RNAi is an antiviral immune response against a dsRNA virus in *Drosophila melanogaster*

Robert A. Zambon, Vikram N. Vakharia and Louisa P. Wu*

*Center for Biosystems Research, University of Maryland Biotechnology Institute, College Park, Maryland 20742, USA.

Summary

**Drosophila melanogaster** has a robust and efficient innate immune system, which reacts to infections ranging from bacteria to fungi and, as discovered recently, viruses as well. The known Drosophila immune responses rely on humoral and cellular activities, similar to those found in the innate immune system of other animals. Recently, RNAi or ‘RNA silencing’ has arisen as a possible means by which *Drosophila* can react to a specific pathogens, transposons and retroviral elements, in a fashion similar to that of a traditional mammalian adaptive immune system instead of in a more generalized and genome encoded innate immune-based response. RNAi is a highly conserved regulation and defence mechanism, which suppresses gene expression via targeted RNA degradation directed by either exogenous dsRNA (cleaved into siRNAs) or endogenous miRNAs. In plants, RNAi has been found to act as an antiviral immune response system. Here we show that RNAi is an antiviral response used by *Drosophila* to combat infection by *Drosophila* X Virus, a birnavirus, as well. Additionally, we identify multiple core RNAi pathway genes, including *piwi*, *vasa intronic gene* (*vig*), **aubergine** (*aub*), **armitage** (*armi*), *Rm62*, *r2d2* and **Argonaute2** (*AGO2*) as having vital roles in this response in whole organisms. Our findings establish *Drosophila* as an ideal model for the study of antiviral RNAi responses in animals.

Introduction

*Drosophila melanogaster* is an excellent model for the study of the innate immune system because of its genetic malleability, its lack of a traditional mammalian adaptive immune system and the conservation of these signalling pathways with those of higher organisms. To date, innate immune responses against bacteria, fungi and viruses have all been identified in flies. These responses have, thus far, been shown to occur via the Toll and IMD immune signalling pathways (reviewed in Brennan and Anderson, 2004; Zambon et al., 2005). *Drosophila* also possesses a robust RNA interference (RNAi) system homologous to the post-transcriptional gene silencing (PTGS)/RNAi systems found in plants and other animals.

The PTGS/RNAi system was initially described in plants over a decade ago during studies of transgenic petunias (van der Krol et al., 1990). Shortly after its initial descriptions, it was found that PTGS was able to induce an immune response against certain viral pathogens in plants. It was found that transgenic plants, which express portions of viral genomic RNA, were specifically resistant to that same virus (Lindbo et al., 1993). Also, the levels of the proteins associated with these viral genome portions were greatly reduced. Viruses were found which carried copies of host genes within their genomes that could cause a reciprocal effect against the host – possibly reducing the associated host gene expression and causing increased susceptibility to viral infection (Kumagai et al., 1995). It was not until later that work in *Caenorhabditis elegans* would identify dsRNA as the initiating factor in these silencing responses and the process would be named RNA interference (RNAi) (Fire, 1999). Further research over the years since this discovery has uncovered the molecular workings of this complex pathway (further reviewed in Lecellier and Voinnet, 2004).

The RNAi pathway occurs in two main steps, the initiation and the execution steps. To initiate RNAi, dsRNA can be introduced by either endogenous or exogenous sources. Endogenous sources include short hairpin RNAs produced by the genome, aberrantly expressed transgenes, and transposons. Exogenous sources of dsRNA include naturally occurring dsRNA, such as that originating from RNA viruses, and dsRNA experimentally produced and introduced (Hannon, 2002). During the initiation phases of RNAi, dsRNAs greater than 23 bp are processed into 21–23 bp dsRNA fragments with 3′ overhanging ends by either Dicer1 or Dicer2, depending upon whether the originating dsRNAs are endogenous, and involved in gene regulation, or non-self dsRNAs respectively (Blaszczyk et al., 2001; Lee et al., 2004). The Dicer proteins are members of the RNAse III gene family and

Received 8 August, 2005; revised 22 November, 2005; accepted 15 December, 2005. *For correspondence. E-mail wul@umbi.umd.edu; Tel. (+1) 301 405 5151; Fax (+1) 301 314 9075.

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contain an N-terminal RNA helicase domain, two RNAse III-like domains, and a C-terminal dsRNA-binding domain. The cleaved length of the produced dsRNAs is caused by the dimerized RNAse III domains in the protein, one of which is inactive and acts simply as a spacer domain. (Blaszczyk et al., 2001). To date, homologues of Dicer have been identified in yeast, plants, worms, flies, mice and humans (Bernstein et al., 2001). These small RNAs are then utilized as templates in the subsequent RNAi execution steps (Tuschl et al., 1999; Hammond et al., 2000; Zamore et al., 2000).

