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Argonaute2 Is the Catalytic Engine of Mammalian RNAi
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a single-stranded RNA should bind fairly readily, opening the claw of the molecule might assist binding the mRNA, after which Argonaute might close on the double-stranded substrate. A possible hinge region exists in the interdomain connector at residues 317 to 320. This hinge could lift the PAZ away from the crescent base, perhaps allowing the RISC loading complex to assist in assembling an active complex (*28*, *29*).

The notion that RISC "Slicer" activity resides in Argonaute itself was tested in a mammalian system, by mutational analysis of hAgo2 (*30*). Conserved active site aspartates in hAgo2 were altered and the mutants lost nuclease activity but retained siRNA binding. This supports the model in which Argonaute itself functions as the Slicer enzyme in the RNAi pathway.

Many questions regarding the details of the mechanism for siRNA-guided mRNA cleavage remain. Several Argonaute protein family members appear to be inactive toward mRNA cleavage despite the presence of the catalytic residues. This situation might be analogous to the case of the Tn5 transposase and its inhibitor, which possess a catalytic domain with an RNase H–like fold. Tn5 inhibitor is a truncated version of the active Tn5 transposase and retains essential catalytic residues. However, there are major conformational differences between the two (*21*). Mutations have been introduced into a catalytically active Ago protein, hAgo2, in the vicinity of the active site, which change residues to corresponding residues in an inactive Ago, hAgo1. These inactivate Ago2 for cleavage, indicating that there are determinants for catalysis beyond simply the catalytic triad and that relatively minor alterations in the PIWI domain can have profound effects on its activity toward RNA substrates. In addition, interactions with other factors may be needed to create a fully active Slicer. The common fold in the catalytic domain of Argonaute family members and transposases and integrases is also intriguing given the relationship of RNAi with control of transposition. Notably, the identification of the catalytic center of RISC awaited a drive toward understanding RNAi at a structural level. Thus, it seems likely that, as in the present example, a full understanding of the underlying mechanism of RNAi will derive from a combination of detailed biochemical and structural studies of RISC.

References and Notes

1. A. Fire *et al*., *Nature* **391**, 806 (1998).
2. S. M. Elbashir, J. Martinez, A. Patkaniowska, W. Lendeckel, T. Tuschl, *EMBO J.* **20**, 6877 (2001).
3. E. Bernstein, A. A. Caudy, S. M. Hammond, G. J. Hannon, *Nature* **409**, 363 (2001).
4. D. P. Bartel, *Cell* **116**, 281 (2004).
5. A. Nykanen, B. Haley, P. D. Zamore, *Cell* **107**, 309 (2001).
6. D. S. Schwarz, G. Hutvagner, B. Haley, P. D. Zamore, *Mol. Cell* **10**, 537 (2002).
7. J. Martinez, A. Patkaniowska, H. Urlaub, R. Luhrmann, T. Tuschl, *Cell* **110**, 563 (2002).
8. S. M. Hammond, E. Bernstein, D. Beach, G. J. Hannon, *Nature* **404**, 293 (2000).
9. L. Cerutti, N. Mian, A. Bateman, *Trends Biochem. Sci.* **25**, 481 (2000).
10. J. J. Song *et al*., *Nature Struct. Biol.* **10**, 1026 (2003).
11. K. S. Yan *et al*., *Nature* **426**, 468 (2003).
12. A. Lingel, B. Simon, E. Izaurralde, M. Sattler, *Nature* **426**, 465 (2003).
13. A. Lingel, B. Simon, E. Izaurralde, M. Sattler, *Nature Struct. Mol. Biol.* **11**, 576 (2004).
14. J. B. Ma, K. Ye, D. J. Patel, *Nature* **429**, 318 (2004).
15. A. M. Friedman, T. O. Fischmann, T. A. Steitz, *Science* **268**, 1721 (1995).
16. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
17. F. Dyda *et al*., *Science* **266**, 1981 (1994).
18. J. Lubkowski *et al*., *Biochemistry* **38**, 13512 (1999).
19. M. Ariyoshi *et al*., *Cell* **78**, 1063 (1994).
20. P. Rice, K. Mizuuchi, *Cell* **82**, 209 (1995).
21. D. R. Davies, I. Y. Goryshin, W. S. Reznikoff, I. Rayment, *Science* **289**, 77 (2000).
22. L. Lai, H. Yokota, L. W. Hung, R. Kim, S. H. Kim, *Struct. Fold Des.* **8**, 897 (2000).
23. K. Katayanagi, M. Okumura, K. Morikawa, *Proteins* **17**, 337 (1993).
24. W. Yang, T. A. Steitz, *Structure* **3**, 131 (1995).
25. U. Wintersberger, *Pharmacol. Ther.* **48**, 259 (1990).
26. J. Martinez, T. Tuschl, *Genes Dev.* **18**, 975 (2004).
27. D. S. Schwarz, Y. Tomari, P. D. Zamore, *Curr. Biol.* **14**, 787 (2004).
28. Y. Tomari *et al*., *Cell* **116**, 831 (2004).
29. J. W. Pham, J. L. Pellino, Y. S. Lee, R. W. Carthew, E. J. Sontheimer, *Cell* **117**, 83 (2004).
30. J. Liu *et al*., *Science* **305**, 1437 (2004); published online 29 July 2004 (10.1126/science.1102513).

