Dicer-2 and R2D2 coordinately bind siRNA to promote assembly of the siRISC complexes

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ABSTRACT

In Drosophila melanogaster, the Dicer-2/R2D2 complex initiates RNA interference (RNAi) by processing long double-stranded RNA (dsRNA) into small interfering RNA (siRNA). Recent biochemical studies suggest that the Dcr-2/R2D2 complex also facilitates incorporation of siRNA into the RNA-induced silencing complex (siRISC). Here we present genetic evidence that R2D2 and Dcr-2 are both required for loading siRNA onto the siRISC complex. Consistent with this, only the Dcr-2/R2D2 complex, but neither Dcr-2 nor R2D2 alone, can efficiently interact with duplex siRNA. Furthermore, both dsRNA-binding domains of R2D2 are critical for binding to siRNA and promoting assembly of the siRISC complexes.

Keywords: RNA interference (RNAi); Dicer-2; R2D2; siRNA; siRISC

INTRODUCTION

RNA interference (RNAi) is an evolutionarily conserved gene-silencing pathway whereby double-stranded RNA (dsRNA) molecules trigger sequence-specific post-transcriptional silencing of cognate mRNA (Hannon 2002; Tomari and Zamore 2005). In general, the RNAi pathway can be initiated by processing long dsRNA or short hairpin RNA into small interfering RNA (siRNA) or microRNA (miRNA), respectively. Both siRNA and miRNA are 21–25 nucleotide (nt) duplexes with 5' 3' phosphate and 3' 2 nt overhang, hydroxyl termini (Tomari and Zamore 2005). However, the majority of miRNA, but not siRNA, duplexes contain mismatches, bulges, or G:U wobble base pairs. Nascent siRNA and miRNA are incorporated into the RNA-induced silencing complex termed siRISC and miRISC. The effector RISC complexes contain single-stranded siRNA or miRNA as guide RNA for sequence-specific cleavage and/or translational repression of complementary mRNA.

Both siRNA and miRNA are generated by Dicer, a family of large multidomain RNase III enzymes (Bernstein et al. 2001). In Drosophila, two distinct Dicer (Dcr) complexes, Dcr-1/Loquacious (Loqs)-PB (also known as R3D1-L [long]) and Dcr-2/R2D2, generate miRNA and siRNA, respectively (Liu et al. 2003; Forstemann et al. 2005; Jiang et al. 2005). Despite sharing extensive sequence homology, Dcr-1 and Dcr-2 enzymes display different ATP requirements and substrate specificities (Lee et al. 2004; Jiang et al. 2005). Dcr-1 is ATP-independent and prefers to process the stem–loop precursor of miRNA (pre-miRNA) (Jiang et al. 2005). On the other hand, Dcr-2 favors long dsRNA as its ideal substrate, and requires ATP hydrolysis for efficient siRNA production (Liu et al. 2003; Jiang et al. 2005). Both R2D2 and Loqs belong to a growing family of dsRNA-binding proteins that function in tandem with specific RNase III enzymes. There are two putative dsRNA-binding domains in R2D2 and three in Loqs. The loqs gene encodes at least two protein isoforms, PA (also known as R3D1-S [short]) and PB, by alternative splicing (Forstemann et al. 2005; Jiang et al. 2005; Saito et al. 2005). While R2D2 does not regulate the siRNA-generating activity of Dcr-2, Loqs-PB greatly enhances Dcr-1’s miRNA-generating activity possibly by increasing its affinity for pre-miRNA (Liu et al. 2003; Jiang et al. 2005; Saito et al. 2005). Recent biochemical studies suggest that the Dcr-2/R2D2 complex also binds duplex siRNA, forms the RISC loading complex (RLC), and facilitates the transfer of siRNA onto Ago2, the catalytic component of siRISC (Liu et al. 2003; Pham et al. 2004; Tomari et al. 2004a). We previously employed an in vitro reconstitution assay to demonstrate that recombinant Dcr-2/R2D2 complex enhances incorporation of siRNA into the effector siRISC complex (Liu et al. 2003). Furthermore, photocross-linking experiments suggest that R2D2 preferentially binds the more stable end of siRNA, whereas Dcr-2 is often found at the less stable end.
with the notion that (S. Kalidas et al., unpubl.). These findings are consistent

