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Distinct Roles for *Drosophila* Dicer-1 and Dicer-2 in the siRNA/miRNA Silencing Pathways

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Summary

The RNase III enzyme Dicer processes RNA into siRNAs and miRNAs, which direct a RNA-induced silencing complex (RISC) to cleave mRNA or block its translation (RNAi). We have characterized mutations in the *Drosophila dicer-1* and *dicer-2* genes. Mutation in *dicer-1* blocks processing of miRNA precursors, whereas *dicer-2* mutants are defective for processing siRNA precursors. It has been recently found that *Drosophila* Dicer-1 and Dicer-2 are also components of siRNA-dependent RISC (siRISC). We find that Dicer-1 and Dicer-2 are required for siRNA-directed mRNA cleavage, though the RNase III activity of Dicer-2 is not required. Dicer-1 and Dicer-2 facilitate distinct steps in the assembly of siRISC. However, Dicer-1 but not Dicer-2 is essential for miRISC-directed translation repression. Thus, siRISCs and miRISCs are different with respect to Dicers in *Drosophila*.

Introduction

Small RNAs influence a wide variety of biological processes by silencing the expression of genes within organisms. These RNAs, with a size of about 22 nucleotides, influence development, genome organization, viral and transposon defense, and disease (Hannon, 2002). There are two classes of small RNAs, and they exert their powers of silencing differently. One class is processed from longer double-stranded (dsRNA) precursor molecules with perfect complementarity. The dsRNAs are cleaved into small interfering RNAs (siRNAs) that are 21–23 nucleotide duplexes. They act as guides for a siRNA-induced silencing complex (siRISC) to target complementary mRNAs. If such an mRNA molecule is found, the base pairing interactions between siRNA and mRNA lead to cleavage of the mRNA molecule and its degradation. A second class of small RNAs, the microRNAs (miRNAs), is processed from stem-loop RNA precursors (pre-miRNAs) that are encoded within plant and animal genomes. The known functions of a few of these miRNAs indicate that they play widespread roles in growth and development (Abrahante et al., 2003; Brennecke et al., 2003; Lee et al., 1993; Lin et al., 2003; Llave et al., 2002; Palatnik et al., 2003; Reinhart et al., 2000). Animal miRNAs silence gene expression primarily by blocking the translation of mRNA transcripts into protein. They act as guides for a multiprotein complex, miRISC, which identifies mRNAs with imperfect complementarity in the 3′ untranslated region of the message.

Recently, it was found that the extent of base pairing between small RNA and mRNA determines the outcome of silencing. An miRNA will direct mRNA cleavage if the target transcript is perfectly complementary in sequence (Hutvagner and Zamore, 2002). Conversely, an siRNA will block protein synthesis if the target transcript has partial complementarity (Doench et al., 2003; Zeng et al., 2003). These observations imply that the extent of base pairing between small RNA and mRNA determines the outcome of silencing. It is unclear whether a single silencing complex is competent to both cleave mRNA and block translation, or whether an miRNA (or siRNA) associates with two biochemically distinct RISC complexes—one able to cleave mRNA and another able to block translation.

Although dsRNAs and pre-miRNAs are structurally distinct, they are both processed into siRNAs and miRNAs, respectively, by the Dicer class of RNase III enzymes (Bernstein et al., 2001; Grishok et al., 2001; Ketting et al., 2001; Lee et al., 2002). Dicer makes staggered cuts in dsRNA to form siRNA duplexes with 3′ overhangs, each strand bearing 5′ phosphate and 3′ hydroxyl termini (Myers et al., 2003; Provost et al., 2002). Dicer exhibits little sequence specificity for cleavage, though it favors processing from the end of a dsRNA substrate (Elbashir et al., 2001). The siRNA product then assembles into a siRISC that retains either the sense or antisense strand of the duplex (Hammond et al., 2000; Nykanen et al., 2001). Dicer also processes pre-miRNA (Hutvagner et al., 2001; Ketting et al., 2001; Lee et al., 2002). However, in the course of assembly into miRISC, one strand is preferentially retained from the siRNA-like duplex. *Dicer* mutants are defective for both transcript destruction and translational repression, suggesting that Dicer is required in both the siRNA and miRNA pathways (Grishok et al., 2001; Ketting et al., 2001; Knight and Bass, 2001). This dual role has made its genetic analysis more complicated.

In addition to dsRNA processing, Dicer appears to play some other, as yet ill-defined role in the siRNA pathway. Dicer functions downstream of siRNA production, as depletion of Dicer in mammalian cells reduces the effectiveness of added siRNAs (Doi et al., 2003). Dicer physically associates with protein components of RISC, and it binds siRNAs tightly in vitro (Doi et al., 2003; Hammond et al., 2001; Liu et al., 2003; Tabara et al., 2002; Tang et al., 2003). In *Drosophila*, this latter interaction is enhanced by an auxiliary dsRNA binding protein, R2D2 (Liu et al., 2003). One interpretation of these results is that Dicer associates with siRNAs and facilitates their activities in siRISC. However, the mechanism of Dicer function within siRISC has remained uncertain. In this paper, we have used a genetic approach in *Drosophila melanogaster* to examine the molecular basis for Dicer function.

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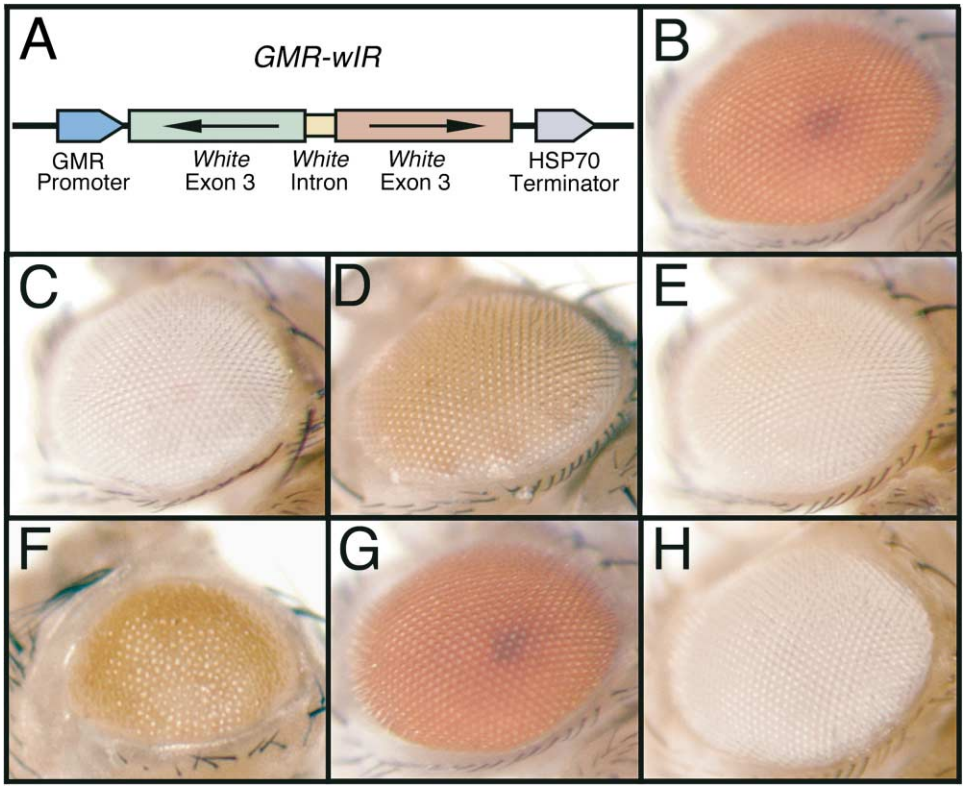

Figure 1. Isolation of RNAi Mutants in *Drosophila*

(A) Structure of the *GMR-wIR* transgene used to induce *white* RNAi in adult eyes. The GMR promoter drives transgene expression specifically in the differentiating eye. The third exon of *white* is repeated in an inverted orientation, separated by a *white* intron. The resulting transcript, after intron splicing, is predicted to create a perfect hairpin dsRNA of length 629 bp.

(B) Eye of a *white*$^+$ adult fly.

(C) Eye of a *white* null mutant fly.

(D) Eye of a *white*$^+$ fly with one copy of *GMR-wIR*. The *white* gene is partially silenced, resulting in a fly with a pale orange eye color.

(E) Eye of a *white*$^+$ fly with two copies of *GMR-wIR*, exhibiting strong loss of *white* gene activity.

(F) Eye of a *white*$^+$ fly carrying one copy of *GMR-wIR* and homozygous for the *Suppressor*(*GMR-wIR*)*D14* mutation, which is a *dcr-1* allele.

(G) Eye of a *white*$^+$ fly carrying two copies of *GMR-wIR* and homozygous for the *Suppressor*(*GMR-wIR*)*A293* mutation, which is a *dcr-2* allele.