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Supporting Online Material

www.sciencemag.org/cgi/content/full/1102514/DC1
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References

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Argonaute2 Is the Catalytic Engine of Mammalian RNAi

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Gene silencing through RNA interference (RNAi) is carried out by RISC, the RNA-induced silencing complex. RISC contains two signature components, small interfering RNAs (siRNAs) and Argonaute family proteins. Here, we show that the multiple Argonaute proteins present in mammals are both biologically and biochemically distinct, with a single mammalian family member, Argonaute2, being responsible for messenger RNA cleavage activity. This protein is essential for mouse development, and cells lacking Argonaute2 are unable to mount an experimental response to siRNAs. Mutations within a cryptic ribonuclease H domain within Argonaute2, as identified by comparison with the structure of an archeal Argonaute protein, inactivate RISC. Thus, our evidence supports a model in which Argonaute contributes "Slicer" activity to RISC, providing the catalytic engine for RNAi.

The presence of double-stranded RNA (dsRNA) in most eukaryotic cells provokes a sequence-specific silencing response known as RNA interference (RNAi) (*1*, *2*). The dsRNA trigger of this process can be derived from exogenous sources or transcribed from endogenous noncoding RNA genes that produce microRNAs (miRNAs) (*1*, *3*).

RNAi begins with the conversion of dsRNA silencing triggers into small RNAs of ~21 to 26 nucleotides (nts) in length (*4*). This is accomplished by the processing of triggers by specialized ribonuclease III (RNase III)–family nucleases, Dicer and Drosha (*5*, *6*). Resulting small RNAs join an effector complex, known as RISC (RNA-induced silencing complex) (*7*). Silencing by RISC can occur through several mechanisms. In flies, plants, and fungi, dsRNAs can trigger chromatin remodeling and transcriptional gene silencing (*8*–*11*). RISC can also interfere with protein synthesis, and this is the predominant mechanism used by miRNAs in mammals (*12*, *13*). However, the best studied mode of

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RISC action is mRNA cleavage (*14*, *15*). When programmed with a small RNA that is fully complementary to the substrate RNA, RISC cleaves that RNA at a discrete position, an activity that has been attributed to an unknown RISC component, "Slicer" (*16*, *17*). Whether or not RISC cleaves a substrate can be determined by the degree of complementarity between the siRNA and mRNA, as mismatched duplexes are often not processed (*16*). However, even for mammalian miRNAs, which normally repress at the level of protein synthesis, cleavage activity can be detected with a substrate that perfectly matches the miRNA sequence (*18*). This result prompted the hypothesis that all RISCs are equal, with the outcome of the RISC-substrate interaction being determined largely by the character of the interaction between the small RNA and its substrate.

RISC contains two signature components. The first is the small RNA, which cofractionated with RISC activity in *Drosophila* S2 cell extracts (*7*), and whose presence correlated with dsRNA-programmed mRNA cleavage in *Drosophila* embryo lysates (*14*, *15*). The second is an Argonaute (Ago) protein, which was identified as a component of purified RISC in *Drosophila* (*19*). Subsequent studies have suggested that Argonautes are also key components of RISC in mammals, fungi, worms, protozoans, and plants (*17*, *20*).

Argonautes are often present as multiprotein families and are identified by two characteristic domains, PAZ and PIWI (*21*). These proteins mainly segregate into two subfamilies, comprising those that are more similar to either *Arabidopsis* Argonaute1 or *Drosophila* Piwi. The Argonaute family was first linked to RNAi through genetic studies in *Caenorhabditis elegans*, which identified Rde-1 as a gene essential for silencing (*22*). Our subsequent placement of a *Drosophila* Argonaute protein in RISC (*19*) prompted us to explore the roles of this protein family. Toward this end, we have undertaken both biochemical and genetic studies of the Ago1 subfamily proteins in mammals.

Mammals contain four Argonaute1 subfamily members, Ago1 to Ago4 [nomenclature as in (*23*); see fig. S1]. We have previously shown that different Argonaute family members in *Drosophila* preferentially associate with different small RNAs, with Ago1 preferring miRNAs and Ago2 siRNAs (*24*). Recent studies of *Drosophila melanogaster* (dm) Ago1 and dmAgo2 mutants have strengthened these conclusions (*25*). To assess whether mammalian Ago proteins specialized in their interactions with small RNAs, we examined Ago-associated miRNA populations by microarray analysis. Ago1-, Ago2- and Ago3-associated RNAs were hybridized to microarrays that report the expression status of 152 human microRNAs. Patterns of associated RNAs were identical within experimental error in each case (Fig. 1A). Additionally, each of the tagged Ago proteins associated similarly with a cotransfected siRNA (Fig. 1C).

