

# Distinct roles for Argonaute proteins in small RNA-directed RNA cleavage pathways

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In mammalian cells, both microRNAs (miRNAs) and small interfering RNAs (siRNAs) are thought to be loaded into the same RNA-induced silencing complex (RISC), where they guide mRNA degradation or translation silencing depending on the complementarity of the target. In *Drosophila*, Argonaute2 (AGO2) was identified as part of the RISC complex. Here we show that AGO2 is an essential component for siRNA-directed RNA interference (RNAi) response and is required for the unwinding of siRNA duplex and in consequence assembly of siRNA into RISC in *Drosophila* embryos. However, *Drosophila* embryos lacking AGO2, which are siRNA-directed RNAi-defective, are still capable of miRNA-directed target RNA cleavage. In contrast, Argonaute1 (AGO1), another Argonaute protein in fly, which is dispensable for siRNA-directed target RNA cleavage, is required for mature miRNA production that impacts on miRNA-directed RNA cleavage. The association of AGO1 with Dicer-1 and pre-miRNA also suggests that AGO1 is involved in miRNA biogenesis. Our findings show that distinct Argonaute proteins act at different steps of the small RNA silencing mechanism and suggest that there are inherent differences between siRNA-initiated RISCs and miRNA-initiated RISCs in *Drosophila*.

[*Keywords*: RNAi; siRNA; miRNA; AGO1; AGO2]

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Double-stranded (ds) RNA induces the sequence-specific posttranscriptional gene silencing of cognate genes in numerous organisms (Cogoni and Macino 2000; Hutvagner and Zamore 2002a; Denli and Hannon 2003). The multidomain ribonuclease III enzyme Dicer excises long dsRNA into duplexes of 21–23 nucleotides (nt) termed short interfering RNAs (siRNAs; Bernstein et al. 2001; Ketting et al. 2001; Knight and Bass 2001), which direct the cleavage of complementary mRNA targets, a process known as RNA interference (RNAi; Fire et al. 1998). Prior to target mRNA recognition, an siRNA duplex goes through an ATP-dependent unwinding process and one strand over the other is often preferentially loaded onto the RNA-induced silencing complex (RISC), the multiple-turnover enzyme complex that mediates endonucleolytic cleavage in the RNAi pathway. The RISC is guided to cleave target mRNAs sharing perfect complementarity across the center of the complementary siRNA strand in the absence of high-energy cofactors (Hammond et al. 2001; Nykanen et al. 2001; Hutvagner and Zamore 2002b; Martinez et al. 2002). siRNAs are not the only products of Dicer. Natural dsRNA-encoding genes, named microRNA (miRNA) genes, encode RNA

products of ~70 nt that are predicted to form imperfect hairpin structures and are processed by Dicer to mature 21–23-nt miRNAs (Grishok et al. 2001; Hutvagner et al. 2001). Only one Dicer enzyme is found in *Caenorhabditis elegans* and humans, therefore indicating that the same Dicer is required for both RNAi and for the processing of miRNA precursors in these organisms (Grishok et al. 2001; Hutvagner et al. 2001; Knight and Bass 2001). The expression of miRNAs is often developmentally regulated, suggesting an important role for miRNAs in the regulation of endogenous gene expression (Lagos-Quintana et al. 2001; Lau et al. 2001; Lee and Ambros 2001; Reinhart et al. 2002; Brennecke et al. 2003; Xu et al. 2003). Target mRNAs containing sequences imperfectly complementary to the miRNA can be subject to translational repression without altering mRNA stability (Lee et al. 1993; Wightman et al. 1993; Olsen and Ambros 1999; Reinhart et al. 2000; Bartel 2004).

Recent findings point to a tight connection between miRNA and RNAi molecular machineries. Both miRNAs and siRNAs have been shown to be capable of target mRNA degradation or translation silencing in mammalian cells and plants (Hutvagner and Zamore 2002b; Llave et al. 2002; Doench et al. 2003; Zeng et al. 2003); additionally, it has been shown that both siRNAs and miRNAs can be loaded onto the same RISC complex

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(Hutvagner and Zamore 2002b; Martinez et al. 2002). These findings imply that, regardless of the maturation process, once the small RNA is loaded, the RISC uses it to degrade or inhibit translation depending on the degree of complementarity between the small RNA and its mRNA target.

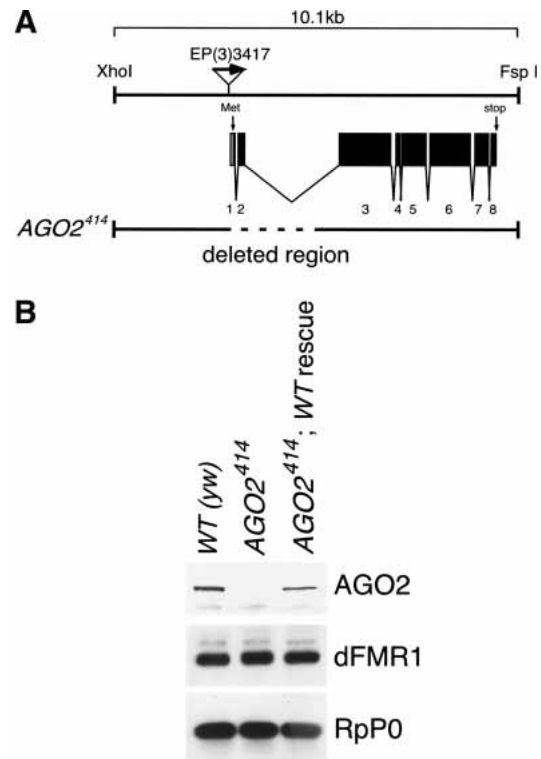
In *C. elegans*, genetic analyses suggested that RDE-1, a member of the Argonaute family of proteins (Carmell et al. 2002), is required for the initiation of RNAi with injected dsRNAs (Grishok et al. 2000), whereas Alg-1 and Alg-2, other Argonaute family members, are required for the accumulation of stable mature miRNAs in *C. elegans*, but not for RNAi driven by dsRNA (Grishok et al. 2001). These results suggest that distinct members of the Argonaute family of proteins may provide specificity to their respective pathways, RNAi and translation inhibition, in *C. elegans*. However, the underlying molecular mechanisms are not well understood.

In *Drosophila*, it has been shown that Argonaute2 (AGO2) protein, a member of the Argonaute family of proteins (Carmell et al. 2002), is essential for RNAi driven by exogenously introduced dsRNA and is the first protein component to be identified as part of the RISC complex in cultured *Drosophila* S2 cells (Hammond et al. 2001). Although AGO2 is known to associate with several proteins such as the *Drosophila* homolog (dFMR1) of the human fragile X mental retardation protein and VIG (Caudy et al. 2002; Ishizuka et al. 2002), the precise role that AGO2 plays in RNAi is not well understood. In this paper, we have produced AGO2 deletion mutant flies and found that embryos lacking AGO2 are siRNA-directed RNAi defective but are still capable of miRNA-directed target RNA cleavage. We also show that AGO2 mutant embryos are impaired in the assembly of siRNA into RISC. In contrast, Argonaute1 (AGO1), another Argonaute protein in fly (Kataoka et al. 2001), is dispensable for siRNA-directed RNA cleavage but is necessary for the accumulation of stable mature miRNAs, and thus impacts on miRNA-directed target RNA cleavage. Our findings suggest that distinct Argonaute proteins act at different steps of the small RNA silencing mechanism, and provide specificity to their respective pathways in *Drosophila*.

