The RNA-Induced Silencing Complex Is a Mg^{2+}-Dependent Endonuclease

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Summary

In the *Drosophila* and mammalian RNA interference (RNAi) pathways, target RNA destruction is catalyzed by the siRNA-guided, RNA-induced silencing complex (RISC). RISC has been proposed to be an siRNA-directed endonuclease, catalyzing cleavage of a single phosphodiester bond on the RNA target. Although 5′ cleavage products are readily detected for RNAi in vitro, only 3′ cleavage products have been observed in vivo. Proof that RISC acts as an endonuclease requires detection of both 5′ and 3′ cleavage products in a single experimental system. Here, we show that siRNA-programmed RISC generates both 5′ and 3′ cleavage products in vitro; cleavage requires Mg^{2+}, but not Ca^{2+}, and the cleavage product termini suggest a role for Mg^{2+} in catalysis. Moreover, a single phosphorothioate in place of the scissile phosphate blocks cleavage; the phosphorothioate effect can be rescued by the thiophilic cation Mn^{2+}, but not by Ca^{2+} or Mg^{2+}. We propose that during catalysis, a Mg^{2+} ion is bound to the RNA substrate through a nonbridging oxygen of the scissile phosphate. The mechanism of endonucleolytic cleavage is not consistent with the mechanisms of the previously identified RISC nuclease, Tudor-SN. Thus, the RISC-component that mediates endonucleolytic cleavage of the target RNA remains to be identified.

Results and Discussion

The RISC Is an Endonuclease
In vitro, 5′ cleavage products are readily detected for both siRNA-programmed [1–9] and microRNA-programmed [4, 10] RISC. In contrast, only stable 3′ cleavage products are detected in vivo in plants [11–13] and in cultured mammalian cells [14]. Although the proposal that RISC is an endonuclease is appealing [5], current evidence is also consistent with a highly processive 5′-to-3′ or 3′-to-5′ exonuclease that stops at the center of the siRNA. In fact, a Staphylococcal family exonuclease, Tudor-SN (TSN), has been identified as a component of RISC in *Drosophila melanogaster*, mammals, and *Caenorhabditis elegans* [15]. The cysteine-alkylating agent, *N*-ethylmaleimide (NEM) reduces nonsequence-specific ribonuclease activity present in *Drosophila* em-

bryo lysates (Figure 1A). NEM also blocks the assembly, but not the activity, of RISC [3, 16]. We assembled siRNA into RISC in *Drosophila* embryo lysate, treated the reactions with NEM, then added either a 5′ or a 3′ ^{32}P-radiolabeled luciferase target RNA and monitored target RNA cleavage. The 3′-radiolabeled target RNA contained a 7-methyl guanosine "cap" at its 5′ end and was 3′ end-labeled with α-^{32}P, 3′-deoxyadenosine 5′ triphosphate (3′-dATP) and yeast poly(A) polymerase (Figure 1A). The 3′-radiolabeled RNA corresponded to the antisense sequence of the 5′-radiolabeled RNA target. The siRNA used in this study generates both sense- and antisense-strand RISCs. The 5′ cleavage product for this antisense target RNA and siRNA has been characterized previously [3, 5].

If RISC is an endonuclease, then the 5′ cleavage product of the 5′-radiolabeled sense target RNA and the 3′ cleavage product of the 3′-radiolabeled antisense target RNA should be the same length. By using the 5′-radiolabeled sense and 3′-radiolabeled antisense target RNAs, we detected both 5′ and 3′ products of siRNA-directed target RNA cleavage. No 3′ cleavage products were detected in the absence of NEM treatment or if dithiothreitol, which quenches NEM, was added before, rather than after, NEM (Figure 1A, "−NEM" and "mock"), likely because both the 3′-radiolabeled target RNA and the 3′ cleavage product are unstable in these conditions. Because the 5′ cleavage product of the sense target and the 3′ cleavage product of the antisense target comigrate, we conclude that RISC is an endonuclease that cleaves at a single, unique site, as first proposed by Tuschl and colleagues [5]. A 3′ cleavage product was also detected for a different siRNA:target pair (Figure S1).

