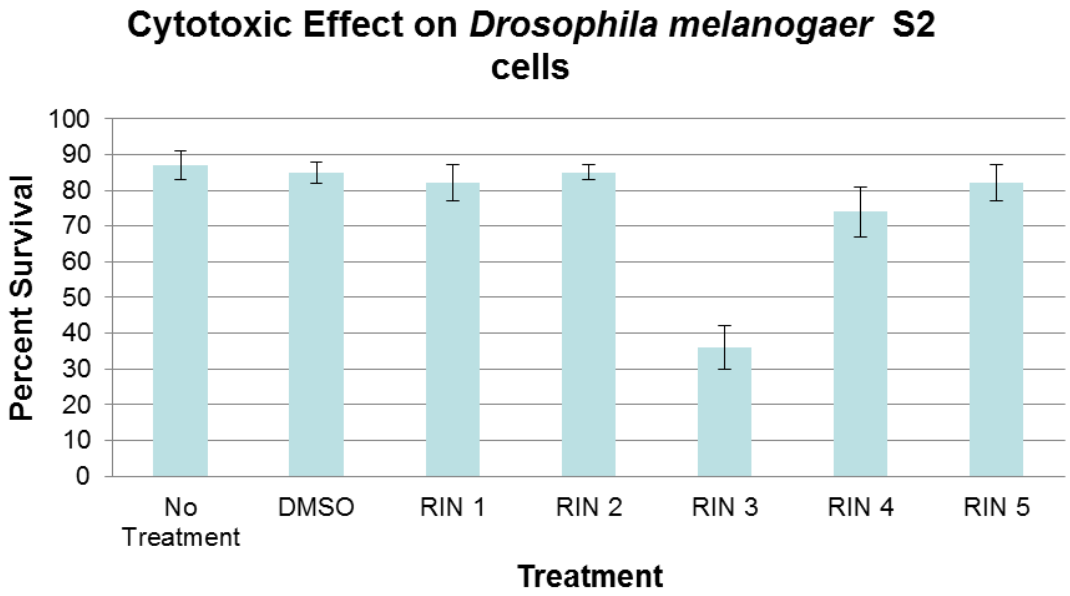
A.


R1gfp
Treatment:
RIN 1
NT
T2
T3
T4
T5
RIN2
RIN3
T8
RIN4
T10
RIN5
RNA1
RNA3
RP49
1 2 3 4 5 6 7 8 9 10 11 12

B.


RIN1
RIN2
RIN3
RIN4
RIN5

C.


Cytotoxic Effect on Drosophila melanogaer S2 cells
Percent Survival
100
90
80
70
60
50
40
30
20
10
0
No Treatment
DMSO
RIN 1
RIN 2
RIN 3
RIN 4
RIN 5
Treatment

D.

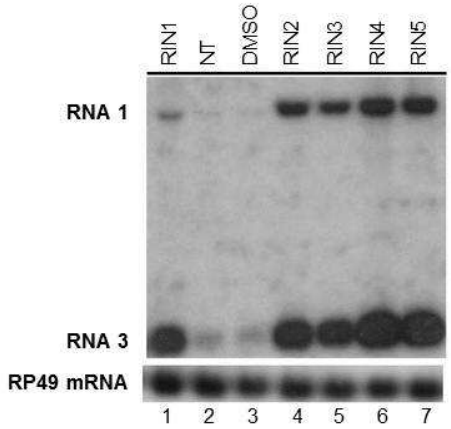

RIN1
NT
DMSO
RIN2
RIN3
RIN4
RIN5
RNA 1
RNA 3
RP49 mRNA
1 2 3 4 5 6 7

Table 2-1 Northern Blot Analysis Buffers

	Buffers for Northern Analysis
10X MOPS Buffer	0.5M 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	3M NaCl, 0.3 M Trisodium citrate
Methylene Blue Solution	0.04% (w/v) Methylene blue, 0.5M Sodium acetate, pH 5.2
1X TE Buffer	10 mM Tris-HCl, 1 mM EDTA, pH 8.0
Stripping Buffer	2% (w/v) SDS, 10 mM Tris, pH 7.4
Pre-hybridization Buffer	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

Chapter 3: Analysis of Function and Mode of Action of RIN Compounds

Abstract

RNA interference, or RNAi, has been well characterized both genetically and biochemically in *Drosophila melanogaster* as well as in other species. The previously described chemical screen resulted in five compounds that were named RIN1 through 5. Here we describe a functional analysis to get at the mode of action of the RIN compounds. Transfection of double-stranded RNA or siRNA targeting cyclin A in RIN treated *Drosophila* S2 cells divided the RIN compounds in two groups; inhibitors of the upstream portions of the RNAi pathway and inhibitors of the downstream portions of the RNAi pathway. Further biochemical studies in *Drosophila* S2 cell and embryo lysates revealed that both RIN1 and RIN2 can block double-stranded RNA processing into siRNAs, called dicing, and RIN5 can block the process of siRNA mediated cleavage of mRNA, called slicing. *In vitro* dicing assays using a recombinant human Dicer protein identified RIN2 as an inhibitor of the Dicer nuclease.

3.1 Introduction

The viral immunity pathway in *Drosophila melanogaster*, which overlaps the exogenous RNA interference pathway, starts with the production of 21 nt small interfering RNAs (siRNAs) by the Dicer-2 (Dcr-2) endoribonuclease from a double-stranded RNA (dsRNA) precursor molecule (Hannon et al., 2002). The processing of the dsRNA by Dicer-2 is aided by the dsRNA binding protein R2D2 which serves to stabilize the dsRNA during the cleavage process (Mlotshwa et al., 2008). The Dicer-2/R2D2/duplex siRNA complex then serves as the first of three protein complexes that serve to load the siRNA into the siRNA induced silencing complex (siRISC) (Liu et al., 2003). The final step of siRISC formation occurs when the siRNA is unwound and one of the strands in then loaded into the Argonaute 2 protein (Ago2) which results in the formation of the mature siRISC (Kim et al., 2007). The loaded siRNA serves as a guide to bring siRISC to a perfectly complementary mRNA target. The siRNA will that bind to the mRNA which then triggers Ago2 to cleave the mRNA via an endonuclease reaction called “slicing” (Okamura et al., 2004). Studies have shown that this exogenous siRNA pathway can be triggered in response to viral dsRNA, transgene driven expression of dsRNA and exogenously introduced dsRNA (Aravin and Tuschl, 2005).

In contrast to the exogenous siRNA pathway, the endogenous siRNA pathway produces siRNAs from convergent transcription of overlapping genes or from highly structured genomic segments that are found in both somatic and gonadal tissue in *D. melanogaster* (Czech et al., 2008; Ghildiyal et al., 2008; Kawamura et al., 2008; Okamura et al., 2008a; Okamura et al., 2008b). As was the case in the exogenous siRNA

pathway, the biogenesis of these endo-siRNAs requires the function of the Dcr-2 protein to cleave the dsRNA into siRNA duplexes. One of the main differences in these two pathways, besides for the source of the dsRNA, is that the endogenous siRNA pathway makes use of the Loqs-PB isoform of the Loquacious dsRNA binding protein as the dsRNA binding protein partner of Dicer-2 as opposed to Loqs-PD, which is used in the exogenous RNAi pathway (Czech et al., 2008; Miyoshi et al., 2010; Okamura et al., 2008).

In *D. melanogaster* the RNAi pathway has been characterized for its genetic components as well as in its biochemical function. It has been previously reported that lysates from *Drosophila* S2 cells can dice dsRNAs into siRNAs as well as slice target mRNAs when incubated with a complementary siRNA (Bernstein et al., 2001; Sontheimer, 2005). Previously characterized translationally active *D. melanogaster* embryo lysates have also been proven to be useful in RNAi studies (Haley et al., 2003). Not only are these lysates active for RNAi, but they allow for the analysis of *Drosophila* genetic mutants to be studied at the biochemical level. These tools have led to the biochemical characterization of genes that have been previously identified to play a role in RNAi (Kawamura et al., 2008; Miyoshi et al., 2008; Pham and Sontheimer, 2005).

Our previous experiments with the RIN compounds indicate that they can affect the antiviral RNAi pathway. As both the exogenous RNAi pathway and antiviral RNAi have been shown to use many of the same components, we used techniques that have been previously used to map the exogenous RNAi pathway to investigate how the RIN compounds affect RNAi (Chotkowski et al., 2008; Galiana-Arnoux et al., 2006; Wang et

al., 2006; Zambon et al., 2006). Using both dsRNA and siRNAs we were able to separate the RIN compounds into two distinct groups; upstream inhibitors of RNAi and downstream inhibitors of RNAi. Biochemical assays revealed that RIN1 and RIN2 had the capacity to block dicing while RIN5 could block slicing *in vitro*. Further investigation of RIN2 identified it as a specific inhibitor of the human Dicer protein in *in vitro* dsRNA processing assays.

3.2 Materials and Methods

3.2.1 *In vitro* synthesis of double-stranded RNA, mRNA and siRNAs

Double-stranded RNAs (dsRNA), mRNAs and siRNAs were all synthesized using Ambion products (Applied Biosystems, Foster City, CA, USA). For dsRNA synthesis, GFP or Cyclin A DNA templates with T7 promoters on both the 5' and 3' ends were synthesized from the pMTGFP plasmid using PCR. 1 µg of the DNA template, 2µl each of the ATP, CTP, UTP and GTP solutions, 2 µl of 10X reaction buffer and 2 µl of the Enzyme mix were mixed together and water added to 20 µl as described by the manufacturer (MEGAscript T7 kit, Product number 1334). For radiolabelled dsRNAs, 1 µl of [α-^{32}P]UTP was added to the reaction mix. The reactions were incubated at 37°C for 4 hours to allow for the transcription reaction to occur. Once reactions have reached completion 1 µl of DNase was added to the reaction to degrade the DNA templates and incubated for an additional 15 minutes at 37°C. 115 µl of nuclease-free water and 15 µl of Ammonium Acetate stop solution was added to the dsRNAs and then the dsRNA was purified using an equal volume of 50:50 phenol/chloroform followed by an equal volume of chloroform. The RNA in the supernatant was then precipitated by adding an equal

volume of isopropanol and incubating it at -20°C overnight. The dsRNA was then spun down at 14,000 rpm at 4°C for 20 minutes. RNA pellets were then washed with 70% ethanol and then dissolved in TE buffer. To degrade any single-stranded RNAs that might be present, the dsRNA were incubated at 65°C and cooled down to room temperature. dsRNAs were then quantified using a Biophotometer and stored at -20°C until used. Radiolabelled capped mRNAs were synthesized in a similar manner to dsRNAs using the Ambion mMessage Machine Kit (Product number 1344). A full length GFP cDNA was synthesized using PCR placing a T7 promoter at the 5'end of the cDNA. In short, 1 µg of the DNA template was mixed with 10 µl of 2X NTP/CAP, 2 µl of 10X reaction buffer, 1 µl [α-^{32}P]UTP, 2 µl of enzyme mix and filled up to 20µl with nuclease free water. Reactions were incubated at 37°C for 2 to 4 hours to allow for the transcription reaction to occur. 1 µl of DNase was added to the reaction to degrade to DNA template and incubated for an additional 15 minutes at 37°C. mRNAs were then purified in the same manner as the dsRNAs were with the exception of the step where single-stranded RNAs were degraded.

siRNAs for RISC cleavage assays were synthesized using the Silencer siRNA construction kit (Ambion, product number 1620). Overlapping sense and antisense oligonucleotides were designed to be complementary to GFP mRNA and each of them were diluted to a concentration of 100 µM. 2 µl of each oligonucleotide was mixed with 2 µl of T7 primer and 6µl of DNA Hyb Buffer and heated at 70°C to anneal the GFP primers to the T7 primer to allow for the transcription of the siRNA precursor. To fill in the primers the two oligonucleotides were hybridized the reactions were mixed with 2 µl

of 10X Klenow reaction buffer, 2 µl of 10X dNTP mix, 4µl of nuclease-free water and 2 µl of exo-klenow and incubated at 37°C for 30 minutes. To synthesize the dsRNA precursor, 2 µl of either the sense or antisense primers from the previous step were mixed with 4 µl of nuclease-free water, 10 µl of 2X NTP mix, 2 µl of 10X T7 reaction buffer and 2 µl of T7 enzyme mix and incubated at 37°C for 2 hours. The sense and antisense reactions were then mixed together and incubated at 37°C for overnight. To eliminate any single-stranded RNA and DNA remaining from the previous reaction 6 µl of digestion buffer, 48.5 µl of nuclease-free water, 3 µl of RNase and 2.5 µl of DNase was added to the transcription reactions and incubated for 2 hours at 37°C. To purify the siRNAs, 400 µl of siRNA binding buffer was added to the digestion mix and incubated for 5 minutes at room temperature and then added to a prewet Filter cartridge and spun at 10,000 rpm to bind the siRNAs to the cartridge. The siRNAs were washed with 500 µl of siRNA wash buffer and then eluted in 100 µl of nuclease-free water by centrifugation at 12,000 rpm for 2 minutes. The siRNAs were then analyzed by gel electrophoresis to confirm size and quantified using a Biophotometer and then stored at -20°C until use.

3.2.2 Transfection of dsRNA and siRNA in S2 cells

Cyclin A dsRNA and siRNAs were transfected into *Drosophila* S2 cells using the Effectene Transfection Kit (QIAGEN Inc. Valencia, CA, USA) according to the manufacturer's protocol. Appropriate amount of cells (1.0-3.5 x 106 cells per well) were seeded into a 12-well plate at a 1 ml final volume per well in Schneider's Insect Medium (Product No. S9895 Sigma-Aldrich, Inc. St. Louis, MO, USA) containing serum and antibiotics with 30µM of RIN's 1 through 5 on the day before transfection. On the day of

transfection, 0.3µg of dsRNA or 0.3µg of siRNA were diluted into a microtube with 2.4 µl of Enhancer and DNA-condensation buffer, Buffer EC, to a final volume of 100µl. The samples were mixed by vortexing for a few seconds and incubated at room temperature for 2-5 minutes. 6 µl of Effectene reagent was added to the DNA-Enhancer solution and the samples were mixed by vortexing for 10 seconds and incubated at room temperature for 10 minutes. The transfection complexes were added to the cells in the 12-well plate. The plate was swirled gently and all the cells were incubated under their normal growth conditions in a 27°C incubator.