## Results

### *AGO2 is essential for RNAi in embryos*

The *Drosophila* AGO2 protein is an essential factor for RNAi as a component of the RISC complex in cultured S2 cells (Hammond et al. 2001). However, the precise role that AGO2 plays in RNAi is not well understood. To gain an insight into the molecular functions of AGO2 in RNAi, we produced fly strains that lack AGO2. To obtain fly strains bearing deletions in AGO2, we mobilized a P[EP]element (Rorth et al. 1998) inserted in the first exon of the AGO2 gene on the EP(3)3417 chromosome to produce several partial deletions of AGO2. One such mutation named *AGO2<sup>414</sup>* was selected for further characterization (Fig. 1A). Western blot analysis with anti-



**Figure 1.** Characterization of the AGO2 deletion mutant *AGO2<sup>414</sup>*. (A) Structure of the AGO2 locus. Exons are indicated with open boxes; the closed portion is the protein-coding region. The translation initiation start site (Met) is located in the first exon. The position of EP(3)3417 is represented as triangle with an arrow pointing in the direction of GAL4-induced transcription. The deleted genomic region of the *AGO2<sup>414</sup>* chromosome is shown as a dotted line. In this mutant, imprecise excision of the EP element generated a 2.3-kb deletion of genomic DNA, which included exons 1 and 2 of the AGO2 gene. Sequence information from the Berkeley Drosophila Genome Project reveals that the AGO2 locus is positioned in a small region on the cytological location 71E1, on the left arm of the third chromosome. (B) Western blotting on protein extracts from adult ovaries. Western blotting was carried out using anti-AGO2 (4D2), anti-dFMR1 (5A11), and antiribosomal protein P0 antibodies. [WT(yw)] Yellow-white wild type; [*AGO2<sup>414</sup>*] *AGO2<sup>414</sup>* homozygote; [*AGO2<sup>414</sup>; WTrescue*] *AGO2<sup>414</sup>* homozygote with two copies of wild-type rescue. The genomic rescue construct contained a 10.1-kb XhoI-FspI fragment of the AGO2 locus in A in which no other known genes are included.

AGO2 antibody revealed that there is no AGO2 protein in homozygous *AGO2<sup>414</sup>* flies (Fig. 1B). We also confirmed the absence of AGO2 mRNA by Northern blot analysis in homozygous *AGO2<sup>414</sup>* flies (data not shown). These results demonstrated that *AGO2<sup>414</sup>* are AGO2 null flies. We found that homozygous *AGO2<sup>414</sup>* flies proceed into adulthood, are fertile, and appear outwardly normal. Because it is not known whether AGO2 is required for RNAi in embryos, we tested for RNAi in vivo by assaying the ability of long dsRNA corresponding to the *fushi tarazu* (*ftz*) gene to produce a *ftz* phenotype (Kennerdell and Carthew 1998) when injected into wild-

type and *AGO2* mutant embryos. We used *AGO2* mutant adult females to make mutant eggs to remove the maternal contribution of *AGO2* (simply called the “*AGO2* mutant embryo” or *AGO2*<sup>414</sup> embryos, hereafter). Wild-type embryos injected with *ftz* dsRNA exhibit segmentation defects in their cuticle (Fig. 2B; Table 1). In contrast, *AGO2* mutant embryos showed a complete absence of interference in response to *ftz* dsRNA, indicating that they are RNAi defective (Fig. 2C). This is consistent with previous findings made with cultured S2 cells (Hammond et al. 2001; Caudy et al. 2002; Ishizuka et al. 2002). To confirm that disruption of the *AGO2* gene is directly responsible for the RNAi-defective phenotype, we transformed mutant flies with a P element containing wild-type *AGO2* genomic sequences (Fig. 1A,B). The RNAi-defective phenotype was ameliorated by the introduction of this *AGO2* minigene (Fig. 2D; Table 1), demonstrating that the RNAi-defective phenotype is caused by the *AGO2* mutation rather than a second site mutation elsewhere on the chromosome.

#### *AGO2* is required for the assembly of siRNA into RISC in embryos

To determine whether *AGO2* is required for the formation of the siRNA duplex or for interference thereafter, we injected synthetic *ftz* siRNA into *AGO2*<sup>414</sup> embryos. siRNA produced a *ftz* phenotype in wild-type embryos (Fig. 3A; Table 1). However, *AGO2*<sup>414</sup> embryos were not responsive to the *ftz* siRNA (Fig. 3B; Table 1). Similarly, in vitro, *AGO2*<sup>414</sup> embryo lysate processed long *ftz* dsRNA into short ~21-nt fragments, similar to lysates from wild-type embryos (Fig. 3C). These findings suggest that *AGO2* is necessary for RNAi after the formation of the siRNA duplex.

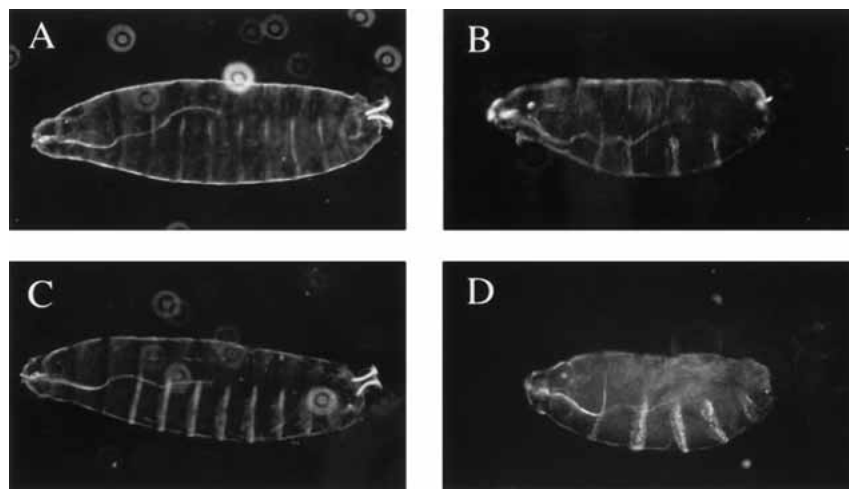
Before target mRNA recognition, the siRNA duplex is unwound (Nykanen et al. 2001), and each RISC contains only one of the two strands of the siRNA duplex (Martinez et al. 2002). We tested whether *AGO2* activity is necessary for this unwinding process. Unwound siRNA