The termini of the products of nucleases often provide clues to the mechanisms of nuclease catalysis. We purified each cleavage product by polyacrylamide gel electrophoresis, and then the purified cleavage products were enzymatically probed to determine if their termini contained hydroxyl or phosphate groups (Figures 1B and 1C). To identify the nature of its 3′ end, the 5′ cleavage product was treated with T4 polynucleotide kinase (PNK) or poly(A) polymerase and 3′-dATP. When treated with PNK, the gel mobility of the 5′ cleavage product was indistinguishable from the untreated sample, suggesting that it does not contain a 3′ phosphate (Figure 1B). (In addition to its 5′ kinase activity, PNK is a 3′ monophosphatase.) In contrast, incubation of the 5′ cleavage product with poly(A) polymerase and 3′-dATP converted it to a slower migrating form, consistent with the addition of a single adenosine to its 3′ end. This result implies the presence of a free 3′ hydroxyl group on the 5′ cleavage product (Figure 1B). We note that while poly(A) polymerase can add 3′-dATP to the 3′ end of an RNA chain, it cannot extend the 3′ hydroxyl group present on the 7-methyl guanosine(5′)ppp(5′) cap (data not shown).

The 3′ cleavage product was treated with calf intestinal phosphatase (CIP), PNK, or CIP then PNK. CIP treatment alone converted the 3′ cleavage product to a

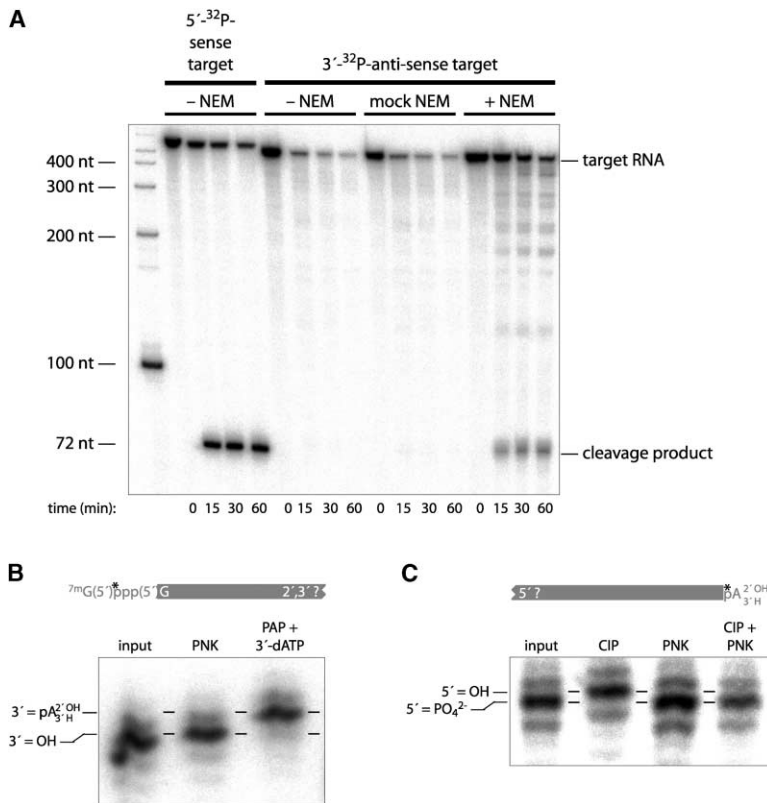*Correspondence: phillip.zamore@umassmed.edu

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B

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slower migrating form compared to the untreated RNA, consistent with the original 3' cleavage product containing one or more 5' phosphate groups. Treatment of the dephosphorylated sample with PNK and ATP restored its mobility to that of the untreated sample. No change was observed when the RNA was treated with PNK alone. Together, these results indicate that the 3' cleavage product begins with a single 5' phosphate group, and the 5' cleavage product ends with a 3' hydroxyl group.