3.2.3 RNA Extraction from S2 cells

Total RNA was isolated from S2 cells with Trizol reagent and previously described in chapter 2.

3.2.4 Northern blot Analysis

Northern blot analysis of total RNA samples was performed as previously described in chapter 2.

3.2.5 *In vitro* Dicer Cleavage Assay

RIN containing S2 cell lysates were generated as followed. Six aliquots of 25 ml of *Drosophila* S2 cells were seeded and cultured in a flask with either no RIN compound or 30µM of one of the RIN compounds overnight in a 27℃ incubator. Cells were then collected by centrifugation at 3000 rpm for 5 minutes and washed 3 times with PBS and then once with Hypotonic buffer (Table 3-1) that had 60mM of KCl. Cells were then spun down again and the Hypotonic buffer was removed and the cell pellet was resuspended in 1 ml of Hypotonic buffer without KCl but containing protease inhibitor

and then put on ice for 10 minutes and then the cell membranes were broken apart in a dounce homogenizer. The cell debris was then separated from the supernatant by centrifugation at 14,000 rpm for 25 minutes at 4°C and then the supernatant was stored in a -80°C incubator until the lysates were used.

For a typical Dicer cleavage reaction 5 µl of S2 cell lysate was mixed with 3 µl of 40x RNAi reaction buffer (Table 3-1), 1 µl of water and 1 µl of 50nM of radiolabelled dsRNA. The reactions were then incubated in 25°C for 3 hours and then quenched with 100 µl of 2x PK buffer (Table 3-1). Reactions were then deprotonated by adding 10 µl of 20 µg/µl of proteinase K (NEB) and 1 µl of 20 µg/µlof a glycogen carrier (Sigma) and incubated at 65°C for 10-60 minutes. The RNA was then purified with a phenol/chloroform extraction and then precipitated with and equal volume of isopropanol and then pelleted. The RNA pellet was then washed with 70% ethanol and then resuspended in 20 µl of formamide loading dye (Table 3-1). A 12% urea denaturing gel was prepared and pre-run for one hour in 0.5x TBE buffer using a Dual Adjustable Slab Gel Unit (C.B.S Scientific Co.). The samples were then loaded into the gel and then the gel was run in 0.5x TBE buffer at 400 V for 4 hours until the bromophenol blue run to the position about 9 cm above the bottom and the Xylene cyanol FF ran out of the gel. Once electrophoresis was completed, the gel was removed gently from the glass plate, rinsed in 0.5x TBE buffer, and placed into a cassette with x-ray film (HyBlot ClTM, DENVILLE Scientific Inc.) and incubated at -80°C overnight. Films were then developed in a Medical film processor QX-70 (KONICA).

3.2.6 *In vitro* RISC Cleavage Assay

To generate *Drosophila* lysates that are active for RNAi, canton-S and *ago2* mutant flies were grown in large populations and then allowed to lay embryos on pineapple agar plates for 2 hours at 27°C. A 50% beach solution was then poured onto the plates to dechorionate the embryos for 5 minutes. The, using a filtration apparatus, embryos were collected onto a piece of filter paper and washed excessively with cold water to rinse away any remaining bleach. Embryos were then harvested with a synthetic-bristle paint brush and put into a microfuge tube and frozen in liquid nitrogen and then lysed in 1 ml of lysis buffer (Table 3-1) containing 5 mM of DTT and 1 mg/ml of complete protease inhibitor per gram of embryos. The lysates were then clarified by centrifugation at 14,000 rpm for 25 minutes at 4°C to separate the embryo debris from the liquid lysate. The liquid was then transferred into a new microfuge tube, flash frozen in liquid nitrogen and stored at -80°C until needed.

To setup the cleavage reaction 5 µl of lysate was mixed with 1 µl of RIN compound, 3 µl of 40x RNAi reaction buffer, 1 µl of 50nM target mRNA and 1 µl of 1000nM siRNA. Mixtures were incubated at 25°C for 3 hours to allow for the cleavage reaction to occur and then quenched with 100 µl of 2x PK buffer. Reactions were then deprotonated by adding 10 µl of 20 µg/µl of proteinase K (NEB) and1 µl of 20 µg/µl of a glycogen carrier (Sigma) and incubated at 65°C for 10-60 minutes. The RNA was then purified with a phenol/chloroform/iso-amyl alcohol (25:24:1) extraction and then precipitated with and equal volume of isopropanol and then pelleted. The RNA pellet was then washed with 70% ethanol and then resuspended in 20 µl of formamide loading dye. An

8% urea denaturing gel was prepared and pre-run for one hour in 0.5x TBE buffer using a Mini Format 1-D Electrophoresis Systems (Bio Rad). The samples were then loaded into the gel and then the gel was run in 0.5x TBE buffer at 100 V for 2 hours. Once electrophoresis was completed, the gel was removed gently from the glass plate, rinsed in 0.5x TBE buffer, and placed into a cassette with X-ray film (HyBlot ClTM, DENVILLE Scientific Inc.) and incubated at -80°C overnight. Films were then developed in a Medical film processor QX-70 (KONICA).

3.3 Results

3.3.1 RIN Compounds can Inhibit Exogenous RNAi in *Drosophila* S2 Cells

Previous results obtained from experiments conducted in R1gfp amplicon system indicated that the RIN compounds could be affected the antiviral RNAi pathway. In order to determine where, and if, in the pathway these molecules carry out their mode of action and whether they can also affect the exogenous RNAi pathway we decided to use long dsRNA and siRNAs targeting cyclin A mRNA and then use cyclin A mRNA levels as a readout of whether exogenous RNAi is being inhibited as well as a preliminary readout of where in the pathway these molecules act. dsRNA was used to assay for the upstream regions of the pathway while siRNAs were used to distinguish chemicals that acted on the downstream portions of the pathway. We first transfected dsRNA targeting nucleotides 121-721 of cyclin A into S2 cells that were pretreated with 30µM of our RIN compounds. 2 days following post transfection cells were collected and RNA was extracted and used for Northern blot hybridization probing for cyclin A mRNA. Treatment of the dsRNA alone showed a strong reduction of the cyclin A mRNA present

in the S2 cells (Figure 3.1a, compare lanes 1 and 2). All the RIN compounds were able to inhibit the exogenous RNAi pathway and restore the abundance of cyclin mRNA to near wild-type levels (Figure 3.1a; compare lanes 1 and 2 to lanes 3-7). This experiment identified that these chemicals are inhibitory to RNAi, but did not give sufficient data to place them in a specific location in the pathway, as a downstream inhibitor would give the same phenotype as an upstream inhibitor in this experimental setup.

To further dissect where in the RNAi pathway these chemicals act, we designed siRNAs targeting nucleotides 591-611 and 2322-2342 of cyclin A mRNA to bypass the siRNA biogenesis step of RNAi (dicing) and used those siRNAs to transfect S2 cells. 2 days post transfection cyclin A mRNA levels were assayed by Northern blot hybridization. The transfection of these siRNAs into the cells led to a reduction in the cyclin A mRNA levels (Figure 3.1b compare lanes 1 and 2). Pretreatment of cells with 30μM of RINs 1, 2 and 3 did not affect the siRNAs ability to knockdown cyclin A mRNA (Figure 3.1b compare lanes 1 and 2 to lanes 3-5). This places RINs 1 through 3 upstream of mRNA targeting by siRNAs. RIN 4 and 5 pretreated cells differed from the previous three compounds in that they were able to block the ability of the siRNA from silencing cyclin A mRNA, visualized by the lack of reduction of cyclin A mRNA levels in RIN4 and RIN5 treated cells (Figure 3.1b compare lanes 1 and 2 to 6 and 7). This places RIN4 and RIN5 in the downstream portions of the exogenous RNAi pathway.

3.3.2 RIN5 Blocks siRNA Mediated mRNA Cleavage (Slicing)

Our initial results indicated that RIN4 and RIN5 were able to inhibit the downstream portions of the RNAi pathway. To assay whether mRNA cleavage induced via the siRISC

complex, or slicing, was being inhibited by either of these chemicals, *in vitro* slicing assays were performed using *D. melanogaster* embryo lysates as has been previously described using a synthetic siRNA (Haley et al., 2003). Once extracts from wild-type embryos were prepared they were treated with 30µM of each RIN and then incubated with a capped and radioactively labeled GFP mRNA as well as an siRNA complementary to nucleotides 350-368 of GFP mRNA, extracts from embryos carried the *ago2*^{414} mutation were used as a cleavage incompetent control. After the incubation period the reactions were run out on an 8% acrylamide gel and exposed to X-ray film and the resulting ~350nt 5' cleavage products were used as a readout to indicate whether the reactions occurred or not in the presence of each RIN compound. Reactions incubated with RINs 1, 2, 3 or 4 showed no change in the amount of the 350nt 5' cleavage product produced from the GFP mRNA (Figure 3.2 compare lane 1 to lanes 2, 3, 4 and 5), which indicated that these compounds did not play an inhibitory role in slicing. Reactions incubated with RIN5 did not produce detectable levels of the 5'cleavage product as was the case in the *ago2*^{414} control (Figure 3.2 lane 1 to lanes 6 and 7). The results of this experiment in conjunction with the previous results indicates that RIN5 can block mRNA cleavage by siRISC, as no 5' cleavage products could be detected in RIN5 treated samples as well as that in RIN5 treated S2 cells cyclin A mRNA could not be silencing by siRNAs targeting cyclin A mRNA. RIN4 on the other hand appears to have a different mode of action then RIN5 as it was able to block siRNA mediated silencing of cyclin A but did not inhibit the slicing reaction itself.

3.3.3 RIN1 and RIN2 Block siRNA biogenesis (dicing)

Experiments conducted in *Drosophila* S2 cells indicated that RIN1, RIN2 and RIN3 played a role in inhibiting the upstream portions of the exogenous RNAi pathway. To test whether any of these RIN compounds can inhibit biogenesis of small RNAs, translationally active cell lysates were prepared from S2 cells that had been treated with 30µM of each RIN compound and *in vitro* Dicer activity assays were performed. In *Drosophila* Dicer-2 is responsible for the biogenesis of viral derived small RNAs (viRNAs), exogenous siRNAs and endogenous siRNAs. Once extracts were made containing 30µM of each RIN they were incubated with a radioactively labeled 500bp dsRNA fragment derived from GFP. After the incubation period the reactions were run out on a 15% acrylamide sequencing gel and exposed on X-ray film for detection of GFP derived siRNAs. The control reaction yielded the expected result of a 21nt long siRNA band indicative of Dicer-2 activity (Figure 3.3a lane 1). In reactions where RIN1 or RIN2 were added to the cell lysates there was a reduction in the siRNAs produced from the dsRNA precursor (Figure 3.3a compare lane 1 to 2 and 3), which signifies that Dicer-2 activity was perturbed in these reaction mixtures. Reactions that were conducted in cell lysates containing RIN3, RIN4 or RIN5 showed no reduction in the amounts of siRNAs produced indicating that these chemicals do not affect the processing of long dsRNA into small RNAs (Figure 3.3a compare lane 1 to lanes 3, 4 and 5). Together with the data from the dsRNA and siRNA transfections and the *in vitro* mRNA cleavage assays it appears as though RIN1 and RIN2 block the biogenesis of siRNAs (dicing) in the upstream portions of the exogenous RNAi pathway while RIN5 blocks mRNA cleavage by siRISC in the

downstream portions of the pathway. Based on the evidence that RIN4 does not perturb either siRNA biogenesis or mRNA cleavage *in vitro* but does inhibit siRNA mediated silencing in S2 cells suggests that RIN4 plays a role between dicing and slicing. On the other hand the data suggests that RIN3 has its mode of action upstream of dicing which explains why it had no effect in the *in vitro* Dicer activity assay while still being able to inhibit dsRNA mediated silencing in S2 cells. This evidence combined with the low cell survival seen in RIN3 treated S2 cells (chapter 2) seems to suggest that RIN3's mode of action may not be an RNAi specific effect but that RIN3 targets a housekeeping gene that can adversely affect the health of the cell when it's function is disrupted.

3.3.4 RIN2 Blocks Recombinant Human Dicer *in vitro*

The previous data suggested that the *D. melanogaster* Dicer-2 protein was a possible target of either RIN1 or RIN2. To answer the question of whether these chemicals inhibited Dicer itself we purchased the commercially available *in vitro* Dicer kit from Genlantis. This kit has been shown to be able to generate siRNAs from a precursor dsRNA and that these siRNAs are functional *in vivo*. The Dicer protein that is provided with this kit is a recombinant human Dicer. The human Dicer protein and *D. melanogaster* Dicer-2 are both class III RNaseIII proteins that are structurally similar as they both contain a helicase domain, a PAZ domain, two RNaseIII domains, a dsRBD and a Domain of unknown function 283 (Hammond, 2005) (Figure 3.3b). The reactions were setup as directed by the manufacturer with a 500bp GFP dsRNA species and the reactions were treated with 30µM of each of the RIN compounds. After a 12-hour incubation period the reactions were run on a 15% sequencing gel and exposed of X-ray

film. RIN1 did not show a reduction of siRNA levels produced in the reaction as compared to the untreated sample (Figure 3.3c, compare lane 1 and 2). RIN4 and RIN5, the chemicals that target the RNAi pathway downstream of siRNA biogenesis, did not have an effect on this reaction as well (Figure 3.3c, compare lane 1 to lanes 4 and 5). RIN2 on the other hand showed a marked decrease in the amount of siRNAs that were produced from the precursor GFP dsRNA (Figure 3.3c compare lanes 1 and 3). The inhibition of siRNA production in the presence of RIN2 also resulted in the accumulation of a precursor dsRNA of approximately 200bp in length, which was not observed in the control sample as well as samples treated with the other RIN compounds where no siRNA biogenesis inhibition occurred. Furthermore, RIN2 showed a dose dependent inhibition to the amount of siRNAs produced, as reactions that were carried out with 15, 30, 45 and 60 µM concentrations respectively yielded decreasing amounts of siRNAs (Figure 3.3d compare lane 1 to lanes 2, 3, 4 and 5 respectively). As Dicer is the only protein present in this reaction we believe Dicer to be target of RIN2 as its ability to produce siRNAs is reduced in the presence of this compound.