**Table 1.** Effects of *ftz* dsRNA and *ftz* siRNA injected into *Drosophila* embryos

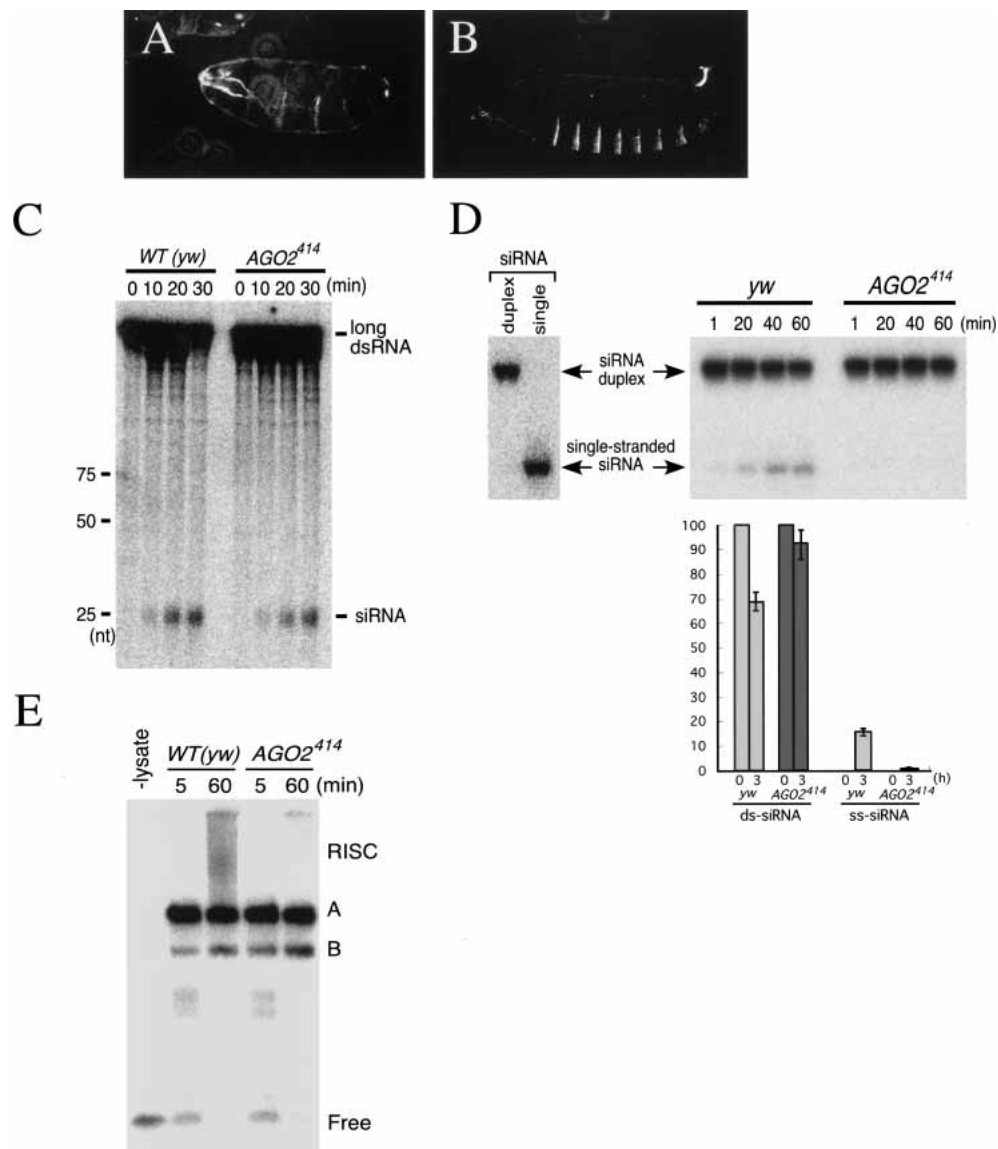
Genotype	Injected RNA	Wild type	<i>ftz</i>
<i>WT(yw)</i>	mock (water)	29	0 (0%)
<i>WT(yw)</i>	<i>ftz</i> dsRNA	5	30 (86%)
<i>AGO2</i> <sup>414</sup>	<i>ftz</i> dsRNA	43	0 (0%)
<i>P[w<sup>+</sup>: AGO2];AGO2</i> <sup>414</sup>	<i>ftz</i> dsRNA	1	22 (96%)
<i>WT(yw)</i>	<i>ftz</i> siRNA	2	30 (94%)
<i>AGO2</i> <sup>414</sup>	<i>ftz</i> siRNA	21	0 (0%)

Numbers indicate embryos with wild-type or *ftz* phenotype after injection. Percentages are given in parentheses. Embryos were scored as *ftz* if they had six or fewer ventral denticle belts. *P[w<sup>+</sup>: AGO2];AGO2*<sup>414</sup> is the genotype of “rescued” flies (*AGO2*<sup>414</sup>; *WT* rescue).

was detected in wild-type embryo lysate, whereas no such activity was detected in *AGO2*<sup>414</sup> embryo lysate (Fig. 3D), suggesting that *AGO2* is required for the unwinding of the siRNA duplex. An alternative explanation of the results could be that the absence of *AGO2* leaves the unwound single-stranded RNA exposed to nucleolytic degradation and therefore the single-stranded RNA could not be detected. However, this is unlikely because the amount of input siRNA duplex left in *AGO2*<sup>414</sup> embryo lysate was not significantly reduced even after 3 h incubation as opposed to that seen in wild-type embryo lysate (Fig. 3D, lower panel), indicating that input siRNA duplex is indeed not unwound in *AGO2* mutant embryo lysate. Using a recently developed RISC assembly assay (Tomari et al. 2004), we next analyzed RISC formation in *AGO2* mutant embryo lysates. ds-siRNA was incubated with embryo lysates in a standard RNAi reaction, and then RISC complexes were resolved by electrophoresis through a native gel. As shown in Figure 3E, three major complexes (B, A, and RISC) were detected in wild-type embryo lysates as reported previously (Tomari et al. 2004). Complexes B and A are thought to be intermediate precursors to active RISC and



**Figure 2.** RNA interference of *ftz* activity in embryos. Representative embryonic phenotypes of wild-type and *AGO2*<sup>414</sup> mutant embryos injected with *ftz* dsRNA are shown. (A) A wild-type embryo injected with water. (B) A wild-type embryo injected with *ftz* dsRNA has a phenotype similar to that of *ftz*. (C) An *AGO2*<sup>414</sup> mutant embryo injected with *ftz* dsRNA has a wild-type phenotype. (D) An *AGO2*<sup>414</sup>; *WT* rescue embryo has a phenotype similar to that of *ftz*.



**Figure 3.** AGO2 is required for interference after the formation of the siRNA duplex. (A) Wild-type embryo injected with synthetic *ftz* siRNA has a phenotype similar to *ftz*. (B) *AGO2*<sup>414</sup> embryo injected with synthetic *ftz* siRNA has a wild-type phenotype. (C) AGO2 is not required for the production of the siRNA duplex. Uniformly <sup>32</sup>P-labeled dsRNA corresponding to the *ftz* gene, incubated in extracts from *yw* and *AGO2*<sup>414</sup> embryos. RNA products of the reaction were analyzed on a polyacrylamide gel. Incubation of lysates with labeled dsRNA generates RNA fragments ~22 nucleotides (nt) long. [WT(*yw*)] Yellow-white wild type; [*AGO2*<sup>414</sup>] *AGO2*<sup>414</sup> homozygote. (D) Analysis of siRNA unwinding in *AGO2*<sup>414</sup> embryo lysate. The siRNA duplex was incubated in the extracts from wild-type (*yw*) and *AGO2*<sup>414</sup> embryos. RNA products of the reaction were analyzed on a native acrylamide gel. The migration patterns of the siRNA duplex, the starting material, and the single-stranded siRNA, the expected product after unwinding, are shown on the left. (Lower panel) The amounts of input siRNA duplex and unwound single-stranded (ss) siRNA at 0 and 3 h incubation are shown. The average of five independent experiments is shown here. (E) Native gel analysis of labeled siRNA-protein complexes from wild-type and *AGO2*<sup>414</sup> embryo lysates. B and A complexes are observed in both lanes from wild-type and *AGO2*<sup>414</sup> reactions. These two complexes correspond to intermediate precursors to RISC (Tomari et al. 2004). The complex corresponding to RISC (Tomari et al. 2004) is not detected in the lane from an *AGO2*<sup>414</sup> reaction.

contain ds-siRNA (Tomari et al. 2004). Both complexes B and A were readily detected in *AGO2* mutant embryo lysates. However, RISC formation was impaired in *AGO2* mutant embryo lysates (Fig. 3E). Together, these results suggest that AGO2 is required in a step(s) in RISC assembly after binding of the siRNA duplex to RISC precursors (complexes B and A).

