Supporting Online Material

Materials and Methods

General Methods

Native gel analysis of RISC assembly and photocrosslinking of 5-iodo-uracil-containing siRNAs were performed as described (1). In photocrosslinking experiments, the siRNA concentration was 25 nM unless otherwise noted, and the photocrosslinked proteins were resolved by 4-20% gradient SDS-polyacrylamide gel electrophoresis (Criterion precast gel system; BioRad). For the photocrosslinking experiments, the incubation time and the UV irradiation time were as follows: Fig. 2 and Fig. 3A, incubation for 1 h and photocrosslinking for 15 min; Fig. 3A, incubation for 10 min and photocrosslinking for 10 min; Fig. 3C, incubation for 1 min and photocrosslinking for 4 min; Fig. 4A and Fig. S4A, incubation as indicated including photocrosslinking for 4 min; Fig. 4B and Fig. S3B, incubation for 86 min and photocrosslinking for 4 min. Drosophila stocks were as reported elsewhere (2-4).

Unwinding assay with increasing concentration of trap RNA

siRNA unwinding was as described previously (5) except that the guide strand of the Pp luciferase-specific siRNA (see siRNA 1 in Supplementary Table 1) was 5’ radiolabeled using T4 polynucleotide kinase, annealed to a two-fold excess of passenger strand and double-stranded siRNA purified by native polyacrylamide gel electrophoresis. 50 nM purified, duplex siRNA was incubated in a standard RNAi reaction with lysate or recombinant proteins (15 nM) at 25°C in the presence of unlabeled guide strand as indicated. Reactions were stopped by the addition of Proteinase K Buffer (100 mM Tris-HCl (pH 7.5), 12.5 mM EDTA, 150 mM NaCl, and 1% (w/v) SDS) containing 1 mg/ml
Proteinase K and 20 µg glycogen, incubated 30 min at 25°C, and the RNA precipitated with three volumes of absolute ethanol at –20°C. The precipitate was collected by centrifugation, washed once with 80% (v/v) ethanol, redissolved in native loading dye (3% w/v Ficoll, 0.04% w/v Bromophenol Blue, 2 mM Tris-HCl (pH 7.4)), and analyzed by electrophoresis at 5 W at 4°C through a native 15% polyacrylamide gel (29:1, acrylamide:bisacrylamide).

**Detection of single-stranded siRNA in RLC**

³²P-radiolabeled siRNA was incubated with lysate, separated by native gel electrophoresis (1), and then fractionated by cutting the gel at 6 mm intervals, excluding the well. The gel slices were soaked in Proteinase K buffer containing 1 mg/ml Proteinase K, 20 µg glycogen, and 100 nM unlabeled RNA corresponding to the sequence of the radiolabeled strand. The eluted RNA was then precipitated with ice-cold ethanol. The RNA was then redissolved in native loading dye and analyzed by electrophoresis through a native 20% polyacrylamide gel.

**Purification of recombinant Dcr-2 and Dcr-2/R2D2**

Sf21 insect cells were infected with His₆-Dcr-2 virus alone or together with His₆-R2D2 virus (4). Cell pellets from one liter culture were lysed in HM buffer (10 mM HEPES, 2 mM Mg(OAc)₂, 5% Glycerol and 5 mM DTT); centrifuged at 100,000 x g for 1 h at 4°C; and the supernatant purified by HisTrap (Amersham) chromatography in HM buffer, eluting with a 0-400 mM imidazole gradient. The peak of recombinant protein was further purified by HiTrap Q (Amersham) chromatography and HiTrap S (Amersham) chromatography. Each column was eluted with a 10-350 mM KCl gradient. The concentration of recombinant protein was determined by quantitative amino acid analysis.
of protein in a slice from an SDS-polyacrylamide gel (Keck Biotechnology Resource Laboratory). Both the stoichiometry of the purified Dcr-2/R2D2 heterodimer (1:1.01) and its elution profile in gel filtration suggest that it was almost exclusively heterodimeric. For photocrosslinking experiments, purified recombinant enzymes were diluted in lysis buffer containing 5 mg/ml BSA (New England Biolabs).