3.4 Discussion

Our laboratory has previously established both Dicer-2 (Dcr-2) and Argonaute 2 (Ago2) as key components of the antiviral RNAi pathway (Li et al., 2002; Wang et al., 2006). Since these discoveries in fruit flies, Dicer and Argonaute proteins have also been implicating in antiviral RNAi in other invertebrate animals including mosquitoes, nematodes and shrimp highlighting the importance of these gene families in antiviral RNAi (Campbell et al., 2008; Khoo et al., 2010; Lu et al., 2005; Sanchez-Vargas et al.,

2009; Schott et al., 2005; Su et al., 2008; Wilkins et al., 2005). In this study the RIN molecules, that have been previously shown to increase Flock house virus (FHV) and Cricket Paralysis Virus (CrPV) replication in *Drosophila melanogaster* S2 cells, were shown to be able to inhibit the exogenous RNAi pathway both in cell culture and in *in vitro* experiments. Moreover, these studies have shown that the RIN compounds can be divided into two distinct functional groups; inhibitors of the upstream portions of the exogenous RNAi pathway and inhibitors of the downstream regions of the exogenous RNAi pathway (Figure 3.4).

This work has shown the versatility and utility of the *Drosophila* in characterizing the function of exogenous RNAi inhibitors. This study has shown that RIN1 and RIN2 are both inhibitors of siRNA biogenesis while RIN5 was shown to be inhibitory to siRNA mediated mRNA cleavage. It has been previously shown in loss-of-function genetic studies that Dicer-2 is required for antiviral silencing in adult fruit flies (Wang et al., 2006). It has also been shown genetically and biochemically that Dicer-2 is required for the processing of long dsRNA into siRNAs in both the antiviral, exogenous and endogenous RNAi pathways (Bernstein et al., 2001; Czech et al., 2008; Tomari and Zamore 2005). Ago2, which acts downstream of siRNA biogenesis, has also been shown to play an important role in the same RNAi pathways that Dicer-2 acts in by binding Dicer-2 derived siRNAs and using them to guide the cleavage of target mRNAs (Kawamura et al., 2008; Kim et al., 2007; Okamura et al., 2004). My data suggests that the RIN compounds are inhibitors of the exogenous RNAi pathway in which Dicer-2 and Ago2 play important roles, which has been attributed to antiviral silencing in *Drosophila*.

The exogenous RNAi pathway has also been shown to be antiviral in *Caenorhabditis elegans* by several groups which indicates that the RIN compounds may be functional in *C. elegans* as well (Lu et al., 2005; Schott et al., 2005; Wilson et al., 2005).

There are several possible applications of the RIN compounds in both basic and applied research fields. One such application will be to identify new intermediates of siRNA loading into RISC. siRNA loading is a multistep process which goes through several intermediates called R1, R2 and R3 before the siRNA can be loading into a mature siRISC and this process requires proteins such as Dcr-2, R2D2 and Ago2 (Pham et al 2004; Pham and Sontheimer 2005). A compound such as RIN4, which currently has no distinct step associated with it, may interact with the RNAi pathway by perturbing the siRISC maturation/loading process and stop said process at a specific, yet currently unidentified step. A RISC loading assay performed in the presence of the RIN compounds could lead to the identification of such an intermediate. Another possible use for these compounds are as novel pesticides against both animal and plant virus vectors. It has been shown that fruit flies carrying mutations in RNAi genes show enhanced disease susceptibility (EDS) to viruses such as CrPV, Drosophila X virus (DXV), Drosophila C virus (DCV), FHV and Vesicular stomatitis virus (VSV) leading to increased virus replication levels as well as earlier and more sever mortality rates (Galiana-Arnoux et al., 2006; Mueller et al., 2010; van Rij et al., 2006; Wang et al., 2006; Zambon et al., 2005). The same results have been shown in several mosquito species that have been challenged by various viruses such as Dengue virus (DENV) Nodamura virus (NoV) and Sinbis virus (SINV) (Campbell et al., 2008; Khoo et al., 2010; Li et al., 2004;

Sanchez-Vargas et al., 2009). As these chemicals have been shown to be able to increase R1gfp viral RNA levels it is reasonable to assume that they may be able to increase virus replication levels in adult fruit flies and well as in related species such as mosquitoes. This increase in viral levels will lead to earlier and increased mortality rates which in turn can stop the spread of viruses to plant and human hosts.

To conclude, this body of work has established that the RIN compounds do in fact inhibit the exogenous RNAi pathway, which was shown both in S2 cell culture and biochemically. We were able to separate the five compounds into two distinct groups; upstream and downstream inhibitors of the RNAi pathway. RIN1 and RIN2 were found to act at the level of siRNA biogenesis while RIN5 was found to inhibit siRNA targeted mRNA cleavage. Although RIN3 was shown to be able to block dsRNA mediated silencing in S2 cells, it seems as though this may be a non-specific effect as cells treated with these compounds are very sick and show a low overall quality of health. RIN4 also performs a yet unknown function as it has been shown to inhibit siRNA mediated silencing but cannot block slicing in *in vitro* reactions. RIN2, but not RIN1, was shown to be not only an inhibitor of dicing, but of the Dicer protein itself by being able to inhibit the human Dicer protein in *in vitro* Dicer activity assays. One possible explanation for this observance is that RIN2 targets a conserved region between Dcr-2 and the human Dicer protein while RIN1 targets a region that is not conserved between the two proteins. Alternatively, RIN1 could be targeting another protein required for small RNA biogenesis in *Drosophila* such as Loq-PD or R2D2 (Marques et al., 2010). Collectively

these findings have established the *Drosophila* system as a model for the identification and validation of chemical inhibitors of the RNAi pathway.

3.5 References

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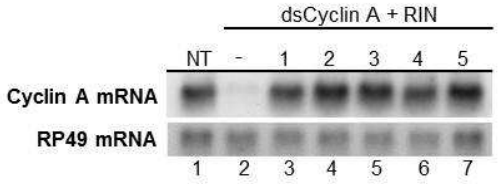
A.



B.

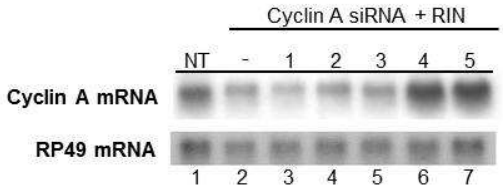


Figure 3.1 dsRNA and siRNA mediated silencing in S2 cells is perturbed by RIN Compounds

Differential susceptibility of an engineered RNAi targeting cyclin A mRNA in S2 cells initiated by dsRNA (A) or siRNA (B) to inhibition by the RIN compounds. S2 cells pretreated with a RIN compound at 30µM or DMSO alone (-) for 12 hours were transfected with either dsRNA or siRNAs and total RNA extracted two days post transfection was analyzed for the accumulation of cyclin A mRNA by Northern blot hybridization. Lane 1 in both (A) and (B) showed the accumulation of cyclin A mRNA in the non-treated S2 cells (NT).

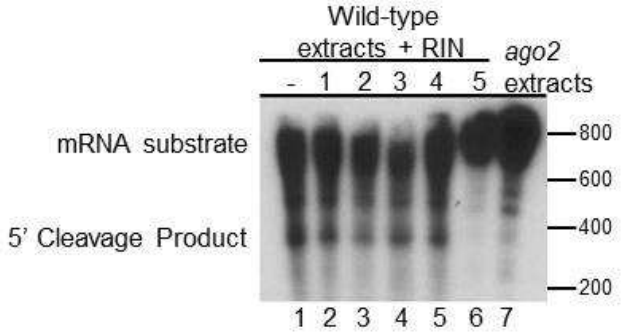
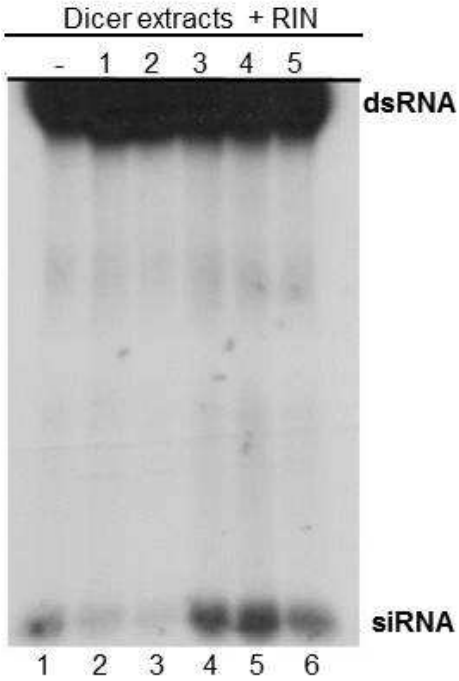


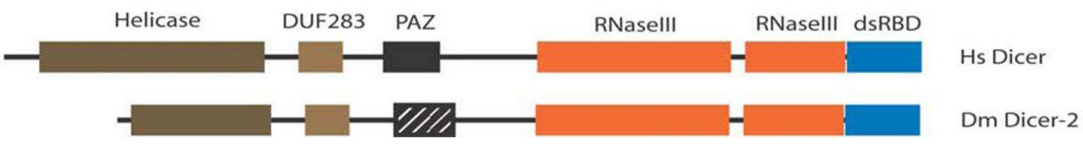
Figure 3.2 *In vitro* mRNA cleavage assay reveals RIN5 inhibition of Slicing
In vitro slicing of a radiolabeled eGFP mRNA by a siRNA-programmed RISC present in the wild type *Drosophila* embryo lysates in the presence of the RIN compounds at 30µM. Embryo lysates from the *ago2* null mutant flies were used as the control (lane 7). The slicing reaction was resolved in a denaturing acrylamide gel electrophoresis (8%).

Figure 3.3 RIN1 and RIN2 Inhibit Dicing while RIN2 inhibits Dicer *in vitro*
In vitro dicing of a radiolabeled 500bp dsRNA into siRNAs by either Dicer extracts from S2 cells(A) or the recombinant human Dicer protein (hDicer) (C and D) in the presence of the RIN compounds at 30μM. 15, 30, 45 and 60μM concentration of RIN2 were used for the dose-response in panel (C). The dsRNA fragments were resolved by denaturing polyacrylamide gel electrophoresis (15%). (B) Domain organization of the Human Dicer protein (Hs Dicer) and *D. melanogaster* Dicer -2 (Dm Dicer-2). The slashes through the PAZ domain of Dicer-2 indicate that there are several mutant residues in this domain which may interfere with RNA binding.

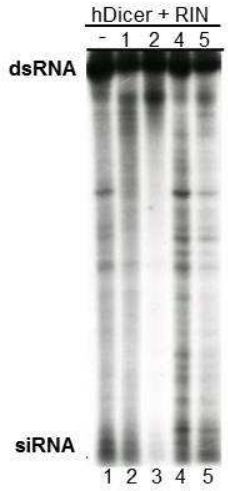
A.


Dicer extracts + RIN
- 1 2 3 4 5
dsRNA
siRNA
1 2 3 4 5 6

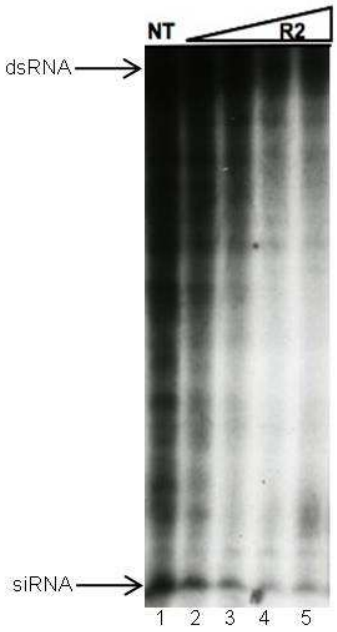
B.


Helicase
DUF283
PAZ
RNaseIII
RNaseIII
dsRBD
Hs Dicer
Dm Dicer-2

C.



D.



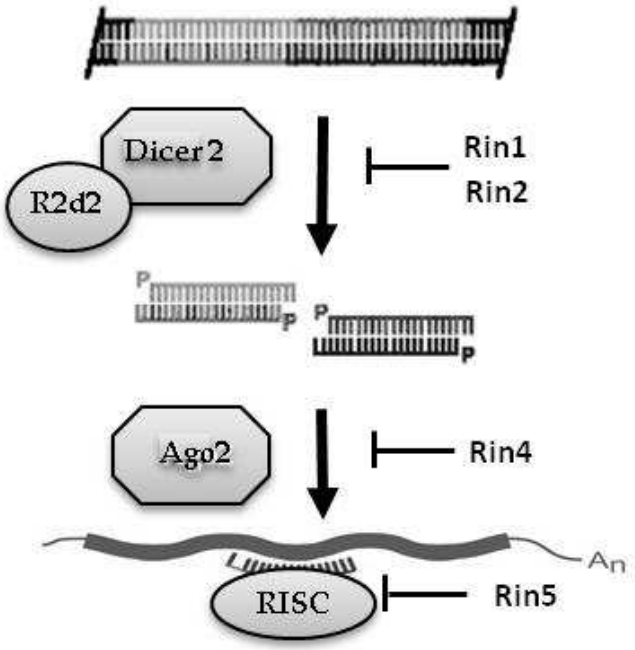


Figure 3.4 Mode of Action Model for the RIN Compounds
The available data places RIN1 and RIN2 at the level of dicing and RIN5 at the level of slicing. RIN4 seems to have its mode of action somewhere in between dicing and slicing, but as of now its point of action is currently unknown. RIN3 seems to play a role upstream of siRNA biogenesis in a non-RNAi related effect and has been left out of this model.

