ARGONAUTE1 is required for efficient RNA interference in Drosophila embryos

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Double-stranded RNA (dsRNA) triggers homology-dependent post-transcriptional gene interference (RNAi) in a diverse range of eukaryotic organisms, in a process mechanistically related to viral and transgene-mediated cosuppression. RNAi is characterized by the conversion of long dsRNA into ≈21–25-nt small interfering RNAs (siRNA) that guide the degradation of homologous mRNA. Many of the genes required for siRNA production and target mRNA degradation are widely conserved. Notably, members of the *Argonaute*-like gene family from *Arabidopsis*, *Caenorhabditis elegans*, *Drosophila*, and *Neurospora* have been genetically and/or biochemically identified as components of the RNAi/cosuppression pathway. We show here that mutations in the *Drosophila Argonaute1* (*AGO1*) gene suppress RNAi in embryos. This defect corresponds to a reduced ability to degrade mRNA in response to dsRNA *in vitro*. Furthermore, *AGO1* is not required for siRNA production *in vitro* nor can the introduction of siRNA bypass *AGO1* mutants *in vivo*. These data suggest that *AGO1* functions downstream of siRNA production.

Many species, across a wide phylogenetic range, respond to aberrant/foreign RNA by degrading endogenous mRNA in a sequence-specific manner (1). This phenomenon, broadly referred to as posttranscriptional gene silencing (PTGS), can be triggered by the introduction of double-stranded RNA (dsRNA) [RNA interference (RNAi)], transformation with sense transgenes (cosuppression/quelling), or viral infection (2). RNAi acts as a cellular defense against parasitic nucleic acids and provides a fortuitous technique for biologists to reduce or eliminate a gene activity (3). RNAi-like mechanisms are also involved in the production of small noncoding RNAs that control developmental timing (4, 5). A better understanding of RNAi may then shed light on genome defense and endogenous developmental pathways.

The molecular mechanisms underlying RNAi are beginning to be elucidated. dsRNA is processed into small double-stranded fragments of 21–25 nucleotides, called small interfering RNA (siRNA; refs. 6–8), by the enzyme Dicer (9–11). These siRNA are then incorporated into a protein complex, called RNA-induced silencing complex (RISC), which degrades homologous mRNA (8, 12). The siRNA signal can also be amplified, possibly by an RNA-dependent RNA polymerase (13, 14). siRNA seems to be the crucial mediator of sequence specificity targeting mRNA for degradation (6, 15–17).

RNAi seems to be an ancient and evolutionarily conserved process. The *Argonaute* gene family was first defined by the *Arabidopsis Argonaute* gene (*AGO1*; ref. 18) and encodes related proteins of unknown molecular function in plants, animals, and fungi. Members of the *Argonaute* gene family, including *rde-1* in *Caenorhabditis elegans* (19), *qde-2* in *Neurospora* (20), *AGO1* in *Arabidopsis* (21), and *piwi* in *Drosophila* (22), are genetically defined as being required for PTGS. The *Drosophila* Argonaute2 (AGO2) protein was biochemically identified as a component of the RISC complex (23). Thus far, AGO2 is the only identified RISC component. The conservation of genes involved in PTGS extends beyond members of the *Argonaute* family. The Dicer enzyme, whose activity was identified in *Drosophila* (9), has homologs in plants (24), *C. elegans* (4, 10, 11), and mammals (5).

Also, RNA-dependent RNA polymerases have been implicated in PTGS in plants (25), *Neurospora* (26), and *C. elegans* (27). Thus, homologous proteins may have similar functions in PTGS throughout a diverse range of eukaryotic organisms.

Based on the evolutionary conservation of genes involved in PTGS, we have begun to address the roles of members of the *Drosophila Argonaute* gene family. We have identified five *Argonaute*-like genes encoded in the *Drosophila* genome. For comparison, the *C. elegans* genome encodes 23 *Argonaute-like* genes. Three of the *Drosophila* genes are maternally expressed, but zygotic expression in the embryo is limited to the presumptive gonad. Thus, they likely have tissue-specific functions. The other two genes, *Argonaute1* (*AGO1*) and *AGO2*, are expressed broadly throughout embryonic development and are better candidates to function in RNAi in embryos. We chose to examine the role *AGO1* plays in RNAi in the *Drosophila* embryo. We show that *AGO1* mutant embryos have a reduced RNAi response when injected with either long dsRNA or siRNA. We also show that *AGO1* is required for the degradation of targeted mRNA *in vitro* but is not required for the Dicer-mediated cleavage of dsRNA. We propose that *AGO1* functions in RNAi and, specifically, functions downstream of siRNA production.

Materials and Methods

***Drosophila* Strains.** Flies were maintained with standard procedures. *P* element insertions in *AGO1*, l(2)k08121, and l(2)k16601 were each out-crossed to w^{1118} and isogenized. The lethality of l(2)k08121 was reverted by precise excision of the *P* element. In addition, the lethality of l(2)k08121 was rescued in a strain containing heat shock Gal4 and UAS-*AGO1* transgenes (28). The lethality of the strain, therefore, is caused by the insertion of the *P* element in *AGO1*. All lethal mutations were balanced over a *CyO* chromosome carrying an *armadillo* (*arm*)-*GFP* transgene that is expressed throughout the embryo.