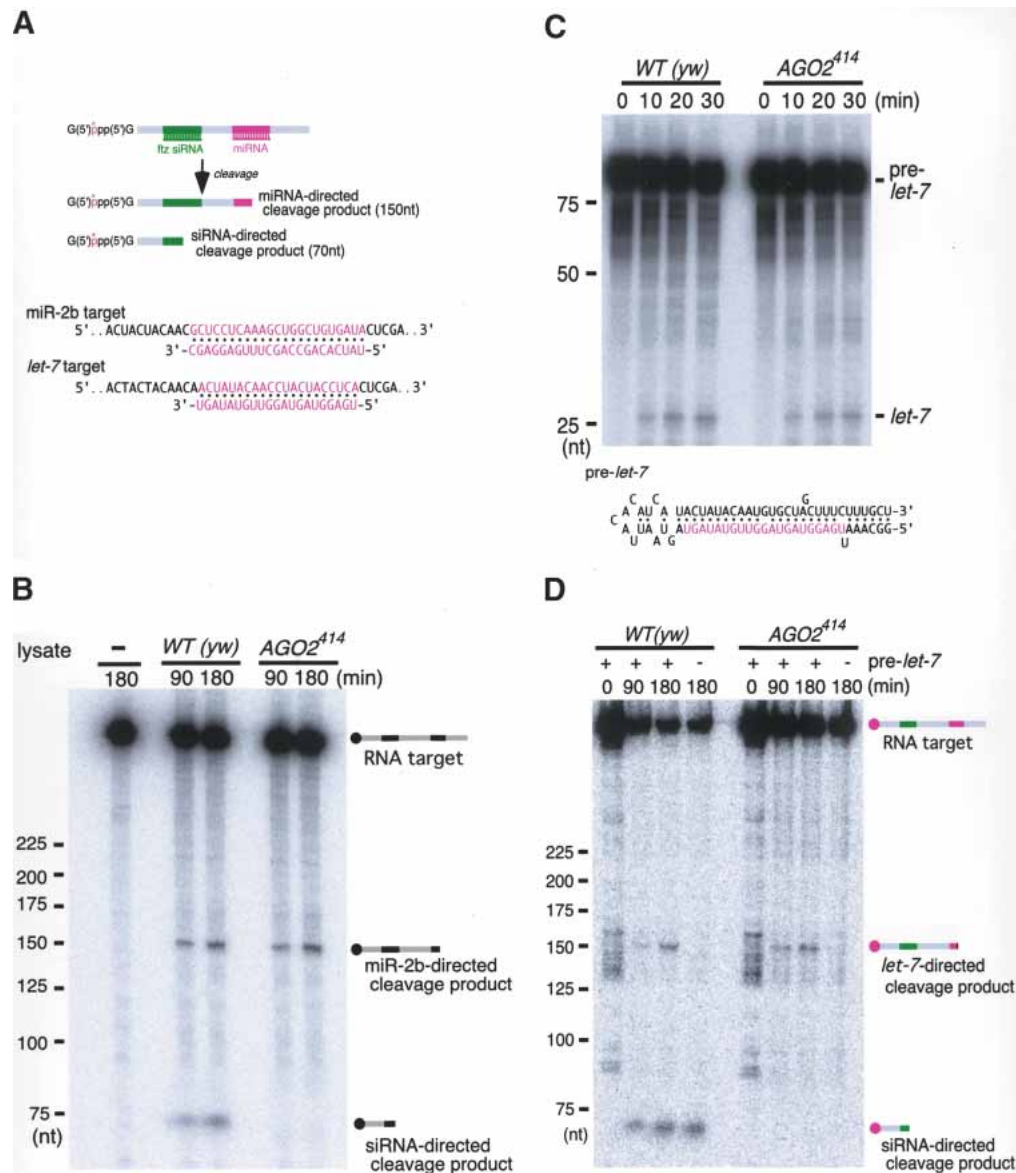
*miRNA-initiated RNA cleavage does not need AGO2 but siRNA-initiated RNA cleavage does*

Recently, it has been demonstrated that both siRNAs and miRNAs are associated with the same RISC and that miRNAs can even direct cleavage of perfectly base-paired substrates in human cells (Hutvagner and Zamore



2002b; Martinez et al. 2002). To see if this is also the case in *Drosophila*, we investigated whether the lack of AGO2 affects the miRNA-mediated cleavage of target RNAs.  $^{32}$ P-radiolabeled target mRNA, containing a sequence fully complementary to an endogenous miRNA, miR-2b, as well as a sequence exactly complementary to one strand of a *ftz* siRNA duplex (Fig. 4A), was incubated with *AGO2*<sup>414</sup> embryo lysate in an in

vitro RNAi reaction. Both siRNA-directed and miRNA-directed cleavage products are seen in the wild-type lysate (Fig. 4B) as shown previously (Schwarz et al. 2002). However, *ftz* siRNA did not direct cleavage in *AGO2*<sup>414</sup> embryo lysate (Fig. 4B); in contrast, miR-2b still directed cleavage of the target RNA in *AGO2*<sup>414</sup> embryo lysates (Fig. 4B). These results demonstrate that although AGO2 is an essential factor in siRNA-directed RNAi,



**Figure 4.** AGO2 is not required for miRNA-initiated RNA cleavage. (A) Schematic drawing of the target RNA and its pairing with *ftz* siRNA and miRNA (miR-2b or *let-7*). (Magenta) miR-2b, or *let-7* and their complementary sequences (below); (green) *ftz* siRNA and its complementary sequence. An asterisk indicates the 5'- $^{32}$ P radiolabel. (B) miRNA-directed target RNA cleavage is catalyzed by embryo lysates with and without AGO2. In vitro RNAi assays were carried out with 14–16-h embryo lysates of *yw* and *AGO2*<sup>414</sup>. Target RNA contains the *ftz* siRNA and the miR-2b target sequences as shown in A. (C) In vitro processing of pre-*let-7* RNAs internally labeled with  $\alpha$ - $^{32}$ P-GTP. Mature *let-7*-generating activities were compared between lysates from 0- to 2-h *yw* and *AGO2*<sup>414</sup> embryos. Both synthetic 21-nt *let-7* RNA (not shown) and in vitro processed *let-7* migrate at ~25 nt relative to the markers. The discrepancy between molecular mass and mobility in polyacrylamide gel electrophoresis is frequently observed for small RNAs (Hutvagner et al. 2001; Lee et al. 2003). (D) In vitro RNAi assays with lysates prepared from 0- to 2-h *yw* and *AGO2*<sup>414</sup> embryos. The RNA target contains *let-7* and *ftz* siRNA target sequences as shown in A.

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AGO2 is dispensable for miRNA-directed RNA cleavage in RNAi.

In this experiment, however, the timings of miRNA loading and siRNA loading into RISC are different. miR-2b is already incorporated into the RISC by the embryo before the lysate is made, whereas siRNA is added to the reaction mixture. We therefore added synthetic *Drosophila melanogaster let-7* precursor RNA to an in vitro RNAi reaction mixture containing the *let-7* complementary target RNA. It should be noted that the sequence of the synthetic *let-7* precursor used here (Fig. 4C, bottom panel) is slightly longer than that of naturally processed pre-*let-7*, which contains short 3' overhangs (Basyuk et al. 2003; Lee et al. 2003). However, the synthetic *let-7* precursor has been previously shown to be processed and produce mature *let-7* with precisely the same 5' end as authentic *let-7* in the *Drosophila* embryo lysates (Hutvagner et al. 2001). In this experiment, the synthetic *let-7* precursor RNA was also converted to mature *let-7* in vitro, not only in the wild-type embryo lysate, but also in *AGO2<sup>414</sup>* embryo lysate (Fig. 4C; data not shown). In the in vitro RNAi assay, target RNA was cleaved within the *let-7* complementary sequence in *AGO2<sup>414</sup>* embryo lysate as efficiently as in wild type (Fig. 4D). Thus, miRNA that is not preloaded by the embryo is still capable of entering into the RNAi pathway without AGO2 activity. These results demonstrate that AGO2 is not required for miRNA production or the loading of miRNAs into the RISC and suggest that AGO2 has a specific role for siRNA activity, likely as a specific unwinding and loading factor of siRNA into RISC as shown earlier, and possibly as a necessary component of an siRNA-initiated RISC.