Table 3-1 Buffers for *in vitro* RNAi Reactions

	Buffers for *in vitro* RNAi Reactions
Hypotonic Buffer	10 mM HEPES pH 7.0, 2 mM $MgCl_2$, 6 mM β-mercaptoethanol
RNAi Reaction Buffer	71 µl water, 20 µl 500 mM creatine phosphate, 2 µl 1M DTT, 2 µl 20U/µl RNasin, 4 µl 100 mM ATP, 6 µl 2U/µl creatine kinase, 16 µl 1M potassium acetate
1X Lysis Buffer	100 mM potassium acetate, 30 mM HEPES – KOH pH 7.4, 2 mM magnesium acetate
2X Proteinase K Buffer	200 mM Tris-Cl pH 7.5, 25mM EDTA pH 8.0, 300mM NaCl, 2% w/v sodium dodecyl sulfate
Formamide Loading Dye	98% w/v deionized formamide, 10 mM EDTA pH 8.0, 0.025% w/v xylene cyanol, 0.025% w/v bromophenol blue

Chapter 4: Biological Activity of RIN Compounds in *Drosophila melanogaster* and *Caenorhabditis elegans*

Abstract

RNA interference, or RNAi, has been well characterized as an antiviral pathway both in *Drosophila melanogaster* and *Caenorhabditis elegans*. Here we show that the RIN compounds modulate the antiviral RNAi response in *C. elegans* as well as *D. melanogaster* S2 cells and adults. Our analysis revealed that these compounds can allow for higher accumulation of both FHV and CrPV in RIN treated S2 cells. Our analysis also indicated that a modified FHV carrying a mutation in the B2 open reading frame can replicate to detectable levels in S2 cells that have been treated with the RIN compounds, similar as in Ago2 knockdown cells. It has been previously shown in worms that antiviral silencing occurs in an RDE-1 dependent manner; here using worms that carry the FR1gfp construct as a transgene we show that the RIN compounds are able to suppress antiviral RNAi in *C. elegans* and allow the viral transgene to replicate to high levels as compared to a non-treated control. Furthermore, like *rde-1* mutant worms, RIN treated worms are able survive feeding of *pop-1* dsRNA, which normally results in embryo lethality.

4.1 Introduction

4.1.1 The Antiviral RNAi Pathway in *Drosophila melanogaster*

Multicellular organisms must contend with pathogens on a daily basis. All organisms have a basal level of defense called the innate immune system to protect themselves from all sorts of invading pathogens. In *Drosophila melanogaster* there are several innate immune pathways which are specialized to fight specific classes of pathogens. The Toll and Imd pathways are triggered by infection of fungal and bacterial pathogens which lead to the activation of antimicrobial peptide effectors via a NF-κB-like signaling cascade (Hoffmann, 2003; Tanji and Ip, 2005). In the case of viruses our laboratory has previously established that RNAi has antiviral function in *Drosophila* S2 cells (Li et al., 2002). In this study FHV was shown to trigger an antiviral response. FHV is in the genus *Alphanodavirus* and in the family *Nodaviridae* and has been used extensively as a model for both viral replication and packaging (Ball and Johnson, 1998). The virus particle encapsulates a bipartite, positive sense RNA genome that is 4.5 kb in length. RNA 1 encodes the RNA dependent RNA polymerase (RdRp) which the virus uses for both replication of the viral genome as well as transcription of viral genes (Schneeman et al., 1998). RNA 2 encodes the pre-capsid protein which after translation is cleaved into its mature form (Schneeman et al., 1998). A third RNA, RNA 3, is not packaged by the virus particle but is transcribed from RNA 1 through the course of replication. RNA 3 is instrumental to the virus' replication cycle as it encodes the B2 protein, a potent viral suppressor of RNAi (VSR) (Li et al., 2002). Studies have shown that FHV infected S2 cells produce viRNAs, a hallmark of active antiviral RNAi, which

can be suppressed by B2 activity (Li et al., 2002; Lu et al., 2005). Interestingly, FR1ΔB2, a B2 deficient FHV RNA 1 amplicon, cannot replicate in *Drosophila* S2 cells. However, when Ago2 is knocked down by dsRNA or when B2 in expressed in trans, FR1ΔB2 can accumulate to high levels indicating that B2 is not required in replication but for suppression of antiviral activity (Li et al., 2002).

An unrelated virus, CrPV, has also been shown to affect and be affected by antiviral RNAi. S2 cells transfected with the FR1gfp construct cannot replicate due to active antiviral RNAi, but in cells that have been depleted for Ago2 it can replicate to high levels (Li et al., 2004). In CrPV infected S2 cells, FR1gfp can replicate to high levels indicating that CrPV can block antiviral RNAi (Wang et al., 2006). Further studies have shown that unlike B2 the CrPV VSR 1A cannot block viRNA biogenesis but can inhibit viRNA activity (Wang et al., 2006; Nayak et al., 2010).

4.1.2 The Exogenous RNAi Pathway in *Caenorhabditis elegans*

Experiments in *Caenorhabditis elegans* were the first to establish dsRNA as the trigger for RNA interference, or RNAi (Fire et al., 1998). Like in *Drosophila melanogaster*, the exogenous RNAi pathway in *C. elegans* is triggered by the introduction of long double-stranded RNA (dsRNA) into the cell. It has been found that there are several means by which dsRNA can be introduced into nematodes, and there have been several gene families that have been shown to mediate uptake of dsRNA. The first gene family are the *fed* genes, FED-1 and FED-2. These genes are involved in the uptake of dsRNA by feeding worms on *E. coli* expressing dsRNA. This dsRNA is then transported to the rest of the animal through the gut (Timmons et al., 2003; Voinnet, 2005). When these genes

are mutated they do not transport dsRNA through the gut, but when the same dsRNA trigger is injected into the pseudo-coelomic fluid RNAi proceeds as normal (Timmons et al., 2003; Voinnet, 2005). A second class of genes involved in the uptake of dsRNA are the *rsd* mutants. As was the case for the *fed* mutants, these genes were also identified in a genetic screen where mutagenized worms were fed on *E. coli* expressing dsRNA. This class can be divided into two groups; *rsd-4* and *rsd-8* mutant were defective for systemic RNAi for both somatic and germline genes while *rsd-2*, *rsd-3*, and *rsd-6* were deficient for systemic RNAi for germline genes but not for somatic genes (Tijsterman et al., 2004). These results indicated that the spread of dsRNA followed a path through the somatic tissues and then to the germline tissues. The third and last class of genes were identified in a screen where the dsRNA was introduced endogenously through a transgene expressing an inverted repeat dsRNA in the pharynx (Voinnet, 2005). The genes found in this screen were named SID-1, SID-2 and SID-3 (Winston et al., 2002). SID-1 is a transmembrane protein that is required for the passive transport of dsRNA. *sid-1* mutant animals were found to be unable to spread dsRNA injected into somatic tissue to the germline, but dsRNA injected straight into the germ line was still able to induce silencing (Winston et al., 2002). However it was found that SID-1 was not required for feeding RNAi, but the *sid-2* mutant was resistant to this form of silencing.

Once the dsRNA gets into the cell, it is recognized and cleaved by the RNase III enzyme Dicer-1 into short interfering RNAs (siRNAs) of approximately 21nt in length (Carmell and Hannon, 2004). Unlike *Drosophila*, which has a separate Dicer protein for miRNA biogenesis and siRNA biogenesis, *C. elegans* has only one Dicer protein that is

responsible for all the small RNA biogenesis that takes place in the various RNAi pathways (Duchaine et al., 2006; Grishok et al., 2001). As is the case in fruit flies, Dicer-1 is aided in this process by the dsRNA binding protein RDE-4 (Grishok, 2005). This gene has been shown biochemically to preferentially bind to long dsRNA both *in vitro* and *in vivo* as well as to associate with DCR-1, RDE-1 and DRH-1 *in vivo* (Parker et al., 2006; Tabara et al 2002). The finding that RDE-1 is present in a dicing complex is interesting as it is the only example of an Argonaute protein being present in a small RNA biogenesis complex. Once this complex produces siRNAs, they are loaded onto RDE-1, one of 27 Argonaute proteins present in *C. elegans*, and guides RDE-1 to its target mRNA and induces cleavage of said mRNA (Yigit et al., 2006).

In the vast majority of animals this is where the pathway stops. But in *C. elegans*, like in plants, the left over 5' cleavage product can be used for the formation of *de novo* dsRNA synthesis (Yigit et al., 2006). The first step in this process starts with the β nucleotidyltransferase RDE-3 binding the 3' end of the 5' cleavage product from the slicing reaction and recruiting an RNA dependent RNA polymerase (RdRP) (Chen et al., 2005). In the germline the RdRP is EGO-2 while in the soma it is RRF-1. These RdRPs then use the cleaved mRNAs as a template for dsRNA synthesis. This new dsRNA is then bound to by a DCR-1 complex containing the PIR-1 phosphatase and cleaves the dsRNA into secondary siRNAs (Aoki et al., 2007; Duchaine et al., 2006; Pak and Fire, 2007; Sijen et al., 2007). These secondary siRNAs are then bound by a subgroup of the Argonaute proteins including CSR-1, PPW-1, PPW-2, SAGO-1 and SAGO-2 (Aoki et al., 2007; Claycomb et al., 2009; Yigit et al., 2006). These secondary siRNAs bound by

this subgroup of Argonautes can then be used to direct silencing of secondary mRNA targets as well as mediate heterochromatin formation and transcriptional silencing (Claycomb et al., 2009; Yigit et al., 2006).

4.1.2 Antiviral RNAi in *C. elegans*

In 2005 three laboratories showed that RNAi in *C. elegans* can be antiviral, two of the laboratories used a primary cell culture system with mammalian virus Vesicular stomatitis virus (VSV) and the third used transgenic worms carrying an amplicon of the insect Flock house virus (FHV) (Lu et al., 2005; Schott et al., 2005; Wilkins et al 2005). In the primary cell culture studies it was found that VSV viral RNA could accumulate to higher levels in the *rde-1, rde-4, rde-3,* and *rrf-1* mutant cell lines (Schott et al., 2005; Wilkins et al., 2005). Furthermore Wilkins et al., 2005 were able to detect VSV specific siRNAs in N2 cells using RNase protections assays. VSV particles were able to be purified from VSV infected cells and plaque assays were performed using mammalian cell culture techniques. It was found that in the RNAi mutants that supported higher levels of virus replication there was also higher production of virus particles (Schott et al., 2005). These results also demonstrated that the virus particles produced in worms was also infective in mammalian cell culture. In the study using transgenic worms carrying the FHV amplicon it was found that both FHV RNA 1 and RNA 2 were able to replicate in adult *C. elegans*. Furthermore, it was found that this replication occurred in an RDE-1 dependent manner as in *rde-1* mutant worms the viral RNAs accumulated to higher levels (Lu et al., 2005). The viral RNAs from worms were also found to be infective in *Drosophila* S2 cells, the natural host of FHV. Researchers were also able to make

transgenes that carried a mutation in the B2 open reading frame (FR1-3ΔB2) rendering it unable to inhibit RNAi. In N2 worms the FR1-3ΔB2 viral amplicon was unable to replicate to detectable levels but was able to replicate in *rde-1* mutant worms (Lu et al., 2005). This proved that the B2 protein was able to inhibit RNAi in worms as B2 was not required in RNAi defective worms, much like in *Drosophila* (Li et al., 2002).

In the study presented here we show that FHV and CrPV accumulation levels in S2 cells were increased in the presence of the RIN compounds. This increase in replication levels was also associated with a decrease in the survival of the treated cells. The decrease in survival was due to the combination of both the RIN compounds and the virus, as the virus on its own or the compound on its own did not decrease cell survival. We found that adult fruit flies infected with FHV and treated with RIN2 or RIN5 were able to accumulate viral RNAs to higher levels than in untreated flies. Not only do they accumulate viral RNAs to higher levels, but the infected flies also display a higher mortality rate as compared to non-treated flies. Finally we expand our analysis of the RIN compounds from fruit flies to nematodes. Worms carrying the FR1gfp transgene that was described in Lu et al., 2009 were used to test whether the RIN compounds are effective in *C. elegans*. Not only were they effective they were also able to inhibit dsRNA knockdown of the *pop-1* gene.

4.2 Materials and Methods

4.2.1 Virus strains, virus propagation and purification

The original CrPV stock was achieved from Dr. Annette Schneemann, and propagated in S2 cells. FHV virions were purified from *Drosophila* S2 cells transfected with both pMTFR1 and pMTFR2 plasmids, as described using the Effectene Transfection Kit (QIAGEN Inc. Valencia, CA, USA) according to the manufacturer's protocol (Li, et al., 2002). Appropriate amount of cells (1.0-3.5 x 10^6 cells per well) were seeded in a 10cm plate in 10mL of Schneider's insect medium (Product No. S9895 Sigma-Aldrich, Inc. St. Louis, MO, USA) containing serum and antibiotics on the day before transfection. On the day of transfection, 1μg pMTFR1 and 1μg pMTFR2 DNAs were diluted into a microcentrifuge tube with the DNA-condensation buffer, Buffer EC, to a total volume of 100μl. After 3.2μl Enhancer was added, the samples were mixed by vortexing for 2 seconds and incubated at room temperature for 2-5 minutes. After 10μl Effectene reagent was added to the solution the samples were mixed by vortexing for 10 seconds and incubated at room temperature for 10 minutes. The transfection complexes were added to the cells and then the plate was swirled gently and then incubated under their normal growth conditions in a 27°C incubator. On the next day, 2μl $CuSO_4$ (0.5M) was added to the plate to induce the transcription of FHV RNA1 and RNA2. The cells were incubated at 27°C for 4-7 days, after which 0.5ml fresh medium was added 3 days post transfection. FHV replication in the *Drosophila* cells induced a cytopathic effect (CPE), which is a characteristic clumping of the cells. The infected cells were observed every day for any

symptoms of FHV infection. Infected cells and media were collected when severe symptom showed up or at day 7 after transfection for further purification and propagation.

Virus particles were released from infected cells by mixing with 0.5% Nonidet P-40 and by three freeze-thaw cycles. The sample was then digested with RNase A (final concentration 10ug/ml), and then pelleted through a 30% sucrose cushion containing 0.05M HEPES (pH 7.0), 5 mM CaCl2, 0.1% β-mercaptoethonal (β-ME), and 0.1% Bovine Serum Albumin (BSA). Ultracentrifugation was performed at 274,000 x g for 2.5 hours at 11°C in a SW 41T1 rotor in a ultracentrifuge. The virus pellet was resuspended in 300-400μl HEPES buffer and the insoluble material was removed by centrifugation at 14,000 rpm at 4°C. The virion quality and concentration was quantified using a Biophotometer (Eppendorf, 50μl for each sample), with an A260/280 ratio of ~ 1.52 indicating purified particles. The virions was aliquoted (100μl /microtube) and stored at -80°C for future study.

Plaque forming units per ml (pfu/ml) of the purified FHV and CrPV 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. CrPV particles were purified by Dr. Aliyari.