RNA Synthesis and Injections. For dsRNA production, regions of the *even-skipped* (*eve*), *fushi tarazu* (*ftz*), and *white* genes were amplified with oligonucleotides that add 5′ T7 promoter sequences (available on request) by using pEve, pGEM F1 (29), and pUAST (28) as templates, respectively. The resulting PCR products were each used as templates for *in vitro* transcription reactions by using the Ambion (Austin, TX) MEGAscript kit following the manufacturer's instructions with or without the addition of 20 µCi of [α-³²P]UTP (Amersham Pharmacia Biotech). The reactions were boiled and allowed to anneal >12 h at room temperature. The resulting RNA was analyzed by agarose gel electrophoresis to verify that it was double stranded and of the appropriate size. dsRNA (21 bp) with 2 base 3′ overhangs (siRNA) corresponding to the *eve* gene (siEVE-2 AACUCCUU-GAACGGCAGCCGC) was purchased from Dharmacon

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(Lafayette, CO). The siRNA was 5′-phosphorylated with T4 polynucleotide kinase (NEB, Beverly, MA). Approximately 100 pl of dsRNA or siRNA (1 μg/μl in H_2O) was injected into syncitial blastoderm embryos. After 40–48 h at 18°C, the surviving embryos were scored for the number of ventral denticle belts. For consistency, any embryo with 7 or 8 visible denticle belts was scored as wild type and embryos with 4, 5, or 6 belts were scored as *eve*. The number of *eve* embryos may be underrepresented, because embryos with a weak phenotype (i.e., only missing a single denticle belt) would have been scored as wild type.

For mRNA production, regions of the *eve* and *white* genes were amplified with a forward oligonucleotide with a 5′ T7 transcription start and a reverse primer that adds 25 A residues to the 3′ end. The PCR products were then used as templates for *in vitro* transcription reactions with the Ambion mMessage kit plus the addition of 20 μCi of [α-^{32}P]UTP. The RNA products were verified by agarose gel electrophoresis.

In situ probes were generated by *in vitro* transcription with a linearized expressed sequence tag clone as a template. Embryo collections, hybridizations, and detection were performed as described by the Berkeley Drosophila Genome Project (www.fruitfly.org/about/methods/RNAinsitu.html) with minor modifications.

Extract Preparation and *in Vitro* RNAi Reactions.

Extracts used for *in vitro* RNAi reactions were prepared as described in Tuschl *et al.* (12) and Zamore *et al.* (7) with some modifications. In short, embryos were collected for 4 h and then aged for an additional 12 h at 25°C to allow for zygotic expression of the *arm-GFP* transgene on the *CyO* balancer chromosome. Homozygous *AGO1^{k08121}* embryos were sorted from their *AGO1^{k08121}/CyO-armGFP* and *CyO-armGFP/CyO-armGFP* siblings following the procedure of Furlong *et al.* (30). Approximately 6,000 embryos were homogenized in 2 ml of lysis buffer (100 mM potassium acetate/30 mM Hepes, pH 7.4/2 mM magnesium acetate/5 mM DTT/0.5 mg/ml Pefabloc SC) with a glass tissue grinder, centrifuged for 25 min at 14,500 × g at 4°C, and flash-frozen in 10-μl aliquots. The total protein concentration in the extracts was determined with a colorimetric Bio-Rad assay.

The cleavage of dsRNA was assessed by incubating 200 ng of labeled dsRNA in a 20-μl reaction containing 5 μg of extract protein for increasing times at 25°C. Reactions were stopped by the addition of formamide tracking dye. Sequence-specific degradation of mRNA was assessed by adding 200 ng of unlabeled dsRNA to a 20-μl reaction containing 5 μg of extract protein. After a 10-min incubation at 25°C, 5 ng of labeled mRNA was added. The reactions were stopped following the procedure of Tuschl *et al.* (12). All reactions were analyzed on a 12% denaturing polyacrylamide gel followed by autoradiography. RNA molecular weight markers were made by T1 RNase (Ambion) digestion of labeled *eve* RNA.

Results

The *Argonaute* Gene Family in *Drosophila*.

Searches of the *Drosophila* genome and expressed sequence tag (EST) databases reveal five separate transcribed members of the *Argonaute* gene family (Fig. 1*A*). *Argonaute-like* genes are defined as encoding two conserved domains: a well conserved region of approximately 300 amino acids, called the PIWI box, near the C-terminal region of the ORF and a more centrally located and less well conserved region of 110 amino acids called the PAZ domain (ref. 31; Fig. 1*A*). The molecular functions of *Argonaute*-like proteins are not known; however, two of the genes, *piwi* and *aubergine* (*aub*)/*sting*, are necessary for proper germ-line development (32–35). *AGO1* is required for viability and embryonic neural growth (36). The AGO2 protein has been implicated in the degradation of target mRNA in response to dsRNA (23). A fifth member,

Argonaute3 (*AGO3*), has not yet been characterized (Fig. 1*A*). Additional information on the EST clones can be found in Table 2, which is published as supporting information on the PNAS web site, www.pnas.org.