#### *AGO1 is necessary for efficient miRNA-directed RNA cleavage*

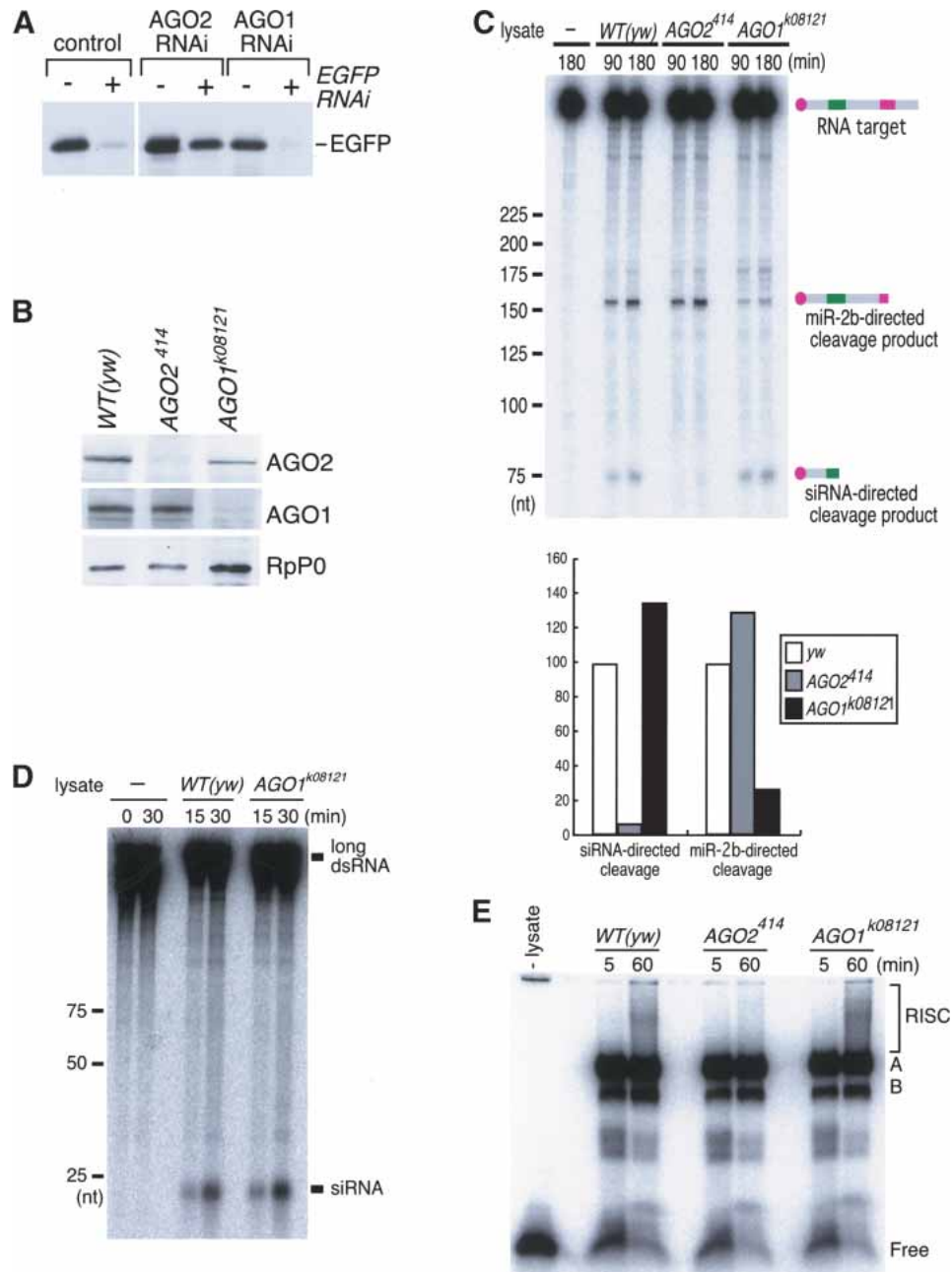
We next tested whether siRNA- and miRNA-directed RNA cleavage pathways have differential requirements for AGO1. Mutations in *AGO1* result in late embryonic/early larval lethality with developmental defects (Kataoka et al. 2001). Although AGO1 has been shown necessary for efficient RNAi in embryos (Williams and Rubin 2002), AGO1 appears to have little or no effect on the efficiency of RNAi in cultured S2 cells (Fig. 5A; see also Caudy et al. 2002). *AGO1<sup>k08121</sup>* is a strong allele (Kataoka et al. 2001). *AGO1<sup>k08121</sup>* was balanced over a *CyO* chromosome carrying a *kruppel (Kr)-Gal4* and a *UAS-GFP* transgene. Homozygous embryos were separated from heterozygous and *CyO-Kr-Gal4-UAS-GFP* homozygous embryos on the basis of GFP expression. Because there is strong reduction of *AGO1* transcripts in *AGO1<sup>k08121</sup>* embryos (Kataoka et al. 2001; data not shown) and AGO1 protein was not detected in lysate from 14- to 16-h staged *AGO1<sup>k08121</sup>* embryos (Fig. 5B), we tested 14–16-h staged *AGO1<sup>k08121</sup>* embryo lysate for its ability to direct cleavage of siRNA or miRNA complementary RNA targets. *ftz* siRNA directed its cleavage in *AGO1<sup>k08121</sup>* embryo lysate as effectively as in wild type (Fig. 5C). *AGO1<sup>k08121</sup>* embryo lysate also

processed long *ftz* dsRNA into short ~21-nt fragments (Fig. 5D). Furthermore, RISC formation directed by siRNA duplex was not impaired in *AGO1<sup>k08121</sup>* embryo lysate as judged by the native gel shift assay (Fig. 5E). These findings suggest that AGO1 is not necessary for the production of the siRNA duplex, siRNA-initiated RISC formation, or siRNA-directed cleavage. However, cleavage of the target RNA directed by miR-2b was suppressed in *AGO1<sup>k08121</sup>* embryo lysate by a factor of five-fold compared with that in wild type (Fig. 5C), suggesting that AGO1 is necessary for efficient target RNA cleavage mediated by miRNAs. Residual miRNA activity seen in *AGO1<sup>k08121</sup>* could be due to some degree of functional complementation among Argonaute family proteins expressed in embryos (Williams and Rubin 2002). Our in vitro experiments with *AGO1<sup>k08121</sup>* mutant embryo lysates argue against AGO1 being required for siRNA-mediated RNAi in embryos when injected with either long dsRNA or siRNA (Williams and Rubin 2002). Although we currently do not understand the discrepancy, our findings are at least consistent with the findings using cultured S2 cells (Fig. 5A; Caudy et al. 2002).

#### *AGO1 is involved in the stable maturation of miRNAs*

We hypothesized that AGO1 might be involved in the maturation and/or function of miRNAs. To directly determine the contribution of AGO1 and AGO2 to miRNA production in *Drosophila* cells, we depleted each from S2 cells by dsRNA soaking (Fig. 6A) and performed Northern blot analyses to monitor the abundance of *bantam* miRNA (miR-ban; Brennecke et al. 2003). A marked reduction of mature miR-ban was observed in AGO1-depleted S2 cells but not in AGO2-depleted S2 cells (Fig. 6A). We also confirmed that the miRNA level was significantly reduced in the *AGO1<sup>k08121</sup>* embryos (Fig. 6B). The accumulation of pre-miR-ban was not observed in both cases (data not shown; see also Fig. 6C). Similar results were obtained when the same RNA preparations were probed for the expression of another miRNA, miR-2b, which is also known to be expressed both in S2 cells and embryos (data not shown). These results suggest that AGO1 is involved in the maturation and/or stability of miRNAs.