**RESULTS**

**Generation of the r2d2^i^ null fly**

We previously purified the siRNA-generating enzyme from *Drosophila* S2 cells and discovered that it consisted of DCR-2 and a novel protein that we named R2D2 (Liu et al. 2003). R2D2 contains tandem dsRNA-binding domains (R2) and forms a heterodimeric complex with Dcr-2 (D2). To study the physiological functions of R2D2, we generated r2d2 deletion flies by imprecise excision of a P-element inserted near the r2d2 locus. One such mutant was named r2d2^i^, and was selected for further characterization (Liu et al. 2003). Based on PCR and sequencing, the r2d2^i^ mutant locus contained a ~4.9-kb deletion, which removed the entire r2d2 open reading frame (ORF) as well as ~1-kb upstream and ~3-kb downstream sequences (Fig. 1A,B). Consequently, no R2D2 protein could be detected in homozygous r2d2^i^ fly lysates by Western blot analysis (Fig. 1C). Therefore, we concluded that r2d2^i^ was a null allele. The r2d2^i^ null flies display several developmental abnormalities and female sterility. All of these phenotypes could be completely rescued by a ~5.6-kb genomic fragment that contained only an intact r2d2 gene (Fig. 1A,C) (S. Kalidas et al., unpubl.). These findings are consistent with the notion that r2d2^i^ null phenotypes are caused by the deletion of the r2d2 gene.

**Dcr-2 stabilizes R2D2 in vivo**

The majority of Dcr-2 and R2D2 proteins form a stable complex in vivo. Both R2D2 and Dcr-2 can be immunodepleted from *Drosophila* S2 cell extracts using anti-R2D2 antibodies. Moreover, our previous studies show that depletion of Dcr-2 by RNAi also diminishes the level of R2D2 protein, whereas RNAi of R2D2 causes a modest reduction in Dcr-2 protein in S2 cells (Liu et al. 2003). Thus, Dcr-2 and R2D2 may stabilize each other in vivo, and either protein alone is less stable. To confirm this in flies, we measured the levels of Dcr-2 and R2D2 proteins in homozygous r2d2^i^ or dcr-2^R416X^ flies by Western blot analysis. The dcr-2^R416X^ mutant is considered a null allele because it introduces a premature stop codon at the amino (N)-terminus of dcr-2 ORF (Lee et al. 2004). While there was little or no change in the level of Dcr-2 in r2d2^i^ mutant extract, neither Dcr-2 nor R2D2 were detected in dcr-2^R416X^ mutant extract (Fig. 2A). Therefore, Dcr-2 is required for the stability of R2D2, but the stability of Dcr-2 does not depend on R2D2 in the fruitfly.

**R2D2 is dispensable for siRNA production**

To determine if R2D2 is required for siRNA production in vivo, we performed dsRNA-processing assays using whole fly or ovary extracts prepared from wild-type and homozygous r2d2^i^ or dcr-2^R416X^ flies. As shown previously, the majority of siRNA-generating activity was removed in dcr-2^R416X^ extract (Fig. 2B; Liu et al. 2003; Lee et al. 2004; Pham et al. 2004). The remaining activity was likely contributed by Dcr-1 (data not shown). By contrast, r2d2^i^ mutant extract was as efficient in siRNA production as wild-type extract (Fig. 2B and data not shown). Thus,

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**Figure 1.** Generation of the r2d2^i^ null mutant. (A, top) A schematic map of the wild-type (WT) r2d2 gene locus. Three open reading frames (ORF) were shown in the 14-kb genomic region. The P-element (EP2450) was inserted ~0.6 kb upstream of the r2d2 ORF. (middle) A ~4.9-kb genomic region, represented by the dash line, was deleted in the r2d2^i^ mutant locus. The deletion was mapped by genomic PCR as illustrated below. (bottom) A 5.6-kb genomic DNA fragment that completely rescued all phenotypes of r2d2^i^ mutant flies. (B) PCR mapping the deletion at the r2d2^i^ mutant locus. Six (I–VI) PCR reactions (A) were performed using genomic DNA template isolated from wild-type (wt, odd lanes) or homozygous r2d2^i^ mutant flies (r2, even lanes). The PCR condition used could not allow amplification of the ~5.9-kb VI PCR product in the wild-type sample. (C) Lysates were prepared from wild-type flies (lane 1) and homozygous r2d2^i^ mutant flies without (lane 2) or with the rescue transgene (lane 3). Western blots were performed with anti-R2D2 and anti-Ras antibodies.
R2D2 is dispensable for siRNA production in vivo. These results are consistent with our previous finding that recombinant R2D2 does not directly regulate the ability of recombinant Dcr-2 to recruit or process dsRNA in vitro.