Based on sequence alignments, the five genes can be subdivided into two subcategories (Fig. 1*B*). Interestingly, the embryonic RNA expression patterns of the five *Drosophila* genes reflect this sequence-based grouping as well. *AGO1* and *AGO2* are maternally deposited and have fairly ubiquitous embryonic expression patterns (Fig. 2 *A–H*). They are, however, more strongly expressed in the ventral and cephalic furrows. Later, *AGO1* is also up-regulated in the developing nervous system. *piwi*, *aub*, and *AGO3*, which are similar in sequence, are also expressed maternally but unlike *AGO1* and *AGO2*, their expression disappears by embryonic stages 10–12. Zygotic transcription is then restricted to the presumptive gonad (Fig. 2 *I–T*), suggesting they have tissue-specific roles during embryo development and are less likely to a part of the general RNAi machinery that is assumed to be ubiquitous.

AGO1 Mutants Are RNAi-Defective.

With the availability of loss-of-function mutations in *AGO1*, we were able to examine its role as a possible component of the RNAi machinery in *Drosophila* embryos. Mutations in *AGO1* result in late embryonic/early larval lethality and have defects in the central and peripheral nervous system (36). Precise excision of the l(2)k08121 *P* element reverted lethality, as did a heat shock-driven *AGO1* cDNA (data not shown). l(2)k08121, an insertion near the transcription start site of two of the *AGO1* isoforms (Fig. 1*A*), was previously shown to be a strong allele (36). Most of our analysis was done in this out-crossed insertion line we refer to as *AGO1^{k08121}*.

We tested for RNAi *in vivo* by assaying the ability of dsRNA corresponding to the *eve* gene to produce an *eve* phenotype when injected into wild-type and *AGO1* mutant embryos. *AGO1^{k08121}* was maintained as a heterozygous stock balanced over a *CyO-armGFP* chromosome. One-quarter of the embryos produced in this stock are *AGO1^{k08121}* homozygous mutants, as identified by the absence of zygotic *GFP* expression. The remaining three-quarters of the embryos, which express *GFP* and represent both heterozygous and *CyO-armGFP* homozygous embryos, are referred to as *AGO1^+*. Wild-type embryos, wild-type embryos injected with control dsRNA corresponding to the *white* gene (Fig. 3*A*), and uninjected *AGO1^{k08121}* mutant embryos have eight ventral denticle belts. By comparison, *eve* mutant embryos or wild-type embryos injected with *eve* dsRNA have a visible reduction in the number of denticle belts (ref. 29; Fig. 3*B*). *eve* dsRNA was injected into *AGO1^{k0812}* and *AGO1^+* embryos. *AGO1^{k08121}* zygotic mutants were less responsive than *AGO1^+* embryos to *eve* dsRNA (Fig. 3 *C* and *D* and Table 1). Specifically, only 8% of *AGO1^{k08121}* zygotic mutants had fewer than 7 denticle belts whereas 38% of their *GFP*-expressing *AGO1^+* siblings exhibited an *eve* phenotype. We also observed a similar reduction in RNAi-induced embryonic phenotypes (12% vs. 34%) by using dsRNA corresponding to the *ftz* gene (Table 1). *AGO1^{k16601}* zygotic mutants, resulting from an independent *P* element insertion 8 nucleotides downstream of the l(2)k08121 insertion site, also exhibited a reduced response to *eve* dsRNA (7% vs. 28%). It should be noted that RNAi is not completely inhibited in *AGO1^{k08121}* mutants, as a small percentage of *eve* embryos are observed after injection with *eve* dsRNA (see *Discussion*). However, the percentage of embryos exhibiting an RNAi-induced phenotype is clearly reduced in *AGO1* mutants compared with wild type.