4.2.2 Propagation and Purification of FHVΔB2 virions

The original FHVΔB2 stock was achieved from Dr. A.L.N. Rao's lab and propagated in S2 cells which Ago-2 was depleted by transfection of Ago-*2*-specific dsRNAs (0.1ug/ml media) one day before viral inoculation. The FHVΔB2 virions were purified

through a 30% sucrose cushion following the protocol for FHV purification described in 4.2.1.

4.2.3 Virus Infection in S2 cells

S2 cells were seeded and prepared as above and incubated in a 27°C incubator for 12 hours. Following the incubation period cells were treated with 30µM of RIN compound dissolved in DMSO to a final concentration of 1% per well and then incubated for another 12 hours at 27°C. Cells were then infected with 1000 pfu of FHV or FHVΔB2 and allowed to replicate for 2 days and 5 days, respectively. For time course analysis samples were taken at the respective time points. For cell survival assays, cells were stained with Evans Blue, and then counted using a hemocytometer. Viral RNA analysis is described below.

4.2.4 Culturing, Growth and dsRNA Treatment of *C. elegans* Strains

Worm strains were maintained on standard Normal Growth Medium (NGM) plates (Table 4-1) seeded with the OP50 *E. coli* strain in a 20°C incubator. For viral RNA analysis or GFP fluorescence visualization worms were prepared as follows. Gravid adult worms were washed off of NGM plates with M9 solution (Table 4-2) and spun down in microfuge tubes at 3000 rpm at room temperature. To release and synchronize the worm embryos, the worms were treated with a 50% bleach solution that dissolves the adult worms while leaving the embryos intact. The embryos were then washed three times in M9 solution and then plates onto NGM plates and placed in a 20°C incubator. Once the worms entered the fourth larval stage they were placed in a 33°C incubator for 3 hours to induce the transcription of the FHV transgene/transgenes (pFR1-3 and pFR2). After the

heat shock was complete the worm plates were placed into a 20°C incubator for 2-3 days to allow for virus replication to occur. After the incubation period was over the worms were collected and used for further analysis. For dsRNA treatment, worms were synchronized as described above and placed on NGM that contained 25µg/ml Carbenicillin, 1mM IPTG and seeded with the *E. coli* expressed *pop-1* dsRNA. Worms were fed on these plates for 2-3 days and then analyzed for the *pop-1* RNAi phenotype. For RIN treatment of worms, RIN compounds were mixed with OP50 and then placed on NGM plates.

4.2.5 RNA Extraction from *C. elegans*

RNA extractions were carried out as follows. Worms were washed off NGM plates using M9 solution, collected in microfuge tubes and spun down at 3000 rpm to collect the worms in a pellet at the bottom of the tube. 500 µl of TRIzol (Invitrogen Life Technologies, Burlington, Ontario, Canada) was added to the tube and homogenized using a Tissue Tearor Variable Speed Homogenizer (Cole-Parmer) and homogenized for 2 minutes. After the worms were homogenized they were left to incubate at room temperature for 10 minutes in the TRIzol and then 100 µl of chloroform was added to the mixture and vortexed at high speed for 20 seconds. The mixture was then centrifuged for 10 minutes at 14,000 rpm on a table top centrifuge and then the supernatant was removed and added to an equal volume (~300 µl) of isopropanol and then incubated at -20°C for 30 minutes to precipitate the RNA. After the incubation period the samples were spun down for 10 minutes at 14, 000 rpm to pellet the RNA. Pellets were then washed once with 70% ethanol and then resuspended in 30 µl of TE buffer. Samples were then

incubated for 10 minutes at room temperature and then for 10 minutes at 65°C to dissolved the pellet in the TE buffer. The RNA quality and concentration was checked by using a Biophotometer.

4.2.3 Northern blot Analysis

Northern blot analysis of total RNA samples was performed as previously described in chapter 2.

4.2.4 Fly Strains and Virus Inoculations

Canton S flies were used in these experiments. Fly stocks and infected flies were reared on standard cornmeal-agar medium at room temperature. Virus particles were diluted in 1 x PBS buffer. Twenty 4-6 day old adult flies of were transferred into a new vial with fresh medium one day before they were infected. Flies were anaesthetized by exposure to CO_2 during the injection process. Approximately 40 nl of a viral suspension (FHV: $3x10^{12}$ pfu/ml) mixed with RIN2 or RIN5 was microinjected into the thorax of adult flies with a microinjector (FemtoJET 5247, Eppendorf, Germany). Injection of the same volume of 1 x PBS buffer or RIN compounds were used as a control. All the infected flies were observed and the survival rate was recorded every day. Survival curves were the average of three independently repeated experiments.

4.2.5 RNA Extraction from Adult Fruit Flies

Total RNA was extracted by using TRIzol according to the manufacturer's directions with some modifications. Approximately 20 adult flies were collected from growth vials and frozen in microtubes in a -80°C freezer. 500 µl of TRIzol reagent was added to each

tube. The flies were then homogenized with a pestle. After homogenization, another 500 µl of TRIzol reagent was added and the samples were mixed and incubated for 5 or 10 minutes at room temperature. The insoluble materials were removed by centrifugation at 14,000 rpm for 10 minutes at 4 °C in a refrigerated centrifuge (Centrifuge 5804R, Eppendorf) and then the supernatant was transferred to a new tube and 200 µl of chloroform was added into each tube. The sample was mixed by vortexing for 15 seconds and then incubated at room temperature for 2 to 3 minutes; the mixture was then spun at 14,000 rpm for 15 minutes at 4°C. The upper aqueous layer was removed and transferred to a new tube and 600µl of isopropyl alcohol was added. The samples were incubated for at least 30 minutes in a -20°C incubator to precipitate the RNA and then spun at 14,000 rpm for 15 minutes at 4°C to pellet the RNA. The supernatant was removed and then the RNA pellets were washed with 1ml of 75% ethanol, air dried for 10-15 minutes and then resuspended in 50µl of RNase-free water. The RNA quality and concentration was checked by using a Biophotometer (50µl for each sample; eppendorf), with an A260/280 ratio of at least 1.75.

4.3 Results

4.3.1 FHV and CrPV Infection in RIN treated S2 cells Results in Cytotoxic Effects

Wild-type FHV particles, containing RNA 1 and RNA 3 (Figure 4.2a; FHV genome structure) were used to infect S2 cells that were treated with 30µM of each of the RIN compounds. Following FHV infection, total RNA was extracted from the infected cells and Northern blot analysis was performed to detect both FHV RNA 1 and RNA 3 levels. RINs 1 and 2 showed a mild increase in viral RNA levels as compared to the non-treated

control (Figure 4.2b; compare lane 1 to lanes 3 and 4). This increase in viral RNA levels in RIN1 or RIN2 treated cells was similar to the increase found in Ago2 depleted cells that were infected with FHV (Figure 4.2b; compare lanes 1 and 2 to lanes 3 and 4). Interestingly, no detectable viral RNA was observed in lanes loaded with RNA from cells treated with either RINs 3, 4 or 5 (Figure 4.2b lanes 5-7). Evans blue staining revealed that nearly all the S2 cells that were treated with RINs 3, 4 or 5 and infected with FHV were dead, with RIN5 treated cell showing only 5% cell survival (Figure 4.2c). To test whether this phenomenon was specific to FHV, similar experiments were done with Cricket paralysis virus (CrPV), a monopartite, plus sense RNA virus related to poliovirus (Figure 4.2d; top panel)(Scotti et al., 1975). S2 cells were treated with 30µM of each RIN compound and subsequently infected with CrPV. In RIN1 or RIN2 treated cells that were infected with CrPV there was a detectable increase in CrPV viral RNA levels as compared to the non-treated control, as shown by Northern blot analysis (Figure 4.2d; compare lanes 2 and 3 to lane 1). Also, as was the case with FHV, no viral RNAs were detected in S2 cells that were treated with either RINs 3, 4 or 5 and infected with CrPV (Figure 4.2d; compare lanes 4, 5 and 6 with lane 1). Put together with the cytotoxic effect data, it appears that RIN treatment of S2 cells in the presence of virus infection resulted in an increase in viral RNA levels, similar to Ago2 depletion. Furthermore, in the case of RINs 3, 4 and 5, the combination of RIN treatment and virus infection led to reduced cell survival, possible due to an intolerable increase in viral RNA levels.

4.3.2 RIN5 Increases FHV Levels in *D. melanogaster* S2 cells

Given the previous data, we wanted to know whether or not the cell death witnessed in S2 cells treated with RIN5 and infected with FHV was due to an increase in viral

replication. To answer this question, FHV particles were diluted 100-fold and then used to infect S2 cells that were treated with 30µM of RIN5. Cell survival and viral RNA levels were monitored over a 2 day time course and compared to FHV only infected cells at each time point. The total RNA that was extracted from these cells was subject to Northern blot analysis to assay the levels of FHV viral RNAs 1 and 3. With RIN5 treatment viral replication was robust and viral RNA levels reached a plateau at 24 hours post infection and maintained those levels until the 48 hour time point (Figure 4.3a). The FHV only cells did not accumulate to the same level as the RIN5 treated cells, even after 48 hours (Figure 2.3a). Samples were also stained with Evans Blue to check for cell survival after RIN5 treatment (Figure 4.3b). Cells that were treated with RIN5 and infected with FHV had their survival drop to 55% after two days, whereas RIN5 only and FHV only cells had cell survival at 85% and above (Figure 4.3c). This confirmed our previous hypothesis that the cell death that was previously observed was due to rampant viral replication in the RIN5 treated cells.

FHV viRNAs levels were also examined in the presence of RIN5. S2 cells were seeded in 10mL dishes, treated with 30µM of RIN5 and then infected with FHV, at the 100-fold dilution that was used in Figure 4.3a. After a 2 day infection period the low molecular weight RNA fractions were collect from the S2 cells and then run out on denaturing sequencing gels and then subject to Northern Blot analysis. In the FHV only treated cells there was very little FHV viRNAs detected (Figure 4.3d; lane 3). In RIN5 treated cells there was abundant viRNA accumulation, similar to the levels detected in cells where Ago2 has been knocked down (Figure 4.3d; compare lanes 1 and 2). The

viRNA phenotype that was detected in RIN5 treated cells is consistent with the data presented in chapter 3 that showed that RIN5 can target the Ago2 mediated slicing reaction.

4.3.3 RIN Treatment can rescue FHVΔB2 in S2 Cells

Previous work from our lab has shown that FHV virus particles can be generated with a mutation in the B2 open reading frame that renders B2 functionless (Aliyari et al., 2008). When this FHV strain in used to infect *D. melanogaster* S2 cells no viral RNAs can be detected but an abundant amount of FHV specific viRNAs can be detected (Aliyari et al., 2008). S2 cells were treated with 30μM of each RIN compound and then infected with FHVΔB2 and allowed to replicate for 72 hours. S2 cells depleted for Ago2 were used as a control. Cells were then collected and RNA isolated for Northern blot analysis for detection of both FHV RNA 1 and RNA 3. In cells that were treated with RINs 1, 2, 3, or 4 there was a slight rescue of FHVΔB2 as compared to the non-treated control (Figure 4.4; Compare lanes 1 through 4 to lane 8). Two concentrations of RIN5 were used to treat S2 cells, 15μM and 30μM, and also infected with FHVΔB2. Both these treatments showed a strong rescue of viral replication as compared to the non-treated control, with the 30μM concentration showing better rescue of FHVΔB2 then the 15μM concentration (Figure 4.4; compare lanes 5 and 6 to lane 8 and lane 5 to lane 6). The RIN5 treatment rescued FHVΔB2 to levels that were similar to those found in Ago2 depleted S2 cells at 15μM and even higher at 30μM (Figure 4.4; compare lanes 5 and 6 to lane 7). This data corroborates the results showing that RIN5 acts at the same level of the RNAi pathway as Ago2 (Figure 4.3d). viRNAs were also analyzed in FHVΔB2 infected cells that were

treated with the RIN compounds. As has been previously reported, S2 cells that were infected with FHVΔB2 showed very abundant viRNA accumulation (Figure 4.4; lane 7). In S2 cells in which Ago2 has been depleted there was abundant viRNA accumulation, however slightly less than in untreated S2 cells (Figure 4.4; compare lanes 7 and 8). RIN5 treated cells that were infected with FHVΔB2 had abundant viRNA accumulation proportional to the high molecular weight viral RNAs that accumulated as compared to Ago2 depleted cells (Figure 4.4; compare lanes 6 to lane 7). In RIN1, 2 or 3 treated cells there was a sharp decrease in viRNA accumulation levels as compared to the non-treated control (Figure 4.4; compare lanes 1, 2 and 3 to lane 8). Again, these results were consistent with the data presented in chapter 3 that showed that RINs 1-3 can inhibit the upstream portions of the RNAi pathway.

4.3.4 RIN2 and RIN5 Treated Adult *D. melanogaster* Infected with FHV Display Enhanced Disease Susceptibility

The results obtained in RIN treated nematodes prompted us to ask if the RIN compounds could inhibit antiviral RNAi in adult *Drosophila melanogaster*. RIN2 and RIN5 were selected for these experiments as the former has been shown to inhibit dicing and the latter inhibits slicing (Chapter 3). Flies were infected with FHV and treated with either 15 or 30μM of the RIN compounds. Mock (HEPES buffer), FHV only and RIN only (30μM) flies were used as controls for the experiment. 20 flies per sample were injected in triplicate and their survival rate was monitored for 16 days post injection. In both the RIN2 and RIN5 only treated flies the chemical by itself had no effect on the survival of the flies as compared to the mock infected samples (Figure 4.5a and 4.5b). Both the low

and the high concentrations of RIN2 and RIN5 infected with FHV had a significant decrease on the survival of the flies as compared FHV only infected flies (Figure 4.5a and 4.5b). In the case of RIN2 there was an approximately a 15% decrease in the survival of the flies at the low concentration and a 30% at the high concentration as compared to FHV only infected flies 16 days post injection (Figure 4.5a). In RIN5 treated flies there was a 20% decrease in the survival of FHV infected flies as compared to FHV only infected flies for both the high and low concentrations (Figure 4.5b). Interestingly there was an immediate decrease in the survival of the flies at the 30µM dose both RIN2 and RIN5 flies infected with FHV only 1 day post injection; 20% in the case of RIN2 and 30% in the case of RIN5 (Figure 4.5a and 4.5b). This could be due to the combination of the sudden loss of the RNAi coupled with the infection of a virulent titer of FHV.