The small RNA fragments are incorporated into the RNA-induced silencing complex (RISC) which is responsible for the execution phase of the RNAi pathway (Blaszczyk et al., 2001; Nykanen et al., 2001). The small dsRNAs are unwound, and the anti-sense strand is then incorporated into the RISC complex with the aid of the protein r2d2 (Schwarz et al., 2002; Liu et al., 2003). This incorporated strand acts as the targeting mechanism for the RISC and directs its action to complementary mRNA. RNAi is also used for gene regulation through the use of endogenously encoded RNA stem loops. These stem-loop structures are processed into micro-RNAs (miRNAs), which can also associate with the RISC complex. miRNA control of gene regulation is required for the control of normal cell processes (reviewed in Pasquinelli et al., 2005) and this may be one of the factors contributing to the lethality of select mutations in the RNAi pathway. Studies of miRNA-mediated gene regulation led to the discovery that variations in the complementarity of the template small RNA determine the final method of inactivation of the target message. Complete matching of the entire template RNA results in degradation of the targeted mRNA, while mismatching at a small number of base pairs (2–3 bp) leads instead to inhibition of translation via a currently unknown mechanism. This difference in final activity could play a role in antiviral immunity because failure to degrade the viral genome would allow its persistence and use in viral replication.

The only components of the RISC complex that have been identified so far are Argonaute2 (AGO2) and the related Argonaute protein family members Fragile X mental retardation protein (FMRP), the Vasa intronic gene (VIG) and Tudor-SN. The Argonaute-related proteins are also called PAZ-Piwi-Domain (PPD) proteins and are characterized by the presence of a PAZ domain and a C-terminal Piwi domain (Cerutti et al., 2000). These domains are believed to function in protein–protein interactions. Members of the Argonaute family have been found to play a role in RNAi in multiple species, including Neurospora crassa, Drosophila, C. elegans and Arabidopsis (Fagard et al., 2000; Bernstein et al., 2001; Parrish and Fire, 2001; Catalanotto et al., 2002). It should be noted that Argonaute family proteins are also involved in other cellular functions, which are distinct from siRNA-based RNAi, such as developmental control and stem cell maintenance (Carmell et al., 2002).

The three Argonaute-related proteins in the RISC complex are suspected to play a role in RNA binding and nuclease functions. Both FMRP and the VIG have RNA binding properties and complex with Tudor-SN (Caudy et al., 2002). Additionally, Tudor-SN is related to micrococcal nuclease. However, the nuclease activity of Tudor-SN has not been identified as the functional nuclease activity of the RISC, but may instead be part of the secondary nuclease activities of the RISC, uncovered in recent research (Schwarz et al., 2004).

The Drosophila genes armitage and aubergine are also essential for RNAi, but have not yet been linked to a specific function in the process. Armitage is necessary for silencing of select mRNAs involved in oogenesis, and armitage mutants display defects in RISC assembly (Cook et al., 2004; Tomari et al., 2004). Aubergine, in addition to being a member of the Argonaute gene family, has been linked to RNAi in the oocyte and colocalizes with known RNAi pathway components (Kennerdell et al., 2002; Findley et al., 2003). Rm62, an orthologue of human p68, unwinds short but not long segments of dsRNA in an ATP-dependent fashion, and has been shown to interact with dFMR1, the Drosophila homologue of the human FMRP1. Rm62 has been found to be necessary for RNAi function (Huang and Liu, 2002; Ishizuka et al., 2002). Piwi, also an Argonaute gene family member, has been shown to control male germ-line stem cell division and has been linked to PTGS (Pal-Bhadra et al., 2002).

The initial identification of the RNAi phenomenon and its antiviral role in plants has resulted in considerable interest into the use of RNAi as an antiviral strategy in animal systems. In mosquitoes, for example, expression of Dengue virus genome fragments in cells or whole mosquitoes was able to inhibit later Dengue virus infection and replication (Gaines et al., 1996; Olson et al., 1996; Adelman et al., 2001). Another study found that infection of Drosophila cells with Flock House virus (FHV) resulted in accumulation of viral genome-specific siRNAs which could promote RNAi-based degradation of viral RNA (Li et al., 2002). Additionally, FHV was found to encode an RNAi inhibitor protein (B2), which can protect the virus from host RNAi responses and promote its survival (Li et al., 2002). The fact that the virus produces this protein suggests that RNAi is imposing selection pressure upon the virus and that the RNAi inhibitor is needed for efficient replication. This advantage would explain how the cost of producing the protein and retaining it within the viral genome is overcome. Work in mammalian cell culture is also uncovering a possible role for RNAi in a mammalian antiviral immune response. The fact that some viruses are susceptible to RNAi when it is induced in cell culture (e.g.
poliovirus, human papillomavirus, HIV) suggests that RNAi can interfere with viral genome expression in certain types of mammalian infections (Gitlin et al., 2002; Jacque et al., 2002; Jiang and Milner, 2002). Whereas other viruses, such as FHV, hepatitis delta virus, and influenza virus are resistant to RNAi, suggesting that these viruses have evolved a means to surmount an RNAi response (Li et al., 2002; 2004; Chang and Taylor, 2003).