Determining R2D2 concentration in vivo and in lysate

*Drosophila* 0-2 h embryos were individually hand collected, dechorionated with 50% (v/v) bleach, and lysed in 1X SDS-PAGE loading buffer. A dilution series of recombinant Dcr-2/R2D2, standard embryo lysate and hand-collected total embryos were loaded on the same SDS-PAGE gel. Quantitative Western analysis with anti-R2D2 antibody (4) was performed as described (6) using recombinant R2D2 concentration standards. The R2D2 bands were quantified using a LAS-3000 (Fuji), and the intraembryonic concentration of R2D2 (~46 nM in vivo in syncitial blastoderm embryos and ~8 nM in lysate) was calculated assuming that the intracellular volume of an embryo is equal to the average volume of water in a single embryo, 7.3 nl (7).

Immunoprecipitation

siRNA with 5-ido-uracil at position 1 on the guide strand was used for crosslinking. Immunoprecipitation by anti-Ago1 (8) and anti-Ago2 antisera (9) was performed as described before (7), except that Protein A paramagnetic beads (Dynabead Protein A; Dynal Biotech) were used instead of Protein A agarose beads.
Capture of crosslinked proteins by tethered 2′-O-Methyl oligonucleotide

$^{32}$P-radiolabeled siRNA c was incubated with embryo lysate for 86 min, then crosslinked by irradiation for 4 min at room temperature. The reaction was then incubated with a 31 nt 2′-O-methyl oligonucleotide that contained a sequence complementary to the siRNA guide strand (5′-biotin-AUGUUGGAGACUUGGGCAAUGUGACUGCUGA-3′), as described (10). The 2′-O-methyl oligonucleotide was tethered to paramagnetic streptavidin beads via a 5′ biotin group.
### Supporting Online Table S1

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<th>Sequence 1</th>
<th>Sequence 2</th>
<th>siRNA</th>
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**Legend to Supporting Online Table S1.**

**Table S1.** siRNA sequences used in this study. siRNAs 1 and 2 target firefly luciferase, and siRNAs a-g target human *sod1*. U indicates 5-iodo-uracil; the 5'-32P radiolabel is marked with an asterisk and highlighted in yellow.
Legends to Supporting Online Figures

**Figure S1.** Recombinant Dcr-2 and R2D2 proteins alone cannot unwind siRNA duplexes. 
(A) Schematic of the assay for measuring unwinding of an siRNA in which the guide strand (red) is 5´-32P-radiolabeled. (B) Analysis of siRNA unwinding. We incubated the recombinant proteins with siRNA, ATP, and increasing concentrations of unlabeled RNA having the same sequence as the radiolabeled strand. The guide strand of the siRNA was 5´ 32P-radiolabeled. The unlabeled RNA served to trap any single-stranded siRNA generated by unwinding. After incubation, the proteins were digested with Proteinase K under conditions that preserve siRNA structure, then the siRNA was analyzed on a non-denaturing gel to resolve single-stranded from double-stranded siRNA. In *Drosophila* embryo lysate, siRNAs are unwound and the passenger strand is destroyed, so a ‘trap’ RNA is not required to detect unwinding. When the siRNA duplex by itself was heat denatured, the presence of increasing amounts of the trap RNA permitted detection of the dissociation siRNA duplex into its component strands. In contrast, no unwinding was detected when physiologically relevant concentrations of Dcr-2 or Dcr-2/R2D2 heterodimer were incubated with the siRNA duplex. The recombinant Dcr-2/R2D2 heterodimer also could not unwind siRNA when the non-radioactive siRNA strand was phosphorylated (11). Both protein preparations were highly active for the processing of long dsRNA into siRNA (11).