Viral RNAs were also analyzed from flies 8 days post infection by Northern blot analysis to detect FHV RNAs 1, 2 and 3. Both the 15 and 30µM concentrations of RIN2 and RIN5 were able to increase the amount of viral RNAs as compared to FHV only infected flies (Figure 4.5c, compare lanes 4, 5, 7 and 8 to lane 2). Furthermore, in RIN5 only treated flies there was a detectable amount of viral RNAs 1, 2 and 3 (Figure 4.5c lane 6). This is likely due to the fact that many *D. melanogaster* fly stocks are persistently infected at a low level with FHV and that RIN5 treatment was able to relieve the repression RNAi had on the virus and allowed FHV to replicate to detectable levels.

4.3.5 RIN compounds can block Antiviral RNAi and the Exogenous RNAi Pathway in *Caenorhabditis elegans*

C. elegans has proven to be a useful tool in studying both RNAi and virus-host interactions. The next step was to determine whether the RIN compounds that were discovered to inhibit both antiviral and exogenous RNAi in S2 cells were effective in nematodes. To do so the FR1gfp system that was used in Lu et al., 2009 was used to determine whether the RIN compounds could inhibit antiviral RNAi in *C. elegans*. The benefit of using this system is that the GFP is expressed from RNA 3 which means that GFP fluorescence can only be visualized once virus replication has occurred. 10 FR1gfp worms in the third larval stage were placed on plates that were seeded with OP50 containing 30µM of either RINs 2, 4 or 5 and then the worms were allowed to grow until they reached the fourth larval stage. The worms were then heat shocked for 2 hours at 36°C to induce transcription of the FR1gfp transgene and then incubated at 20°C for 2 more days to allow for the viral RNA to replicate. Untreated FR1gfp worms and *rde-1* worms with the FR1gfp transgene were used as controls. Virus derived GFP fluorescence was visualized using a Leica MZIII Pursuit stereo microscope and images were captured with a 4MP RGB/gray SPOT camera. Worms treated with RINs 2, 4 or 5 had varying expression levels of GFP in both GFP intensity and location of fluorescence. Worms treated with RINs 2 and 5 had GFP fluorescence throughout the whole body but the intensity of the GFP was stronger in RIN2 treated worms then in RIN5 treated worms (Figure 4.6a). RIN4 had strong GFP expression only in the pharynx with weaker GFP fluorescence in the remainder of the body (Figure 4.6a). Untreated worms had no detectable GFP fluorescence (Figure 4.6a). Worms were also treated with two other

chemical concentrations, 60 and 100µM, using the same procedure described above. RINs 2, 4 and 5 showed a does dependent response in GFP fluorescence; the higher the RIN concentration the stronger the intensity of the GFP fluorescence (Figure 4.6b). RINs 2 and 4 treated worms showed a steady increase in GFP fluorescence from 30 to 100µM while GFP fluorescence in RIN5 treated worms reach a plateau at 60µM (Figure 4.6).

These resulted led us to ask if the RIN compounds could inhibit exogenous RNAi in *C. elegans* as in *Drosophila melanogaster*. To address this, gravid N2 worms were bleached and the resulting embryos were cleaned and then 10 embryos were placed onto plates with bacteria expressing dsRNA that targets the *pop-1* gene was laced with each of the RIN compounds. It has been previously reported that the offspring of worms fed of dsRNA targeting *pop-1* fail to develop to adulthood, arresting in the embryo (Kostic and Roy 2002). Worms were then allowed to grow, feed and lay eggs on the RIN treated plates and then the adult worms were removed from the plates and the F1 generation was allowed to grow for 2 more days. After the 2 day period the plates were collected and the percent survival of the worms was calculated as compared to *rde-1*, untreated N2 worms and RIN treated worms that were not fed on *pop-1* dsRNA. As was the case in S2 cells, RIN3 alone showed high mortality rates as compares to untreated worms while the other compounds did not show such an effect (Figure 4.6c). RINs 1, 2, 4 and 5 were able to rescue the embryonic lethal *pop-1(RNAi)* phenotype to varying degrees with RIN1 and RIN5 being the highest at 82% and 84% survival. RIN4 had the lowest survival rate at 46% (Figure 4.6c). RIN2 had an intermediate ability to resist *pop-1(RNAi)* with a survival rate of 67%. As was the case in fruit flies, the RIN compounds can also inhibit exogenous

RNAi in nematodes which indicates that these compounds may be used as a means of control against nematode pests.

4.4 Discussion

Previous work from our lab has shown that when viruses are infected in RNAi defective cells that the viral load and RNA increases dramatically (Aliyari et al., 2008; Li et al., 2002). The same is true in adult flies defective in RNAi that have been infected with virus particles (van Rij et al., 2006; Wang et al., 2006; Zambon et al., 2006). In the work presented here we show that S2 cells that were treated with the RIN compounds allowed for higher accumulation of FHV and CrPV viral RNAs. In cells treated with RINs 3, 4 or 5 virus replication reached levels that induced cytopathic effects in the infected cells. This phenotype was not seen in FHV only infected cells or in cells that were only treated with RIN compounds. Further experiments showed that when RIN5 treated cells are infected with FHV the viRNA phenotype was the same as in FHV S2 cells where Ago2 has been depleted (Figure 4.3d). This is consistent with the idea that RIN5 acts in the downstream portions of the RNAi pathway as Ago2 has been shown to act downstream of siRNA biogenesis being involved in siRNA recruitment in siRISC as well as RNA cleavage (Okamura et al., 2004; Tomari and Zamore, 2005). More work has shown that FHVΔB2 replication can be rescued to varying degrees in RIN treated S2 cells, further evidence that the RINs can inhibit the antiviral RNAi pathway, as FHVΔB2 has only been shown to be able to replicate in RNAi deficient organisms (Aliyari et al 2008; Li et al., 2002; Lu et al., 2005). In RIN1, 2, 3 or 4 treated S2 cells infected with FHVΔB2 the viRNA accumulation levels were reduced as compared to untreated or

dsAgo2 treated cells indicated that viRNA biogenesis was compromised in these cells (Figure 4.4). This phenotype is in agreement with the idea that dicing of the dsRNA viral precursor, mediated by Dcr-2, was perturbed in these cells (Wang et al., 2006). In *D. melanogaster* Dcr-2 is also responsible for siRNA biogenesis in both the endogenous and exogenous RNAi pathways (Czech et al., 2008; Lee et al., 2004; Miyoshi et al., 2010; Okamura et al., 2008). Based on previous data presented in chapter 3, it is possible that RIN4 inhibits the accumulation of secondary siRNAs in *Drosophila.* Seeing that very low levels of viRNAs were detected in FHVΔB2 infected cells treated with RIN4 and combined with the fact that RIN4 has no effect on dicing or slicing, it is hypothetically possible that RIN4 can block secondary siRNA biogenesis. A possible target of this function could be D-elp1, a subunit of RNA polymerase II which has been shown to be responsible of *de novo* dsRNA formation which can then be used for secondary siRNA production (Lipardi and Paterson, 2009). More work will need to be done to establish if this is the case or not. The RNA levels which were detected in S2 cells treated with RIN5 rivaled those in Ago2 depleted cells. This finding suggests that RIN5 can be used as a tool for the propagation of FHVΔB2, which normally required transfection of dsAgo2 to purify the particles. The usage of RIN5 could cut the costs of the propagation of this virus as generation and transfection of dsRNA can be costly.

Previous studies have shown that viral RNAs can accumulate to high levels in adult flies and nematodes that carry mutations in key RNAi genes (Lu et al., 2005; Lu et al., 2009; van Rij et al., 2006; Wang et al., 2006; Zambon et al., 2006). The replication of FR1gfp in RIN treated *C. elegans* serves as additional evidence that the RIN compounds

do in fact block antiviral RNAi as well as that RNAi is an antiviral mechanism in nematodes. Further experiments showed that RIN treated nematodes are resistant to feeding RNAi providing additional evidence that the RIN compounds can inhibit the exogenous RNAi pathway. The results the RIN compounds had in nematodes prompted the investigation of the effectiveness of them in adult fruit flies where it was found that RIN2 and RIN5 can subvert antiviral RNAi. These phenotypes were in agreement with previous studies where RNAi mutant flies injected with virus particles supported higher levels of viral RNA replication (Chotkowski et al., 2008; Galiana-Arnoux et al., 2006; Wang et al., 2006; Zambon et al., 2006).

The decrease in survival of RIN treated fruit flies infected with FHV is akin to the decrease in the survival witnessed in flies infect with FHV as well as other insect viruses (Chotkowski et al., 2008; Galiana-Arnoux et al., 2006; Wang et al., 2006; Zambon et al., 2006). When viruses including CrPV, DXV, FHV and WNV are injected in RNAi mutant flies there is an increase in viral RNAs and well as a decrease in the survival of the flies. Potential uses of the RIN compounds are as pesticides, but not in the traditional sense. Since flies infected with FHV and treated with RIN2 or RIN5 show enhanced disease susceptibility it is reasonable to hypothesize that the treatment insect pests with these chemicals, or a combination of them, can render their antiviral immunity defunct and allow for naturally occurring viruses to kill the pests by allowing them to replicate to higher levels. An advantage of using this method is that the chemical itself does not kill the pest but a naturally occurring virus does, which may limit the pest from acquiring resistance to the chemicals. This idea is further supported by the fact that RIN5 treated

fruit flies that were not infected with FHV accumulated viral RNAs (Figure 4.5c lane 6). This is due to the fact that many virus stocks are persistently infected with a low level of virus and that RIN5 treatment removed the limitations placed by RNAi and allowed the virus to replicate to detectable levels.

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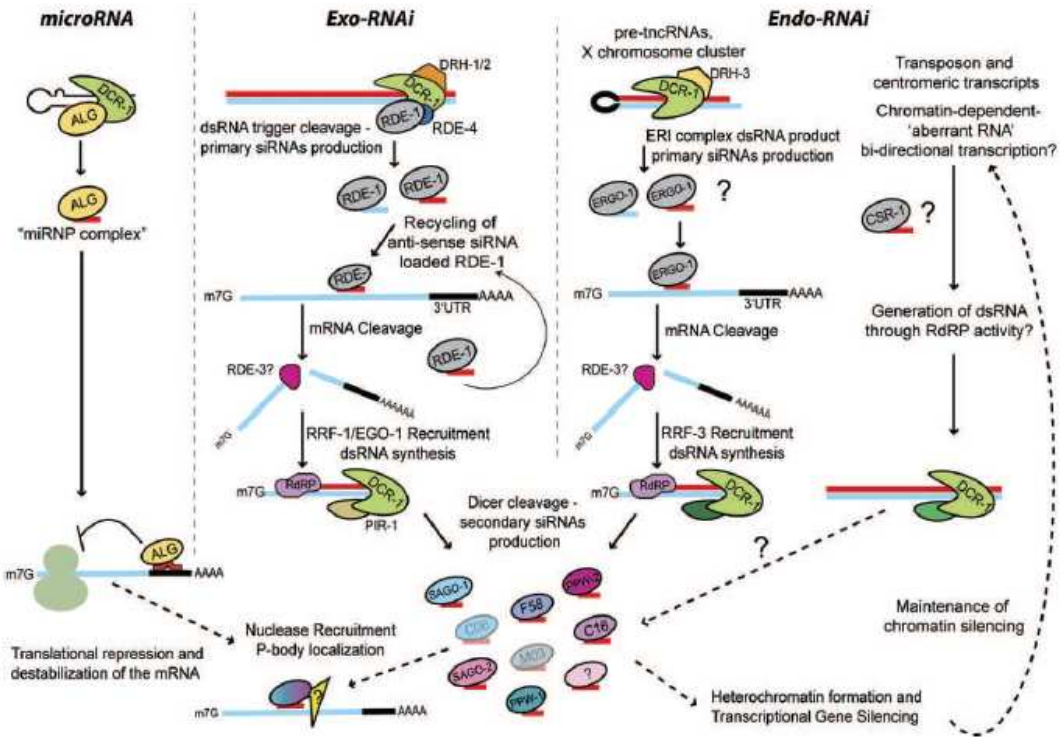


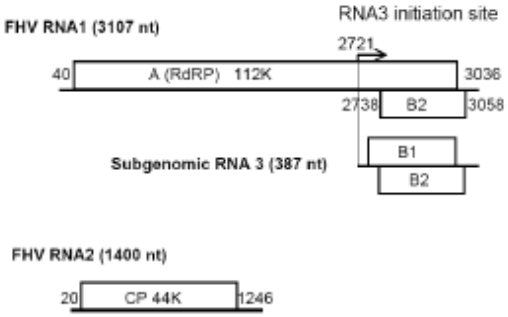
From Yigit et al., 2006

Figure 4.1 Small RNA Pathways in *C. elegans*

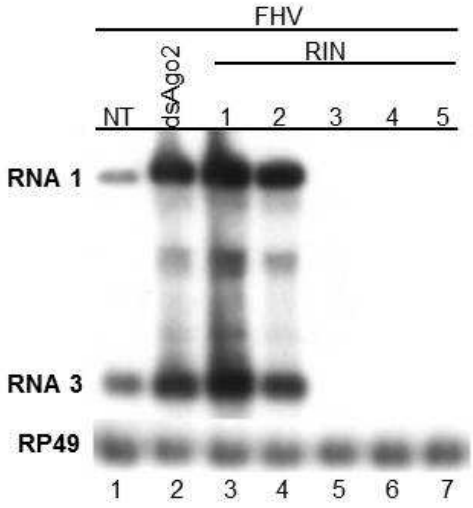
On the left panel is a diagram of the microRNA pathway which requires DCR-1 and the miRNA specific ALG class of Argonaute proteins. Once the miRNA's are processed and loaded into either ALG-1 or ALG-2 they can cause translational repression which leads to the destabilization of the mRNA target. The center panel depicts the exogenous RNAi pathway. This pathway produces siRNAs via DCR-1, RDE-1, RDE-4 and either DRH-1 or DRH-2. Once siRNAs are produced they mediate cleavage of target RNAs. On the right is the endo-siRNA pathway which also requires DCR-1 and DRH-3 as opposed to DRH-1 or 2. All these small RNA pathways compete for access the DCR-1 protein.