(H) Eye of a *white*$^+$ fly carrying one copy of *GMR-wIR* and homozygous for the *Enhancer*(*GMR-wIR*)*A28* mutation on the right arm of chromosome II.

Results

Genetic Identification of *dicer-2*

To identify new and essential components of the siRNA pathway, we screened for EMS-induced mutations that result in reduced or enhanced RNAi activity in *Drosophila melanogaster*. We screened animals that had homozygous mutant compound eyes, while all other tissues, including germline, were heterozygous. Such genetic mosaics were constitutively generated due to eye-specific mitotic recombination between heterozygous sister chromosomes (Newsome et al., 2000; Stowers and Schwarz, 1999). This approach enabled us to recover mutations from the F1 germline that otherwise were homozygous lethal or sterile to the animal. The mosaic F1 animals also carried a transgene (*GMR-wIR*) that silenced expression of the endogenous *white* gene, by driving eye-specific synthesis of a hairpin dsRNA corresponding to an exon of *white* (Figure 1A). *GMR-wIR* reduces eye pigmentation from its normal red color to a paler variation (Figures 1B–1E). Since one copy of *GMR-wIR* does not completely silence *white*, we were able to compare the eye pigmentation of *GMR-wIR* in wild-type animals (pale orange) with those of mutants, looking for enhanced (white) and suppressed (red) phenotypes (Figures 1F–1H). We kept flies (2% of those screened) whose eye colors deviated from pale orange.

Our screens of three major autosomal arms of *Drosophila* have identified more than 15 loci that when mutated result in stronger pigmentation in a *GMR-wIR* background. One such locus, identified in the screen of the right arm of the second chromosome, was a homozygous viable complementation group consisting of 39 alleles. Noncomplementation was based on a strongly suppressed eye color phenotype in the presence of *GMR-wIR* (Figure 1G). To genetically map the locus, we used *Drosophila* single nucleotide polymorphism (SNP) markers (Berger et al., 2001). Mapping placed the locus within a 568 kb interval of 54C, an interval that contains a Dicer gene. *Drosophila* contains two genes in the Dicer family, *dicer-1* (*dcr-1*) and *dicer-2* (*dcr-2*) (Figure 2A). Most Dicer orthologs contain a DExH-type ATP-dependent RNA helicase domain at their amino termini, and a PAZ domain that is also found in some protein compo-

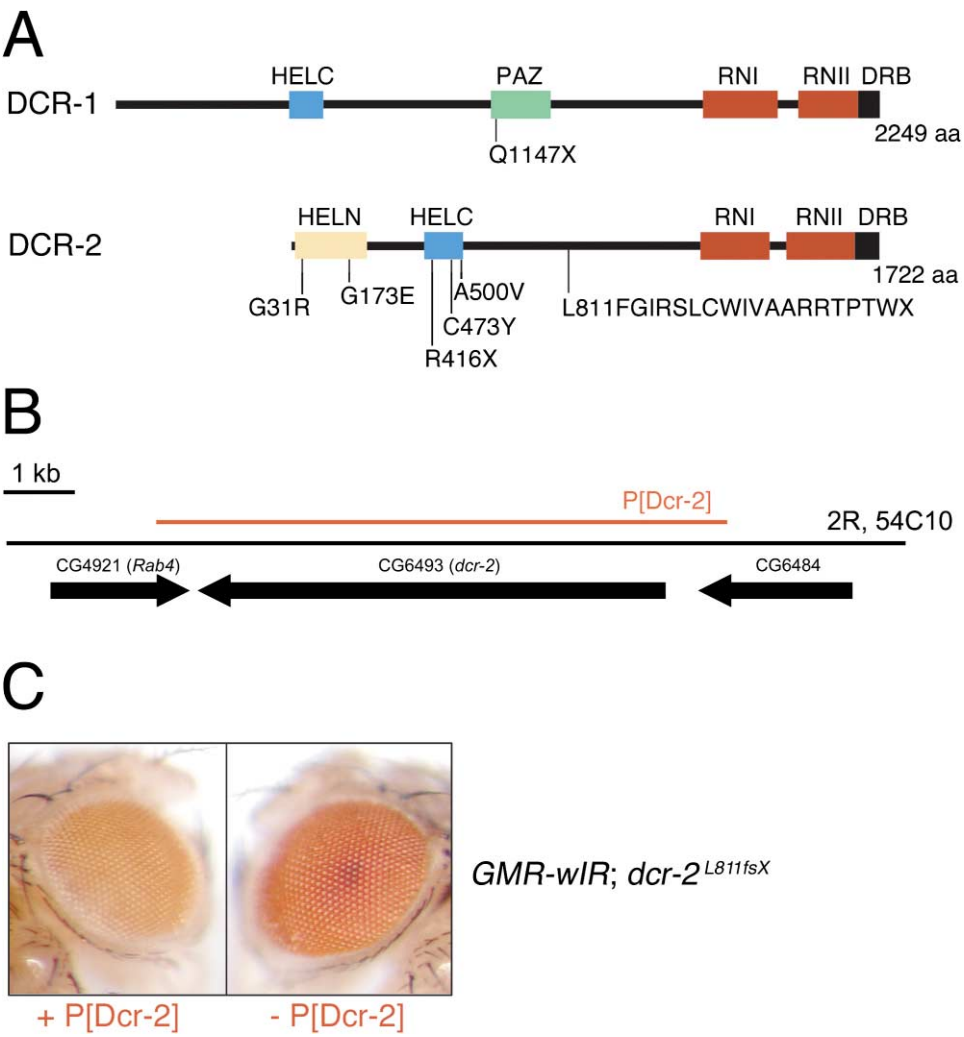

Figure 2. Mutations in *dcr-1* and *dcr-2* Block Gene Silencing by *GMR-wIR*

(A) Schematic of *Drosophila* Dcr-1 and Dcr-2 proteins. Highlighted are: within Dcr-1, the PAZ domain; within Dcr-2, the DExH box- (HELN) and C-helicase domains (HELC); the tandem repeats of RNase III (RN) domains; and a C-terminal dsRNA binding domain (DRB). Indicated is the nonsense codon within *dcr-1* coding sequence that is found in the *Suppressor(GMR-wIR)D14* mutant, and which we rename *dcr-1*^{Q1147X}. Also shown are the changes in *dcr-2* amino acid sequence of six mutations within a complementation group that includes the *Suppressor(GMR-wIR)A293* mutant, which is renamed *dcr-2*^{L811fsX}.

(B) Genomic organization of the *dcr-2* locus and transformation construct. *dcr-2* is located between the genes *Rab4* and CG6484. A 7.2 kb PCR fragment encompassing the putative *Dcr-2* transcription unit is shown as a red line drawn above the genomic map.

(C) Transformation rescue of *dcr-2*. Flies carrying *GMR-wIR* and homozygous mutant for *dcr-2*^{L811fsX} with (left) or without (right) one copy of the 7.2 kb *Dcr-2* rescue transgene.

nents of RISC. Interestingly, *Drosophila* Dcr-1 lacks a functional helicase domain, whereas Dcr-2 lacks a PAZ domain. This suggests that the two enzymes might have different or complementary biochemical activities. However, like other members of the Dicer family, both Dcr-1 and Dcr-2 contain two RNase III domains and a dsRNA binding domain at their carboxy-termini.

The *dcr-2* gene is located within the interval that contained our complementation group of suppressor mutations. We sequenced the *dcr-2* gene in six independent mutants, and each mutant had base changes that significantly altered the predicted protein product (Figure 2A). Two alleles contained premature stop codons that would produce truncated proteins, lacking the RNase III domains essential for dsRNA processing activity. These two mutations likely represent null alleles of *dcr-2*. To confirm that the mutant complementation group corresponded to *dcr-2*, we performed transformation rescue with a 7.2 kb genomic fragment that spans the *dcr-2* transcription unit (Figure 2B). This fragment completely rescued the phenotype associated with a homozygous *dcr-2* null mutation (Figure 2C).