These termini are not consistent with a 2' hydroxyl group on the target RNA acting as a nucleophile to attack the scissile phosphate; such a mechanism would be expected to leave a 2',3' cyclic phosphate and a 5' hydroxyl. Control experiments demonstrate that 2',3' cyclic phosphate and 5' hydroxyl termini are stable in the in vitro RNAi reaction but are nonetheless not found on the cleavage products of RISC (data not shown). After 1 hr incubation in NEM-treated lysate in standard RNAi conditions, an 80 nt, 5'-7-methyl guanosine cap-radiolabeled RNA bearing a 2',3'-cyclic phosphate at its 3' terminus was unaltered. Similarly, a 3'-radiolabeled RNA bearing a 5' hydroxyl group but otherwise identical to the 3' cleavage product described in this manuscript was not converted to a 5' phosphorylated form. Thus, the 5' phosphate and 3' hydroxy termini we observed on the cleavage products of RISC are unlikely to result from modification after the initial cleavage reaction.

RISC Exhibits Dependence on Divalent Cations
Instead of the 2' hydroxyl group, a water or hydroxide ion may serve as a nucleophile for endonucleolytic

cleavage by RISC. Such enzyme mechanisms often use divalent cations to assist in catalysis. To test whether a divalent metal ion is required for RISC endonuclease function, siRNA was incubated with *Drosophila* embryo lysate to assemble RISC, and then RISC assembly was inactivated with NEM. Finally, the siRNA-programmed RISC was mixed with target RNA and EGTA (to chelate calcium), EDTA (to chelate magnesium), or 1,10-phenanthroline (to chelate zinc). Compared to a reaction with no chelator added, the reactions containing either EGTA or 1,10-phenanthroline showed no decrease in cleavage efficiency. In contrast, reactions that contained EDTA showed a marked decrease in cleavage efficiency at 2 mM chelator; at 5 mM and 10 mM EDTA, no cleavage was detected (Figure 2A).

RISC function could be rescued by adding additional Mg^{2+} after EDTA, demonstrating that inactivation of RISC by EDTA reflected Mg^{2+} chelation, not an unrelated effect (Figure 2B). Furthermore, EDTA did not cause RISC disassembly, because RISC could be incubated with the RNA target in the presence of EDTA for 1 hr prior to the addition of Mg^{2+} (Figure 2C). RISC was assembled, the assembly was quenched with NEM, then EDTA and target RNA were added. The reaction was incubated for 1 hr; no target cleavage occurred. Next, Mg^{2+} was added, and the incubation continued for another hour. Addition of Mg^{2+} resurrected RISC activity. We note that RISC could not have fully disassembled in the presence of EDTA and then reassembled when Mg^{2+} was added because the initial NEM treatment inactivates RISC assembly [3, 16]. Of course, these data alone cannot exclude that RISC was partially disassem-
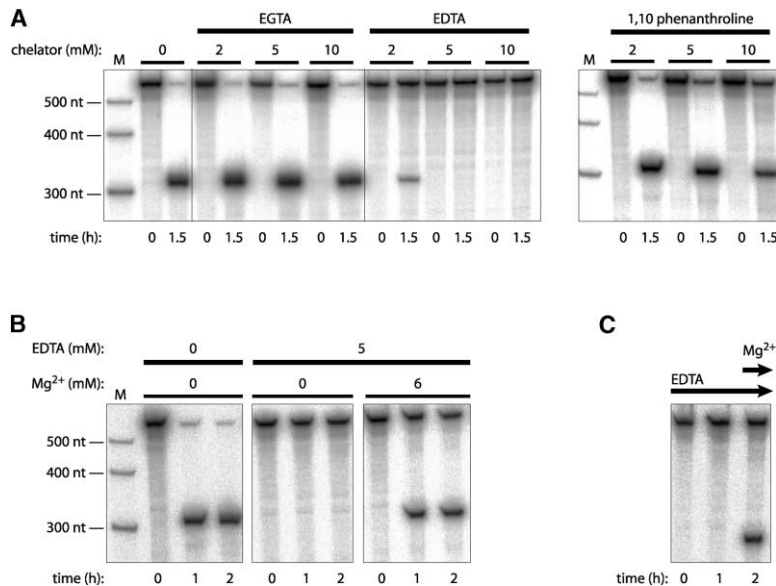
Figure 2. Endonucleolytic Cleavage by siRNA-Programmed RISC Requires Mg²⁺