Figure 4.2 FHV and CrPV Replication in RIN Treated S2 cells
The genome organization of FHV (A) and CrPV (D). S2 cells pretreated for 12 hours with 30µM of RIN compound and infected with FHV (B) or CrPV (D). Total RNA was extracted 2 days post infection for Northern blot analysis by a probe specific for the B2 region of FHV or to ORF 1 of CrPV. (C) Evans blue staining with a 1% solution reveals that RIN3, 4 or 5 treated S2 cells infected with FHV have greatly reduced rates of survival.

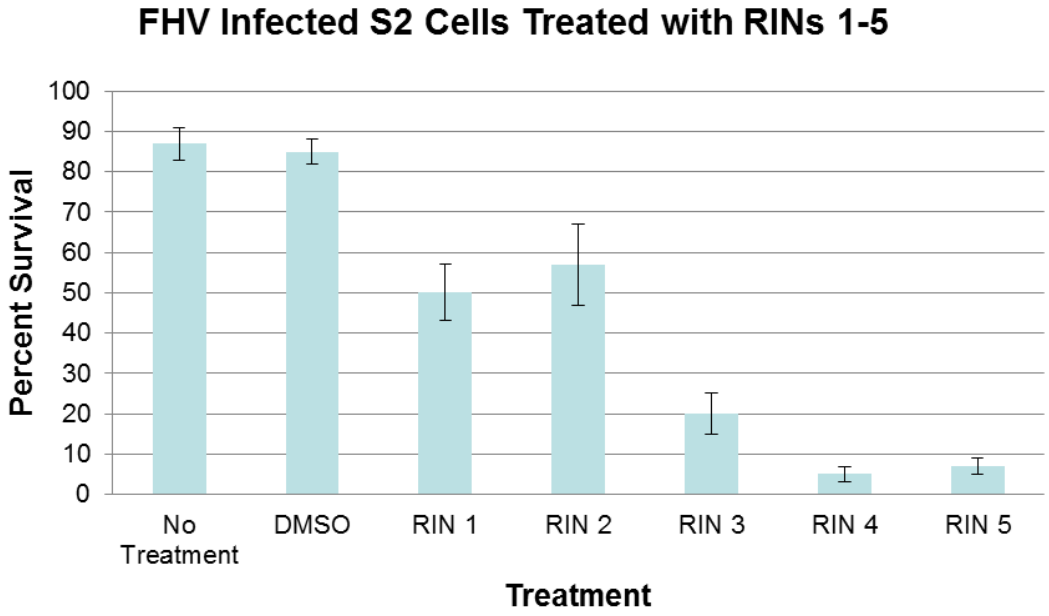
A.


FHV RNA1 (3107 nt)
RNA3 initiation site
2721
40
A (RdRP) 112K
3036
2738
B2
3058
B1
Subgenomic RNA 3 (387 nt)
B2
FHV RNA2 (1400 nt)
20
CP 44K
1246

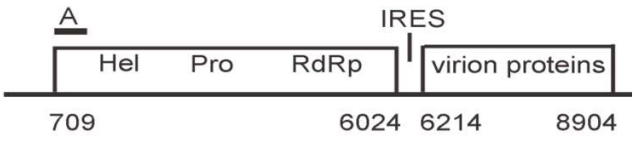
B.


FHV
RIN
dsAgo2
NT
1
2
3
4
5
RNA 1
RNA 3
RP49
1
2
3
4
5
6
7

C.


FHV Infected S2 Cells Treated with RINs 1-5
Percent Survival
100
90
80
70
60
50
40
30
20
10
0
No Treatment
DMSO
RIN 1
RIN 2
RIN 3
RIN 4
RIN 5
Treatment

D.


A
IRES
Hel
Pro
RdRp
virion proteins
709
6024
6214
8904

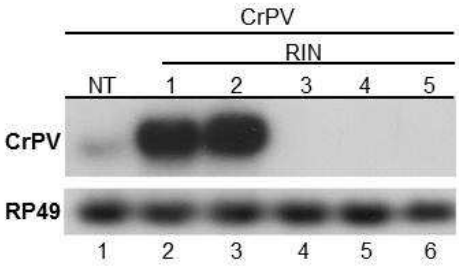
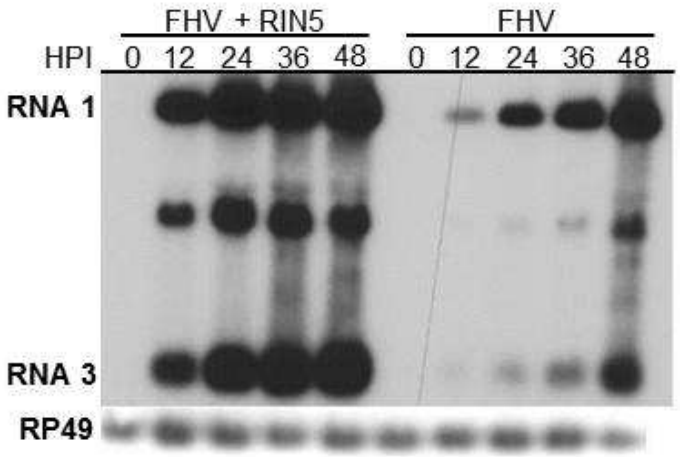

CrPV
RIN
NT
1
2
3
4
5
CrPV
RP49
1
2
3
4
5
6

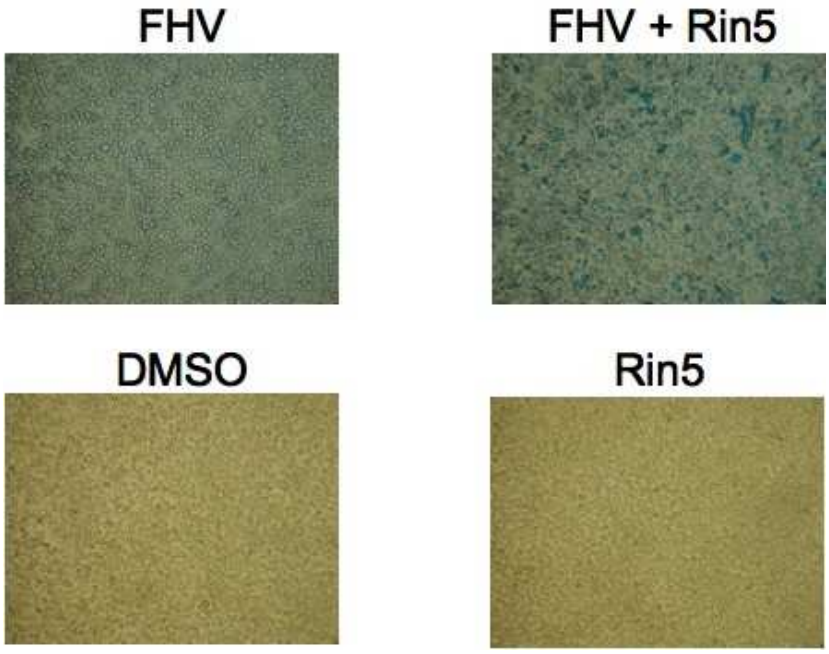
Figure 4.3 Dilution of FHV particles indicates Cell Death due to Abundant Viral RNA Accumulation in RIN5 Treated Cells

(A) Northern blot of S2 cells pretreated for 12 hours with 30µM of RIN5 and infected with an FHV inoculum diluted 1 to 100 from the experiment in figure 2.2. (B) and (C) S2 cells infected with FHV and either treated with DMSO or RIN5 were stained with a 1% Evans blue solution for cell survival studies. Graphs represent the average of three independent experiments with error bars representing the standard deviation. (D) viRNA analysis of RIN5 treated cells infected with the same inoculum of FHV used in (A). The filter was probed using oligonucleotides corresponding to FHV RNA 3.

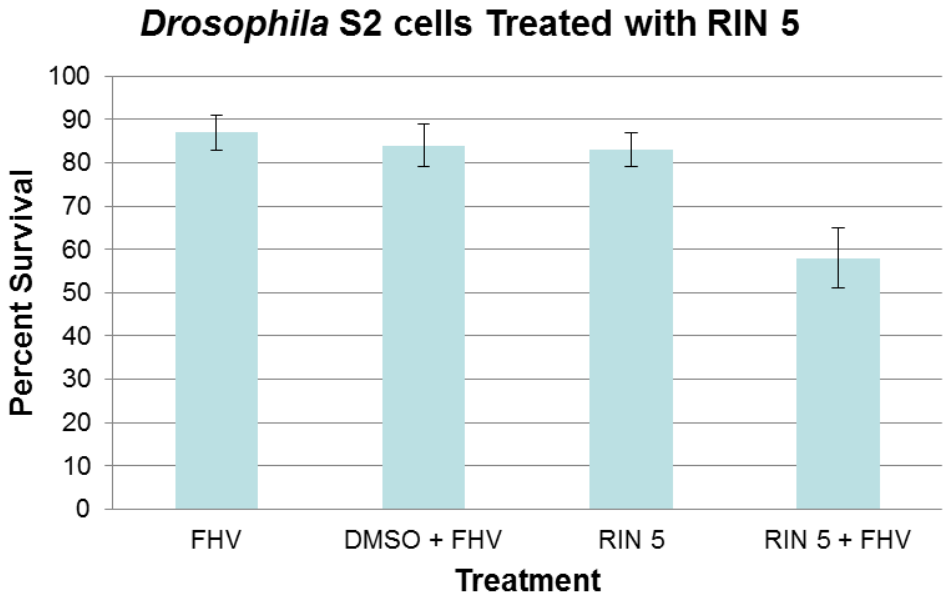
A.


FHV + RIN5
FHV
HPI
0 12 24 36 48
0 12 24 36 48
RNA 1
RNA 3
RP49

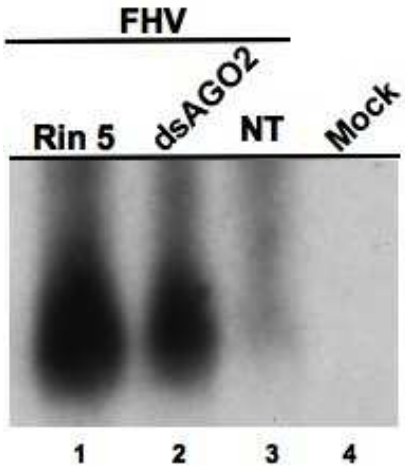
B.


FHV
FHV + Rin5
DMSO
Rin5

C.


Drosophila S2 cells Treated with RIN 5
Percent Survival
100
90
80
70
60
50
40
30
20
10
0
FHV
DMSO + FHV
RIN 5
RIN 5 + FHV
Treatment

D.


FHV
Rin 5
dsAGO2
NT
Mock
1
2
3
4

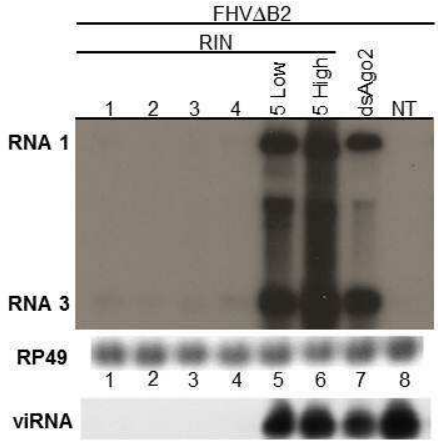
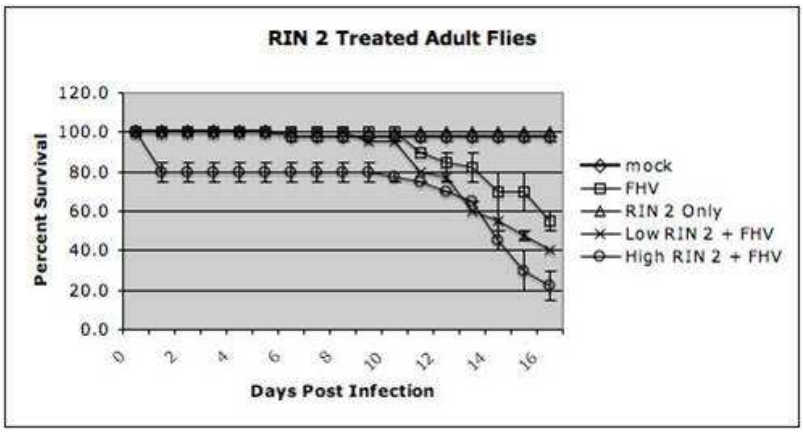


Figure 4.4 FHVΔB2 Infection on RIN treated S2 cells
30µM pretreatment of S2 cells for 12 hours infected with FHVΔB2. Total RNA was collected and size fractionated to separate high and low molecular weight RNAs. Low molecular weight RNAs were analyzed by a 15% polyacrylamide sequencing gel while high molecular weight on an agarose gel. Both filters were probed with a probe complementary to FHV RNA 3.

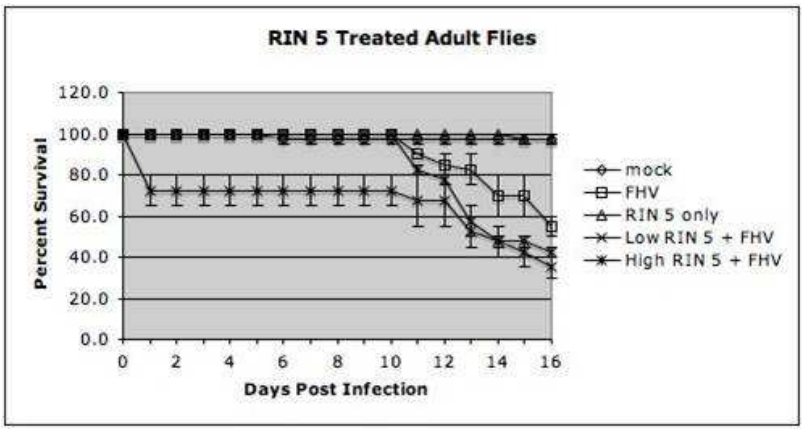
Figure 4.5 RIN2 and RIN5 treated adult *Drosophila melanogaster* and infected with FHV particles display Enhanced Disease Susceptibility

Survival curves of RIN2 (A) and RIN5 (B) treated flies at both 15 and 30µM infected with FHV. Flies were infected and their survival was tracked over a 16 day time course. Mock infected, FHV only and RIN only treated flies were used as controls. (C) Total RNA was extracted from RIN treated, FHV infected flies 8 days post infection and used for Northern blot analysis to detect RNAs 1, 2 and 3. RP49 was used for a control to insure equal loading.