While these and other experiments using RNAi specifically induced against the virus provide some support that RNAi acts as a semi-adaptive innate immune defence in animals, they also raise the question of whether or not the host endogenous RNAi response can provide protection in whole organisms similar to that observed when antiviral RNAi is induced in cell culture or whole animals.

To examine a possible role for RNAi as an antiviral innate immune response, we use a model system that was developed using Drosophila X Virus (DXV), a bisegmented dsRNA birnavirus (Zambon et al., 2005). Drosophila infected with this virus develop acute anoxia sensitivity and die within 2 weeks of symptom onset. The anoxia sensitivity to DXV infection phenotype can be used as a means to identify mutants that are more susceptible to virus. From screening known immunity mutants, it was found that the Toll pathway, but not the IMD pathway, plays a key antiviral role in Drosophila. By screening Drosophila lines having mutations in key components of the RNAi pathway, we have determined that the RNAi pathway is also an essential part of the antiviral response in Drosophila.

Results

To determine whether RNAi might play an antiviral role in Drosophila, we examined all available adult viable Drosophila lines affecting known or predicted RNAi pathway genes. To do this, we screened 14 fly lines with different mutations in the RNAi pathway at 3, 7 and 10 days post infection (d.p.i.) for alterations in anoxia sensitivity-induced death caused by infection with DXV. Of the RNAi pathway mutants, all but three lines were found to be highly susceptible to viral infection. The criteria for susceptibility were defined from our previous pilot screen as having survival rates outside one standard deviation of the screen average at two of the three time points. This correlates well with a significant difference from wild type as determined by having a P-value below 0.05, as determined by log-rank analysis. The fact that 11 out of the 14 mutants are more susceptible to infection is striking (Fig. 1), and suggests that the RNAi pathway is a key antiviral defence.

VIG and AGO2 which encode essential proteins in the RISC complex (Hammond et al., 2001) were both identified as having increased sensitivity to viral infection. We found that vig<sup>ET07816</sup> mutants had a dramatic increase in viral sensitivity at all time points, with approximately 80% of the flies dying by 7 d.p.i. (P ≤ 0.001). AGO2<sup>ET04479</sup> mutants displayed similar increases in anoxia sensitivity during DXV infection compared with the average (P ≤ 0.001). Of note, flies heterozygous for the deficiency, D(3L)Bk10, which deletes the genomic region encompassing AGO2, also resulted in an increase in sensitivity (P ≤ 0.001). As a heterozygote, flies with this deficiency would be predicted to express half the amount of AGO2 as wild type. Consistent with this, the increase was shifted to the later time points (7 and 10 d.p.i.), as compared with the earlier onset observed in the homozygous AGO2 mutant line. This suggests that the host antiviral response is severely sensitive to the amount of AGO2 present and that it may be a limiting component for RISC formation.

In addition to the RISC-associated genes identified above, lines with mutations for aubergine, r2d2, armitage, and Rm62 were also found to have increased susceptibility to viral infection (P ≤ 0.001). Mutants in aubergine (aub<sup>HN</sup> and aub<sup>KG03638</sup>), an Argonaute1 homologue that colocalizes with RNAi pathway components (Tomari et al., 2004), displayed dramatic increases in viral susceptibility following infection. Similarly, a mutation in the r2d2 protein (r2d2<sup>2</sup>), which chaperones miRNA/siRNA incorporation into the RISC complex (Liu et al., 2003), also resulted in a similar sensitivity. Two of three examined alleles of armitage (armi<sup>721</sup> and armi<sup>KG04664</sup>), which plays a role in RISC assembly (Tomari et al., 2004), had similar increases in viral infection sensitivity, although they did cause the weakest increase in viral sensitivity of all mutants observed. The third armi allele (armi<sup>KG04646</sup>) showed a response within the wild-type range. This allele is due to a P-element insertion approximately 65 bp upstream of the armi start site. The armi<sup>KG04646</sup> allele has not been well-characterized and our results indicate that it is likely a weak mutation as compared with the other two armitage alleles. Mutants in Rm62 (Rm62<sup>ET01086</sup>), a Drosophila orthologue of human p68 which unwinds short dsRNAs in an ATP-dependent manner (Huang and Liu, 2002), also displayed a dramatic increase in viral infection sensitivity (P ≤ 0.001). Lastly, piwi (piwi<sup>KG03643</sup>) mutants were also identified as having increased sensitivity to viral infection, resulting in significantly higher mortality at 7 and 10 d.p.i. (P ≤ 0.001). The specific role of piwi in Drosophila RNAi is not yet fully understood, but piwi has been shown to be important in the RNAi pathway (Pal-Bhadra et al., 2002). piwi was originally identified for its role in germ line stem cell division (Cox et al., 2000; Pal-Bhadra et al., 2002); how this process might relate to its function in RNAi is currently not understood.