R2D2 is required for loading siRNA onto siRISC

To determine if R2D2 is required for loading siRNA onto the siRISC complex, we performed (duplex) siRNA-initiated RISC assays by using wild-type, r2d21, and dcr-2R416X ovary extracts. As shown in Figure 2C, dcr-2R416X extract was completely defective in this assay. Interestingly, this was also the case for r2d21 extract. However, unlike dcr-2R416X extract, r2d21 extract could efficiently process dsRNA trigger into siRNA. This defect of r2d21 mutant could be fully recovered by the r2d21-rescuing transgene. Thus, R2D2 is a bona fide component of the Drosophila RNAi pathway. Moreover, these data suggest that R2D2 functions downstream of siRNA production, for instance, by facilitating siRNA transfer from Dcr-2 to siRISC.

To determine if R2D2 is required for loading siRNA onto the siRISC complex, we performed (duplex) siRNA-initiated RISC assays by using wild-type, r2d21, and dcr-2R416X ovary extracts. As shown previously (Lee et al. 2004; Pham et al. 2004), the siRNA-initiated RISC activity was abolished in dcr-2R416X extract and was efficiently rescued by supplementing the recombinant Dcr-2/R2D2 complex (Fig. 3B).

R2D2 is required for loading siRNA onto siRISC

To determine if R2D2 is essential for RNAi, we performed long dsRNA-initiated RISC (sequence-specific mRNA cleavage) assays using wild-type, r2d21, and dcr-2R416X ovary extracts. As shown in Figure 2C, dcr-2R416X extract was completely defective in this assay. Interestingly, this was also the case for r2d21 extract. However, unlike dcr-2R416X extract, r2d21 extract could efficiently process dsRNA trigger into siRNA. This defect of r2d21 mutant could be fully recovered by the r2d21 rescuing transgene. Thus, R2D2 is a bona fide component of the Drosophila RNAi pathway. Moreover, these data suggest that R2D2
Dicer-2 and R2D2 coordinately bind siRNA

Dcr-2 and R2D2 coordinately bind the siRNA duplex

Previous biochemical studies have suggested that the Dcr-2/R2D2 complex binds the siRNA duplex, forms the RISC loading complex (RLC), and facilitates incorporation of siRNA into the siRISC complex (Liu et al. 2003; Pham et al. 2004; Tomari et al. 2004a). To determine if either R2D2 or Dcr-2 could bind the siRNA alone, we performed native gel-shift assays by incubating the 5′-radiolabeled synthetic siRNA duplex with recombinant R2D2, Dcr-2, or the Dcr-2/R2D2 complex proteins. As shown in Figure 4, neither Dcr-2 nor R2D2 could efficiently interact with siRNA in this assay. A distinct mobility shift was observed only when Dcr-2 and R2D2 were combined in the reaction. Because of the MBP-tag, the siRNA shift generated by Dcr-2/MBP-R2D2 was slightly higher than that of the Dcr-2/R2D2 complex. These results suggest that Dcr-2 and R2D2 coordinately bind the siRNA duplex to promote assembly of the siRISC complex. Indeed, both Dcr-2 and R2D2 can be efficiently photocross-linked to radiolabeled siRNA by ultraviolet light (Liu et al. 2003; Tomari et al. 2004b).

Both dsRNA-binding domains of R2D2 are critical for siRNA binding

R2D2 contains two putative dsRNA-binding domains (dsRBDs) at the amino (N) terminus. To further analyze these domains, we generated three mutant R2D2 proteins by replacing two highly conserved alanine (A) residues with lysines (K) in the first (m1), second (m2), or both (dm) dsRBDs of R2D2 as previously described (Fig. 5A) (Liu et al. 2003). These mutant forms of R2D2 were also produced and purified as MBP fusion proteins from E. coli. To determine if these R2D2 mutants could bind long dsRNA, we used poly (inosine) (I):poly (cytosine) (C) dsRNA-conjugated agarose beads (Amersham) to pull down purified wild-type or mutant MBP–R2D2 proteins. As shown in Figure 5B, while R2D2m2 was precipitated as efficiently as wild-type R2D2, neither R2D2m1 nor R2D2dm could interact with long dsRNA. These results indicated that the first, but not the second, dsRBD of R2D2 was capable of binding long dsRNA. Alternatively, the second dsRBD of R2D2 might be covered by the C-terminal region of unknown function, thereby rendering it inaccessible to long dsRNA. However, the latter scenario was unlikely because we obtained the same results after removing the C-terminal 107 amino acids from wild-type and mutant MBP–R2D2 (data not shown).