We performed a number of control experiments to confirm that the decrease in RNAi-induced phenotypes was a specific effect of *AGO1* reduction-of-function. Of the embryos homozygous for a l(2)k08121 revertant chromosome that were injected with *eve* dsRNA, 33% exhibited an *eve* phenotype (Table 1).
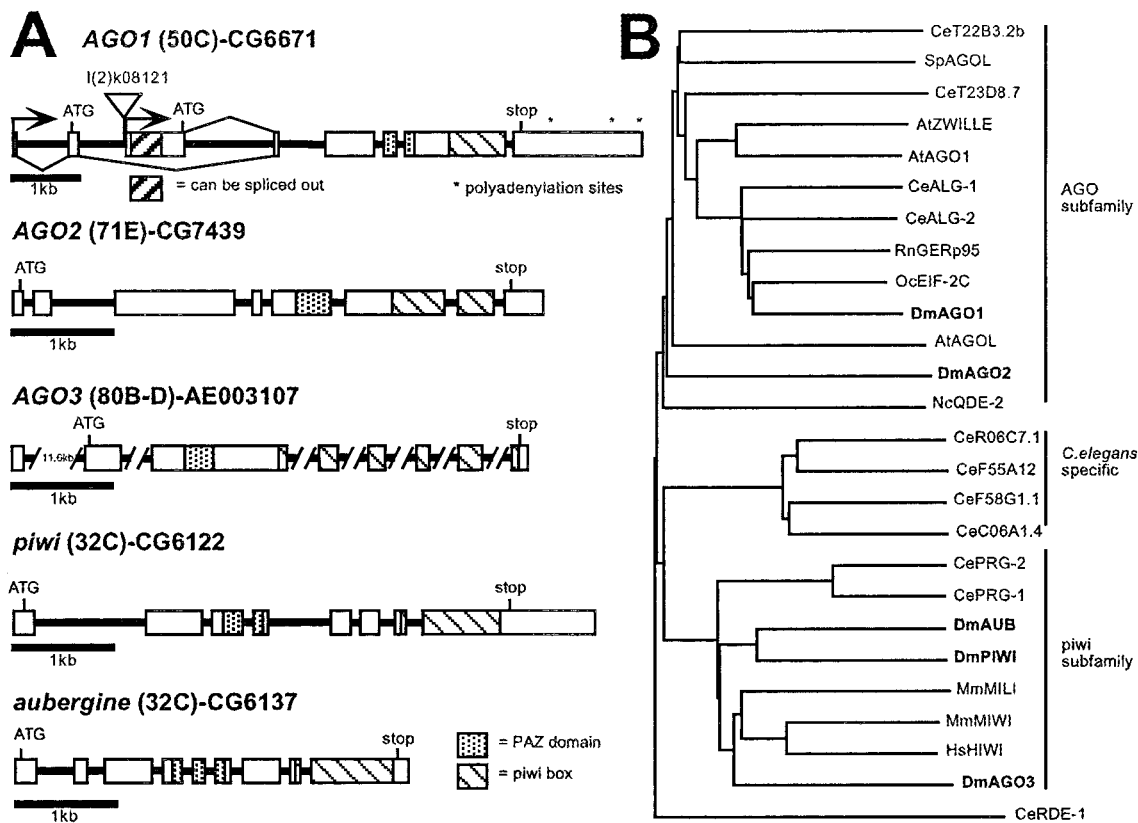
Fig. 1. The *Argonaute* gene family. (*A*) Genomic organization and cytological location of the five *Drosophila Argonaute-like* genes. Exons are shown as boxes and introns are shown as solid lines. The PAZ and PIWI domains are defined by Cerutti *et al.* (31). The *AGO1* gene has two transcriptional start sites, resulting in two different ORFs differing in the first 65 amino acids. The *AGO1 P* element insertion l(2)k08121 is shown. l(2)k16601, an independent insertion in AGO1, is located 8 base pairs downstream from the l(2)k08121 insertion site. *AGO3* is located near the centromere at 80B-D. Only one of the seven introns has been completely sequenced. The curated gene name, CG, is given for all except *AGO3*. The GenBank accession no. for a genomic scaffold sequence that contains *AGO3* is listed instead. (*B*) Phylogenetic grouping of the *Drosophila Argonaute*-like proteins and representatives from other organisms. The tree was constructed with the full-length protein sequences with CLUSTALX and a BLOSUM protein weight matrix. Dm, *Drosophila melanogaster*; Ce, *C. elegans*; At, *Arabidopsis thaliana*; Sp, *Schizosaccharomyces pombe*; Mm, *Mus musculus*; Hs, *Homo sapiens*; Oc, *Oryctolagus cuniculus*; Rn, *Rattus norvegicus*. The *Drosophila* sequences are shown in bold.