It was interesting to find that the elimination of Dicer2 (dcr-<sup>2</sup>811tsu) or a heterozygous mutant of Dicer1 (dcr-<sup>1</sup>01147X/TM3) caused no significant increases in suscepti-
RNAi is an antiviral immune response in Drosophila. The ability to viral infection as compared with the wild-type range ($P = 0.88$ and $P = 0.44$ respectively). The Dicer2 and Dicer1 genes are essential for the generation of the 21–23 bp fragment siRNAs and miRNAs respectively (Lee et al., 2004). They have been shown to have some overlap in function (Lee et al., 2004). Our results support this hypothesis because single mutations affecting either of these viral RNAi pathway RNases fails to cause a deleterious effect on viral immunity. It is important to note that we only examined heterozygous mutants of Dcr1, which could account for the lack of effect on the viral susceptibility in these lines.

The mutation in spindle-E ($spn-E$), which is involved in intracellular mRNA localization and has been associated with RNAi (Gonzalez-Reyes et al., 1997; Kennerdell et al., 2002), did cause a dramatic increase in sensitivity at 10 d.p.i. However, the difference from wild type at 3 and 7 d.p.i. was not severe enough to be considered significant ($P = 0.88$).

These results suggest that, individually, $spn-E$, $dcr-1$ and $dcr-2$ are not necessary for an antiviral RNAi response in the organism, and that redundancy exists in the RNAi pathway at these select steps. In contrast, the dose effect of the heterozygous $AGO2$ deficiency suggests that the
activity level of the RISC complex is critical and its decrease results in a dramatically reduced ability to use RNAi against the DXV genome. This is further supported by previous research showing that the most effective way to reduce RNAi in tissue culture is by knockdown of Ago2 (Li et al., 2002). As RNAi is, in general, less than 100% effective at eliminating a gene transcript, this suggests that AGO2 protein levels are vitally important for RNAi function.

The susceptibility of multiple mutants in the RNAi pathway indicates that this pathway is playing a key role in resistance against DXV infection in Drosophila. Also, the results suggest that Piwi may play a larger role in antiviral-directed RNAi than previously known.

We next wanted to determine if the increased viral sensitivity in our mutant lines correlated with higher viral titer in these flies. To do this, we used reverse transcription polymerase chain reaction (RT-PCR) against a 62 bp region of DXV segment B to examine viral titer levels in the mutants relative to wild-type Drosophila infected with DXV. We have previously found increased viral titer at earlier time points correlates well with an earlier onset of anoxia-induced death in other mutant lines with increased viral susceptibility (Zambon et al., 2005). At 3 d.p.i., we found that all the RNAi lines with increased sensitivity to viral infection also had increased levels of viral titer as compared with that found in wild-type flies infected with DXV. The increased viral levels ranged from 13 to 40 times that of wild-type levels at the same time point (data not shown). These increased viral titers are similar to titer levels found 24 h later in DXV-infected wild-type flies. This suggests that the RNAi pathway is essential for effective inhibition of viral replication in wild-type animals.

One reason for the use of DXV in our studies is it would theoretically be completely susceptible to RNAi because of the dsRNA nature of its genome and replicative intermediates. During DXV replication, plus-sense ssRNA is extruded from the viral capsid into the cytoplasm. This RNA is then translated and eventually used in viral genome generation. These genome copies, once packaged inside the viral capsid, are believed to be protected from RNAi. Because of this, and our findings that flies impaired in RNAi are more susceptible to viral infection, we next wanted to determine if inducing RNAi could render cells resistant to DXV infection. To accomplish this, we generated a dsRNA fragment from the coding region (bps 337–791) of the DXV RNA-dependent RNA polymerase (RdRp) VP1 gene, which is essential for viral replication, and knocked down the gene in Drosophila S2 cells. Viral protein levels during infection were then examined in media samples taken every 24 h post infection by Western Blot using a polyclonal antibody against DXV (Fig. 2). In untreated DXV-infected S2 cells, DXV protein was detectable at 2 d.p.i. and reached a plateau by 4 d.p.i., similar to the plateau observed when examining viral titer via Q-PCR methods in cell culture. Over the course of 8 days, however, DXV protein in RNAi-treated DXV-infected S2 cells remained largely undetectable, only appearing faintly at 7 d.p.i.

These results demonstrate, for the first time, that a birnavirus is susceptible to replication inhibition by genome-directed RNAi. Additionally, the appearance of viral protein at days 7 and 8 suggests that RNAi is not 100% efficient under DXV infection conditions and/or the capsid of a mature virion can protect the viral genome from degradation. This capsid-provided protection would allow for continued production of ssRNA even under highly induced and specific RNAi conditions, overcoming the RNAi reaction by mass generation of plus-sense RNAs. It is important to note that because our system uses 300–500 bp dsRNA instead of the 21–23 bp siRNAs used in mammalian RNAi systems, it is highly unlikely that viral mutations and selection are the cause of the eventual virus evasion of RNAi (Gitlin et al., 2005).

