RNA interference (RNAi) silences gene expression through small interfering RNAs (siRNAs) and microRNAs (miRNAs). In Drosophila melanogaster (1), Dicer-2 (Dcr-2) produces siRNAs, whereas Dicer-1 (Dcr-1) recognizes precursors of miRNAs. The small RNAs are assembled with an Argonaute ( Ago) protein into related effector complexes, such as RNA-induced silencing complex (RISC), to guide specific RNA silencing (1).

RNA silencing provides an antiviral mechanism in plants and animals (2–6). Plant viruses have evolved diverse strategies for evading the RNA silencing immunity, and expression of viral suppressors of RNAi (VSRs) is essential for infection and virulence (6). However, it is unknown whether antiviral silencing in plants is targeted by plant VSRs. Bacterial and fungal infections of D. melanogaster induce Toll and immune deficiency (Imd) pathways, leading to transcriptional induction of antimicrobial peptide effectors via nuclear factor-kB (NF-kB)–like signaling processes (7). However, it has been unclear whether either pathway plays a role in Drosophila innate immunity against viruses (8, 9). Our previous work in cell culture has indicated that RNAi might mediate viral immunity in D. melanogaster (3). Here, we investigated whether RNAi indeed provides protection against virus infection in Drosophila embryos and adults.

Flock house virus (FHV) contains an RNA genome (10) divided among two plus-strand molecules, RNAs 1 and 2. RNA2 (R2) encodes the single virion structural protein, whereas RNA1 (R1) encodes protein A, the viral RNA–dependent RNA polymerase (RdRP), and B2, a VSR (3, 4, 11) expressed after RNA1 replication from its own mRNA, RNA3 (fig. S1). In the absence of R2, R1 replicated autonomously, accumulated to high levels, and produced abundant RNA3 in wild-type (WT) D. melanogaster embryos 30 hours after injection with R1 transcripts synthesized in vitro (Fig. 1, lane 2). No FHV RNAs accumulated in WT embryos injected with R1fs transcripts that contain a frameshift mutation in the RdRP open reading frame (ORF) (Fig. 1, lane 1). FHV RNAs were also not readily detected in WT embryos injected with a second mutant of R1, R1AB2, which does not express the VSR (Fig. 1, lane 3). However, abundant accumulation of R1AB2 (Fig. 1, lane 9) but not FR1fs (Fig. 1, lane 7) occurred in mutant Drosophila embryos that carried a homoygous null mutation in ago-2 (ago-2<sup>14</sup>), which is essential for RNAi in Drosophila (11, 12, 13). These data indicated that viral RNA replication in Drosophila embryos triggers an RNAi-mediated virus clearance in an Ago-2–dependent manner and that effective RNAi suppression by B2 is necessary to achieve normal accumulation of FHV RNAs.

In Drosophila, Ago-2 acts downstream of Dicer-2 (Dcr-2) to recruit siRNAs, the products of Dcr-2 activity, into the siRNA-dependent RISC (siRISC) (1, 14). Thus, a genetic requirement for ago-2 in FHV RNA clearance implicates Dcr-2 in the RNAi antiviral effector mechanism. To test this hypothesis, we injected R1, R1fs, and R1AB2 transcripts into embryos carrying a homozygous dcr-2 null mutation, dcr-2<sup>L811fsX</sup>. Northern blot hybridizations showed that, although FHV RNAs remained undetectable in dcr-2<sup>L811fsX</sup> embryos injected with R1fs (Fig. 1, lane 4), viral RNA accumulation was rescued in the dcr-2<sup>L811fsX</sup> embryos injected with R1AB2 transcripts (Fig. 1, lane 6). This result shows that Dcr-2 is required to initiate RNAi-mediated clearance of FHV RNAs in Drosophila embryos.

To investigate whether the RNAi pathway protects Drosophila from virus infection, we injected adult flies of either WT or dcr-2<sup>L811fsX</sup> and ago-2<sup>14</sup> genotype with purified FHV virions. The FHV isolate was of low virulence in WT flies, because about 50% of infected flies survived 15 days postinoculation (dpi) (Fig. 2A) despite a detectable virus load (Fig. 2B, lanes 1 to 6). Inoculation with the same dose of FHV resulted in 60% mortality by 6 dpi and 95% by 15 dpi in dcr-2<sup>L811fsX</sup> flies (Fig. 2A). Mock inoculation with buffer had little effect on either WT or dcr-2<sup>L811fsX</sup> flies for as long as observations were made. Both Northern and Western blot analyses revealed that the virus accumulated more rapidly and to much greater levels in dcr-2<sup>L811fsX</sup> than WT flies (Fig. 2, B and C). Thus, dcr-2 mutants exhibit enhanced disease susceptibility to FHV in comparison with WT flies, demonstrating that Dcr-2 is also required to mount an immune response that protects adult Drosophila against FHV infection.