Dcr-2 Functions Upstream and Downstream of siRNA Production In Vivo

We asked if the phenotype associated with *dcr-2* mutants resulted from a defect in dsRNA processing. We examined the levels of siRNAs generated from *GMR-wIR* in the eyes of wild-type and *dcr-2* mutants. The *dcr-2* null mutants exhibited a large reduction in siRNA

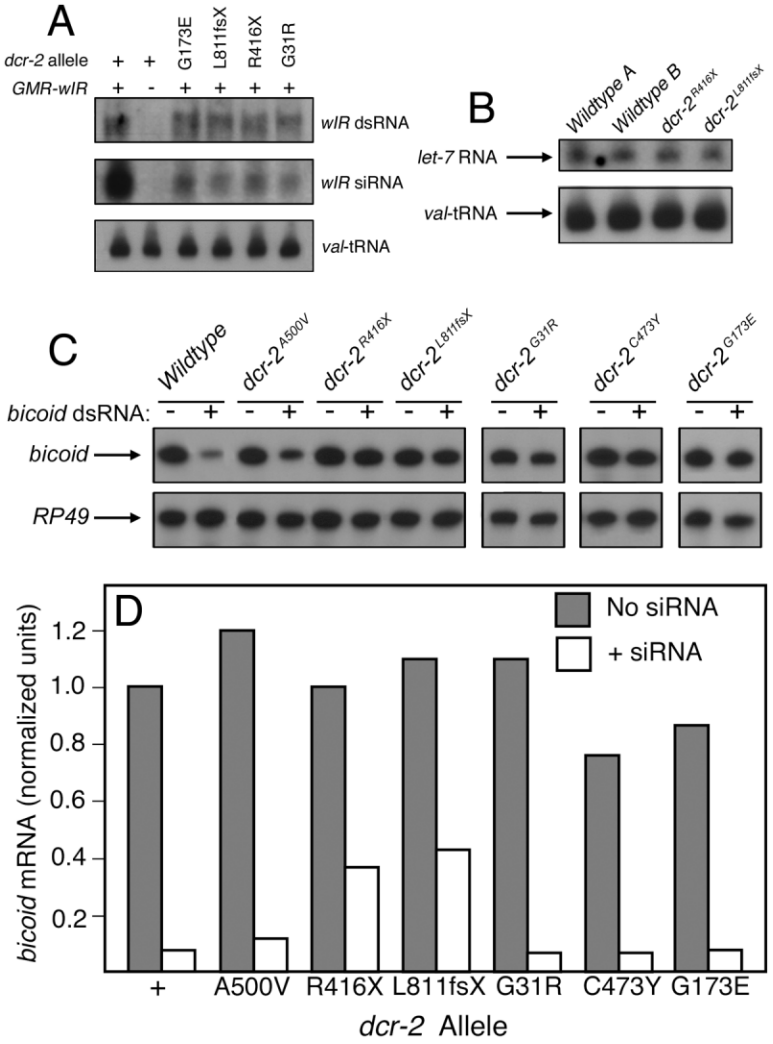

Figure 3. Dcr-2 Is Required for Both dsRNA Processing and siRNA-Dependent Transcript Degradation

(A) Northern blot analyses of total RNA isolated from heads of wild-type and *dcr-2* mosaic flies with genotypes as indicated. The presence or absence of *GMR-wIR* in flies is indicated as + or −, respectively. The top image shows a blot probed for hairpin RNA expressed from the *GMR-wIR* transgene. The middle image shows a blot probed for siRNAs generated from *GMR-wIR* hairpin RNA while the bottom image shows the same blot after stripping and reprobing for $tRNA^{val}$ as a loading control.

(B) Northern blot for the miRNA *let-7* isolated from wild-type and *dcr-2* homozygous mutant flies. Wild-type A is the parental strain that was originally subjected to mutagenesis; Wild-type B is Canton S strain. The blot was reprobed for $tRNA^{val}$ to control for loading.

(C) Levels of mRNA transcripts in wild-type or *dcr-2* mutant eggs injected with *bicoid* dsRNA or buffer. Shown are levels of *bicoid* and the heterologous transcript *RP49*, as determined by semiquantitative RT-PCR.

(D) Levels of mRNA in wild-type or *dcr-2* mutant eggs injected with *bicoid* siRNA or buffer. Shown are levels of *bicoid* transcript normalized to the corresponding levels of *RP49* in each sample, as determined by semiquantitative RT-PCR.

levels when compared to wild-type (Figure 3A). This reduction did not result from instability or low-level expression of *GMR-wIR* dsRNA, since *GMR-wIR* precursor RNAs were present at levels comparable to wild-type. These data indicate that Dcr-2 plays a major role in dsRNA processing. Interestingly, substitution mutants in the Dcr-2 helicase domain were as impaired for siRNA production as null mutants. One of these, the *dcr-2*^{G31R} mutant, changes one of the invariant GXGXXG residues in the ATP binding site of the helicase domain. Thus, Dcr-2 requires a functional helicase domain for dsRNA processing.

Flies homozygous for null *dcr-2* alleles are viable and fertile, and are morphologically normal in external appearance. Since miRNAs are indispensable for growth and development in *Drosophila*, the *dcr-2* phenotype suggests that Dcr-2 is not essential for pre-miRNA processing. To address this, we examined levels of the miRNA *let-7* in *dcr-2* null mutants. The *dcr-2* mutants exhibited mature *let-7* RNA levels comparable to those of wild-type controls (Figure 3B). This confirms that Dcr-2 is not required for the processing of pre-miRNAs.

Previous work showed that RNAi is established in the *Drosophila* female germline (Kennerdell et al., 2002). To examine whether Dcr-2 is required for mRNA degradation in eggs, we injected *dcr-2* mutant eggs with dsRNA corresponding to the *bicoid* gene, which is maternally expressed. Subsequently, we assayed *bicoid* mRNA levels by RT-PCR. Wild-type eggs displayed rapid reduction in *bicoid* transcript abundance after dsRNA injection (Figure 3C). In contrast, *dcr-2* null mutant eggs showed no significant reduction in *bicoid* transcript abundance, indicating that *dcr-2* is required for effective RNAi in the female germline. A similar effect was observed in *dcr-2* mutants bearing substitutions in the Dcr-2 helicase domain.

We next asked whether the RNAi defect in *dcr-2* eggs was simply due to defective siRNA production. To test this hypothesis, we injected eggs with a synthetic siRNA corresponding to the *bicoid* gene and subsequently assayed *bicoid* transcript levels. Wild-type eggs exhibited loss of *bicoid* mRNA in response to siRNA injection (Figure 3D). In contrast, *dcr-2* null mutant eggs exhibited an impaired RNAi response to siRNA. Five-fold more *bicoid* mRNA was present in *dcr-2* mutant eggs compared to wild-type eggs after siRNA treatment (Figure 3D). This result indicates that Dcr-2 also functions downstream of siRNA production in the RNAi pathway. Inter-

estingly, substitution mutants in the Dcr-2 helicase domain were unimpaired for siRNA-dependent RNAi, suggesting that a functional helicase activity is not required for Dcr-2 to mediate its downstream function.

Dcr-1 Is Required for miRNA- and siRNA-Dependent Gene Silencing

Our experiments established an important though not absolute role for Dcr-2 in *Drosophila* RNAi. Since another Dicer (Dcr-1) is present in *Drosophila*, it is possible that it has a redundant function with Dcr-2. The *dcr-1* gene is located at 94C4 on the third chromosome, and we found a mutation that mapped by linkage and complementation analysis to that region. The coding sequence of *dcr-1* in the mutant contained a premature stop codon such that the truncated product lacks the PAZ and RNase III domains (Figure 2A). Moreover, *dcr-1* mRNA is not detectable in the mutant as determined by RT-PCR and Northern blot analysis, suggesting that the transcript is unstable when truncated Dcr-1 protein is produced (Figures 4A and 4B). This transcript null *dcr-1*^{Q1147X} mutant exhibited an eye color phenotype when assayed in a *GMR-wIR* genetic background (Figure 1F). The mutant appeared to partially suppress silencing by *GMR-wIR*, with patches of dark orange eye color. In addition, the eye was half its normal size, the organization of ommatidial facets was disrupted, and sensory bristles were missing over the eye surface. Other bristles, which flank the eye surface, were sometimes absent or exhibited hyperplasia.

Despite an effect on *white* gene silencing, the *dcr-1* mutant had normal levels of *wIR* siRNAs (Figure 4C). This observation is consistent with Dcr-2 processing the great majority of *wIR* dsRNA (Figure 3A). It is further consistent with a central role for Dicer helicase activity in dsRNA processing, since Dcr-1 lacks a DExH-box helicase domain. If the *dcr-1* mutant has normal dsRNA processing, why is it partially disrupted for gene silencing? To answer this, we generated clones of homozygous *dcr-1* mutant germ cells in heterozygous females, and then injected *dcr-1* mutant eggs with either dsRNA or siRNA complementary to *bicoid* transcripts. Loss of *bicoid* mRNA was measured as a consequence. *dcr-1* mutant eggs exhibited an impaired RNAi response to dsRNA and siRNA (Figure 4E). Six-fold more *bicoid* mRNA was present in *dcr-1* mutant eggs compared to wild-type eggs after either dsRNA or siRNA treatment. This result indicates that Dcr-1 acts downstream of siRNA production in the RNAi pathway. Dcr-1 plays an important though not absolute role in siRNA-dependent RNAi. Since Dcr-2 is also required downstream of siRNAs, these data suggest that Dcr-1 and Dcr-2 function might be partially redundant in some downstream activity.