**Figure S2.** (A) Experimental scheme for detecting siRNA unwinding in the RLC. (B) Four independent trials showing that RLC contains a small amount of single-stranded siRNA. Either the guide or the passenger stand was 32P-radiolabeled for a luciferase siRNA (identical to siRNA 1, but with uracil in place of 5-iodo-uracil), which is slow to assemble RISC, allowing Complex B to be detected, or for a sod1 siRNA (identical to...
siRNA $b$, but with dT in place of 5-iodo-uracil), which assembles RISC more rapidly. The $x$-axis shows the gel slice number, the $y$-axis gives the relative amount of double-stranded or single-stranded siRNA in each band, normalized to the peak of the double-stranded siRNA in RLC. The right upper graph (luciferase siRNA with guide strand labeled) is presented also in Fig. 1C. The sample in the sixth gel slice for luciferase siRNA with a radiolabeled passenger strand was lost.

**Figure S3.** The ~130 kDa protein photocrosslinked to the $^{32}$P-radiolabeled guide strand is Ago2. (A) Immunoprecipitation of proteins photocrosslinked to the siRNA containing a 5-iodo-uracil at position 1 (p1) of the guide strand. p1 siRNA can be photocrosslinked to two different proteins (~110 kDa and ~130 kDa). The ~130 kDa photocrosslinked protein detected with this siRNA comigrated with that observed with the 5-iodo-uracil at p20 (siRNA $c$) (11). The ~110 kDa band was immunoprecipitated with anti-Ago1 antisera (8), and ~130 kDa band with anti-Ago2 antisera (9). (B) ~130 kDa protein photocrosslinked to siRNA $e$ (which contains a 5-iodo-uracil at p20 of the guide strand) was not detected in lysates lacking both maternal and zygotic Ago2 ($ago2^{414}$). Both wild-type and $ago2^{414}$ (3) lysates were prepared from overnight collections of embryos.

**Figure S4.** (A) Lysates from wild-type (11) and $ago2^{414}$ overnight embryo collections were incubated with siRNA $b$ and $e$ in the presence of ATP, and the binding of proteins near the 3' end of the siRNA passenger and guide strands monitored by photocrosslinking. Indicated times include the four minutes during which the sample was UV irradiated at room temperature. In wild-type lysates, binding of Dcr-2 and R2D2 decreased with time, accompanied by a corresponding increase in binding of Ago2 (11). In $ago2^{414}$ lysates, Dcr-2 and R2D2 binding did not decrease with time. (B) RISC assembly assay using guide-
strand 5′-32P-radiolabeled luciferase siRNA (identical to siRNA 1, but with uracil in place of 5-iodo-uracil). (C) The peaks of RISC, RLC and complex B were excised from the agarose gel in (B) and analyzed to determine the structure of the siRNA in the gel slice. Double-stranded and single-strand siRNA from this analysis were separated on a native polyacrylamide gel. No single-strand siRNA was detected in the RLC formed in ago2 #14 embryo lysates.

References for Supporting Online Materials

2. Y. S. Lee et al., Cell 117, 69 (2004).
11. Data not shown.
Tomari et al., Figure S1

A

unwinding

B

[trap], nM

0 125 1250

0 125 1250

0 125 1250

unwinding

native gel

+ +

+ +

+ +

heat denatured embryo lysate buffer only

double-stranded single-stranded

Dcr-2 only Dcr-2/R2D2 heterodimer
Tomari et al., Figure S2

A

incubate siRNA duplex with lysate (2 min) → run native gel → RISC → measure ds and ss in each slice (unwinding assay)

B

luciferase siRNA $^{32}$P-radiolabeled guide strand

luciferase siRNA $^{32}$P-radiolabeled passenger strand

sod1 siRNA $^{32}$P-radiolabeled guide strand

sod1 siRNA $^{32}$P-radiolabeled passenger strand

gel slice number
Tomari et al., Figure S3

A

1/5th input

IP

α-Ago1

α-Ago2

Ago2

Ago1

B

wild-type

gao2^{414}

Ago2

R2D2

α*-Ago2

α*-Ago1

OH

p

p*

AB