Once we had determined that RNAi-based inhibition was able to limit DXV replication, we wanted to determine if specific RNAi knockdown of the RNAi pathway would result in increased viral replication. To do this, we utilized the S2 cell system so that we could look at cell autonomous effects and confirm our in vivo results. It was previously noted that the RNAi pathway can be effectively knocked down by using RNAi to target Ago2, one of the genes identified in our screen (Li et al., 2002). The fact that loss of Ago2 is more effective at inhibiting the RNAi pathway than loss of either of the Dicer RNAases suggests...
little to no redundancy of Ago2 compared with other RNAi components. Additionally, Ago2's role as part of the RISC may be causing its reduction to result in a bottleneck for all RNAi activity. This allowed for an examination of both the effects of Ago2's knockdown specifically as well as the likely knockdown of the RNAi pathway as a whole. We found that DXV protein levels in the Ago2/RNAi knockdown cell culture reproducibly increased to detectable levels 24 h prior to the detection in untreated DXV-infected S2 cell cultures (Fig. 3). To ensure that our results were gene specific and not a non-specific dsRNA-induced response, we performed the above experiments using dsRNA against the LacZ gene and found that while RNAi could suppress LacZ expression, it had no effect on DXV protein levels over the course of a DXV infection (data not shown). This suggests that the RNAi response must be gene specific. Also, these results suggest that DXV does not encode a general RNAi pathway inhibitor because RNAi against LacZ can still occur during a DXV infection. However, further examination will be required to confirm this conclusively.

In summation, our results demonstrate that the RNAi pathway is being utilized in an antiviral capacity during a DXV infection in Drosophila, causing a reduction in both DXV replication and protein production. Additionally, the results in the cell culture knockdown of the RNAi pathway correlate well with the increased viral titers observed in the whole organism. Our studies demonstrate that in cell culture, as well as in animals, the endogenous RNAi pathway is limiting DXV replication during infection, and this is critical for increased host survival.

In our experiments, we screened a collection of RNAi pathway mutant Drosophila lines. AGO2, vig, aub, armi, r2d2, piwi and Rm62 mutant fly lines were all identified as having increased sensitivity to viral infection. Additionally, all identified lines displayed increased viral titer levels at 3 d.p.i. Our previous research into the antiviral function of the Toll pathway shows a similar increase in viral titers at early time points in select mutants, suggesting that this RNAi response is being utilized alongside a Toll-based response to restrict viral replication. These results indicate that the RNAi pathway is essential for an antiviral immune response in vivo.

The simplest explanation is that the RNAi pathway is required to generate siRNAs that incorporate into RISC and act to degrade viral genomes to defend the animal against viral infection. This is consistent with what has been observed in plants and in animal tissue culture. The fact that Dicer2 mutants failed to show increased susceptibility indicate either that Dicer2 is not important or that it acts in a redundant fashion with Dicer1 for an antiviral siRNA response. However, as we were not able to assess a Dicer1 homozygous mutant, an alternate possibility is that the antiviral RNAi pathway may instead be acting through Dicer1 and that the RNAi pathways mutants’ increased susceptibility could be due to defects in endogenous miRNA gene regulation. The possibility that the Dicers are functioning redundantly or that miRNAs are important in an antiviral immune response will be interesting questions to explore in the future.

Additionally, we determined that RNAi against the RdRp of DXV is highly efficient at restricting viral replication in cells. This suggests that an RNAi response mounted against DXV is being used by Drosophila to restrict virus propagation. However, our results indicate that virus replication is only being restricted by RNAi, not completely halted, as the virus is not being fully cleared. This is apparent by the detection of DXV proteins 7 d.p.i. in cell culture despite continuous addition of microgram quantities of dsRNA to induce RNAi against the DXV RdRp. This strongly suggests that RNAi is only slowing virus replication and not completely clearing the viral infection. This slowing of replication could provide the time necessary for a more synergistic host response by allowing other immune responses, such as the Toll-related responses, to be mounted against the virus prior to the infection reaching a terminal point.

Four main pieces of evidence are generally cited as support for RNAi as an antiviral mechanism in plants, the system in which RNAi as an antiviral response was first established. Our work provides the final piece of evidence needed to fulfil these criteria for animal systems as well. First, infection by viruses induces a strong RNAi

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response. As observed with FHV, infection by a virus induces a strong RNAi response in Drosophila cells, as can be seen by the increased amount of virus-specific siRNA post infection (Li et al., 2002).

Second, induced targeted RNAi is able to suppress viral replication. Many examples exist of such studies showing targeted RNAi is able to suppress viral replication in animals, including FHV (S2 cells), Taura Syndrome virus (Litopenaeus vannamei) and hepatitis C (Huh7.5 cells) (Li et al., 2002; Randall and Rice, 2004; Robalino et al., 2004). Studies using the O'nyong-nyong virus, a dsRNA Alphavirus, in Anopheles gambiae, have also shown a similar outcome (Keene et al., 2004). Their work shows that in vivo co-injection of virus and dsRNA complementary to the virus genome results in reduced viral titers and spread of virus infection through the infected organism, as compared with injection of virus alone. Our work with DXV shows, similarly, that induced targeted RNAi against the DXV virus genome is able to suppress DXV replication in Drosophila cells as well.