Previous studies have used tagged siRNAs to affinity purify Argonaute-containing RISC (*17*). These preparations, containing mixtures of at least two mammalian Argonautes, were capable of cleaving synthetic mRNAs that were complementary to the tagged siRNA. We examined the ability of purified complexes containing individual Argonaute proteins to catalyze similar cleavages. Unexpectedly, irrespective of the siRNA sequence, only Ago2-containing RISC was able to catalyze cleavage (Fig. 1B and fig. S2). All three Ago proteins were similarly expressed and bound similar amounts of transfected siRNA (Fig. 1, C and D).

These results demonstrated that mammalian Argonaute complexes are biochemically distinct, with only a single family member being competent for mRNA cleavage. To examine the possibility that Ago proteins might also be biologically specialized, we disrupted the mouse Ago2 gene by targeted insertional mutagenesis (fig. S3 and Fig. 2A) (*26*). Intercrosses of Ago2 heterozygotes produced only wild-type and heterozygous offspring, strongly suggesting that disruption of Ago2 produced an embryonic-lethal phenotype.

Ago2-deficient mice display several developmental abnormalities beginning approximately halfway through gestation. Both gene-trap and in situ hybridization data of day 9.5 embryos show broad expression of Ago2 in the embryo, with some hot spots of expression in the forebrain, heart, limb buds, and branchial arches (Fig. 2, F and G). The most prominent phenotype is a defect in neural tube closure (Fig. 2, D and E), often accompanied by apparent mispatterning of anterior structures, including the forebrain (Fig. 2, C and D). Roughly half of the embryos display complete failure of neural tube closure in the head region (Fig. 2E), while all embryos display a wavy neural tube in more caudal regions. Mutant embryos also suffer from apparent cardiac failure. The hearts are enlarged and often accompanied by pronounced swelling of the pericardial cavity (Fig. 2C). By day 10.5, mutant embryos are severely developmentally delayed compared with wild-type and heterozygous littermates (Fig. 2B). This large difference in size, like the apparent cardiac failure, may be accounted for by a general nutritional deficiency caused by yolk sac and placental defects (*27*), as histological analysis reveals abnormalities in these tissues.

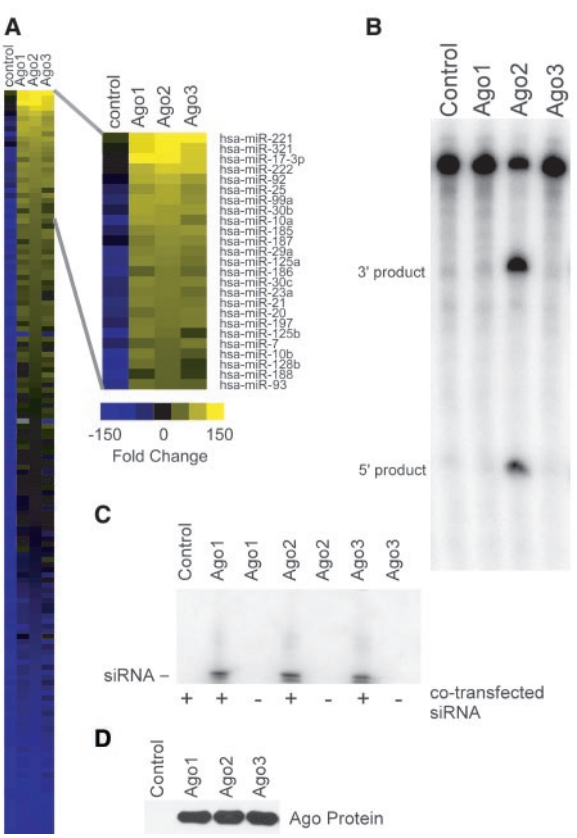

Fig. 1. Only mammalian Ago2 can form cleavage-competent RISC. (**A**) The miRNA populations associated with Ago1, Ago2, and Ago3 were measured by microarray analysis as described in (*44*). The heat map shows normalized log-ratio values for each data set, with yellow representing increased relative amounts and blue indicating decreased amounts relative to the median. The top 25 log ratios are shown in the expanded region. In each panel, "control" indicates parallel analysis of cells transfected with a vector control. (**B**) The 293T cells were transfected with a control vector or with vectors encoding myc-tagged Ago1, Ago2, or Ago3, along with an siRNA that targets firefly luciferase. Immunoprecipitates were tested for siRNA-directed mRNA cleavage as described in (*44*). Positions of 5′ and 3′ cleavage products are shown. (**C**) Immunoprecipitates as in (B) were tested for in vivo siRNA binding by Northern blotting of Ago immunoprecipitates (*44*). (**D**) Western blots of transfected cell lysates show similar levels of expression for each recombinant Argonaute protein.

Not all Argonaute proteins are required for successful mammalian development (*28*, *29*). Thus, it is unclear why Ago2 should be required for development, while other Ago proteins are dispensable. Ago subfamily members are expressed in overlapping patterns in humans (*30*). In situ hybridization demonstrates overlapping expression patterns for Ago2 and Ago3 in mouse embryos (Fig. 2F and fig. S4). Considered together with the essentially identical patterns of miRNA binding, our results suggest the possibility that the ability of Ago2 to assemble into catalytically active complexes might be critical for mouse development. Although most miRNAs regulate gene expression at the level of protein synthesis, recently miR196 has been demonstrated to cleave the mRNA encoding HoxB8, a developmental regulator (*31*). Evolutionary conservation of an essential cleavage-competent RISC in organisms in which miRNAs predominantly act by translational regulation raises the possibility that target cleavage by mammalian miRNAs might be more important and widespread than previously appreciated.