(A) Standard in vitro RNAi reactions were assembled and preincubated for 1 hr at 25°C; treated with NEM to block further RISC assembly; and then 2, 5, or 10 mM chelator was added together with 5′-radiolabled target RNA. Reactions were incubated for an additional 1.5 hr.

(B) Reactions were assembled as in (A) but in one set, additional magnesium acetate was added.

(C) Reactions were assembled as in (A) with 5 mM EDTA. After 1 hr incubation of RISC with target RNA in the presence of EDTA, magnesium acetate was added and incubation continued for another hour.

bled to an intermediate state whose maturation does not require any NEM-sensitive factors and then reassembled to the active form upon Mg²⁺ addition. However, such a possibility is inconsistent with our observation below that a nonbridging oxygen of the scissile phosphate is likely a ligand for at least one divalent cation.

The Scissile Phosphate Is a Mg²⁺ Ligand

RISC cleaves its cognate mRNA target across from siRNA nucleotides 10 and 11, measured from the 5′ end of the siRNA guide strand [5, 6]. That is, the scissile phosphate of the target RNA lies between nucleotides 11 and 12, where nucleotide 1 is the target base paired to the twenty-first nucleotide of the siRNA guide strand (Figure 3A). To test whether one or more phosphate groups on the target RNA might bind Mg²⁺ during endonucleolytic cleavage, we prepared five differnet target RNAs, each bearing a single phosphorothioate substitution (Figure 3A). Phosphorothioate linkages contain a sulfur atom in place of one of the two nonbridging oxygens of the phosphodiester bond [17] (Figure 3B). Sulfur, unlike oxygen, does not bind well to Mg²⁺ [17, 18]. Although phosphorothioate linkages are chiral, we did not resolve the Rp and Sp isomers but, instead, analyzed the racemic mixture for each substrate RNA. Of the five singly substituted phosphorothioate target RNAs, only the RNA with a phosphorothioate between nucleotides 11 and 12 was detectably impaired for cleavage (Figure 3C). This position corresponds to the scissile phosphate originally identified by Tuschl and colleagues [5, 6]. Although the phosphorothioate-substituted target RNA contained both Rp and Sp isomers, cleavage was blocked by much more than 50%. Therefore, our data suggest that both the *pro*-Rp and *pro*-Sp nonbridging oxygens of the scissile phosphate of the RNA target play a role in siRNA-directed endonucleolytic cleavage of mRNA.

Substitution of a nonbridging oxygen with sulfur might block RISC activity simply because sulfur is larger than

oxygen. Alternatively, one or both nonbridging oxygens may be a ligand for Mg²⁺. Such a Mg²⁺ ion might play a role in generating the nucleophile (e.g., hydroxide ion) at the active site or in stabilizing the transition state. If a nonbridging oxygen acts as a Mg²⁺ ligand, then sulfur substitution should be rescued by Mn²⁺, which binds more strongly to sulfur than does Mg²⁺ [17, 18]. Addition of Mn²⁺, but not Mg²⁺ or Ca²⁺, partially rescued the effect of phosphorothioate substitution at the scissile phosphate (Figure 3D). Rescue by Mn²⁺ was specific for the phosphorothioate-substituted RNA target; addition of 2 mM Mn²⁺ did not increase the rate of cleavage of a target RNA containing only phosphodiester linkages (Figure S2). Efficient cleavage occurred only in the presence of a complementary siRNA; in the absence of siRNA, Mn²⁺ did not induce target cleavage (Figure 3E). The simplest explanation for our results is that RISC is a Mg²⁺-dependent endonuclease in which at least one nonbridging oxygen of the scissile phosphate directly interacts with the divalent cation.