To determine if either or both dsRBDs of R2D2 was necessary for siRNA binding, we performed siRNA gel-shift assays using recombinant Dcr-2 in combination with wild-type or mutant MBP–R2D2 proteins. As shown in Figure 5C, mutations in either dsRBD abolished the ability of MBP–R2D2 to cooperate with Dcr-2 for siRNA binding. More importantly, none of these mutant MBP–R2D2 proteins could rescue the siRNA-initiated RISC activity in r2d2−1 null ovary extract (Fig. 5D). It should be noted that Dcr-2 interacted with mutant R2D2 proteins as efficiently as with wild-type control (data not shown; Liu et al. 2003). Together, our results indicate that both dsRBDs of R2D2

FIGURE 4. Dcr-2 and R2D2 coordinately bind siRNA duplex. The gel-shift assays were performed by incubating 5′-radiolabeled siRNA duplex and 10-pmol recombinant Dcr-2/R2D2 complex (lane 1), Dcr-2 (lane 2), MBP-R2D2 (lane 3), Dcr-2 + MBP-R2D2 (lane 4), R2D2 (lane 5), and Dcr-2 + R2D2 (lane 6). The arrowhead points to free siRNA. Two arrows refer to the Dcr-2/MBP-R2D2/siRNA and Dcr-2/R2D2/siRNA complexes, respectively.
MBP-R2D2 proteins (lanes 2–4) and 100% bound proteins (lanes 5–8) were resolved by SDS-PAGE followed by coomassie staining. (C) The gel-shift assays were performed in buffer alone (lane 1) or with 3.5-pmol recombinant Dcr-2 alone (lane 2) or in combination with 12.5-pmol wild-type or various mutant MBP-R2D2 (lanes 3–6); 12.5-pmol wild-type MBP-R2D2 alone (lane 7). (D) The siRNA-initiated RISC assays were performed with buffer control (lane 1); 32 μg of wild-type ovary extract (lane 2); 32 μg of r2d2 mouse extract alone (lane 3), or supplemented with 12.5-pmol wild-type or mutant MBP-R2D2 proteins (lanes 4–7).
How do Dcr-2 and R2D2 facilitate siRNA loading onto siRISC?

It has been reported that Dcr-2 is required for siRNA-initiated RISC assembly in vivo (Lee et al. 2004; Pham et al. 2004). Here we present genetic evidence that R2D2 is also required for loading siRNA onto the siRISC complex in vivo. It is possible that the Dcr-2/R2D2 complex helps recruit the siRNA duplex to Ago2 for siRISC assembly (Liu et al. 2003; Tomari et al. 2004b). However, it remains unclear exactly how the Dcr-2/R2D2 complex facilitates incorporation of siRNA into the siRISC complex. While newly synthesized siRNA is double-stranded, siRNA exists as a single-stranded form in an active siRISC complex (Martinez et al. 2002). Thus, the nascent siRNA duplex must be unwound during siRISC assembly. It is reasonable to speculate that the Dcr-2/R2D2 complex facilitates unwinding of the siRNA duplex, thereby promoting incorporation of single-stranded siRNA into the siRISC complex. Dcr-2 is a candidate for the siRNA-unwinding helicase because it carries a putative DExH helicase domain and physically contacts the siRNA end that is easier to unwind (Tomari et al. 2004b). However, two dcr-2 mutations in the helicase domain have been isolated that do not affect the siRNA-initiated RISC activity, suggesting that a functional helicase activity is not required for Dcr-2 to promote siRISC assembly (Lee et al. 2004). In addition, recombinant Dcr-2 or Dcr-2/R2D2 complex cannot unwind the siRNA duplex in vitro (Tomari et al. 2004b). Alternatively, the Dcr-2/R2D2 complex may recruit an unknown unwinding helicase to unwind the siRNA duplex. In C. elegans, the DCR-1/RDE-4 (an R2D2 homolog) complex is associated with two highly related RNA helicases, DRH-1 and DRH-2, that are necessary for RNAi (Tabara et al. 2002). Several RNA helicases, such as Armitage and Dmp68, have also been implicated in the Drosophila RNAi pathways (Ishizuka et al. 2002; Tomari et al. 2004a).