Numerous studies have indicated that experimentally triggered RNAi in mammalian cells proceeds through siRNA-directed mRNA cleavage because in many, but not all, cases, reiterated binding sites are necessary for repression at the level of protein synthesis [see, for example (*13*, *32*, *33*)]. If Ago2 were uniquely capable of assembling into cleavage-competent complexes in mice, then embryos or cells lacking Ago2 might be resistant to experimental RNAi. To address this question, we prepared mouse embryo fibroblasts (MEFs) from E10.5 embryos from Ago2 heterozygous intercrosses. Reverse transcription polymerase chain reaction (RT-PCR) analysis and genotyping revealed that we were able to obtain wild-type, mutant, and heterozygous MEF populations. Importantly, MEFs also express other Ago proteins, including Ago1 and Ago3 (Fig. 3A). Ago2-null MEFs were unable to repress gene expression in response to an siRNA (Fig. 3B and fig. S5). This defect could be rescued by the addition of a third plasmid that encoded human Ago2 but not by a plasmid encoding human Ago1 (Fig. 3B). In contrast, responses were intact for a reporter of repression at the level of protein synthesis, mediated by an siRNA binding to multiple mismatched sites (*32*) (Fig. 3C).

Because Ago2 is exceptional in its ability to form cleavage-competent complexes, we set out to map the determinants of this capacity. Deletion analysis indicated that an intact Ago2 was required for RISC ac-

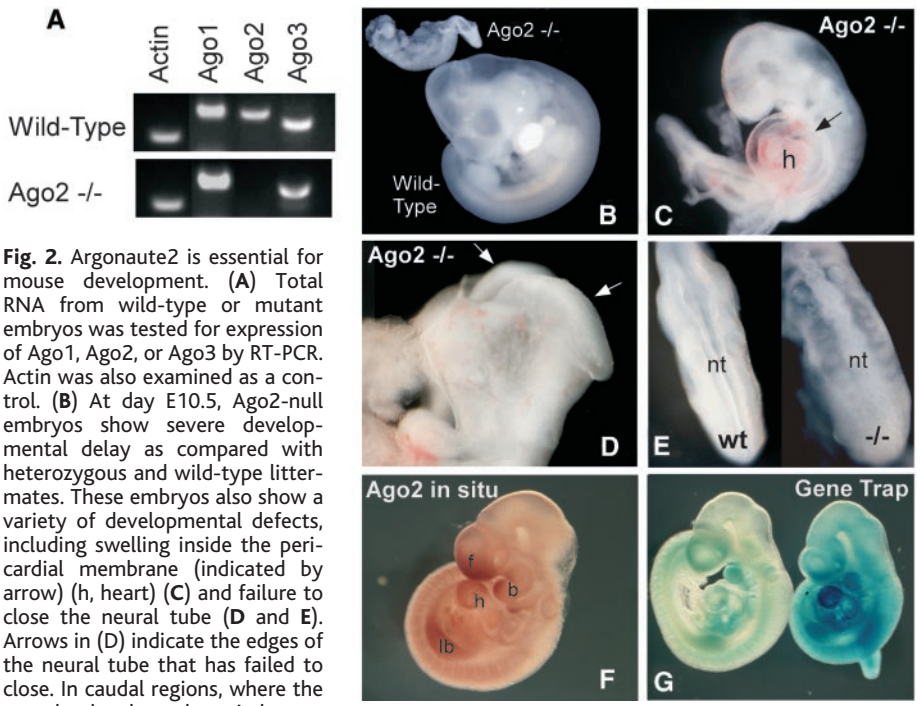

Fig. 2. Argonaute2 is essential for mouse development. (**A**) Total RNA from wild-type or mutant embryos was tested for expression of Ago1, Ago2, or Ago3 by RT-PCR. Actin was also examined as a control. (**B**) At day E10.5, Ago2-null embryos show severe developmental delay as compared with heterozygous and wild-type littermates. These embryos also show a variety of developmental defects, including swelling inside the pericardial membrane (indicated by arrow) (h, heart) (**C**) and failure to close the neural tube (**D** and **E**). Arrows in (D) indicate the edges of the neural tube that has failed to close. In caudal regions, where the neural tube does close, it has an abnormal appearance, being wavy as compared with wild-type embryos (E) (compare wild-type and Ago2 −/−). Ago2 is expressed in most tissues of the developing embryo as measured by in situ hybridization (**F**) or by analysis of an Ago2 gene-trap animal (**G**). In (F), f is forebrain, b is branchial arches, h is heart, and lb is limb bud, all of which are relative hot spots for Ago2 mRNA. In (G), the left embryo shows similar patterns when staining for the gene-trap marker, β-galactosidase, proceeds for only a short period. Longer incubation (G, right) gives uniform staining throughout the embryo.