**Effects of pdTp Inhibitor
on Endonucleolytic Cleavage**

The Tudor-SN (TSN) protein is the only purified protein component of RISC that displays single-stranded, RNA-specific ribonuclease activity. TSN is a component of the RISC in *Drosophila*, *C. elegans*, and mammals [15] and is present in *Drosophila* embryo lysates (G. Hutvág-ner and P.D.Z., unpublished data). Three lines of evidence suggest that the nuclease activity of TSN does not mediate siRNA-directed endonucleolytic cleavage of target RNA. First, Staphylococcal nuclease homologs are not known to be endonucleases nor are they expected to leave 3′ hydroxy and 5′ phosphate termini after cleavage. In fact, RNA or DNA hydrolysis by members of this class of nucleases yields 3′-phosphomono-nucleotides and dinucleotides [19–21], inconsistent with the 3′ hydroxy terminus we observe for the 5′ cleavage product of RISC. Second, as a member of the Staphylococcal nuclease family, TSN is expected to require Ca²⁺

A


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                   13 12 11 10 9  8
siRNA: 3´-UGAUAUGUU G  G  A  U  GAUGGAGU-5´
                    • • • • • • • • •   •   •  • • • • • • • •
target: 5´-⁷mGᵖpppG...ACUAUACAApCpCpUpApCUACCUCA...-3´
                      9  10 11 12 13 14
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B
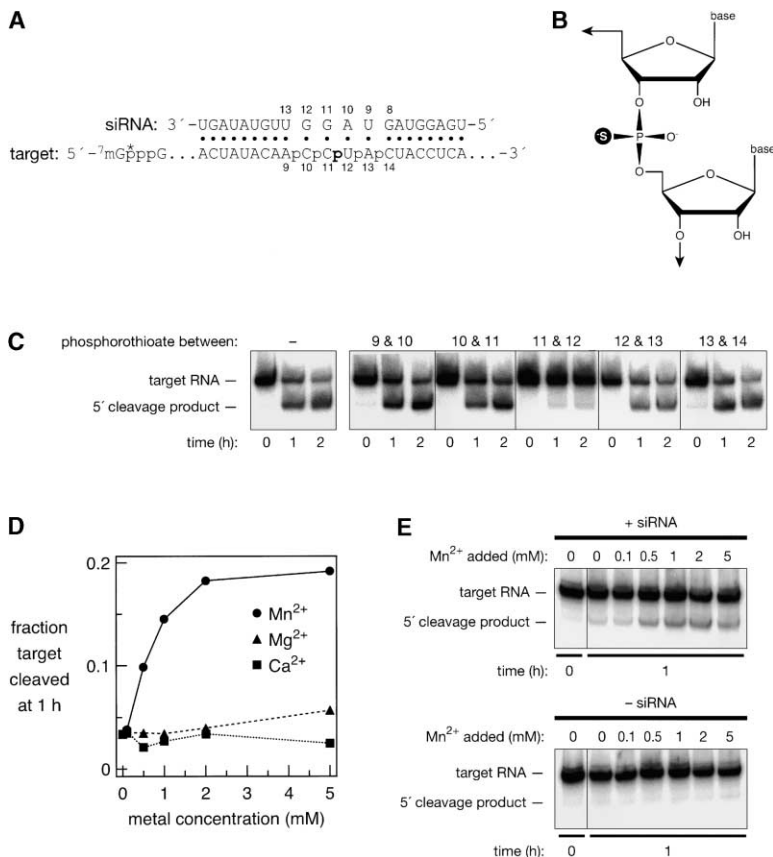

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D

E

Figure 3. A Nonbridging Oxygen of the Scissile Phosphate Binds at Least One Mg^{2+} Ion

(A) Scheme for single phosphorothioate substitution. The phosphorothioate-substituted phosphates in the target are indicated by "p" and the scissile phosphate is in bold.