Third, many viruses have a means of evading total replication suppression by RNAi in the host during infection. This is demonstrated by the FHV's genome-encoded RNAi inhibitor and by DXV's ability to eventually continue replication, despite strong genome-specific RNAi pressure in S2 cells (Li et al., 2002). Hence, similar evidence for virus evasion of RNAi exists in animal models.

Fourth, and last, is that mutations in the RNAi pathway should cause increased susceptibility to viral infection in whole organisms. While it has been demonstrated that RNAi knockdown of one specific part of the RNAi pathway, specifically AGO2, reduces an antiviral response in mosquitoes, our studies broaden that observation by examining flies mutant for multiple RNAi pathway components and determining that these mutations affect virus susceptibility in vivo. There are several limitations inherent to the RNAi knockdown of AGO2 as a means to examine the RNAi pathway. First, RNAi itself does not result in total elimination of a gene product, as would occur with a null mutant for an examined gene. The effectiveness of injected dsRNA-induced RNAi to reduce the given gene product in vivo is often dependent on both the natural turnover rate of the protein and the ability of the targeted or relevant tissue to take up the dsRNA. Second, the use of RNAi to knockdown the RNAi pathway can bias results because producing the desired phenotype (loss of the pathway) depends on activation or use of the same pathway. Our studies examine how true mutants in RNAi genes are affected by viral infection, and hence, provide the final crucial evidence that demonstrates that RNAi is acting as an effector against viral infection in vivo. Taken as a whole, our studies add to the cited evidence to provide the same support for RNAi as an antiviral mechanism in animals.

In addition, our results define another aspect of the Drosophila antiviral immune response. Previously, we have shown that the Toll pathway is required for an effective immune response against DXV in Drosophila. Similarities in susceptibility to viral infection observed between mutant Drosophila deficient in the Toll pathway and the RNAi pathway suggest that both are required for an effective antiviral response against DXV infection because mutations affecting either pathway result in dramatic increases in the viral titer and earlier susceptibility to viral infection. Our previous work suggests that the Toll antiviral effects occur systemically (Zambon et al., 2005). In Drosophila, unlike in plants and worms, RNAi effects are not transmitted to neighbouring cells and occur only in cells in which dsRNA is initially present or taken up (Van Roessel et al., 2002). Because of this, we believe that RNAi is acting on a cell autonomous level and not systemically, limiting viral replication on a cell-to-cell basis and slowing infection progression. This slowing may provide more time for a Toll-mediated response to occur, possibly systemically, against virus-infected cells and their surrounding tissue.

In conclusion, we have shown that DXV replication is highly sensitive to RNAi-based silencing, although the virus can eventually overcome the inhibition. Additionally, and more importantly, we have demonstrated that Drosophila utilize RNAi as an antiviral immune response against a dsRNA virus and shown that D. melanogaster is an excellent model for the study of antiviral RNAi responses in both whole organisms and cell culture.

**Experimental procedures**

**Viral infection of flies**

All flies used were 3- to 5-day-old adults and reared at 22°C on standard yeast/agar media. Oregon R flies were used as WT. dcr-2Δ2110 and dcr-1Δ1147X/TM3 flies were obtained from Richard Carthew (North-western University). piwi06843 flies were obtained from Haifan Lin (Duke University). All other fly lines were obtained from the Bloomington Drosophila Stock Center. Flies were injected using a Drummond Nanoject or WPI PicoPump with approximately 30 nl of a 10⁵-fold dilution of purified DXV. This same concentration was used for the pilot screen (Zambon et al., 2005) and for all subsequent viral infections. Purified DXV was generated from an initial stock provided by Peter Dobos (University of Guelph, Guelph, Canada). Control injections were done with double-distilled H₂O.

**Anoxia sensitivity survival assay**

Assaying was performed via 15 min of CO₂ exposure at 3, 7 and 10 d.p.i. in a sealed chamber. Following anoxia, flies were then assayed for survival. Flies were transferred to new vials every 3 days. All flies were base-lined to water-injected Drosophila of the same line to control for wound effects. Fly lines were selected as significantly altered if their survival at two of the three time
points examined fell outside one standard deviation of the average survival curve and which had a P-value equal to or less than 0.05 as determined by a Mantel-Cox log-rank analysis. The average wild-type survival and range was developed in our previous pilot screen (Zambon et al., 2005) from 50 examined mutant fly lines and the OregonR wild-type fly line. The sample size for each RNAi mutant tested was no fewer than 50 flies per line.