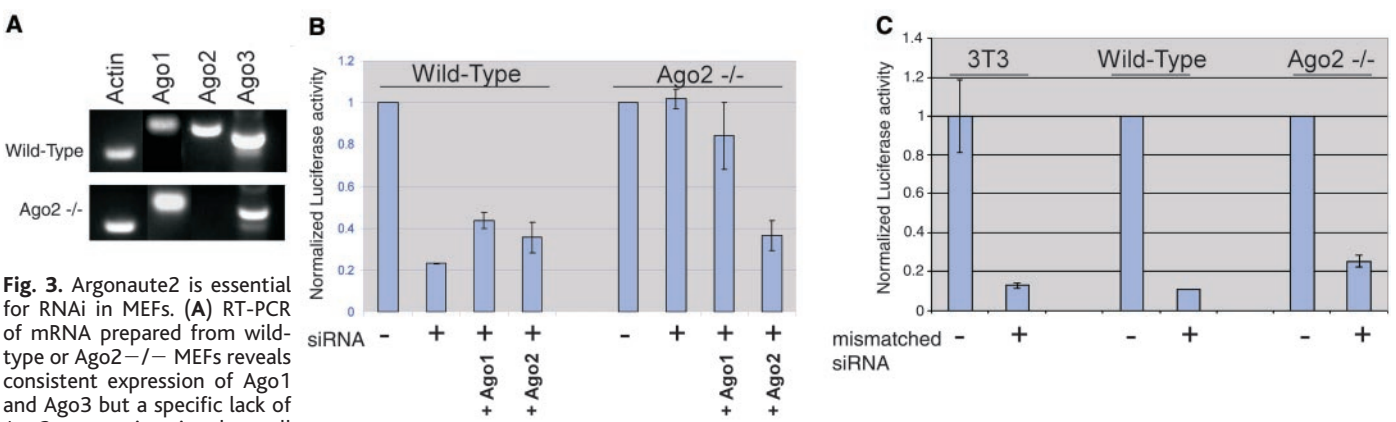

Fig. 3. Argonaute2 is essential for RNAi in MEFs. (**A**) RT-PCR of mRNA prepared from wild-type or Ago2−/− MEFs reveals consistent expression of Ago1 and Ago3 but a specific lack of Ago2 expression in the null MEF. Actin mRNA serves as a control. (**B**) Wild-type and mutant MEFs were cotransfected with plasmids encoding *Renilla* and firefly luciferases, either with or without firefly siRNA. Ratios of firefly to *Renilla* activity, normalized to 1 for the no-siRNA control, were plotted. For each genotype, the ability of Ago1 and Ago2 to rescue suppression was tested by cotransfection with expression vectors encoding each protein as indicated. (**C**) NIH-3T3 cells, wild-type MEFs, or Ago2 mutant MEFs were tested as described in (B) (except that *Renilla*/firefly ratios are plotted) for their ability to suppress a reporter of repression at the level of protein synthesis. In this case, the *Renilla* luciferase mRNA contains multiple imperfect binding sites for a CXCR4 siRNA. Cells were transfected with a mixture of firefly and *Renilla* luciferase plasmids with or without the siRNA.

tivity (fig. S6). We therefore used the sequence of highly conserved but cleavage-incompetent Ago proteins as a guide to the construction of Ago2 mutants. A series of point mutations included H634P, H634A, Q633R, Q633A, H682Y, L140W, F704Y, and T744Y. Whereas all of these mutations retain siRNA-binding activity and most retain cleavage activity, changes at Q633 and H634 have a profound effect on target cleavage (Fig. 4). Both the Q633R and H634P mutations, in which residues were changed to corresponding residues in Ago1 and Ago3, abolished catalysis. Changing H634 to A also inactivated Ago2, whereas a similar change, Q633A, was permissive for cleavage. Thus, even relatively conservative changes can negate the ability of Ago2 to form cleavage-competent RISC.

Several possibilities could explain a lack of cleavage activity for Ago2 mutants. Such mutations could interfere with the proper folding of Ago2. However, this seems unlikely because those same residues presumably permit proper folding in closely related Argonaute proteins, and mutant Ago2 proteins retained the ability to interact with siRNAs. Alternatively, cleavage-incompetent Ago2 mutants could lose the ability to interact with the putative Slicer. Finally, Ago2 itself might be Slicer, with our conservative substitutions altering the active center of the enzyme in a way that prevents cleavage.