Production of both siRNAs and miRNAs require Dicers, which interact with RISC components (Hammond et al. 2001; Tabara et al. 2002), suggesting that Dicer action is coupled to loading small RNAs onto the RISC. Two Dicers, Dicer-1 and Dicer-2, have been identified in *Drosophila*. siRNA production is associated with Dicer-2, but not Dicer-1 (Liu et al. 2003). Dicer-2, together with the dsRNA-binding protein R2D2, facilitates siRNA loading onto RISC (Liu et al. 2003). We found that the targeted destruction of only one of the two Dicers, Dicer-1, leads to an accumulation of the miR-ban precursor in S2 cells (Fig. 6C), consistent with the recent genetic studies showing that Dicer-1 is required for miRNA processing (Lee et al. 2004). The amount of pre-miR-ban was further increased after pro-

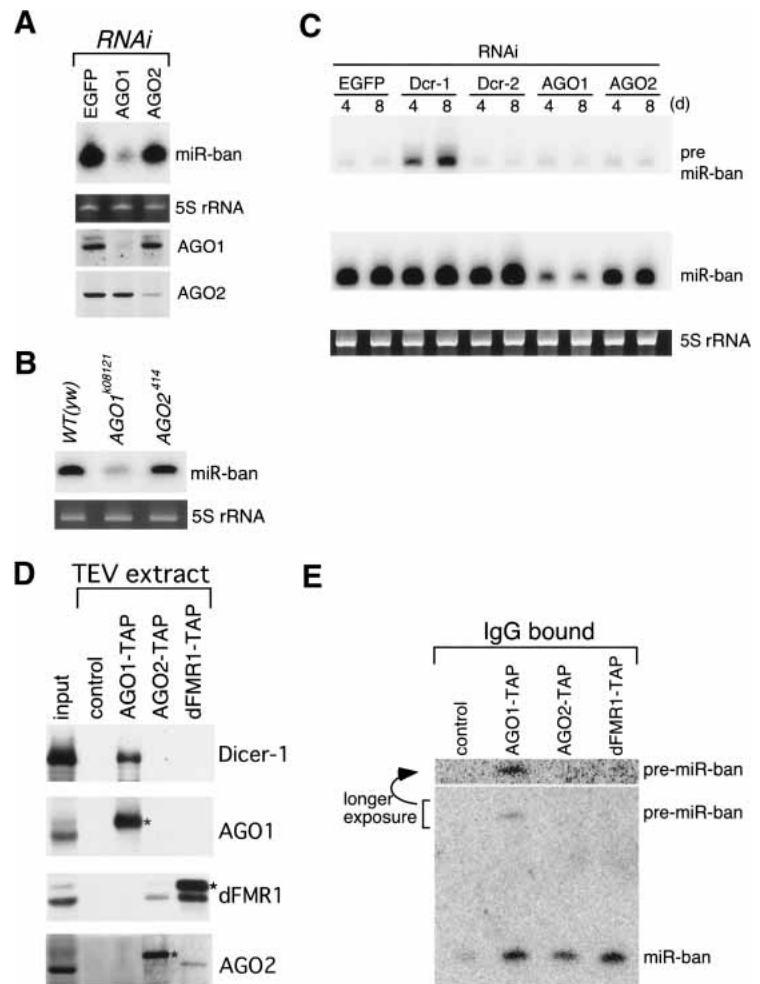


**Figure 5.** AGO1 is required for efficient miRNA-initiated RNA cleavage. (A) When AGO2 is suppressed by introducing specific dsRNA in S2 cells expressing EGFP, the ability of the cells to silence EGFP by RNAi is severely reduced. In contrast, when AGO1 is suppressed, the EGFP silencing effect is unaffected, indicating that AGO1 is not essential for the RNAi pathway in S2 cells. (B) Lysates were prepared from 14- to 16-h *yw*, *AGO2<sup>414</sup>* mutant, and *AGO1<sup>k08121</sup>* mutant embryos. Western blots were performed using the antibodies against AGO2 (upper), AGO1 (middle), and ribosomal protein P0 (lower) as a loading control. (C) In vitro RNAi assays with lysates prepared from 14- to 16-h *yw*, *AGO2<sup>414</sup>*, and *AGO1<sup>k08121</sup>* embryos. The RNA target was the same as that used in Figure 4B. The amount of specifically cleaved bands was quantified and normalized to that of bands in wild-type embryos. (D) AGO1 is not required for the production of the siRNA duplex. Uniformly <sup>32</sup>P-labeled dsRNA corresponding to the *ftz* gene, incubated in extracts from *yw* and *AGO1<sup>k08121</sup>* embryos. RNA products of the reaction were analyzed on a polyacrylamide gel. Incubation of lysates with labeled dsRNA generates RNA fragments ~22 nt long. [WT(*yw*)] Yellow-white wild type; (*AGO1<sup>k08121</sup>*) *AGO1<sup>k08121</sup>* embryos. (E) RISC formation is not impaired in *AGO1<sup>k08121</sup>* embryo lysates.

longed Dicer-1 knockdown for 8 d. In contrast, prolonged suppression of AGO1 expression did not result in the accumulation of pre-miR-ban (Fig. 6C). These findings suggest that mature miRNAs are processed from pre-

miRNAs by Dicer-1 and are stabilized by AGO1. We next asked whether AGO1 is physically associated with Dicer-1 by purifying AGO1-associated complexes from S2 cells using a TAP method (Rigaut et al. 1999). It was

**Figure 6.** AGO1 is involved in miRNA biogenesis. (A) AGO1 is required for stable, mature miRNA production. Expression of AGO1, AGO2, or EGFP (control) was suppressed by RNAi in S2 cells. Western blots (lower panels) show that the Argonaute proteins are indeed down-regulated. RNAs were obtained from each cell and subjected to Northern blots to analyze the amounts of miR-ban using a specific probe. (B) The miRNA level in the wild-type, *AGO1*, and *AGO2* mutant embryos. Northern blot analysis on the *AGO1* mutant (*AGO1<sup>k08121</sup>*) shows a marked reduction of mature miR-ban. (C) Dicer-1 and AGO1 are related to miRNA processing and stabilization, respectively. Expression of either Dicer-1 (*Dcr-1*), Dicer-2 (*Dcr-2*), *AGO1*, *AGO2*, or EGFP (control) was reduced by RNAi in S2 cells for indicated days, then total RNAs were obtained from each cell and subjected to Northern blots to visualize mature and pre-miR-ban. (D) AGO1 associates with Dicer-1. TEV extract prepared from S2 cells expressing AGO1-TAP, AGO2-TAP, or dFMR1-TAP, or the parental S2 cells (control), were subjected to Western blots using antibodies against Dicer-1, AGO1, dFMR1, and AGO2. Protein bands with asterisks indicate AGO1, AGO2, and dFMR1 with a CBP tag, a converted form of a TAP tag after TEV cleavage. (E) AGO1 associates with both pre-miRNA and mature miRNA. Northern blots show that all AGO1-TAP, AGO2-TAP, and dFMR1-TAP complexes contain mature miR-ban, but the precursor is associated only with the AGO1-TAP complex.



found that AGO1 was associated with Dicer-1 (Fig. 6D). This interaction of AGO1 with Dicer-1 seems to be quite specific for AGO1 because Dicer-1 was not detected in AGO2- and in dFMR1-associated complexes under the conditions used for the purification (Fig. 6D). RNA preparations from AGO1-, AGO2-, and dFMR1-associated complexes were then probed for the presence of miR-ban. AGO2- and dFMR1-associated complexes contained the mature form of miR-ban (Fig. 6E). Only the AGO1-associated complex contained the pre-miR-ban and mature forms (Fig. 6E). The interaction of AGO1 with Dicer-1 and pre-miRNA further suggests that AGO1 is involved in miRNA biogenesis.