Generation of dsRNA

For the anti-DXV VP1 dsRNA generation, a cDNA clone of DXV genome segment B was created using two separate reverse transcription reactions utilizing the following primers: 5'-GAG CTC TAA TAC GAC TCA TTA TAG GAA AAT AAT TGG CGG CCG ATA AGG-3'; 5'-GGT CAG AGA TAG GTA AGC CAC-3'; 5'-GTG AGT TAC GTG CCC AGC CAG CAT GTC CAT C-3' 5'-CTG CAG GGA GCC GCC CAA TTT ACA TTT GG-3'. The reactions generated two 1.6 kb fragments with overlapping regions containing an MfeI restriction site. Both were then restriction digested with MfeI and ligated to form a full-length cDNA clone, which was subcloned into pCR2.1-TOPO using the Invitrogen TOPO-TA Kit as described by the manufacturer. A dsRNA generating DNA fragment was then created from this dsDNA clone using primers flanking a 470 bp central region of VP1, the viral RdRp, which had 5' flanking T7 promoters: Forward: 5'-TAATACGACTCACTATAGGCGTACCAAGGGGTGTTTGGAT-3', Reverse: 5'-TAATACGACTCACTATAGGCGTACCAAGGGGTGTTTGGAT-3'. This fragment was also cloned into pCR2.1-TOPO as above.

To generate an anti-AGO2 dsRNA, Drosophila RNA was isolated from S2 cells using STAT-60 buffer according to the manufacturer's protocol (Isotex Diagnostics), then reverse transcription and PCR against 1 kb fragment of the respective gene was performed. Primers used contained 5' flanking T7 promoters, and are as follows: Forward: 5'-TGTATAAGGTACCGCATACATAGGGCAATCGTTTGCCTTTGCGT-3', Reverse: 5'-TGTAATACGACTCATACTATTATAGGCTACCATCGT-3'. The reactions were then subcloned into pCR2.1-TOPO using the Invitrogen TOPO-TA Kit as above.

The pCR2.1 vectors containing the dsRNA generation templates were then amplified by PCR, using primers noted above to generate specific short linear DNA templates to generate ssRNA with the help of the Ambion MegaScript T7 Kit, as per manufacturer's instructions. The resulting ssRNA was ethanol-precipitated and resuspended in DEPC-treated H2O. The ssRNA was then heated to 65°C for 30 min, cooled (1°C min\(^{-1}\)) to 4°C, and stored at −20°C. The dsRNA was then fractionated by gel electrophoresis and visualized to confirm uniformity and size.

Reverse transcription and quantitative real-time PCR

RNA was isolated from adult flies by homogenizing flies in STAT-60 buffer according to the manufacturer's protocol (Isotex Diagnostics). Quantitative RT-PCR was then performed using a PE Biosystems 5700 GeneAmp Sequence detection system and Invitrogen Lux Primers. Specific LUX primers were designed against DXV strand B and used to quantify relative viral titer. Ribosomal protein 49 (rp49) was used as a control in all experiments. The following primers were used: for DXV, left primer 5'-GGA GTT GAA GCC ACG GTT TG-3', right primer 5'-GAC GAT CTT GCC AGT TGG CTC ATC G(FAM)C-3'; for rp49, left primer 5'-CAC GAT AGC ATA CAG GCC CAA GAT CG(FAM)G-3', right primer 5'-GCC ATT TGT GCG ACA GCT TAG-3'.

RNAi in S2 cells

S2 cells were grown in Drosophila SFM medium (Invitrogen). Cells were plated in 6 well plates at 1 × 10^6 cells in 2 ml SFM. Twenty micrograms of appropriate dsRNA was immediately added and plates were then incubated at room temperature for 60 min. Two milliliters of SFM with Penicillin (50 units ml\(^{-1}\) and Streptomycin (50 µg ml\(^{-1}\)) was then added to each well and plates were incubated at room temperature for 72 h to allow for protein turnover before DXV infection. Transfection of dsRNA was not found to have an improved effect on RNAi and was therefore not done. Cells were then infected with DXV at a moi of −1. Samples were taken every 24 h. At the time of infection, and every 3 days subsequent, 15 µg of anti-RdRp dsRNA was added to each experimental well to ensure that DXV replication was still occurring under RNAi conditions, and not limited by degradation of the RNAi inducing dsRNA.

Western blotting

Purified DXV was used to produce rabbit antisera (Duncroft, Lovettsville, VA). Antiserum was adsorbed with acetone-treated powder prepared from S2 cells and OregonR adult Drosophila, and utilized at 1:1000 dilution in Western blots. Peroxidase-conjugated AffiniPure Goat Anti-Rabbit IgG (Jackson ImmunoResearch Laboratories) was utilized as a secondary antibody at 1:1000 dilution. Western blotting was performed as per standard protocol, utilizing ECL Western Blotting Detection Reagents (Amersham Biosciences).