The last possibility predicted that we might reconstitute an active enzyme with relatively pure Ago2 protein. We immunoaffinity purified Ago2 from 293T cells and attempted to reconstitute RISC in vitro. Incubation with the double-stranded siRNA produced no appreciable activity, whereas Ago2 could be successfully programmed with single-stranded siRNAs to cleave a complementary substrate (Fig. 5A). Formation of the active enzyme was unaffected by first washing the immunoprecipitates with up to 2.5 M NaCl or 1 M urea. A 21-nt single-stranded DNA was unable to direct cleavage (Fig. 5A). Programming could be accomplished with different siRNAs that direct activity against different substrates (fig. S7). RISC is formed though a concerted assembly process in which the RISC-loading complex (RLC) acts in an adenosine triphosphate (ATP)–dependent manner to place one strand of the small RNA into RISC (*34–36*). In vitro reconstitution occurs in the absence of ATP, which suggests that Ago2 could be programmed with siRNAs without a need for the normal assembly process (Fig. 5A). However, in vitro reconstitution of RISC still requires

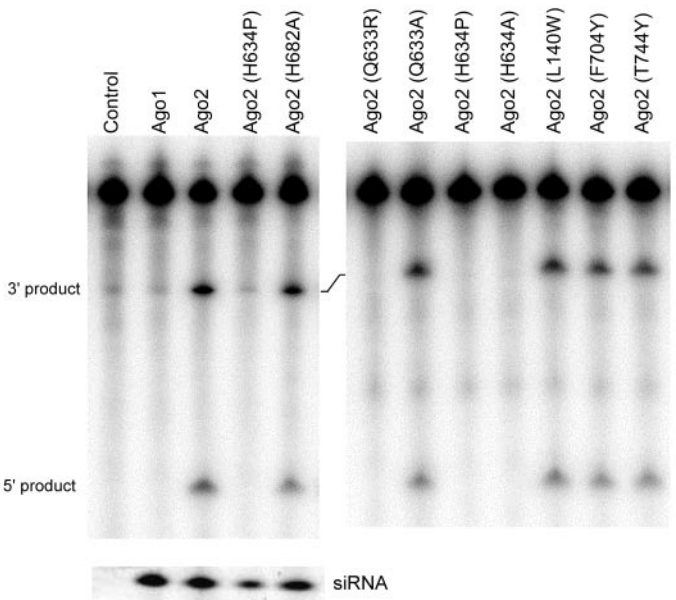

Fig. 4. Mapping the requirements for assembly of cleavage-competent RISC. Ago1, Ago2, or mutants of Ago2 were expressed as myc-tagged fusion proteins in 293T cells. In all cases, expression constructs were cotransfected with a luciferase siRNA. Western blotting indicated similar expression for each mutant. Immunoprecipitates containing individual proteins were tested for cleavage activity against a luciferase mRNA (*44*). Positions of 5′ and 3′ cleavage products are indicated. SiRNA binding was examined for each mutant by Northern blotting of immunoprecipitates or by staining of immunoprecipitates with Sybr Gold (Molecular Probes, Eugene, Oregon). Representatives for these assays are shown. In no case did we detect a defect in interaction of mutants with siRNAs.