(B) An Sp phosphorothioate linkage.

(C) In vitro RNAi reactions were programmed with a *let-7* siRNA duplex. The target RNA contained a 21 nt sequence with complete complementarity to *let-7*. The effect on target cleavage of substituting each phosphate indicated in (A) was assessed. Only when the scissile phosphate was replaced by a phosphorothioate was target cleavage impaired.

(D) The inhibition of cleavage observed when the scissile phosphate was replaced with a phosphorothioate could be rescued by Mn^{2+}, but not by Ca^{2+} or additional Mg^{2+}. Mn^{2+} and Ca^{2+} reactions also contained 1.2 mM Mg^{2+}; indicated Mg^{2+} concentrations are the concentration added in addition to the 1.2 mM basal level contained in a standard RNAi reaction. Triangles, Mg^{2+}; circles, Mn^{2+}; squares, Ca^{2+}.

(E) Rescue of cleavage by Mn^{2+} requires siRNA.

for activity [22]. Our data show that Mg^{2+}, but not Ca^{2+}, is required for siRNA-directed endonucleolytic cleavage. Third, 2′-deoxythymidine 5′,3′-bisphosphate (pdTp), a general inhibitor of staphylococcal nucleases [22], inhibits TSN activity [15] but does not inhibit endonucleolytic cleavage by RISC (Figure 4). We assembled siRNA into RISC and then incubated it with target RNA in the presence of 50 or 100 μM pdTp, 50 or 100 μM 2′-deoxythymidine 3′-monophosphate (dTp), or no inhibitor (Figure 4). 5′ cleavage products were efficiently formed in all conditions even though the pdTp concentration was more than 2000-fold higher than RISC and ∼2-fold

greater than the K$_i$ reported for inhibition of Staphylococcus nuclease [23]. 100 μM pdTp was shown previously to block TSN activity and RISC-directed target RNA degradation in vitro [15]. Our data suggest that if the nuclease activity of TSN functions in target RNA destruction, the protein must act after the siRNA-directed, Mg^{2+}-dependent endonucleolytic cleavage of the target RNA.
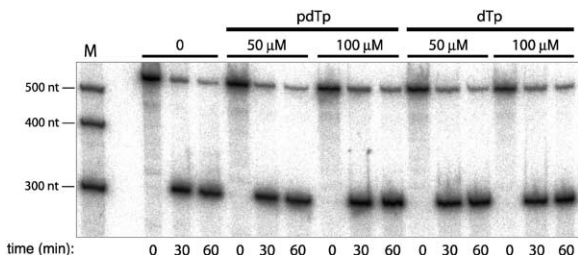
Figure 4. Endonucleolytic Cleavage by siRNA-Programmed RISC Is Unaltered by the TSN Inhibitor pdTp (2′-deoxythymidine 5′,3′-bisphosphate)

Standard RNAi reactions were carried out in the absence or presence of 50 or 100 μM pdTp or in the presence of 50 or 100 μM dTp (2′-deoxythymidine 3′-monophosphate). An *sod1*-specific siRNA and *sod1* target RNA were used.

References

1. Tuschl, T., Zamore, P.D., Lehmann, R., Bartel, D.P., and Sharp, P.A. (1999). Targeted mRNA degradation by double-stranded RNA in vitro. Genes Dev. *13*, 3191–3197.

2. Zamore, P.D., Tuschl, T., Sharp, P.A., and Bartel, D.P. (2000). RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell *101*, 25–33.

3. Nykänen, A., Haley, B., and Zamore, P.D. (2001). ATP requirements and small interfering RNA structure in the RNA interference pathway. Cell *107*, 309–321.

4. Hutvágner, G., and Zamore, P.D. (2002). A MicroRNA in a multiple-turnover RNAi enzyme complex. Science *297*, 2056–2060.

5. Elbashir, S.M., Lendeckel, W., and Tuschl, T. (2001). RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev. *15*, 188–200.