## Discussion

In mammals, there appear to be no real distinctions between the siRNA and miRNA pathways downstream of Dicer (Hutvagner and Zamore 2002b). In contrast, genetic studies in *C. elegans* have shown that distinct Argonaute homologs appear to be dedicated to distinct RNAi and translation repression pathways (Grishok et al. 2001). Using target RNA cleavage assays, we show that siRNA-directed RNA cleavage depends on AGO2

but does not require AGO1, whereas miRNA-directed RNA cleavage depends on AGO1 but does not need AGO2 in *Drosophila* embryos. Our results suggest that maturation and the function of siRNAs and miRNAs have differential requirements for Argonaute proteins in *Drosophila*. These findings also suggest that Argonaute proteins regulate entry points of small RNAs to RISC but may not act as determinants for target RNA cleavage or translation repression. AGO2 is an essential component of RNAi and part of the RISC complex (Hammond et al. 2001). We show that the particular function of AGO2 lies downstream of siRNA duplex production in the RNAi pathway (Fig. 3). It has been shown that duplex siRNAs are incorporated in RISC precursors and ATP-dependent unwinding of siRNAs converts RISC precursors into active RISC that then degrades the specific target mRNAs (Hammond et al. 2000; Nykanen et al. 2001; Tomari et al. 2004). Thus, AGO2 is thought to function at some or all of these steps in RNAi. In this study, we demonstrate that both the unwinding of siRNA duplex and the assembly of siRNA into functional RISC are impaired in *AGO2* mutant embryos (Fig. 3). Therefore, these results indicate that AGO2 functions at a step(s) in RISC assembly after binding of the siRNA duplex to



RISC precursors. Recently, Liu et al. (2003) demonstrated that Dicer-2 not only associates with siRNA production, but also, together with R2D2, facilitates siRNA loading onto RISC in *Drosophila*. These data suggest that AGO2, together with Dicer-2 and R2D2, assembles siRNA into functional RISC in *Drosophila* embryos.

AGO2<sup>414</sup> flies that we produced are developmentally normal, which provides circumstantial evidence that AGO2 is not important for development and by inference not essential for utilization and function of miRNA that often regulates the developmental pathways (Bartel 2004). In contrast, our findings that AGO1 is required for the stable production of mature miRNAs might explain the fact that AGO1 is essential for normal development, particularly in the nervous system (Kataoka et al. 2001). The physical association of AGO1 with Dicer-1 and pre-miRNA suggests that AGO1 is involved in miRNA biogenesis. In fact, recent genetic studies of *Dicers* in *Drosophila* have shown that mutations in *Dicer-1* block processing of miRNA precursors (Lee et al. 2004). Together, these results suggest that in *Drosophila*, distinct pathways exist for siRNA and miRNA production and their concomitant assembly into RISC complexes. Do AGO1 and AGO2 functional specificities reflect physically distinct miRNA-associated RISC and siRNA-associated RISC, or differential processing and/or loading of small RNAs into generic RISC? Although there is no definitive evidence that there is a generic RISC for both small RNAs, recent studies have shown that the RISC containing active miRNAs and the RISC involved in siRNA-directed RNAi are very similar, if not identical, as endogenous miRNAs can cleave mRNAs with perfect complementarity (Hutvagner and Zamore 2002b; Llave et al. 2002), and exogenously introduced siRNAs can translationally repress mRNAs bearing imperfectly complementary binding sites (Doench et al. 2003; Zeng et al. 2003). Our findings show that the miRNA-associated RISC that cleaves RNA does not need AGO2, whereas siRNA-associated RISC does. This argues that there are inherent differences between siRNA-initiated RISC and miRNA-initiated RISC in *Drosophila*. Physically, they might be the same generic complex (for instance, both containing AGO2) but one does not need AGO2 activity. It is also possible that they might be physically distinct with respect to Argonaute proteins. AGO1 is not present in AGO2-associated complexes (Fig. 6D) and can be biochemically separated from siRNA-loaded RISCs (Caudy et al. 2002). Therefore, AGO1 and AGO2, conceivably, are not incorporated into the same RISC. However, miRNAs and siRNAs are found, to some extent, in both AGO1 and AGO2 complexes (Fig. 6E; see also Caudy et al. 2002), suggesting that association of Argonaute proteins with small RNAs is preferential rather than absolutely specific. Alternatively, a fraction of small RNAs might be exchangeable between the two complexes, probably during recycling of their silencing function.

The recent study has shown that miRNA-associated RISCs and siRNA-associated RISC are different with respect to *Dicers* in *Drosophila* (Lee et al. 2004). Our re-

sults clearly suggest that in conjunction with *Dicers*, Argonaute proteins regulate the formation of siRNA-associated RISC or miRNA-associated RISC, as their particular functions lie downstream of *Dicers* in a step(s) in the assembly of small RNAs into RISC.

## Materials and methods

### *Drosophila* strains

Flies were maintained under standard procedures. P element insertion in AGO2 EP{3}3417 was out-crossed to *yw* to clean up the genetic background. AGO1<sup>k08121</sup> (Kataoka et al. 2001) was balanced over a *CyO* chromosome carrying a *Kruppel*-Gal4 and a UAS-GFP transgene (*CyO*-*Kr*-Gal4-UAS-GFP chromosome).

### Generation of AGO2 mutants

EP{3}3417 contains the EP transposon (Rorth et al. 1998) in the 5' UTR region of the AGO2 locus. To isolate new AGO2 alleles, we generated 400 independent lines from the cross of virgin EP{3}3417 females with males possessing  $\Delta 2-3$  transposase. Genomic DNA from these lines were prepared and used as PCR templates to screen lines that carried a deletion within the AGO2 locus. The breakpoints of the deletion were determined by sequencing the PCR products. AGO1<sup>k08121</sup> was maintained as a heterozygous stock balanced over a *CyO*-*Kr*-Gal4-UAS-GFP chromosome. One-quarter of the embryos produced in this stock were AGO1<sup>k08121</sup> homozygous mutants, as identified by the absence of zygotic GFP expression. The remaining three-quarters expressed GFP and represented both heterozygous and *CyO*-*Kr*-Gal4-UAS-GFP homozygous embryos.

### RNAi in embryos

For dsRNA production, a region of the *fushi tarazu* (*ftz*) gene was amplified with oligonucleotides 5'-ATCCGATATGGC CACCAC-3' and 5'-CGCTCTGGGGAAGAGAGTAA-3' by using an embryonic cDNA pool as a template. The PCR product was cloned into the pGEM-T vector (pGEM-T-*ftz*) and the insert was amplified with T7 and SP6 primers. The resulting PCR product was used as a template for in vitro transcription reactions using MEGAScript T7 and SP6 kits (Ambion) according to the manufacturer's instructions. Equimolar concentrations of sense and antisense RNAs were boiled and allowed to anneal >12 h at room temperature. siRNA (21 bp) with 2 base 3' overhangs corresponding to the *ftz* gene (siFTZ 5'-UGCCUACUAU CAGAACACC-3') was purchased from Dharmacon. The siRNA was 5'-phosphorylated with T4 polynucleotide kinase (PNK; TaKaRa). dsRNA or siRNA (1 mg/mL in H<sub>2</sub>O) was injected into syncytial blastoderm embryos (Kennerdell and Carthew 1998). After 20–24 h at room temperature, the surviving embryos were scored for the number of ventral denticle belts. For consistency, any embryos with four, five, or six belts were scored as *ftz*. For cuticle analysis, embryos were incubated at room temperature under oil for 20–24 h. After dissection from their vitelline membranes, embryos were washed and fixed, and their cuticles were prepared as described (Kennerdell and Carthew 1998).