Recent studies also suggest an alternative model for separation of siRNA strands and activation of the siRISC complex (Matranga et al. 2005; Rand et al. 2005). After the Dcr-2/R2D2 complex recruits duplex siRNA to Ago2, the PIWI domain of Ago2 cleaves the passenger strand and facilitates the formation of an active siRISC complex containing only the guide strand. It is likely that the orientation of siRNA binding by the Dcr-2/R2D2 complex allows Ago2 to access and cleave only one of the two siRNA strands. Therefore, the Dcr-2/R2D2 complex determines which strand of siRNA duplex becomes the guide strand or passenger strand. The two mechanistic models of siRISC assembly are not mutually exclusive. In either model, the Dcr-2/R2D2 complex plays a critical role in facilitating the strand separation of the duplex siRNA. Since not all Ago proteins possess the slicer activity, there must be more than one mechanism for siRNA loading and RISC activation.

MATERIALS AND METHODS

Generation, analysis, and transgenic rescue of the r2d2 mutant null fly

The r2d2 mutant fly was generated by mobilizing a P-element inserted ~0.6 kb upstream of the r2d2 ORF in the EP2450 stock (Bloomington stock center). Candidate mutants were selected based on loss of the red-eye marker carried by the P-element, bred to homozygosity, and screened by PCR for lack of the r2d2 ORF. To further map the deletion in the r2d2 mutant, we performed six PCR reactions with the primers listed below using a genomic DNA template isolated from wild-type or homozygous r2d2 flies (Fig. 1). The PCR fragment VI from r2d2 mutant DNA was sequenced to determine the precise boundary of deletion at the r2d2 mutant locus.

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<th>Primer Set</th>
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<th>PCR Conditions</th>
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<tr>
<td>P1:5'- P2:5'</td>
<td>GATCCATCATGCGCTGAAT-3'</td>
<td>GCATTTTGGCG-3'</td>
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<tr>
<td>P3:5'- P4:5'</td>
<td>ACCGACACCTATGGAATCC-3'</td>
<td>ATCAACATGG-TGGAAATAA-3'</td>
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<tr>
<td>P5:5'- P6:5'</td>
<td>CGCACCATGTTGATTTAATGC-3'</td>
<td>AACGGTTGCTCCTGCTGTAC-3'</td>
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<tr>
<td>P7:5'- P8:5'</td>
<td>ACGAGTTCGTCTGCTGAC-CGGAATATGCC-3'</td>
<td>GCCAATATGCC-3'</td>
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<tr>
<td>P9:5'- P10:5'</td>
<td>TTGATAGAGCGCTCTCTCGT-3'</td>
<td>GGAGATGTGTA-3'</td>
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To construct the r2d2 rescuing transgene, we cloned into pCasepeR4, a 5.6-kb genomic fragment that only contained an intact r2d2 gene. Because this DNA fragment also carried a large portion of an upstream ORF CG14536, to avoid complication, we introduced a frameshift mutation at the N terminus of ORF CG14536 by filling in a unique SacII site. Transgenes inserted into the third chromosome were crossed into the r2d2 mutant background to perform the rescuing experiments.

Antibodies

The anti-Dcr-2 and anti-R2D2 antibodies were previously described (Liu et al. 2003). The anti-Dcr-1 antibody was a generous gift from Dr. Gregory Hannon, whereas the anti-Ras antibody was purchased from Calbiochem.

Purification of recombinant Dcr-2 and R2D2 proteins

The His-tagged recombinant Dcr-2 and Dcr-2/R2D2 complexes were produced and purified from insect cells as previously described (Liu et al. 2003). The R2D2 ORF was cloned as a Ncol–NotI fragment into pMBP-parallel. Different R2D2 mutants were generated by Quickchange (Stratagene). Both wild-type and mutant MBP–R2D2 proteins were produced and purified from BL21 bacteria. In brief, after the bacteria culture reached OD600 of ~0.8 at 37°C, IPTG was added to a 1-mM concentration to induce expression of MBP–R2D2 proteins for 5–6 h at 25°C. Cells were harvested and lysed by sonication in Buffer T (20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 5 mM β-mercaptoethanol) freshly supplemented with complete protease inhibitor cocktail (Roche).
Recombinant MBP-R2D2 proteins were purified using an Amylose–Agarose column (NEB) followed by SP- and Q-Sepharose chromatography (Amersham).