Dcr-1 is essential to generate mature miRNAs. We demonstrated this role by analyzing miRNA levels in *dcr-1* mutant eggs. No mature miRNAs belonging to the *miR-2* group were detected in *dcr-1* mutant eggs (Figure 4D). Thus, Dcr-1 is critical for miRNA production whereas Dcr-2 is required primarily for siRNA production.

Although Dcr-1 and Dcr-2 preferentially produce different types of small RNAs, both are required for efficient siRNA-dependent mRNA degradation. Does this dual requirement extend to the miRNA pathway as well? To test this possibility, we used a genetic assay for miRNA-dependent gene silencing in *dcr-1* and *dcr-2* mutants. Several classes of motifs are present in the 3′ UTR regions of the *E(spl)* and *Bearded* genes (Lai et al., 1998; Lai and Posakony, 1997). The 3′UTR motifs are complementary to a variety of miRNAs, and they mediate post-transcriptional repression of gene expression (Lai, 2002). A series of reporter transgenes was constructed that mimics this posttranscriptional repression (Lai et al., 1998; Lai and Posakony, 1997). The reporter genes contain a constitutive promoter from *armadillo*, *lacZ* coding sequence, and the 3′UTR from the *Bearded* or *E(spl)m8* gene. When the reporter contains a wild-type *Bearded* 3′UTR, its expression in the developing eye disc is very weak (Figure 4F). It is somewhat more strongly expressed in the eye disc posterior to the morphogenetic furrow and is equally weak in the anterior eye disc and antennal disc. When the reporter contains a *Bearded* 3′UTR with its three B motifs mutated, expression is ubiquitously strong in the eye and antennal discs, confirming that the B motifs mediate a silencing effect on gene expression (Figure 4G).

We then examined expression of a wild-type reporter gene in clones of mutant *dcr-2* cells that were generated in the developing eye disc. Clones expressed the reporter at levels indistinguishable from wild-type tissue, indicating that Dcr-2 is not required for this gene silencing mechanism (Figure 4H). In contrast, expression of a wild-type reporter gene in clones of mutant *dcr-1* cells was much stronger than in wild-type tissue (Figures 4I and 4J). The derepressive effect of the *dcr-1* mutation requires intact B motifs in the *Bearded* 3′UTR, since mutant clones did not affect expression of a reporter gene with mutated B motifs (Figure 4K). These results argue that *dcr-1* but not *dcr-2* is necessary for post-transcriptional gene silencing that is mediated by a miRNA mechanism. This conclusion is also validated by other mutant phenotypes associated with each gene. Loss of *dcr-1* has profound effects on *Drosophila* development within both somatic- and germ-lineages (data not shown), whereas loss of *dcr-2* appears to have little or no effect on development.

The *dcr-1* mutant clones exhibited an interesting pattern of reporter expression. Clones in the antennal disc and eye disc, anterior to the morphogenetic furrow, exhibited little or no derepression of the reporter gene (Figure 4J). Typically, only a few mutant cells in each clone had high levels of reporter gene expression. No overt cell differentiation occurs in this region of the eye disc. In contrast, almost all eye disc clones posterior to the furrow, where cell differentiation actively occurs, exhibited extensive derepression. Many, if not most, mutant cells in a clone exhibited this behavior. A boundary of reporter gene expression within a clone could be detected if the clone was bisected by the furrow. This boundary coincided with the morphogenetic furrow (Figures 4L–4N). Three interpretations seem possible. First, different sets of miRNAs repress the reporter in different regions of the eye disc, one set of which requires Dcr-1 and one set of which does not. However, all of these miRNAs would have to act through the 3′UTR binding sites, since a mutated reporter is constitutively derepressed (Figure 4G). Thus, we do not favor this interpre-

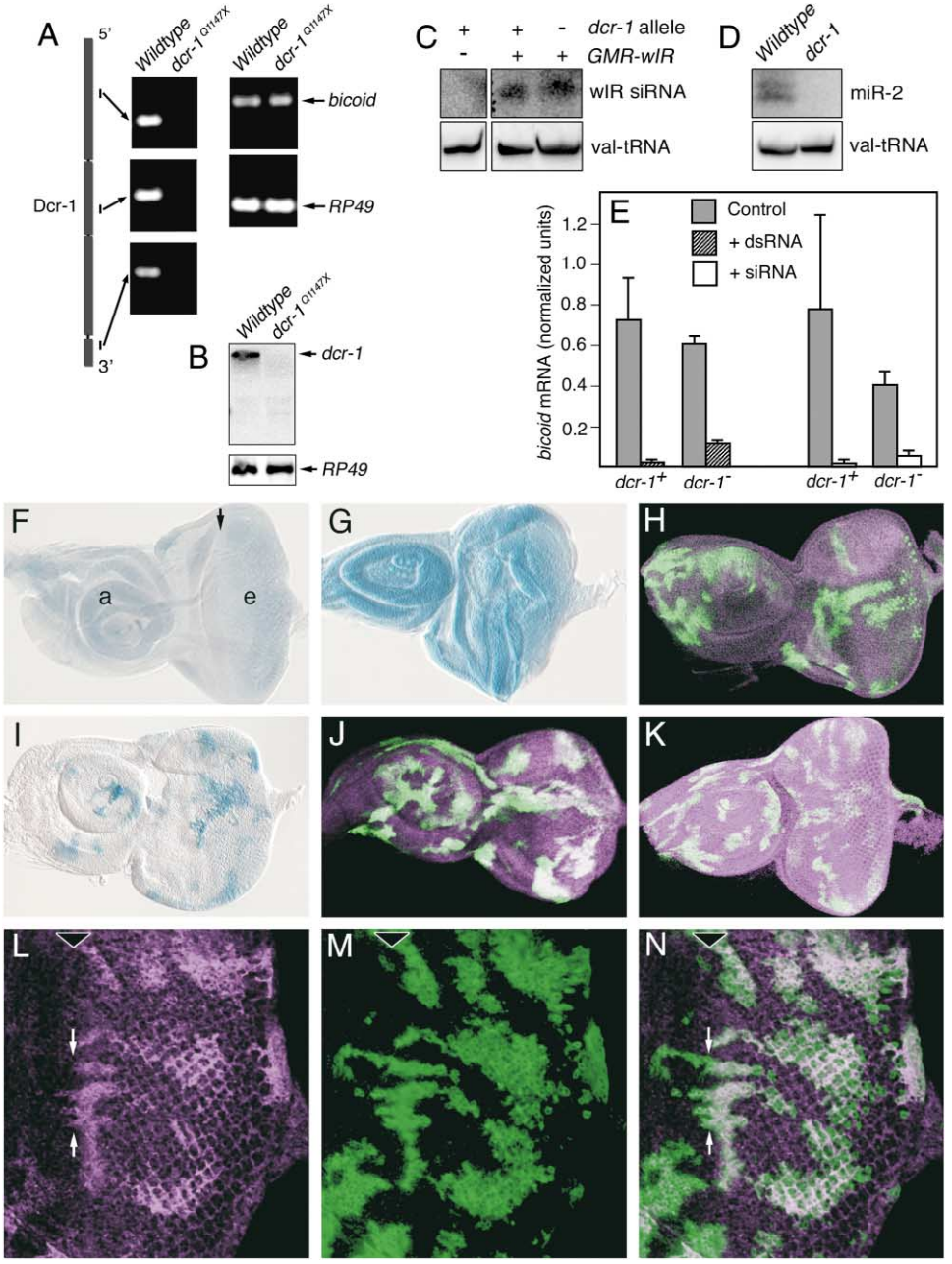

Figure 4. Dcr-1 Is Required for Both the miRNA and siRNA Pathways

(A) RT-PCR analysis of *dcr-1* mRNA abundance in *dcr-1*^{Q1147X} homozygous mutant eggs. PCR amplification of RT products was performed on three different regions of the *dcr-1* transcription unit, as shown on the left. Boxes represent exons and lines represent introns. As controls, PCR was also performed on *bicoid* and *RP49* RT products.

(B) Northern blot of *dcr-1* mRNA isolated from wild-type and *dcr-1*^{Q1147X} mutant eggs, with *RP49* mRNA blotted as a loading control.

(C) Northern blot of total RNA isolated from heads of wild-type (+) and mosaic flies bearing homozygous *dcr-1*^{Q1147X} mutant (–) eyes. The presence or absence of *GMR-wIR* in flies is indicated. The top image shows a blot probed for *wIR* siRNAs while the bottom image shows the same blot reprobed for tRNA^{val}.

(D) Northern blot of total RNA isolated from wild-type and homozygous *dcr-1*^{Q1147X} mutant eggs. Shown is the blot probed for five related miRNAs of the *miR-2* group, and for tRNA^{val}.