Similarly, $AGO1^{k08121}$ embryos containing the heat shock-driven *AGO1* transgene that rescues lethality also had a near wild-type response to dsRNA (29%). Therefore, the *P* element insertion in *AGO1* causes the reduced RNAi response exhibited in $AGO1^{k08121}$ embryos.
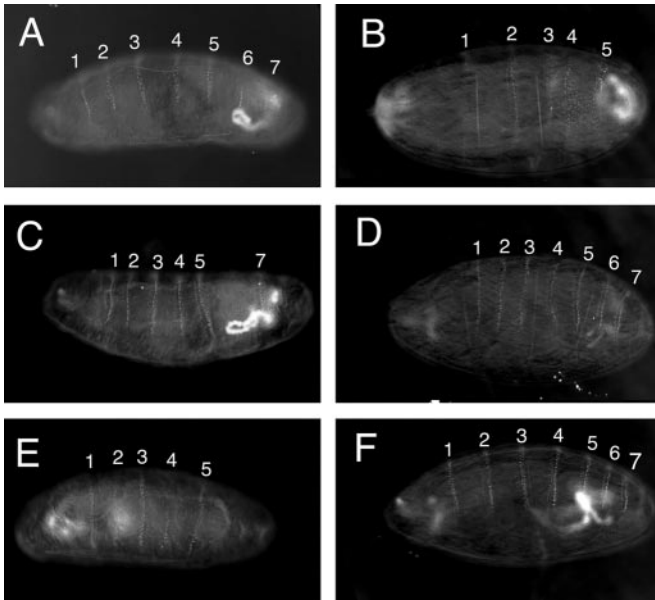
AGO1 Mutants Are Defective in Degrading Targeted mRNA. As a first step toward understanding the molecular function of *AGO1*, we used an *in vitro* extract to narrow down the step(s) in RNAi that are affected in *AGO1* mutants. Previous *in vitro* analysis of RNAi in *Drosophila* relied on extracts, prepared from either early syncitial embryos (12) or S2 cells (8), which were capable of processing dsRNA into siRNA and then degrading mRNA in a sequence-specific manner. We prepared extracts from older 12–16-h cellular embryos, when the zygotic expression of the *armGFP* clearly distinguishes *CyO*-containing embryos from $AGO1^{k08121}$ embryos, by following a procedure similar to Zamore *et al.* (7).

We assessed the ability of extracts prepared from $AGO1^{k08121}$ and *GFP*-expressing embryos, laid by an $AGO1^{k08121}/CyO$-*armGFP* balanced stock, to degrade target mRNA in response to dsRNA. mRNA incubated in the extracts without dsRNA for 1 h were only modestly degraded (Fig. 4, lanes 1–8). Extracts prepared from $AGO1^+$ embryos and preincubated with *eve* dsRNA efficiently degrade *eve* mRNA (lanes 9 and 10). Similarly, *white* dsRNA could target *white* mRNA for degradation
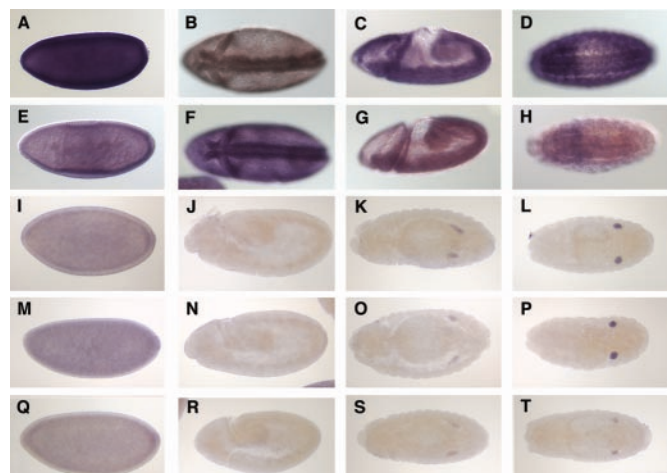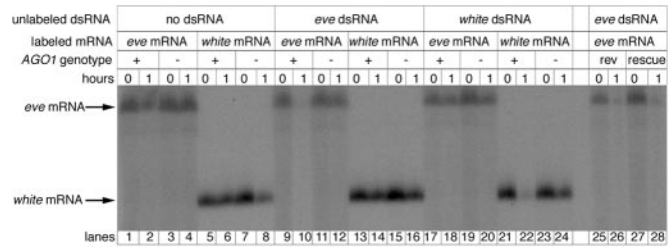

Fig. 2. Embryonic expression patterns of the five *Drosophila Argonaute-like* genes. (*A–D*) AGO1, (*E–H*) AGO2, (*I–L*) AGO3, (*M–P*) piwi, and (*Q–T*) aub. (*A, E, I, M,* and *Q*) Stage 4–5 embryos. (*B* and *F*) Stage 6 embryos initiating gastrulation. (*C* and *G*) Stage 8 embryos. (*D* and *H*) Stage 15–16. (*J, N,* and *R*) Stage 10–12 embryos. (*K, O,* and *S*) Stage 14. (*L, P,* and *T*) Stage 15–16. Anterior is to the left. Lateral views in *A, C, E, G, I, J, M, N, Q,* and *R*. Ventral view in *B, D, F,* and *H*. Dorsal view in *K, L, O, P, S,* and *T*.

Fig. 4. Degradation of targeted mRNA in response to dsRNA is reduced in *AGO1* mutants. Gel of uniformly ^{32}P-labeled mRNA that was incubated in extracts prepared from *AGO1*^{k08121} (−) and *GFP*-expressing *AGO1*$^+$ (+) embryos. Before the addition of labeled mRNA, the extracts were incubated with or without unlabeled dsRNA. Samples collected just after the addition of mRNA (0) or after a 1-h incubation (1) are in neighboring lanes. There is some nonspecific degradation of the mRNA after 1 h in the embryo extracts as seen in the ''no dsRNA'' controls. However, the amount of mRNA is greatly reduced in (+) extracts preincubated with homologous dsRNA. mRNA is unaffected in extracts prepared from *AGO1* mutants. Extracts prepared from a viable revertant line derived from l(2)k08121 (rev) are able to degrade targeted mRNA. Similarly, extracts prepared from *AGO1*^{k08121} embryos rescued with an *AGO1* cDNA (rescue) degrade mRNA targeted with homologous dsRNA.