A.


RIN 2 Treated Adult Flies
Percent Survival
120.0
100.0
80.0
60.0
40.0
20.0
0.0
0
2
4
6
8
10
12
14
16
Days Post Infection
mock
FHV
RIN 2 Only
Low RIN 2 + FHV
High RIN 2 + FHV

B.


RIN 5 Treated Adult Flies
Percent Survival
120.0
100.0
80.0
60.0
40.0
20.0
0.0
0
2
4
6
8
10
12
14
16
Days Post Infection
mock
FHV
RIN 5 only
Low RIN 5 + FHV
High RIN 5 + FHV

C.

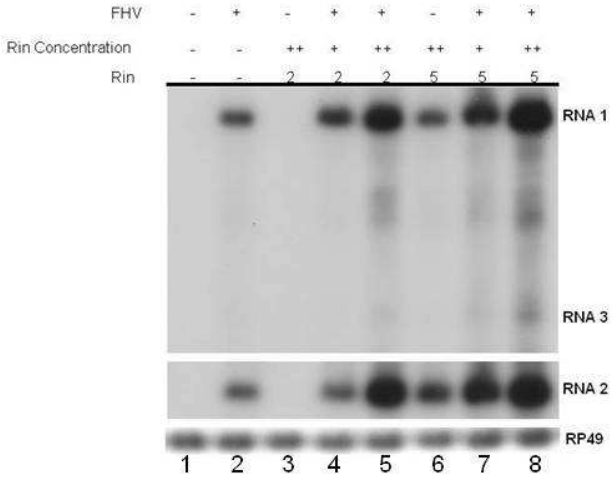
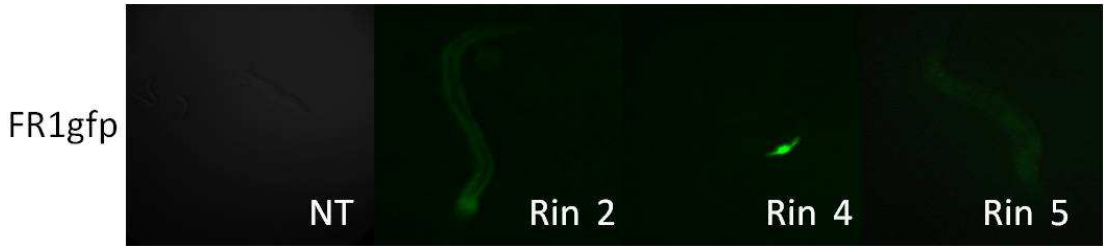

FHV - + - + + - + +
Rin Concentration - - ++ + ++ ++ + ++
Rin - - 2 2 2 5 5 5
RNA 1
RNA 3
RNA 2
RP49
1 2 3 4 5 6 7 8

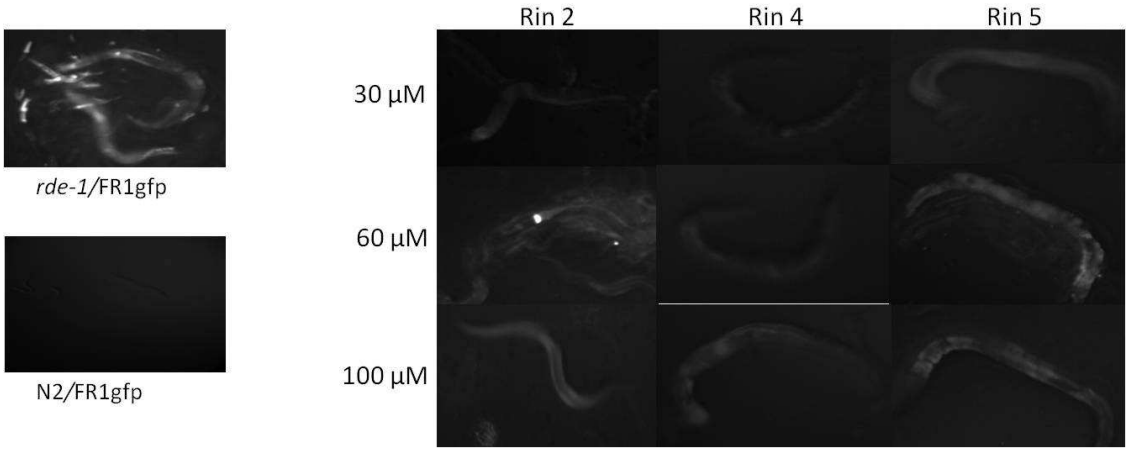
Figure 4.6 RIN treated worms phenocpy RNAi mutants

(A) Green fluorescence detected in FR1gfp worms treated with 30µM of RINs 2, 4 or 5 two days post heat induction. (B) Green fluorescence detected in FR1gfp worms treated with 30µM, 60µM and 100µM of RINs 2, 4 or 5 two days post heat induction shows a dose dependent response. (C) N2 worms on *pop-1*(RNAi) feeder and treated with 30µM of RIN compounds phenocopy rde mutant animals. Bar graphs display the average of three independent experiments with error bars indicating the standard deviation.

A.


FR1gfp
NT
Rin 2
Rin 4
Rin 5

B.


rde-1/FR1gfp
N2/FR1gfp
Rin 2
Rin 4
Rin 5
30 µM
60 µM
100 µM

C.

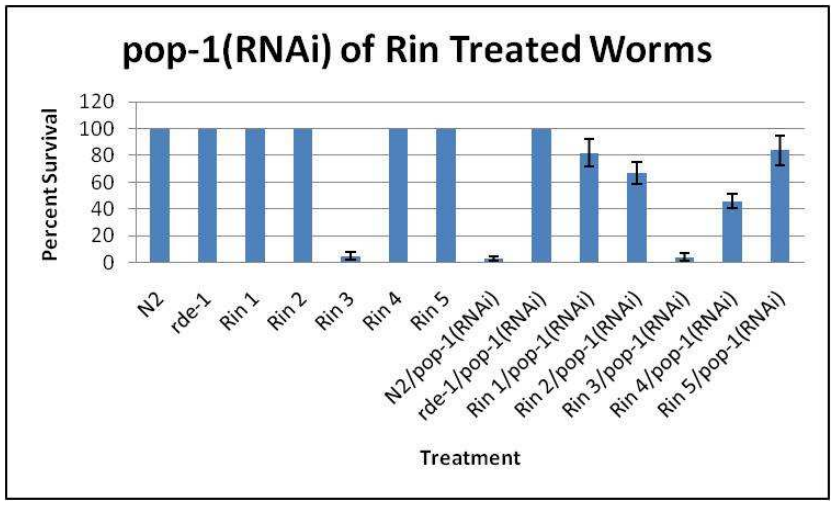

pop-1(RNAi) of Rin Treated Worms
Percent Survival
120
100
80
60
40
20
0
N2
rde-1
Rin 1
Rin 2
Rin 3
Rin 4
Rin 5
N2/pop-1(RNAi)
rde-1/pop-1(RNAi)
Rin 1/pop-1(RNAi)
Rin 2/pop-1(RNAi)
Rin 3/pop-1(RNAi)
Rin 4/pop-1(RNAi)
Rin 5/pop-1(RNAi)
Treatment

Table 4-1 Small RNA Blot Analysis Buffers

	Buffers for Small RNA Blot Analysis
Stacking Polyacrylamide 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.
Denaturing Polyacrylamide gel	12 ml Polyacrylamide:Bis (19:1 40% w/v); 1 ml 5x TBE; 14.4g urea; add ddH2O to 30 ml, dissolved by stirring using a magnetic bar; 300 µl 10% APS, 15 µl TEMED added in the end
5x TBE	0.45M Tris-borate, 10 mM EDTA, pH 8.0

Chapter 5. Conclusions and Future Directions

5.1 Conclusions

In this study genetic and chemical genetic approaches were taken in order to investigate the role of RNA interference in both *Drosophila melanogaster* and *Caenorhabditis elegans*. My data indicates that both of these approaches can be powerful tools in deciphering the RNAi pathway in these organisms.

Firstly, the work presented here demonstrated that the R1gfp cell line is a suitable platform for conducting high-throughput screening of small molecules to identify compounds that can allow for the replication of a VSR deficient viral amplicon, which cannot replicate under normal circumstances due to the activity of antiviral RNAi. Using cellular, genetic and biochemical assays I was able to show that the compounds identified in said screen, named RIN, are also inhibitors of the exogenous RNAi pathway in *Drosophila*, which has strong overlap with the antiviral RNAi pathway as both pathways use the Dcr-2/R2D2/Ago2 pipeline for generation and utilization of small RNAs. Treatment of *Drosophila* S2 cells with RIN compounds allowed for an increase in RNA levels of both Flock house virus and Cricket paralysis virus in infected cells. These infected cells also showed a decrease in survival when infected with virus as compared to the uninfected controls. Through the combination of genetic and biochemical approaches the RIN compounds were positioned in the RNAi pathway at distinct stages, with RIN3 working upstream of small RNA biogenesis (dicing), RIN1 and RIN2 being able to block dicing, RIN4 having it's mode of action between dicing and slicing and finally RIN5 blocking slicing itself. The above data also highlighted that exogenous RNAi and

antiviral RNAi targets have differing susceptibility to the RNAi pathway. The use of the RIN compounds in this study showed a different susceptibility of endogenous mRNA and viral RNA targets to the core RNAi machinery. This is observed by the fact that the five RIN compounds were similarly effective in suppressing RNAi targeting cyclin A mRNA whereas RIN1 and RIN2 were less effective at inhibiting RNAi against FHV as compared to RIN5.

Following experiments conducted in cell culture and biochemically active lysates, RIN treatments were conducted in the whole animal models *D. melanogaster* and *C. elegans*. In adult flies, RIN treatment followed by FHV infection led to both a decrease in the survival of the flies as well as an increase in viral RNA levels. These results phenocopied the results found in virus infected RNAi mutant flies, further confirming that these compounds inhibit the RNAi pathway (Galina-Arnoux 2006; van Rij et al., 2006; Wang et al., 2006). The *C. elegans* strain developed to express the FR1gfp amplicon was treated with the RIN compounds. The RIN compounds allowed for the replication of FR1gfp in a dose dependent manner which was visualized by GFP fluorescence. Wild-type N2 worms fed pop-1 dsRNA were also exposed to RIN treatment. It was found that as in *rde-1* mutant worms, the RIN treated worms were resistant to dsRNA feeding, with each RIN granting a varying degree to resistance (Tabara et al., 2002). The experiments in *C. elegans* highlight that the RIN compounds target conserved steps in the RNAi pathway which explains the ability of the molecules to cross the species barrier.

The second part in this work expands upon genetic analysis of antiviral RNAi in *C. elegans* that was initiated in our laboratory (Lu et al., 2005). The analysis presented here added RDE-4 to the antiviral pathway as well as RRF-1 and SID-1, genes involved of the amplification and spreading of the silencing signal, respectively. Not only was the antiviral RNAi pathway expanded upon, but it was also discovered that nematodes engineered to carry and replicate both FHV RNA 1 and RNA 2 can produce FHV particles that were infective to S2 cells. It is also of note that it is the particle itself that is infective as virus isolates were treated with RNase to insure that all unpackaged RNAs were degraded.

5.2 Future Directions

5.2.1 RIN Treatment in Mosquitoes

It has been previously demonstrated that RNAi plays an antiviral role in a variety of mosquito species (Campbell et al., 2008; Khoo et al., 2010; Li et al., 2004; Sanchez-Vargas et al., 2009). As there is much in common between RNAi in fruit flies and mosquitoes it is reasonable to hypothesize that the RIN compounds would have a similar effect in mosquitoes as in fruit flies. This hypothesis is strengthened by the fact the RIN compounds had an inhibitory effect in *C. elegans*, a further distantly related species to fruit flies then mosquitoes are. Preliminary experiments can be conducted in *Anopheles gambiae* 4a-2s4 cells transfected with the pONR1-ΔB2 plasmid which encodes Nodamura virus (NoV) RNA 1 with a mutation in the B2 open reading frame. Normally the pONR1-ΔB2 plasmid cannot replicate in 4a-2s4 cells, but can replicate in cells where the PAZ domain of Ago1 or Ago2 have been knocked down with dsRNA. Accumulation

of viral RNA 1 in RIN treated 4a-2s4 cells would give preliminary evidence that the RIN compounds could inhibit antiviral RNAi in mosquitoes. Further experiments would include RIN treatment in adult mosquitoes. There is an available SINV infection system present in *Ae. Aegypti* which can be used to test the RIN compounds. After treatment with the RINs, mosquitoes can be collected and RNA levels quantified via Northern blot or qPCR. An increase in viral RNA levels would mean that inhibition of the RNAi pathway was successful. Furthermore, survival studies can be done to see investigate if the increase in RNA levels causes lethality in the animals. If that were the case it would highlight the potential these chemicals have as a method of control pathologically important mosquito species.

5.2.2 RIN Treatment in Mammalian cell lines

Our initial results showed us that RIN2 has the ability to block the human Dicer protein in *in vitro* dicing assays. Future experiments should be conducted in mammalian cell lines to test whether not only RIN2, but all the RIN compounds can block RNAi *in vivo*. A simple and efficient method to do this would be a transfection based assay where cells are co-transfected with a plasmid that expresses eGFP mRNA as well as eGFP specific siRNAs. In untreated cells low to no GFP fluorescence should be detected as the siRNA would knock down the GFP mRNA. If the RIN compounds have an effect in mammalian cells then there will an increase in the GFP fluorescence from those cells. The difference in GFP fluorescence levels can be further confirmed by performing either Northern blot analysis or qPCR to detect eGFP mRNA levels in the cells.

Further analysis in Baby Hamster Kidney (BHK) cells can be done to assay whether or not RNAi can be antiviral in mammalian cells. It has been shown that NoV can replicate very well in the BHK cell line (Ball and Johnson 1998). BHK cells would be infected with NoV and treated with the RIN compounds. Cells would be monitored over a 4 day time course to see if cell survival is affected. After the 4 day time course cells would be collected and viral RNAs would be analyzed via Northern blot analysis or qPCR. An increase in in viral RNA levels would indicate that the RIN compounds have a similar effect in BHK cells that they do in S2 cells. This would be strong support to the idea that RNAi plays an antiviral role in mammalian systems.

5.3 References

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