(E) Levels of *bicoid* transcripts in wild-type or *dcr-1*^{Q1147X} mutant eggs injected with *bicoid* dsRNA, siRNA, or buffer. Shown are levels of *bicoid* transcript normalized to the corresponding levels of *RP49* mRNA in each sample, as determined by semiquantitative RT-PCR. Error bars represent standard deviation values.

(F–N) Eye-antennal discs from larvae expressing a *Bearded* or *E(spl)m8* reporter gene. All discs are oriented with anterior to the left.

(F) Reporter gene containing wild-type *Bearded* 3′UTR. Eye disc (labeled e) with the eye morphogenetic furrow indicated with an arrow, and antennal disc (labeled a) were stained with Xgal.

(G) Reporter gene containing the *Bearded* 3′UTR with mutated B motifs. The Xgal reaction time for this disc was one-tenth the length of time for the disc in (F).

tation. Second, Dcr-1 might not be essential in anterior disc cells because Dcr-2 or another factor substitutes if Dcr-1 is missing. Third, anterior disc cells may contain miRNAs that were originally generated in *dcr-1*$^{+}$ progenitor cells, and may therefore not require *dcr-1*. Passage of the morphogenetic furrow may trigger miRNA turnover, resulting in renewed dependence on *dcr-1* posterior to the furrow.

Dcr-1 and Dcr-2 Are Required for siRISC Assembly

The RNAi pathway can be divided into discrete biochemical steps: dsRNA processing, maintenance of siRNA 5′ phosphate termini, siRNA loading into siRISC, and siRISC-based cleavage of target mRNA. We confirmed that Dcr-2 but not Dcr-1 is required for dsRNA processing by incubating radiolabeled dsRNA substrate in lysates made from mutant embryos and monitoring siRNA formation (Figure 5A). Labeled 21–23 nucleotide RNAs were readily detectable from reactions with wild-type and *dcr-1* lysates, but were greatly reduced in reactions with *dcr-2* lysate.

Our genetic experiments suggested a role for Dcr-1 and Dcr-2 downstream of dsRNA processing. Therefore, we assayed lysates made from mutant embryos for steps downstream of siRNA production. siRNAs require 5′ phosphate termini for proper association with RISC, and the 5′ phosphates are maintained by a kinase that recognizes siRNAs (Nykanen et al., 2001). Both wild-type and *dcr-2* mutant lysates efficiently converted synthetic siRNAs bearing 5′ hydroxyl groups into 5′-phosphorylated forms (Figure 5B), indicating that the mutant lysates have normal end-maintenance activity.

Nykanen et al. (2001) previously reported that siRNAs are incorporated into a 350 kDa complex when incubated with embryo lysate. The complex is converted to active siRISC using ATP hydrolysis. We incubated radiolabeled siRNA with wild-type or *dcr-2* mutant embryo lysates in the presence of ATP, and then size-fractionated the products by gel filtration chromatography. siRNA was predominantly associated with a ~350 kDa complex in wild-type lysate (Figure 5C). However, most of the siRNA in the *dcr-2* mutant lysate fractionated as if unbound by proteins, indicating that Dcr-2 is essential for siRNA entry into functional complexes. Thus, *dcr-2* lysates should be defective for siRNA-directed mRNA cleavage. We coincubated siRNA complementary to a radiolabeled target mRNA with embryo lysate. Incubation in wild-type lysate produced a truncated mRNA whose length was consistent with it being the 5′ cleavage product (Figure 5D). Incubation in *dcr-2* lysate generated 5- to 15-fold less cleavage product. This indicates that Dcr-2 is required for mRNA target cleavage by a siRNA, and is consistent with our in vivo observations (Figure 3D).

To examine siRISC formation in *dcr-1* mutant lysate, we used native gel electrophoresis. Gel filtration chromatography requires large lysate volumes, which we were unable to obtain from *dcr-1* embryos. Pham et al. (2004 [this issue of *Cell*]) have developed a gel electrophoretic method to characterize siRNA complexes. The R1 gel complex corresponds to Dcr-2 and R2D2 proteins bound to labeled siRNA (Figure 5E). R2 complex appears to be an intermediate that links R1 to a third complex, R3. The R3 complex corresponds to a siRISC that is competent to cleave cognate mRNA (Pham et al., 2004). To address the role of Dcr-1 in complex formation, we looked for complexes in a *dcr-1* mutant lysate (Figure 5E). R1 complex was detected, but its mobility was slightly shifted and more heterogeneous. No complex with comparable mobility to R2 was detected. The data indicate that proper formation of the R2 intermediate from the R1 precursor complex is dependent upon Dcr-1.

Site-Directed Mutagenesis of the Dcr-2 RNase III Domains

Bacterial RNase III is an antiparallel dimer containing a deep cleft within the catalytic domain (Blaszczyk et al., 2001). At each end of the cleft lies a symmetric cluster of acidic residues that are conserved among RNase III enzymes (Figures 6A and 6B). Some of the residues at each end coordinate a divalent metal ion (Mg^{2+}), which is essential for the nucleophilic attack on the RNA phosphodiester bonds at each active site (Blaszczyk et al., 2001). The metal ion makes a bidentate interaction (inner-sphere and outer-sphere) with an invariant glutamate residue. In one model of catalysis, the nucleophile is used twice, thereby cleaving both strands (Nicholson, 2003). In another model, based on a crystal structure, each active site uses two separate clusters of residues to cleave the two phosphodiesters of the RNA helix (Blaszczyk et al., 2001). One cluster involves the metal ion, and the other cluster acts independent of the metal ion.

Dicers contain two catalytic domains, which based on the structure of bacterial RNase III, might fold into a pseudodimer structure or might associate within a dimeric holoenzyme. In either scenario, the catalytic repeats contain many of the invariant acidic residues implicated in RNase III catalysis. Moreover, like bacterial RNase III, Dicers cleave dsRNA to produce fragments

(H) Reporter gene containing wild-type *Bearded* 3′UTR is expressed (purple) at the same intensity in unmarked wild-type cells as in *dcr-2* mutant cells, which are marked with GFP in green. Reporter expression is visualized with anti-β-galactosidase.
(I) Wild-type reporter expression, visualized with Xgal, is strongly variegated in discs with *dcr-1*^{Q1147X} mutant clones. The clones are unmarked.
(J) Wild-type reporter expression, as detected with anti-β-galactosidase (purple), is stronger within *dcr-1*^{Q1147X} clones, which are marked with GFP (green).
(K) A reporter gene containing the *Bearded* 3′UTR with mutated B motifs is expressed (purple) within clones of *dcr-1*^{Q1147X} cells (green) at a level comparable to unmarked wild-type tissue.
(L–N) *dcr-1*^{Q1147X} clones upregulate the wild-type reporter gene posterior to the furrow (marked with arrowhead).
(L) Reporter expression as determined by anti-β-galactosidase.
(M) Clones of *dcr-1*^{Q1147X} mutant cells marked by GFP.
(N) Merged image to show clones that span across the furrow (arrows) and show upregulation specifically posterior to the furrow.