Fig. 3. *AGO1* mutant embryos have a reduced response to *eve* dsRNA. Representative embryonic phenotypes of wild-type, *GFP*-expressing *AGO1*$^+$, and *AGO1*^{k08121} mutant embryos injected with dsRNA are shown. (*A*) *w*^{1118} embryo injected with *white* dsRNA showing no alteration in the number of ventral denticle belts. (*B*) *w*^{1118} embryo injected with *eve* dsRNA. Denticle belt 4 is fused with 5, and 6 is missing. (*C*) *AGO1*$^+$ embryo injected with *eve* dsRNA. Belt 6 is missing. (*D*) *AGO1*^{k08121} embryo injected with *eve* dsRNA showing no alteration in the number of denticle belts. (*E*) *AGO1*$^+$ embryo injected with *eve* siRNA. Belt 4 is partially formed whereas belt 6 is missing. (*F*) *AGO1*^{k08121} embryo injected with *eve* siRNA showing no alteration in the number of denticle belts. The denticle belts corresponding to each abdominal segment are labeled, except 8, which is not in the plane of focus.

(lanes 21 and 22). This process is sequence-specific because *eve* dsRNA does not affect the stability of *white* mRNA nor does *white* dsRNA affect the stability of *eve* mRNA (lanes 13 and 14

and 17 and 18), suggesting that our embryo extracts faithfully reproduce RNAi *in vitro*. Extracts prepared from *AGO1*^{k08121} embryos, however, did not degrade mRNA when preincubated with a homologous dsRNA (lanes 11 and 12 and 23 and 24). The ability to degrade mRNA in a sequence specific manner was restored in extracts prepared from the revertant line (lanes 25 and 26) and in extracts prepared from the line where the *AGO1*^{k08121} insertion is rescued by the expression of an *AGO1* cDNA (lanes 27 and 28). The reduced RNAi observed in living *AGO1*^{k08121} embryos correlates with the lack of dsRNA-triggered mRNA degradation *in vitro*.

AGO1 Acts Downstream of siRNA Production. To determine whether *AGO1* is required for the initial Dicer-mediated cleavage of dsRNA into siRNA, we injected synthetic *eve* siRNA into

Table 1. Effects of dsRNA injected into *Drosophila* embryos

Genotype*	Injected RNA	Wild type[†]	*eve*[†]	Expected[‡]
w^{1118}	*eve* dsRNA	44	66 (60%)	
w^{1118}	*eve* siRNA	156	54 (26%)	
CyO-armGFP/Adv	*eve* dsRNA	55	32 (37%)	
Adv/Adv	*eve* dsRNA	30	10 (25%)	
CyO-armGFP/AGO1^{k08121}	*eve* dsRNA	91	56 (38%)	
AGO1^{k08121}	*eve* dsRNA	55	5 (8%)	23
CyO-armGFP/AGO1^{k16601}	*eve* dsRNA	31	12 (28%)	
AGO1^{k16601}	*eve* dsRNA	13	1 (7%)	4
CyO-armGFP/AGO1^{k08121}	*ftz* dsRNA	39	20 (34%)	
AGO1^{k08121}	*ftz* dsRNA	17	2 (12%)	6
AGO1^{k08121} revertant	*eve* dsRNA	34	17 (33%)	
AGO1^{k08121} [*hsAGO1*]	*eve* dsRNA	32	13 (29%)	
CyO-armGFP/AGO1^{k0812}	*eve* siRNA	157	61 (28%)	
AGO1^{k08121}	*eve* siRNA	75	1 (1%)	21
w^{1118}	*white* dsRNA	61	0	
CyO-armGFP/AGO1^{k08121}	*white* dsRNA	39	0	
AGO1^{k08121}	*white* dsRNA	12	0	

*Zygotic genotype. Genotypes listed as *CyO-armGFP/AGO1*^{k08121} were scored as *GFP*-positive and represent a mixed population including *AGO1*^{k08121}/*CyO-armGFP* and *CyO-armGFP/CyO-armGFP* embryos.

[†]Number of embryos with wild-type or *eve* phenotype after injection. Embryos were scored as *eve* if they had 6 or fewer ventral denticle belts. Embryos were scored as wild type if they had 7 or 8 denticle belts (see *Materials and Methods*). Percentages are given in parentheses.

[‡]The expected number of *eve* embryos based on the percent penetrance from the above row assuming *AGO1* mutants have no affect on RNAi.