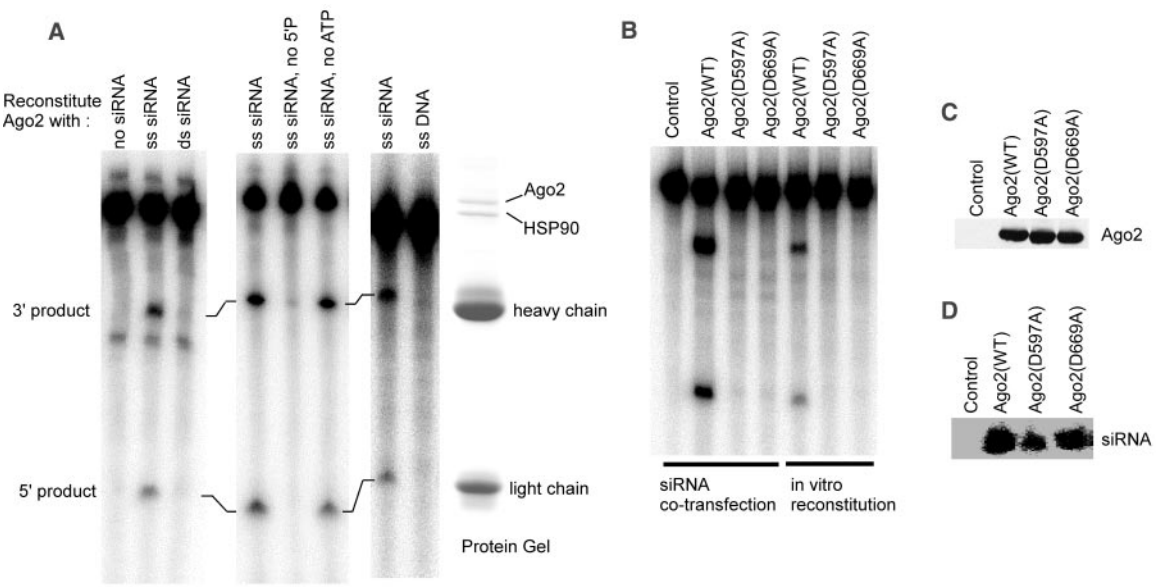

Fig. 5. Argonaute2 is a candidate for Slicer. (**A**) Ago2 protein was immunoaffinity purified from transiently transfected 293T cells. The preparation contained two major proteins (protein gel), in addition to heavy and light chains. These were identified by mass spectrometry as Ago2 and HSP90. Immunoprecipitates were mixed (*44*) in vitro with single- or double-stranded siRNAs or with a 21-nt DNA having the same sequence as the siRNA. Reconstituted RISC was tested for cleavage activity with a uniformly labeled synthetic mRNA. Positions of 5′ and 3′ cleavage products are noted. Where indicated, the siRNA was not 5′ phosphorylated and, in one case, ATP was not added to the reconstitution reaction. (**B**) Ago2 or Ago2 mutants were assembled into RISC in vivo by cotransfection with siRNAs, followed by immunoaffinity purification or by in vitro reconstitution, mixing affinity-purified proteins with single-stranded siRNAs. These mutants were tested for activity against a complementary mRNA substrate. 5′ and 3′ cleavage products are as in (A). (**C** and **D**) Both mutant proteins were expressed at levels similar to wild-type Ago2 and bound siRNAs as readily. Ago2 (H634P) and Ago2 (Q633R) behave similarly in this assay.

the essential characteristics of an siRNA. For example, single-stranded siRNAs that lack a 5′ phosphate group cannot reconstitute an active enzyme.

Although consistent with the possibility that the catalytic activity of RISC is carried within Ago2, these results do not rule out the possibility that a putative Slicer copurifies with Ago2. To demonstrate more conclusively that Ago2 is Slicer, we turned to the crystal structure of an Argonaute protein from an archebacterium, *Pyrococcus furiosus* (*37*). This structure revealed that the PIWI domain folds into a structure analogous to the catalytic domain of RNase H and avian sarcoma virus (ASV) integrase. The notion that such a domain would lie at the center of RISC cleavage is consistent with previous observations. RNase H and integrases cleave their substrates, leaving 5′ phosphate and 3′ hydroxyl groups through a metal-catalyzed cleavage reaction (*38*, *39*). Notably, previous studies have strongly indicated that the scissile phosphate in the targeted mRNA is cleaved via a metal ion in RISC to give the same phosphate polarity (*40*). Our in vitro data are consistent with the reconstituted RISC also requiring a divalent metal (fig. S8).

The active center of RNase H and its relatives consists of a catalytic triad of three carboxylate groups contributed by aspartic or glutamic acid (*38*, *39*). These amino acid residues coordinate the essential metal and activate water molecules for nucleolytic attack. Reference to the known structure of RNase H reveals two aspartate residues in the archeal Ago protein present at the precise spatial locations predicted for formation of an RNase H–like active site (*37*). These align with identical residues in the human Ago2 protein (fig. S9). Therefore, to test whether the PIWI domain of Ago2 provides catalytic activity to RISC, we changed the two conserved aspartates, D597 and D669, to alanine, with the prediction that either mutation would inactivate RISC cleavage. Consistent with our hypothesis, the mutant Ago2 proteins were incapable of assembling into a cleavage-competent RISC in vitro or in vivo, despite retaining the ability to bind siRNAs (Fig. 5, B to D).

Considered together, our data provide strong support for the notion that Argonaute proteins are the catalytic components of RISC. First, the ability to form an active enzyme is restricted to a single mammalian family member, Ago2. This conclusion is supported both by biochemical analysis and by genetic studies in mutant MEFs. Second, single amino acid substitutions within Ago2 that convert residues to those present in closely related proteins negate RISC cleavage. Third, the structure of the *P. furiosis* Argonaute protein reveals provocative structural similarities between the PIWI domain and the RNase H domains, providing a hypothesis for the method by which Argonaute cleaves its substrates. We tested this hypothesis by introducing mutations in the predicted Ago2 active site. It is extremely unlikely that such mutations could affect interactions with other proteins, because they are buried within a cleft of Ago.

Our studies indicate that the Argonaute proteins that are unable to form cleavage-competent RISC differ from Ago2 at key positions that do not include the putative metal-coordinating residues themselves. However, we cannot yet, based either on biochemical or structural studies, provide a precise explanation for the catalytic defects in these proteins. It is conceivable that Ago1 and Ago3 fail to coordinate the catalytic metal or that the structure of the active site is distorted sufficiently that a bound metal is unable to access the scissile phosphate. Alternatively, catalytic mechanisms with two metal ions have been proposed for RNase H (*38*, *39*), which leaves open the possibility that catalytically inert Ago family members might lack structures essential to bind the second metal ion.

The relationship between the nuclease domain in PIWI and conserved nuclease domains in viral reverse transcriptases, transposases, and viral integrases has potential evolutionary implications. In *Drosophila*, plants, and *C. elegans*, the RNAi pathway has a major role in controlling parasitic nucleic acids such as viruses and transposons (*41*–*43*). The fact that the RNAi machinery shares a core structural domain with viruses and transposons suggests that this nucleic acid immune system may have arisen in part by pirating components from the replication and movement machineries of the very elements that RNAi protects against. This hypothesis is made even more poignant by considering the role of RNA-dependent RNA polymerases in RNAi, their functional relationship to viral replicases, and the possibility that the siRNAs themselves might first have served as primers that enable such replicases to duplicate primordial genomes.