### RNAi in S2 cells

dsRNAs (~700 bp long) of EGFP, AGO2 and AGO1, Dicer-1, and Dicer-2 were produced by in vitro T7 transcription, denatured

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by heating, and reannealed in water. The dsRNA of AGO1 or AGO2 was introduced into S2 cells expressing EGFP by soaking (Clemens et al. 2000). After 3 d, cells were harvested and divided into half. To one half, AGO1 or AGO2 dsRNA was again introduced. To the other half, AGO1 or AGO2 dsRNA plus EGFP dsRNA were introduced into the cells. All cell pools were incubated for another 4 d and subjected to Western blot analyses using anti-EGFP antibody. In Figure 6, to start RNAi for AGO1, AGO2, or EGFP in S2 cells expressing EGFP, dsRNA for each gene was introduced into cells by soaking. After 4 d incubation or after double transfection of siRNA or dsRNA and prolonged incubation for 8 d, total RNAs were prepared from each sample and Northern blotting was performed using a bantam antisense oligo labeled with gamma-<sup>32</sup>P-ATP.

#### *In vitro* RNAi assays

To prepare *ftz* RNA with miR-2b or *let-7* target site, we amplified a region of the *ftz* gene from pGEM-T-*ftz* with the following primer pairs: 5'-ACAACATGCATCACCCACAGCCT-3' and 5'-AGAGCTCGAGTATCACAGCCAGCTTTGAGGAGCGTTGTAGTAGTAGCAGCTCTCCGAG-3' (for the miR-2b target) or 5'-AGAGCTCGAGTGAGGTAGTAGTTGTATAGTTGTTGTAGTAGTAGCAGCTCTCCGAG-3' (for the *let-7* target). The resulting PCR products were cloned into the NsiI-SalI site of pGEM-T-*ftz* as an NsiI-XhoI fragment (pGEM-T-*ftz*-2b or pGEM-T-*ftz*-*let-7*). The target RNAs were transcribed with a MEGAScript SP6 kit from PCR templates prepared with SP6 and 3' (5'-CGCCGGGTGATGTATCTATT-3') primers and pGEM-T-*ftz*-2b or pGEM-T-*ftz*-*let-7*. RNA was radiolabeled at the 5'-G cap by guanylyltransferase (Ambion). In a 20- $\mu$ L reaction, 500 ng RNA, 40 U RNasin (Promega), 0.2 U inorganic pyrophosphatase (Sigma), and 5  $\mu$ L of  $\alpha$ -<sup>32</sup>P GTP (800 mCi/mMole) were incubated in 50 mM Tris-HCl (pH 7.9), 12.5 mM MgCl<sub>2</sub>, 2 mM EDTA, 60 mM KCl, 1 mg/mL BSA, and 25 mM DTT. The reaction mixture was incubated for 2 h at 37°C, and the radiolabeled RNA was then PAGE-gel purified. Pre-*let-7* RNA was transcribed from a synthetic deoxynucleotide template with T7 RNA polymerase in the presence or absence of  $\alpha$ -<sup>32</sup>P-GTP (4). Extract used for *in vitro* RNAi assays were prepared as described in Tuschl et al. (1999) and Hutvagner and Zamore (2002b) with some modifications. In short, embryos were collected for 2 h and immediately homogenized (0–2-h embryo extract), or then aged for 14 h at room temperature and homogenized (14–16-h embryo extract). Homozygous *AGO1*<sup>k08121</sup> embryos were sorted from their *AGO1*<sup>k08121</sup>/*CyO*-*Kr*-Gal4-UAS-GFP and *CyO*-*Kr*-Gal4-UAS-GFP/*CyO*-*Kr*-Gal4-UAS-GFP siblings under fluorescent microscopy. Approximately 100 mg embryos were homogenized in 100  $\mu$ L of lysis buffer (100 mM potassium acetate, 30 mM HEPES at pH 7.4, 2 mM magnesium acetate, 5 mM DTT, and 0.5 mg/mL Pefabloc SC), centrifuged for 25 min at 14,600  $\times$  g at 4°C, and flash-frozen in 10- $\mu$ L aliquots. Total protein concentration in the extracts was determined with a colorimetric Bio-Rad assay. Cleavage of dsRNA or processing of pre-*let-7* was assayed by incubating 1000 cpm of labeled dsRNA or pre-*let-7* in a 10- $\mu$ L reaction containing 15  $\mu$ g of extract proteins for increasing times at room temperature. Reactions were stopped by the addition of stop buffer (100 mM Tris-HCl at pH 7.9, 50 mM NaCl, 50 mM EDTA, 1% SDS, and 100  $\mu$ g/mL proteinase K). For miR-2b target RNA cleavage reactions, 100 nM *ftz* siRNA duplex was incubated with 2000 cpm of cap-labeled miR-2b target RNA in 14–16-h (Figs. 4, 5) embryo lysate under standard conditions for 90 or 180 min at room temperature. For *let-7* target RNA cleavage reactions, 100nM *ftz* siRNA duplex and 100 nM *in vitro* transcribed pre-*let-7* RNA were incubated with 2000 cpm of cap-

labeled *let-7* target RNA in 0–2-h embryo lysate under standard conditions for 90 or 180 min at room temperature.

#### *siRNA unwinding assay*

siRNA unwinding assay was performed as described in Nykanen et al. (2001) with some modifications. The 5' end of the antisense strand siRNA was <sup>32</sup>P-labeled with PNK. RNAi reactions (10  $\mu$ L) were quenched with 40  $\mu$ L of stop buffer and a 100 molar excess of Pp-luc-antisense RNA; incubated for 15 min at 25°C; and immediately ethanol precipitated, redissolved in 4  $\mu$ L of 1 $\times$  loading buffer (Takara), and immediately analyzed by electrophoresis at 4°C through 15% native polyacrylamide gel. siRNA sequences were 5'-UCGAAGUAUUCGCGUACGUG-3' (Pp-luc-antisense) and 5'-CGUACGCGAAUACUUCGAAA-3' (Pp-luc-sense).

#### *RISC assembly assay*

RISC assembly assay was performed as described in Tomari et al. (2004). <sup>32</sup>P-radiolabeled siRNA duplex was incubated with embryo lysates at room temperature. After incubation, the samples were adjusted to 6% (w/v) glycerol and resolved by submarine native agarose gel electrophoresis. Gels were dried under vacuum onto Hybond-N+ nylon membrane (Amersham Bioscience).

#### *Northern blot analysis*

RNA was isolated from S2 cells or the IgG bound fractions with ISOGEN (Nippon gene). Northern blot was performed as described (Ishizuka et al. 2002), except that the RNA was run on 12% acrylamide-denaturing gels and transferred onto Hybond-N+ membrane. The hybridizations were performed at 42°C in 0.2 M sodium phosphate (pH 7.2) and 7% SDS and washed at 42°C in 2 $\times$  saline sodium citrate and 0.1% SDS.

#### *TAP purification*

The expression of AGO1-TAP, AGO2-TAP, or dFMR1-TAP in S2 cells was induced by adding copper ion into the medium. After overnight incubation, the cytoplasmic lysate was prepared in a buffer containing 10 mM HEPES (pH 7.0), 200 mM KOAc, 2 mM MgAc, 0.1% NP40, 2  $\mu$ g/mL leupeptin, 2  $\mu$ g/mL pepstatin, and 0.5% aprotinin. AGO1-TAP, AGO2-TAP or dFMR1-TAP, and associated materials to the TAP-tagged fusion protein were bound to IgG beads. After extensive washing, the bound fraction was eluted by treatment with TEV protease. The polyclonal antibodies against Dicer-1 and AGO1 were kind gifts from G. Hannon and T. Uemura, respectively. The AGO2 and dFMR1 monoclonal antibodies (4D2 and 5A11, respectively) were produced by injecting AGO2 or dFMR1 peptides into mice, followed by fusing the mouse spleen cells to myeloma cells to obtain hybridomas producing the specific antibodies.

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## Distinct roles for Argonaute proteins in small RNA-directed RNA cleavage pathways

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