6. Elbashir, S.M., Martinez, J., Patkaniowska, A., Lendeckel, W., and Tuschl, T. (2001). Functional anatomy of siRNAs for mediating efficient RNAi in Drosophila melanogaster embryo lysate. EMBO J. *20*, 6877–6888.

7. Martinez, J., Patkaniowska, A., Urlaub, H., Lührmann, R., and Tuschl, T. (2002). Single-stranded antisense siRNA guide target RNA cleavage in RNAi. Cell *110*, 563–574.

8. Schwarz, D.S., Hutvágner, G., Haley, B., and Zamore, P.D. (2002). Evidence that siRNAs function as guides, not primers, in the *Drosophila* and human RNAi pathways. Mol. Cell *10*, 537–548.

9. Chiu, Y.L., and Rana, T.M. (2003). siRNA function in RNAi: a chemical modification analysis. RNA *9*, 1034–1048.

10. Tang, G., Reinhart, B.J., Bartel, D.P., and Zamore, P.D. (2003). A biochemical framework for RNA silencing in plants. Genes Dev. *17*, 49–63.

11. Xie, Z., Kasschau, K.D., and Carrington, J.C. (2003). Negative feedback regulation of Dicer-Like1 in *Arabidopsis* by microRNA-guided mRNA degradation. Curr. Biol. *13*, 784–789.

12. Llave, C., Xie, Z., Kasschau, K.D., and Carrington, J.C. (2002). Cleavage of *Scarecrow-Like* mRNA targets directed by a class of *Arabidopsis* miRNA. Science *297*, 2053–2056.

13. Palatnik, J.F., Allen, E., Wu, X., Schommer, C., Schwab, R., Carrington, J.C., and Weigel, D. (2003). Control of leaf morphogenesis by microRNAs. Nature *425*, 257–263.

14. Holen, T., Amarzguioui, M., Wiiger, M.T., Babaie, E., and Prydz, H. (2002). Positional effects of short interfering RNAs targeting the human coagulation trigger Tissue Factor. Nucleic Acids Res. *30*, 1757–1766.

15. Caudy, A.A., Ketting, R.F., Hammond, S.M., Denli, A.M., Bathoorn, A.M., Tops, B.B., Silva, J.M., Myers, M.M., Hannon, G.J., and Plasterk, R.H. (2003). A micrococcal nuclease homologue in RNAi effector complexes. Nature *425*, 411–414.

16. Tomari, Y., Du, T., Haley, B., Schwarz, D.S., Bennett, R., Cook, H.A., Koppetsch, B.S., Theurkauf, W.E., and Zamore, P.D. (2004). RISC assembly defects in the *Drosophila* RNAi mutant armitage. Cell *116*, 831–841.

17. Eckstein, F. (1985). Nucleoside phosphorothioates. Annu. Rev. Biochem. *54*, 367–402.

18. Pecoraro, V.L., Hermes, J.D., and Cleland, W.W. (1984). Stability constants of Mg2+ and Cd2+ complexes of adenine nucleotides and thionucleotides and rate constants for formation and dissociation of MgATP and MgADP. Biochemistry *23*, 5262–5267.

19. Cunningham, L., Catlin, B.W., and Privat de Garilhe, M. (1956). A deoxyribonuclease of Micrococcus pyogenes. J. Am. Chem. Soc. *78*, 4642–4645.

20. Reddi, K.K. (1958). Action of micrococcal phosphodiesterase on tobacco mosaic virus nucleic acid. Nature *182*, 1308.

21. Reddi, K.K. (1960). Mode of action of micrococcal phosphodiesterase. Nature *187*, 74–75.

22. Cuatrecasas, P., Fuchs, S., and Anfinsen, C.B. (1967). Catalytic properties and specificity of the extracellular nuclease of Staphylococcus aureus. J. Biol. Chem. *242*, 1541–1547.

23. Cuatrecasas, P., Fuchs, S., and Anfinsen, C.B. (1967). The interaction of nucleotides with the active site of staphylococcal nuclease. Spectrophotometric studies. J. Biol. Chem. *242*, 4759–4767.