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References

Adelman, Z.N., Blair, C.D., Carlson, J.O., Beaty, B.J., and Olson, K.E. (2001) Sindbis virus-induced silencing of dengue viruses in mosquitoes. Insect Mol Biol 10: 265–273. Bernstein, E., Caudy, A.A., Hammond, S.M., and Hannon, G.J. (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature 409: 363–366. Blaszczyk, J., Tropea, J.E., Bubunenko, M., Routzahn, K.M., Waugh, D.S., Court, D.L., and Ji, X. (2001) Crystallographic and modeling studies of RNase III suggest a mechanism for double-stranded RNA cleavage. Structure (Camb) 9: 1225–1236. Brennan, C.A., and Anderson, K.V. (2004) Drosophila: the genetics of innate immune recognition and response. Annu Rev Immunol 22: 457–483. Carmell, M.A., Xuan, Z., Zhang, M.Q., and Hannon, G.J. (2002) The Argonante family: tentacles that reach into RNAi is an antiviral immune response in Drosophila 887
RNAi, developmental control, stem cell maintenance, and tumorigenesis. *Genes Dev* **16**: 2733–2742.

Catalanotto, C., Azzalin, G., Macino, G., and Cogoni, C. (2002) Involvement of small RNAs and role of the qde genes in the gene silencing pathway in *Neurospora*. *Genes Dev* **16**: 790–795.

Caudy, A.A., Myers, M., Hannon, G.J., and Hammond, S.M. (2002) Fragile X-related protein and VIG associate with the RNA interference machinery. *Genes Dev* **16**: 2491–2496.

Cerutti, L., Mian, N., and Bateman, A. (2000) Domains in gene silencing and cell differentiation proteins: the novel PAZ domain and redefinition of the Piwi domain. *Trends Biochem Sci* **25**: 481–482.

Chang, J., and Taylor, J.M. (2003) Susceptibility of human hepatitis delta virus RNAs to small interfering RNA action. *J Virol* **77**: 9728–9731.

Cook, H.A., Koppetsch, B.S., Wu, J., and Theurkauf, W.E. (2004) The *Drosophila* SDE3 homolog armitage is required for oskar mRNA silencing and embryonic axis specification. *Cell* **116**: 817–829.

Cox, D.N., Chao, A., and Lin, H. (2000) *piwi* encodes a nucleoluspeal factor whose activity modulates the number and division rate of germine stem cells. *Development* **127**: 503–514.

Dengue type 2 virus in mosquito cells by expression of the RDE1/AGO1 homolog, Aubergine, in nuage. *Cell* **116**: 358–363.

Cook, H.A., Koppetsch, B.S., Wu, J., and Theurkauf, W.E. (2004) The *Drosophila* SDE3 homolog armitage is required for oskar mRNA silencing and embryonic axis specification. *Cell* **116**: 817–829.

Cox, D.N., Chao, A., and Lin, H. (2000) *piwi* encodes a nucleoluspeal factor whose activity modulates the number and division rate of germine stem cells. *Development* **127**: 503–514.

Dengue type 2 virus in mosquito cells by expression of the RDE1/AGO1 homolog, Aubergine, in nuage. *Cell* **116**: 358–363.

Cox, D.N., Chao, A., and Lin, H. (2000) *piwi* encodes a nucleoluspeal factor whose activity modulates the number and division rate of germine stem cells. *Development* **127**: 503–514.
RNAi is an antiviral immune response in Drosophila. RNA 7: 1397–1402.
Parrish, S., and Fire, A. (2001) Distinct roles for RDE-1 and RDE-4 during RNA interference in Caenorhabditis elegans. RNA 7: 1397–1402.
Pasquinelli, A.E., Hunter, S., and Bracht, J. (2005) MicroRNAs: a developing story. Curr Opin Genet Dev 15: 200.
Ran dall, G., and Rice, C.M. (2004) Interfering with hepatitis C virus RNA replication. Virus Res 102: 19–25.
Robalino, J., Browdy, C.L., Prior, S., Metz, A., Parnell, P., Gross, P., and Warr, G. (2004) Induction of antiviral immunity by double-stranded RNA in a marine invertebrate. J Virol 78: 10442–10448.
Schwarz, D.S., Hutvagner, G., Haley, B., and Zamore, P.D. (2002) Evidence that siRNAs function as guides, not primers, in the Drosophila and human RNAi pathways. Mol Cell 10: 537–548.
Schwarz, D.S., Tomari, Y., and Zamore, P.D. (2004) The RNA-induced silencing complex is a Mg²⁺-dependent endonuclease. Curr Biol 14: 787–791.
Tuschl, T., Zamore, P.D., Lehmann, R., Bartel, D.P., and Sharp, P.A. (1999) Targeted mRNA degradation by double-stranded RNA in vitro. Genes Dev 13: 3191–3197.
Van Roessel, P., Hayward, N.M., Barros, C.S., and Brand, A.H. (2002) Two-color GFP imaging demonstrates cell-autonomy of GAL4-driven RNA interference in Drosophila. Genesis 34: 170–173.
Zambon, R.A., Nandakumar, M., Vakharia, V.N., and Wu, L.P. (2005) The Toll pathway is important for an antiviral response in Drosophila. Proc Natl Acad Sci USA 102: 7257–7262.
Zamore, P.D., Tuschl, T., Sharp, P.A., and Bartel, D.P. (2000) RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21–23 nucleotide intervals. Cell 101: 25–33.