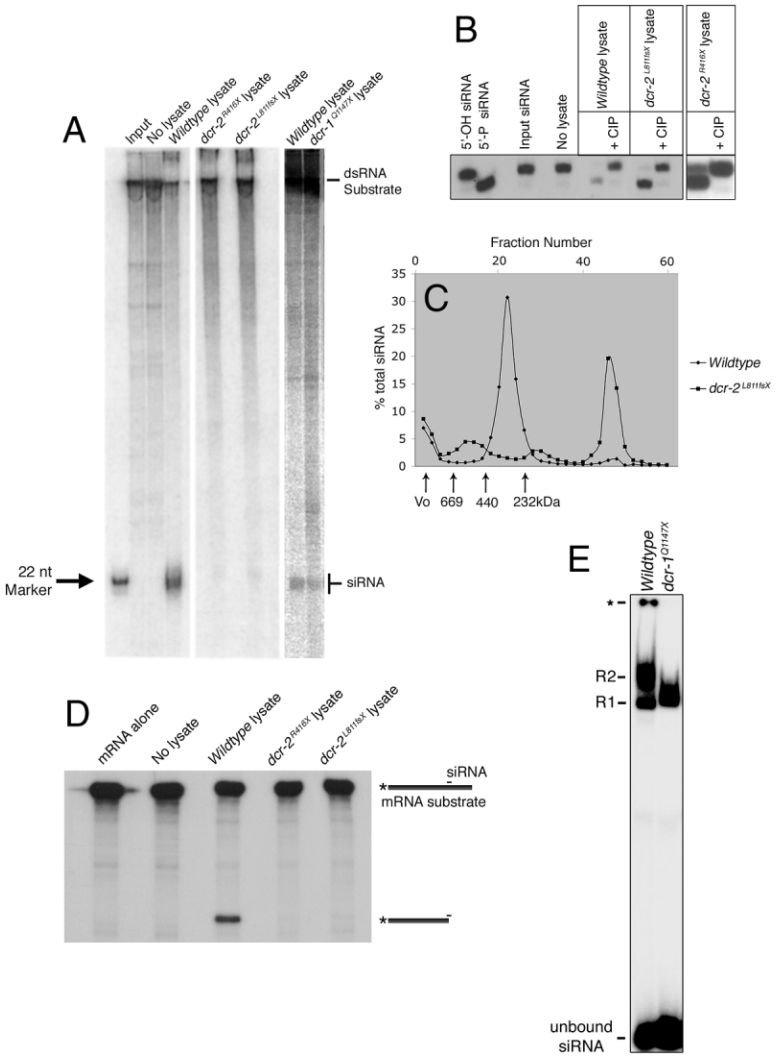

Figure 5. Dcr-1 and Dcr-2 Are Required for siRISC Formation and Activity

(A) Processing of siRNA from dsRNA substrate by lysates from wild-type, *dcr-1*, or *dcr-2* mutant embryos. Incubation of lysate with labeled dsRNA and ATP generates RNA fragments of approximately 22 nucleotides in length. This activity is not detected in *dcr-2* mutant lysates.

(B) siRNA phosphorylation activity is normal in *dcr-2* mutant lysates. Dephosphorylated siRNA was incubated with lysate from wild-type or *dcr-2* mutant embryos. A portion of the treated siRNA sample was posttreated with calf intestinal phosphatase (CIP) to remove any phosphate that might have been added during lysate incubation. 5′-phosphorylated and nonphosphorylated siRNAs were resolved by denaturing gel electrophoresis.

(C) Association of labeled siRNA into a 350 kDa complex is inhibited in *dcr-2* mutant embryo lysate. Labeled siRNA was incubated with wild-type or *dcr-2*^{L811fsX} mutant lysate, and complexes were resolved by Superdex-200 gel filtration chromatography. Shown are plots of siRNA levels as eluted from the column, and shown below are elution positions of proteins used as size standards. The major peak from *dcr-2* lysate corresponds in size to unbound siRNA.

(D) Directed cleavage of labeled mRNA by a complementary synthetic siRNA is impaired in lysates from *dcr-2* mutant embryos. The 5′-labeled cleavage product is readily detected in wild-type lysate whereas it is reduced in *dcr-2* lysates. The 3′ cleavage product is not visible because it contains no radiolabel.

(E) Native gel analysis of labeled siRNA-protein complexes from wild-type and *dcr-1*^{Q1147X} embryo lysates. R1 and R2 complexes are observed in the lane from a wild-type reaction; siRNA indicated (*) corresponds to material too large to resolve in the gel. This material and R2 are not detected in the lane from a *dcr-1*^{Q1147X} reaction.

with 3′ overhangs, and with 5′-phosphate and 3′-hydroxy termini in a reaction that requires a divalent metal ion. On this basis, the chemistry of phosphodiester hydrolysis is likely to be similar. Accordingly, we mutated certain invariant residues within Dcr-2 that we predicted would specifically disrupt phosphodiester hydrolysis. E1371 and E1617 in the first and second RNase III repeats, respectively, are homologous to the E residue that extensively interacts with Mg^{2+} in the bacterial holoenzyme (Figure 6A). An E→K mutant in *E. coli* RNase III fails to cleave dsRNA, but still binds the dsRNA substrate (Dasgupta et al., 1998). To elucidate the functions of the homologous residues in Dcr-2, we generated single and double E1371K and E1617K substitution mutants of *dcr-2* and transformed the mutant genes into a *Drosophila* strain null for *dcr-2*. We then tested for their ability to silence *white* expression in a *GMR-wIR* background (Figures 6E–6G). The single mutants gave barely detectable silencing activity, while the double mutant gave no detectable silencing. Thus, E1371 and E1617 are essential for Dcr-2 activity in vivo.

Two other conserved acidic residues in bacterial RNase III form an interdomain bridge near each metal binding site, but do not coordinate the metal (Figure 6B and Blaszczyk et al., 2001). Interestingly, all known Dicers have acidic residues in homologous positions of the first domain repeat, but have nonacidic residues in the homologous positions of the second domain repeat (Figure 6A). To test the functionality of the residues in the first domain, we introduced an E1210V or E1237A substitution into the *dcr-2* gene. The same substitutions at the homologous positions of *E. coli* RNase III abolish activity (Blaszczyk et al., 2001). However, both *dcr-2* point mutants fully rescued the *dcr-2* null phenotype (Figures 6D and 6H), indicating that E1210 and E1237 are not critical for Dcr-2 activity. These results are consistent with the notion that the nonacidic partner residues in the second repeat normally render these clusters nonfunctional.

Dcr-2 RNase III Activity Is Not Required for mRNA Cleavage

Our genetic and biochemical analyses support the idea that siRISC activity is dependent on Dcr-1 and Dcr-2.

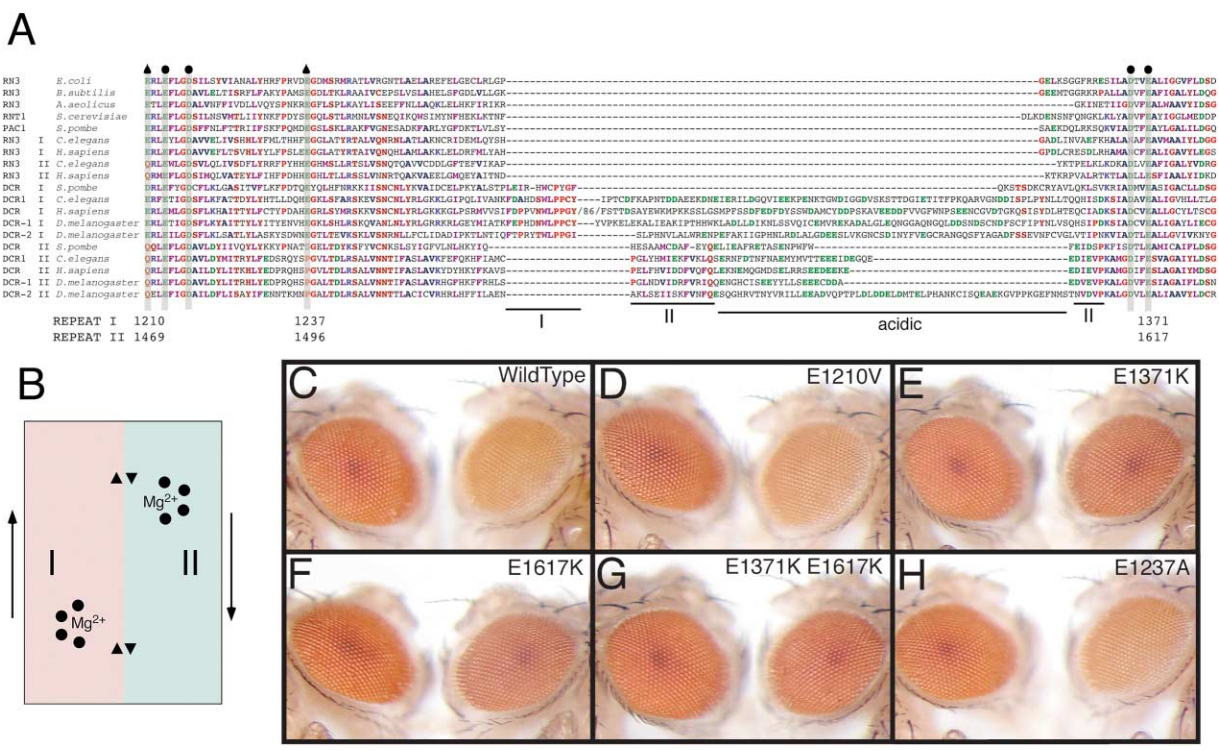

Figure 6. The RNase III Domains of Dcr-2

(A) Sequence alignment of nuclease domains from RNase III proteins. Aligned residues are color-coded: small hydrophobic (black), bulky hydrophobic (purple), uncharged polar (brown), acidic (green), basic (blue), helix breakers glycine and proline (red). Four conserved acidic residues in each domain that coordinate a metal ion in *A. aeolicus* RNase III are marked with circles at the top of the alignment. The last of these residues, corresponding to E1371 and E1617 in repeats I and II of *Drosophila* Dcr-2, makes both inner- and outer-sphere bonds with the metal. Two conserved residues that form a second acidic cluster in *A. aeolicus* RNase III are marked with triangles at the top of the alignment. In Dcr-2, residues 1210 and 1237 within repeat I fit the consensus, but residues 1469 and 1496 within repeat II are nonacidic. The RNase III domains in Dicer proteins have several features that diverge from other RNase III proteins. There is an acid-rich region as indicated below the alignment, plus conserved sequence motifs unique to repeat I (indicated as I) and repeat II (indicated as II). Following motif I in human Dicer are 86 amino acids that are part of the acid-rich region and are not shown.