Williams and Rubin
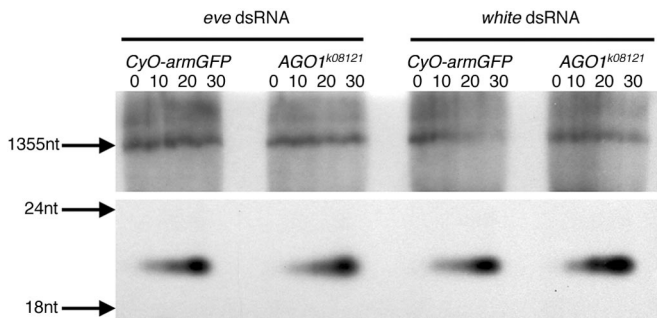
Fig. 5. Production of ≈21-nt fragments is unaffected in *AGO1* mutants. Gel of uniformly ^{32}P-labeled dsRNA, corresponding to either the *eve* or *white* gene, which was incubated in extracts from *GFP*-expressing *AGO1*$^+$ embryos and *AGO1*^{k08121} mutant embryos for 0, 10, 20, and 30 min. *Upper* is the top part of the gel showing the full-length input RNA. *Lower* shows the lower part of the same gel showing that both *eve* dsRNA and *white* dsRNA were cleaved into ≈21-nt fragments.
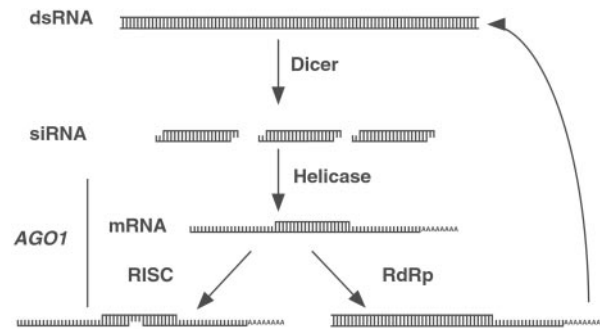

Fig. 6. Proposed step(s) during RNAi where *AGO1* may function. RNAi is initiated by the Dicer-mediated cleavage of dsRNA into ≈21-nt siRNA. siRNA is then used to target mRNA for degradation by the RISC complex. In another step, which may or may not be mediated by RISC, siRNA can act as a primer for second-strand synthesis of RNA complementary to the targeted mRNA. The newly dsRNA may then become a substrate for Dicer. Based on our results, we proposed that *AGO1* functions downstream of siRNA production. Our experiments do not address whether *AGO1* is specific for mRNA degradation or whether it is required for the RNA-dependent RNA polymerase (RdRp) amplification of the siRNA.

embryos laid by *w*^{1118} and *AGO1*^{k08121}/*CyO-armGFP* females and scored them for the number of denticle belts. siRNA was capable of producing an *eve* phenotype in wild-type embryos, albeit at a lower penetrance than longer dsRNA (26% vs. 60% for *w*^{1118} and 28% vs. 38% for *GFP*-expressing embryos; Fig. 3 *E* and *F* and Table 1). This lower penetrance may be the result of incomplete phosphorylation of the 5′ ends of the siRNA, which is required for efficient degradation of mRNA (38–40) or may reflect a lower potency of the siRNA. *AGO1*^{k08121} mutants, however, produced only 1% *eve* embryos after injection with *eve* siRNA. Therefore, *AGO1*^{k08121} mutants have a reduced response to synthetic siRNA, suggesting that *AGO1* functions downstream of siRNA production.

We then assayed the ability of extracts from *AGO1*^{k08121} mutant embryos and their *AGO1*$^+$, *GFP*-expressing siblings to process dsRNA into short ≈21-nt fragments. Extracts prepared from both *AGO1*^{k08121} zygotic mutants and *AGO1*$^+$ embryos were able to cleave dsRNA corresponding to both the *eve* and *white* genes into ≈21-nt fragments (Fig. 5). The rate of siRNA production was similar in both *AGO1*^{k08121} and *AGO1*$^+$ extracts. Taken together, these experiments indicate that wild-type *AGO1* activity is required after the Dicer-mediated production of siRNA.

Discussion

A Family of *Argonaute*-like Genes in *Drosophila*. The *Drosophila* genome encodes five *Argonaute*-like proteins. Consistent with their embryonic expression patterns, *piwi* and *aub* are defined by mutations that affect aspects of germ-line development. *piwi* is required to maintain germ-line stem cells perhaps by regulating a somatically derived stem cell promoting signal (33). Recently, *piwi* has also been shown to affect transgene-mediated cosuppression in *Drosophila* (22). *aub* is required for embryo patterning, by regulating *oskar* and *gurken* translation, and for pole cell formation (32, 35). *aub* is also required for *Su(Ste)*-mediated suppression of *Stellate* in the testis (34). Interestingly, this regulation seems to involve an RNAi-like mechanism (41). The related *AGO3* gene has an embryonic expression pattern very similar to *piwi* and *aub*, suggesting that it may have a role in gonad and/or germ-line development as well.