References and Notes

1. G. J. Hannon, *Nature* **418**, 244 (2002).
2. A. Fire *et al*., *Nature* **391**, 806 (1998).
3. G. Hutvagner, P. D. Zamore, *Curr. Opin. Genet. Dev.* **12**, 225 (2002).
4. A. Hamilton, O. Voinnet, L. Chappell, D. Baulcombe, *EMBO J.* **21**, 4671 (2002).
5. E. Bernstein, A. A. Caudy, S. M. Hammond, G. J. Hannon, *Nature* **409**, 363 (2001).
6. Y. Lee *et al*., *Nature* **425**, 415 (2003).
7. S. M. Hammond, E. Bernstein, D. Beach, G. J. Hannon, *Nature* **404**, 293 (2000).
8. M. F. Mette, W. Aufsatz, J. van der Winden, M. A. Matzke, A. J. Matzke, *EMBO J.* **19**, 5194 (2000).
9. I. M. Hall *et al*., *Science* **297**, 2232 (2002).
10. T. Volpe *et al*., *Science* **297**, 1833 (2002).
11. M. Pal-Bhadra, U. Bhadra, J. A. Birchler, *Mol. Cell* **9**, 315 (2002).
12. P. H. Olsen, V. Ambros, *Dev. Biol.* **216**, 671 (1999).
13. D. P. Bartel, *Cell* **116**, 281 (2004).
14. T. Tuschl, P. D. Zamore, R. Lehmann, D. P. Bartel, P. A. Sharp, *Genes Dev.* **13**, 3191 (1999).
15. P. D. Zamore, T. Tuschl, P. A. Sharp, D. P. Bartel, *Cell* **101**, 25 (2000).
16. S. M. Elbashir, J. Martinez, A. Patkaniowska, W. Lendeckel, T. Tuschl, *EMBO J.* **20**, 6877 (2001).
17. J. Martinez, A. Patkaniowska, H. Urlaub, R. Luhrmann, T. Tuschl, *Cell* **110**, 563 (2002).
18. G. Hutvagner, P. D. Zamore, *Science* **297**, 2056 (2002).
19. S. M. Hammond, S. Boettcher, A. A. Caudy, R. Kobayashi, G. J. Hannon, *Science* **293**, 1146 (2001).
20. M. A. Carmell, G. J. Hannon, *Nature Struct. Mol. Biol.* **11**, 214 (2004).
21. L. Cerutti, N. Mian, A. Bateman, *Trends Biochem. Sci.* **25**, 481 (2000).
22. H. Tabara *et al*., *Cell* **99**, 123 (1999).
23. M. A. Carmell, Z. Xuan, M. Q. Zhang, G. J. Hannon, *Genes Dev.* **16**, 2733 (2002).
24. A. A. Caudy, M. Myers, G. J. Hannon, S. M. Hammond, *Genes Dev.* **16**, 2491 (2002).
25. K. Okamura, A. Ishizuka, H. Siomi, M. C. Siomi, *Genes Dev.* **18**, 1655 (2004).
26. B. Zheng, A. A. Mills, A. Bradley, *Nucleic Acids Res.* **27**, 2354 (1999).
27. S. J. Conway, A. Kruzynska-Frejtag, P. L. Kneer, M. Machnicki, S. V. Koushik, *Genesis* **35**, 1 (2003).
28. W. Deng, H. Lin, *Dev. Cell* **2**, 819 (2002).
29. S. Kuramochi-Miyagawa *et al*., *Development* **131**, 839 (2004).
30. T. Sasaki, A. Shiohama, S. Minoshima, N. Shimizu, *Genomics* **82**, 323 (2003).
31. S. Yekta, I. H. Shih, D. P. Bartel, *Science* **304**, 594 (2004).
32. J. G. Doench, C. P. Petersen, P. A. Sharp, *Genes Dev.* **17**, 438 (2003).
33. M. Kiriakidou *et al*., *Genes Dev.* **18**, 1165 (2004).
34. A. Nykanen, B. Haley, P. D. Zamore, *Cell* **107**, 309 (2001).
35. J. W. Pham, J. L. Pellino, Y. S. Lee, R. W. Carthew, E. J. Sontheimer, *Cell* **117**, 83 (2004).
36. Y. Tomari *et al*., *Cell* **116**, 831 (2004).
37. J.-J. Song *et al.*, *Science* **305**, 1434 (2004). Published online 29 July 2004; 10.1126/science.1102514.
38. B. R. Chapados *et al*., *J. Mol. Biol.* **307**, 541 (2001).
39. W. Yang, T. A. Steitz, *Structure* **3**, 131 (1995).
40. D. S. Schwarz, Y. Tomari, P. D. Zamore, *Curr. Biol.* **14**, 787 (2004).
41. R. F. Ketting, T. H. Haverkamp, H. G. van Luenen, R. H. Plasterk, *Cell* **99**, 133 (1999).
42. T. Sijen, R. H. Plasterk, *Nature* **426**, 310 (2003).
43. E. Sarot, G. Payen-Groschene, A. Bucheton, A. Pelisson, *Genetics* **166**, 1313 (2004).
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