(B) Schematic representation of *A. aeolicus* RNase III structure (Blaszczyk et al., 2001). Two RNase III domains are dimerized in antiparallel orientation to form two active sites that cleave dsRNA. Each active site contains one Mg^{2+} ion coordinated by four invariant acidic amino acids (circles). A second acidic cluster (triangles) does not coordinate a metal ion but is required for catalysis.

(C–H) In each image, *dcr-2*^{L811fsX} mutant flies (left) are shown with *dcr-2*^{L811fsX} mutant flies carrying one copy of a genomic *Dcr-2* transgene (right). All flies carry the *GMR-wIR* transgene to induce *white* RNAi. The *Dcr-2* transgene sequence is wild-type (C), or with amino acid substitutions E1210V (D), E1371K (E), E1617K (F), E1371K E1617K (G), E1237A (H).

Pham et al. (2004) have observed Dcr-1 and Dcr-2 proteins in siRISC that is competent for target cleavage. We can imagine at least three functions that Dicer could play in siRISC. One, Dicer may stably associate with siRISC after having passed an siRNA molecule to other RISC factors, but has no further role in siRISC activity. This is unlikely since siRNAs can UV-crosslink to Dcr-1 and Dcr-2 in assembled siRISC (Pham et al., 2004). Alternatively, Dicer may use its dsRNA binding activity to retain double-stranded siRNA or a siRNA/mRNA duplex within siRISC. Finally, the RNase III domain of Dicer may be responsible for RNA cleavage by siRISC. To test this latter possibility, we examined the E1371K and E1617K variants of Dcr-2 for siRISC activity.

Lysates were prepared from mutant embryos in which *dcr-2*^{E1371K}, *dcr-2*^{E1617K}, or *dcr-2*$^{E1371K\ E1617K}$ genes were expressed in place of the endogenous *dcr-2* gene. Lysates were incubated with siRNA duplexes and a labeled mRNA substrate, and siRNA-directed cleavage of the substrate was monitored by 5′ product formation (Figures 7A and 7B). All three mutants exhibited normal mRNA cleavage activity in vitro. To demonstrate that the mutant proteins are nevertheless defective for RNase III activity, we tested them for dsRNA processing. Neither *dcr-2*^{E1371K} nor *dcr-2*^{E1617K} lysates were able to support dsRNA cleavage to form siRNAs (Figure 7C). These data indicate that siRISC activity is unaffected when Dcr-2 RNase III activity is specifically impaired.

Discussion

Dcr-1 and Dcr-2 generate different classes of small RNAs in *Drosophila*. Dcr-1 processes pre-miRNAs while Dcr-2 processes dsRNAs. This specificity may reflect the distinct structural properties of the two types of substrates. miRNA precursors are imperfectly paired stem loops, whereas siRNA precursors are typically long dsRNA helices with at least one blunt end. Dcr-1 might preferentially bind and attack imperfectly paired helices characteristic of miRNAs. Dcr-2 might prefer dsRNA

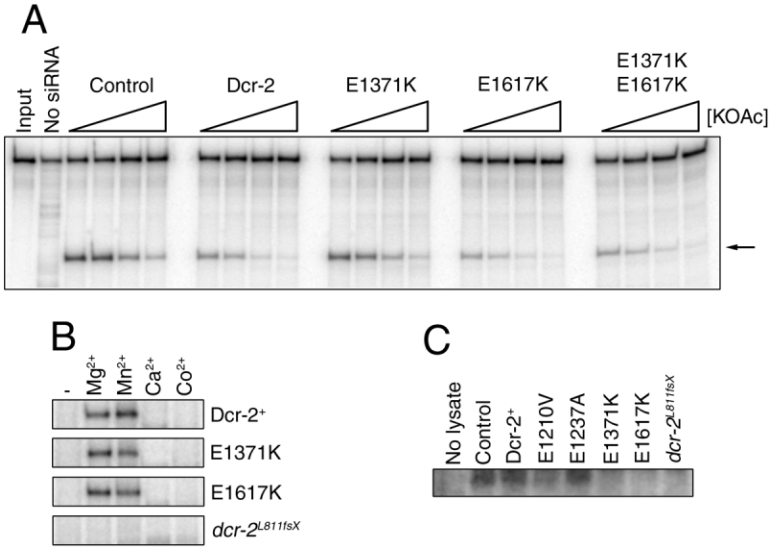

Figure 7. Dcr-2 RNase III Activity in RNAi

(A) mRNA cleavage reactions as directed by exogenous siRNA. Shown are the mRNA substrate and the 5′ cleavage product (arrow) formed in a manner dependent upon siRNA addition. Lysates were prepared from Canton S (Control) or *dcr-2*^{L811fsX} mutant eggs carrying two copies of a *Dcr-2* transgene. The transgene was wild-type or with amino acid substitutions as indicated at the top. Reactions were performed in varying concentrations of KOAc ranging from 50 mM to 200 mM. All lysates were prepared with extraction buffer containing 50 mM KOAc.

(B) Levels of mRNA 5′ cleavage products produced from reactions with different divalent cations. The lysates were prepared from *dcr-2*^{L811fsX} mutant eggs or those carrying two copies of the *Dcr-2* transgene as shown at the right. All lysates were prepared with buffer containing no divalent cation. Chloride salts of different divalent metals were then supplemented to 1 mM in the mRNA cleavage reaction. Shown at the top are the divalent cations tested.

(C) Levels of siRNAs produced from dsRNA precursor in the presence of embryo lysates. Lysates were prepared from Canton S (Control), *dcr-2*^{L811fsX}, or mutants carrying two copies of the *Dcr-2* transgene as shown on top. Cleavage of radioactive dsRNA into siRNAs was carried out with lysates that were normalized for Dcr-2 abundance, as determined by Western blotting for Dcr-2. Shown are the labeled siRNA products of each reaction.

with perfect complementarity. Another difference between siRNA and miRNA precursors is their abundance. Typically, dsRNA substrates for siRNA processing are highly abundant in cells, resulting from viral infection or promiscuous transcription. Precursors of miRNAs are expressed from endogenous genes and are not highly abundant. Dcr-1 and Dcr-2 could utilize one substrate over another according to differences in their kinetic and thermodynamic properties for each type of precursor. Finally, Dcr-1 and Dcr-2 might commit to different substrates because they contain different biochemical activities. For example, only Dcr-2 contains a DExH helicase domain and only Dcr-1 contains a PAZ domain. The PAZ domain might help link Dcr-1 to miRNA precursor molecules as they are shuttled from the cell's nucleus. Our experiments clearly indicate that Dcr-2 DExH helicase activity is required for dsRNA processing. The RNA helicase domain might be needed to move Dcr-2 along the dsRNA substrate or displace Dcr-2 from the dsRNA substrate upon cleavage.

In contrast to their processing specificities, both Dcr-1 and Dcr-2 are required for siRNA-directed transcript cleavage and gene silencing. In both cases the requirement is not absolute, arguing that there is some overlapping redundancy between them. We find that both Dcr-1 and Dcr-2 are required for assembly of siRNA into siRISC, but play distinct roles. We demonstrate that Dcr-2 is required to form a stable siRNA-protein complex, which contains Dcr-2 and R2D2 (Liu et al., 2003; Pham et al., 2004). This complex initiates siRISC assembly. Dcr-1 has a distinct role from Dcr-2. Dcr-1 is not necessary to form a stable initiator complex but instead functions to form a stable intermediate in siRISC assembly. It is likely that Dcr-1 is directly involved since Dcr-1 protein directly associates with siRNA in initiator complexes, intermediate complexes, and assembled siRISC (Pham et al., 2004). We suggest that Dcr-1 within the initiator complex facilitates the stable association of other factors and formation of an intermediate complex.