AGO1 is an essential gene that plays a role in neuronal growth (36). The related AGO2 protein was identified as part of the RISC protein complex that degrades targeted mRNA in response to dsRNA (23). Both *AGO1* and *AGO2* transcripts are maternally deposited and expressed throughout embryonic development. Consistent with its mutant phenotype, *AGO1* is up-regulated in the embryonic nervous system (36). Unlike *piwi*,

aub and *AGO3*, *AGO1*, and *AGO2* are expressed outside the gonad and thus may have more general somatic functions.

Possible Roles for *AGO1* in RNAi. Based on its sequence similarity to the *C. elegans rde-1*, the *Neurospora qde-2*, and the *Arabidopsis Argonaute* genes, *AGO1* is a likely candidate to function in RNAi. This idea is also supported by its sequence similarity to the *Drosophila* AGO2 and its wide embryonic expression pattern. With both *in vivo* embryo injections and *in vitro* assays, we have shown that *AGO1* zygotic mutants are compromised for RNAi.

AGO1 mutant embryos still exhibit some RNAi activity, however. There are several possible explanations for this observation. First, *AGO1*^{k08121} may not be a null allele. The *P* element is inserted near the transcriptional start site for two of the isoforms and in the second intron of a third isoform (Fig. 1*A*), leaving the possibility that a functional mRNA could be produced; however, there is a strong reduction of all *AGO1* transcripts in the l(2)k08121 allele (36). Secondly, because *AGO1* and *AGO2* have similar expression patterns and both may function in RNAi, they may share functional redundancy. Partial redundancy has been demonstrated for two related *Argonaute*-like genes during *C. elegans* and *Arabidopsis* development (4, 18, 37). An interesting and untested idea is that *AGO1* and *AGO2* may have some redundancy during early embryogenesis but then later tissue-specific differences in their expression patterns would uncover the lethality associated with *AGO1* mutants. This model would be consistent with the continued up-regulation of *AGO1* mRNA, especially in the ventral nerve cord, near the end of embryogenesis. Third, the maternal contribution of *AGO1* mRNA might provide some level of *AGO1* activity, although it cannot support wild-type levels of RNAi.

AGO1 is not required for siRNA production. This model is supported by the cleavage of dsRNA into ≈21fragments in *AGO1* mutants and the inability to bypass *AGO1* function by injection of synthetic siRNA. Therefore, *AGO1* functions at some step(s) between siRNA production and target mRNA degradation (Fig. 6).

However, there are still several possible steps in which *AGO1* may function (38). Recent studies of *C. elegans rde-1* mutants have shown that *in vitro* extracts are capable of processing dsRNA into siRNA, but siRNA was greatly reduced *in vivo*. This work suggested that wild-type *rde-1* activity is required to stabilize siRNA (42, 43). Thus, *AGO1*/*rde-1* may protect siRNA from a nuclease. *AGO1* may help to incorporate siRNA into a

functional RISC or, like AGO2, is itself a component of RISC. Such activity could include maintaining the required 5′ phosphate group on siRNA, acting as a scaffold to assemble the multiprotein complex, unwinding siRNA/strand selection for base pairing to target mRNA, or acting as a component of the nuclease that cleaves mRNA. These possible functions for *AGO1* are not exclusive. For example, siRNA could be stabilized by incorporation into RISC. However, the biochemical activity or *in vivo* partners of the AGO1 protein are not known.

Developmental Roles of *AGO1*? *AGO1* mutants are late embryo/early larval lethal and exhibit defects in the embryonic nervous system (36). This finding is not surprising because other components of the RNAi pathway have been shown to function during development. *Dicer/dcr-1* and the *rde-1* homologs *alg-1* and *alg-2* are required for the production of the small temporal RNA (stRNA) in *C. elegans* (4, 5). stRNAs, encoded by the *lin-4* and *let-7* genes (44–49), are 21–22-nt single-stranded RNAs that function by base pairing to the 3′ untranslated regions and inhibiting translation of genes that control developmental timing. stRNAs are initially made as ≈70-nt primary transcripts that can fold into a hairpin structure. The double-stranded stem portion of the RNA is cleaved and processed into the functional 21–22-nt stRNA. Recent studies have uncovered a large and diverse population of endogenous microRNA (50–52) that share many of the characteristics of *lin-4/let-7*. These observations raise the possibility that small RNA represents a common mode of gene regulation and their production/usage requires a mechanism similar to RNAi.

The neuronal defect in *AGO1* mutants is particularly intriguing. Lai and Posakony (53) have proposed that short regions of RNA:RNA duplex formation between the 3′ untranslated regions (UTRs) of the proneural genes and members of the *Enhancer of split complex E(spl)* may represent a level of gene regulation during neurogenesis. Most recently, it was noted that several *Drosophila* microRNAs have regions of complementarity to negative regulatory elements in the 3′ UTRs of multiple members of the *Bearded* and *E(spl)* complexes (54). Because members of these gene families regulate development of the nervous system, it would be very interesting if the developmental defects in *AGO1* mutants are the results of a defect in microRNA processing or use.

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