The dsRNA- and pre-miRNA-processing assays

The dsRNA- and pre-miRNA-processing assays were performed essentially as previously described (Liu et al. 2003; Jiang et al. 2005). The uniformly radiolabeled 250-bp dsRNA was made by in vitro transcription using the Riboprobe T7 Kit (Promega). A 61-nt pre-let7 RNA was 5’-end labeled by γ-32P by T4 polynucleotide kinase (NEB) followed by PAGE purification. For dsRNA-processing assay, 10^5 cpm dsRNA was incubated with fly extracts or recombinant proteins in 1× buffer 12 (100 mM KOAc, 10 mM HEPES (pH 7.4), 2 mM Mg(OAc)₂, and 5 mM DTT). The pre-miRNA-processing assay used 4 × 10^4 cpm pre-miRNA in 1× buffer 16: 100 mM KOAc, 10 mM HEPES (pH 7.4), 10 mM Mg(OAc)₂, and 5 mM DTT. Typically, 10-µL reactions were performed at 30°C for 30 min in the presence of an ATP-regenerating system: 1 mM ATP, 30 mM creatine phosphate (Fluka), and 30 U creatine phosphokinase (Sigma). The reaction was stopped by addition of 200 µL 0.3 M NaOAc, phenol/chloroform extracted and ethanol precipitated, resolved on a 16% denaturing 9 M Urea polyacrylamide gel.

The siRNA gel-shift assay

Both strands of siRNA were radiolabeled at the 5’ end with [γ-32P] ATP by T4 polynucleotide kinase, annealed in 1× annealing buffer (100 mM KOAc, 30 mM HEPES (pH 7.4), 2 mM Mg(OAc)₂), and passed through a G25 column (Amersham). In a 10-µL reaction, 5 × 10^5 cpm siRNA was incubated with fly ovary extracts or recombinant proteins at 30°C for 30 min in 1× buffer 12. After addition of 1.2 µL 50% glycerol, the reaction was resolved on a 5% native PAGE, and exposed to X-ray film.

The dsRNA-, pre-miRNA, and siRNA-initiated RISC assays

All RISC (sequence-specific mRNA cleavage) assays were performed using an mRNA substrate containing a perfect sense or antisense let7 target site as previously described (Liu et al. 2003; Jiang et al. 2005). The mRNA was radiolabeled at the 5’ G cap by guanylyl transferase (Ambion) followed by PAGE purification. The 130-bp dsRNA trigger contained essentially four repeats of let7 sequence (Liu et al. 2003; Jiang et al. 2005). Typically, 2.5 × 10^8 cpm mRNA was incubated with fly ovary extracts for 1 h at 30°C in 1× buffer 12 with the presence of ATP-regenerating system. The dsRNA, pre-let7, or let7 siRNA triggers were respectively used at 250 nM, 250 nM, and 25 nM final concentrations.

Embryo injections

A 500-bp GFP dsRNA was transcribed by Megascript (Ambion) and processed by a recombinant Dcr-2/R2D2 complex to generate GFP siRNAs followed by PAGE purification. Wild-type or r2d2 mutant green fluorescent embryos were injected with GFP siRNAs at a concentration of 0.24 µg/µL (Liu et al. 2003). Embryos were viewed and photographed 48 h after injection.

ACKNOWLEDGMENTS

We thank Dr. Gregory Hannon for reagents, Zhengzheng Li, Courtney Karner, and Tim Rand for technical assistance, and Drs. Zain Paroo and Gaya Amarasinghe for discussion and critical reading of the manuscript. Q.L. is a W.A. “Tex” Moncrief Jr. Scholar in Medical Research and a Damon Runyon Scholar, supported by the Damon Runyon Cancer Research Foundation (DRS-43). This work is also supported by a Welch grant (1-I608) to Q.L. and an NIH grant (GM070648) to D.S.

Received March 30, 2006; accepted May 4, 2006.

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RNA 2006 12: 1514-1520

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