Each Dicer has distinct qualities with regards to siRISC assembly and yet they are somewhat redundant. They may function analogously to TBP binding to a TATA sequence, initiating assembly of a transcription complex. Each component alone (Dicer or siRNA) is not sufficient to start assembly, but the combination provides enough interaction energy to drive the process. Dcr-1 and Dcr-2 remain closely associated with siRNA in assembled siRISC. This could also be analogous to TBP, which remains a component of the assembled transcription complex. However, in the process of siRISC assembly, siRNA duplex is unwound and a single strand is retained. Once this strand finds a perfect mRNA complement, the transition back to a dsRNA state initiates RNA cleavage. Clearly, Dcr-2 is not directly required for siRNA duplex unwinding, since the RNA helicase activity of Dcr-2 is not necessary for siRNA-dependent mRNA cleavage. One tantalizing notion is that the nuclease activities of the Dicers are used for cleavage of the mRNA strand within the hybrid duplex. But if so, it does not require the RNase III activity of both Dicers, since point mutants that abolish Dcr-2 RNase III activity still promote mRNA cleavage. One possibility is that a nonconventional catalytic site within Dcr-2 cleaves the hybrid duplex, or that Dcr-1 can efficiently cleave the duplex in place of Dcr-2. Alternatively, the Dicers might hold a siRNA-mRNA duplex in place for attack by the cleavage enzyme. The position of the mRNA cleavage site is highly exact, corresponding to the bond precisely ten bases from the corresponding 5′ end of the siRNA strand (Elbashir et al., 2001).

Our genetic analysis demonstrates that Dcr-1 but not Dcr-2 is required for gene silencing by miRNAs. Loss of

dcr-2 has a profound effect on dsRNA processing but no significant effect on *Drosophila* development. Although this suggests that endogenous dsRNAs do not play a critical role in development, it is possible that Dcr-1 has weak dsRNA processing activity and in a *dcr-2* mutant this weak activity might process enough endogenous dsRNA to fulfill possible developmental functions. Loss of *dcr-1* derepresses miRNA target genes and causes profound changes in development and patterning. We cannot definitely say whether Dcr-1 and mature miRNAs form effector complexes, analogous to those formed by Dcr-1 and siRNAs. However, Dicer coimmunoprecipitates with miRNAs (Lee et al., 2003), suggesting that a similar mechanism is at work. Because siRISC contains Dcr-2, and because Dcr-2 is dispensable for miRISC function, it argues that siRISCs and miRISCs are inherently different. This might correlate with the functional differences seen between siRISC and miRISC with regard to mRNA cleavage and translation. That said, the capacity for Dcr-1 to act in both siRNA and miRNA pathways could explain how small RNAs of either class can cleave mRNA or block its translation, depending on the degree of complementarity between the small RNA and the mRNA target (Hutvagner and Zamore, 2002; Doench et al., 2003; Zeng et al., 2003). Dcr-1 could recruit a miRNA into a RISC with cleavage activity, and Dcr-1 could recruit a siRNA into a RISC that represses translation.

Experimental Procedures

Genetic Screen and Mosaic Analysis

The *GMR-wIR* transgene has been described (Lee and Carthew, 2003). *y w eyFLP; FRT* males, isogenized for their second or third chromosome, were mutagenized with ethylmethanesulfonate and crossed to *GMR-wIR; FRT GMR-hid cl/Balancer* females. The *FRT* elements used were: *40A* for 2L, *42D* for 2R, and *82B* for 3R. The presence of the eye-specific apoptosis transgene *GMR-hid* on each sister chromosome ensured that only homozygous mutant eye cells survived to adulthood. F1 females were examined for altered eye pigmentation, and positive flies were mated to *Balancer* males. F2 *GMR-wIR; FRT */Balancer* males were then testcrossed to *y w eyFLP; FRT GMR-hid cl/Balancer* females, and F3 progeny that exhibited the mutant phenotype were used to establish balanced stocks. Mosaic adults bearing homozygous mutant eyes were generated en masse by crossing mutant stocks to *y w eyFLP; FRT GMR-hid cl/Balancer* flies. Adult females bearing homozygous mutant germ cells were generated en masse by crossing mutant stocks into a *hsFLP; FRT P[OvoD]* background, and inducing mitotic recombination as described (Chou and Perrimon, 1996).

Genetic Mapping and Complementation Analysis

To test for complementation with chromosome deletions, mutants were testcrossed to flies that carried deficiencies within the relevant chromosome arm. Heterozygotes were then examined for noncomplementing phenotypes in a *GMR-wIR* background. To generate recombinants for linkage analysis, each of the mutants was crossed to an appropriate *y w eyFLP; EP* line (Berger et al., 2001) to obtain *y w eyFLP; FRT */EP* females. *FRT EP* recombinant progeny were testcrossed to score for the presence or absence of the mutant phenotype. They were then scored for SNP marker genotypes as described (Berger et al., 2001). For complementation grouping of mutants, lines were crossed to each other, and then judged for noncomplementation in a *GMR-wIR* background.

Sequencing and RNA Analysis

Six alleles in the *dcr-2* complementation group were analyzed for sequence changes in the coding region of *dcr-2* cDNAs that were generated from homozygous mutant embryos. Genomic DNA from heterozygous *dcr-1* mutant flies was purified and *dcr-1* coding sequence was determined. To analyze *dcr-1* mutant RNA, total RNA from homozygous mutant eggs was subjected to RT-PCR analysis or Northern blotting.

RNAi Assay in Eggs

bicoid dsRNA was described previously (Kennerdell et al., 2002). Two DNA-RNA chimeric oligonucleotides 5′-ACGAGCAAGAAGAC GACGCdTdT-3′ (*bicoid* sense) and 5′-GCGUCGUCUUCUUGCUC GUdTdT-3′ (*bicoid* antisense) were chemically synthesized and annealed to form a siRNA duplex. *bicoid* dsRNA or siRNA was injected into eggs collected from wild-type, *dcr-1* mosaic females, or *dcr-2* homozygous females. After injection, they were incubated at 25°C for 90 min, and quantitative RT-PCR analysis was performed with total RNA as described (Kennerdell et al., 2002).

Northern Blot Analysis

To detect hairpin RNA expressed from *GMR-wIR*, total head RNA denatured with glyoxal was electrophoresed in agarose, transferred to neutral membrane, and hybridized with a DNA probe. To detect small RNAs, total RNA was electrophoresed, transferred to membrane, and hybridized as described (Hamilton and Baulcombe, 1999). Sense RNA probes were used for detection of *GMR-wIR* siRNAs. For detection of *let-7* and *miR-2* miRNAs and $tRNA^{val}$, 5′-end labeled DNA oligonucleotides were used as Northern probes: *let-7*, 5′-ACTATACAACCTACTACCTCA-3′; *miR-2* mixture, 5′-GCY CMTCAAAGCTGGCTGTGATA-3′; $tRNA^{val}$, 5′-TGGTGTTTCCGCCC GGGAA-3′.

Transgenics

To construct the *dcr-2* rescue transgene, a 7.2 kb genomic DNA fragment was cloned into pW8. Germline transformants were generated by standard procedures. Site-directed mutagenesis was performed on the 7.2 kb genomic fragment, after which it was cloned into pW8 and transformed into *Drosophila*. We confirmed the nucleotide sequences of all constructs by DNA sequencing.

Microscopy

Clones of homozygous *dcr* mutant cells were induced in imaginal discs with *ey-FLP*, and mutant cells were marked with GFP using the MARCM system (Lee and Luo, 1999). Mosaic animals were constructed with one copy of a reporter transgene in the background. X-gal activity staining and immunohistochemistry of mosaic eye-antennal discs were performed as described (Xu et al., 2000).

mRNA Cleavage, dsRNA Processing, and Phosphorylation Assays

Extracts were prepared from Canton S, *dcr* mutants, and transgenic strains as described (Tuschl et al., 1999). Starting material was either 0–2 hr embryos or 0–6 hr unfertilized eggs. For some experiments, lysis buffers were missing divalent salts, as specified in the figures and legends. These modifications had little effect on the mRNA cleavage activity of the resulting lysates. Reagents and protocols used for all mRNA and dsRNA cleavage assays were as described (Pham et al., 2004). Reagents and protocols used for the siRNA phosphorylation assay were as described (Nykanen et al., 2001). For lysates prepared from transgenic embryos, relative Dcr-2 levels were determined by Western blot, and assays were conducted with lysate amounts normalized for constant Dcr-2.

Gel-Filtration Chromatography and Native Gel Electrophoresis

Radiolabeled siRNA duplexes were prepared as described (Nykänen et al., 2001). siRNAs were incubated in 200 μL reaction mixtures (containing Canton S or $dcr\text{-}2^{L811fsX}$ embryo extract) that were supplemented with 10% (v/v) glycerol and incubated for 60 min at 25°C. The mixtures were then chromatographed over Superdex-200 (Nykänen et al., 2001) with modifications (Pham et al., 2004). An RNA aliquot from each fraction was separated by electrophoresis and radioactive signal was quantified by Phosphorimager. Native gel electrophoresis assays with radiolabeled siRNA duplex were performed as described (Pham et al., 2004), except that extract concentrations were reduced due to the limited quantities of *dcr-1* mutant

lysate that could be obtained. Detection of stable R3 complex is highly dependent on extract concentration (data not shown), accounting for its absence in wild-type lysate in the experiment shown in Figure 5E.

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