R2D2 contains tandem double-stranded RNA (dsRNA)–binding domains and forms a heterodimer with Dcr-2 in vivo that is required for siRNA loading into RISC (1, 15). We found that flies homozygous for a loss-of-function mutation in r2d2 exhibited a phenotype of enhanced disease susceptibility to FHV infection similar to that of dcr-2<sup>L811fsX</sup> (Fig. 2). Thus, R2D2 also participates in the innate immunity pathway that protects adult flies from FHV infection. Notably, although FHV accumulated to extremely high levels in both dcr-2 and r2d2 mutant flies, abundant viral siRNAs were detected only in r2d2 mutant flies, and viral siRNAs were below the level of detection in dcr-2<sup>L811fsX</sup> flies (Fig. 2D). Thus, FHV infection is detected by Dcr-2, leading to production of FHV siRNAs. However, R2D2 is not required for the production but is essential
for the function of viral siRNAs, which is consistent with the genetic requirements for processing the artificially introduced dsRNA 

To investigate whether the RNAi pathway in Drosophila is specific against nodaviruses and not other classes of RNA viruses, we assessed the susceptibility of WT, dcr-2L811fsX, and r2d2 mutant flies to cricket paralysis virus (CrPV). CrPV contains a nonsegmented plus-strand RNA genome but belongs to a group of picorna-like viruses (16). CrPV is substantially more virulent than FHV in Drosophila, because injection of CrPV at much lower titers resulted in mortality of 70% of WT flies by 15 dpi (Fig. 3A). We found that CrPV also induced enhanced disease susceptibility in both dcr-2 and r2d2 mutant flies (Fig. 3A). About 60% of the infected mutant flies were dead by 6 dpi, and more than 95% were dead by 15 dpi (Fig. 3A). In addition, Northern blots indicated that the virus accumulated more rapidly and to greater levels in the mutant flies (Fig. 3B). Thus, both dcr-2 and r2d2 are required for protection of Drosophila against CrPV.

CrPV infection of cultured S2 cells induced antiviral silencing, illustrated by detection of CrPV-specific siRNAs (Fig. 4A). Antiviral silencing against FHV in S2 cells induced by FR1gfp as described previously (11) was suppressed by CrPV superinfection, leading to derepression of green fluorescent protein (GFP) (Fig. 4B, left). Two ORFs are encoded by the CrPV RNA genome (16) (fig. S2). We did not observe suppression of antiviral silencing in S2 cells cotransfected with a plasmid expressing either the entire downstream ORF of CrPV or its N-terminal 140 codons (Fig. 4C, pA in lane 1). However, the suppressor activity was not detected after a frameshift mutation was introduced into pA (Fig. 4C, lane 2), thus identifying the N-terminal fragment of 140 amino acids of the CrPV nonstructural polyprotein as a VSR.

In D. melanogaster, Imd signaling is stimulated by Gram negative (Gram–) bacterial infection, whereas Toll signaling is triggered by Gram positive (Gram+) bacterial infection (7, 17). To determine whether loss of the RNAi pathway initiated by Dcr-2 had an impact on the Toll and Imd signaling processes, we subjected WT, dcr-2L811fsX, and r2d2 mutant flies to immune challenge by inoculation with Escherichia coli (Gram+) or Micrococcus luteus (Gram–). Northern blot hybridizations detected substantial transcriptional induction of the antimicrobial peptide gene Diptericin A as described (17). Similar induction patterns for Diptericin A were observed in dcr-2L811fsX and r2d2 mutant flies inoculated with Gram+ bacteria (fig. S3). Furthermore, we found that induction of either Attacin A or Drosomycin by Gram+ and Gram– bacteria was also not altered in dcr-2L811fsX and r2d2 mutant flies as compared to WT flies (fig. S3). These results indicate that induction of antimicrobial peptide genes via Toll and Imd signaling pathways is not compromised in dcr-2L811fsX and r2d2 mutant flies.
Nodaviruses and the polio-like CrPV belong to two different superfamilies of animal RNA viruses. We demonstrate that the same set of RNAi pathway genes is required for Drosophila defense against FHV and CrPV and that both viruses encode a potent VSR. Furthermore, a genetic requirement for general counterdefensive strategy used by insect species gene vir-1 was unaltered in the dcr-2 and r2d2 mutants, as shown by our recent work. Because the Toll and Imd pathways are highly conserved in vertebrates (7), the Drosophila model established for RNAi may also be useful for the analyses of the innate antiviral immunity in vertebrates.

Fig. 4. Induction and suppression of antiviral silencing by CrPV. (A) Accumulation of the genomic RNA and siRNAs of CrPV in infected S2 cells. (B) pFR1gfp directed transcription of a recombinant FHV RNA1 in which the coding sequence for B2 was replaced by that of GFP. S2 cells were transfected with pFR1gfp alone (middle) or pFR1gfp plus either dsRNA-targeting Ago-2 (right) or CrPV superinfection (left). (C) Identification of CrPV RNAi suppressor. Cells were cotransfected with pFR1gfp and a plasmid as indicated on top of each lane (fig. S2), and total RNA was analyzed for the accumulation of or pFR1gfp plus either dsRNA-targeting Ago-2 (right) or CrPV superinfection (left). (b), the first 107 codons of the upstream ORF; V2, VP2; V3, VP3; V4, VP4; V0, VP0 (precursor for VP3 and VP4); A, the first 140 codons of the upstream ORF; ΔA, a frameshift mutant of A; and b, the first 107 codons of the upstream ORF. NS1 is an RNAi suppressor of influenza A virus as described previously (11).

References and Notes
8. C. Wilkins et al.
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