

# The slicing activity of miRNA-specific Argonautes is essential for the miRNA pathway in *C. elegans*

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## ABSTRACT

**Among the set of Argonautes proteins encoded by metazoan genomes, some have conserved amino acids important for catalytic or slicing activity. The functional significance of these residues in microRNA (miRNA)-specific Argonautes in animals is still unclear since miRNAs do not induce site-specific cleavage of targeted messenger RNAs (mRNAs), unlike small interfering RNAs (siRNAs). Here, we report that miRNA-specific ALG-1 and ALG-2 Argonautes from *Caenorhabditis elegans* possess the slicing activity normally implicated in the siRNA-silencing pathway. We also find that ALG-1/2 can bind and use a Dicer-processed miRNA duplex to target mRNAs, suggesting an ability to displace RNA strands. Importantly, the slicing activity of ALG-1 or ALG-2 is essential for the miRNA pathway *in vivo*, as shown by the accumulation of truncated miRNA precursors and altered miRNA-induced silencing complex (miRISC) formation. Taken together, our data demonstrate that the slicing activity of Argonautes contributes to a new and unexpected step in the canonical miRNA pathway that occurs prior to miRISC loading in animals.**

## INTRODUCTION

Argonaute proteins act at different steps of the microRNA (miRNA)-mediated gene-silencing pathway. They form a complex with Dicer and interact with miRNAs to form the miRISC: the effector complex that targets complementary messenger RNA (reviewed in (1)). All members of this protein family share signature domains: PAZ, Mid and PIWI domains (2). Although the PAZ and Mid provide the small RNA-binding capacity to the protein (3–6), the PIWI domain can confer a catalytic or slicing activity to a specific subset of Argonautes that possesses three

conserved amino acid residues (DDH) in the catalytic center (reviewed in (7)). In humans, Argonaute-encoding genes are phylogenetically grouped into two different clades called the Argonaute-like and the Piwi-like proteins that are, respectively, required for the miRNA and PIWI-interacting RNA (piRNA) pathways (reviewed in (2)). In the nematode *Caenorhabditis elegans*, specific Argonaute genes found in the animal genome form a worm-specific protein clade called the WAGOs, which are important for endogenous (endo-) and exogenous (exo-) RNA interference (RNAi) pathways (8). Although a number of observations support the role of slicing Argonautes in the piRNA pathway as well as the exo- and endo-RNAi pathways (9–16), the importance of this enzymatic activity for miRNA-specific Argonautes remains unclear in animals.

Recently, two studies reported that the mouse and zebrafish slicer Ago2 is essential for the production of only one specific miRNA that is not subject to canonical Dicer-dependent biogenesis pathway (17,18). Aside from this unique case, current hypotheses suggest that the slicer activity of specific Argonaute proteins could be involved in the formation of miRISC, wherein they could cleave perfectly paired small RNA duplexes and promote the degradation of the passenger strand (11,14,16). It has also been proposed that the slicer activity of human Ago2 could cleave the precursor miRNA molecule to stimulate production of the mature form by Dicer (19). Thus far, there is no *in vivo* evidence that supports the functional importance of Argonaute slicer activity in the canonical biogenesis of miRNAs.

To address the implications of the slicing activity of miRNA-specific Argonautes in animals, we set out to characterize *C. elegans* ALG-1 and ALG-2: the two Argonautes that carry a DDH motif and are essential for the miRNA pathway (20). Here, we demonstrate that ALG-1 and ALG-2 Argonautes possess slicer activity and that this activity is essential for viability. We also determine that the slicing activity of ALG-1 and ALG-2 is important to coordinate Dicer cleavage and thus is required for the formation of miRISC in *C. elegans*.

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## MATERIALS AND METHODS

### Recombinant ALG-1 and ALG-2 protein preparation and purification

BL-21 codon plus RIPL bacteria (Stratagene), transformed with Glutathione S-transferase (GST)-tagged ALG-1 and ALG-2 plasmids, were grown at 37°C in Luria Broth (LB) media containing ampicillin and chloramphenicol. When optical density reached 0.4, 1 mM Isopropylthio- $\beta$ -galactoside (IPTG) was added to the media and the cultures were grown overnight at 16°C. The bacterial pellet was resuspended in cold phosphate buffered saline 1 $\times$  with protease inhibitor cocktail from Roche and 100  $\mu$ M of phenylmethylsulfonyl fluoride (PMSF). The pellet was disrupted using a Cell Disruption machine (Constant System). The lysate was then incubated for 1 h at 4°C in the presence of 0.25% Empigen (Sigma) and cleared by centrifugation. GST-tagged proteins were purified using Glutathione Sepharose 4B column (GE Healthcare) according to the manufacturer's instructions. It is important to take into consideration that a significant amount of RNA molecules from the expression systems (either bacteria or insect cells) remain associated to purified recombinant Argonautes (data not shown) (21,22) and thus might impinge on the efficiency of small RNA loading onto the proteins.

### Preparation of RNA molecules for slicing and binding assays

Synthetic RNAs (Sigma) were phosphorylated and gel purified using a denaturing acrylamide gel. To produce duplexes, an equivalent amount of purified phosphorylated single-stranded RNAs (ssRNAs) were annealed as described in (23). To confirm the absence of ssRNA in double-stranded RNA (dsRNA) duplexes, the nature of the samples were assessed by running a fraction of sample on a native acrylamide gel along with a single-stranded phosphorylated RNA as a size control.

### Slicing and binding assays

The slicing assays were performed at 20°C following the protocol used in (16). The RNA-binding experiments were performed as described in (24).

### C. elegans methods

*C. elegans* strains were grown under standard conditions (25). Transgenic lines carrying *alg-1 wt*, *alg-1 AAA*, *gfp::alg-2 wt* or *gfp::alg-2 AAA* were produced using standard procedure (26). Transgenes carrying *alg-1 wt* and *alg-1 AAA* genes were introduced in *alg-1(gk214)* mutant and transgenes expressing tagged *alg-2 wt* and *AAA* genes introduced in *alg-2(ok304)* animals.

To generate RNAi knockdown population, synchronized L1 *C. elegans* population were exposed to bacteria expressing either control, *alg-1* or *alg-2* dsRNA molecules for 4 days at 15°C.

### RNA isolation and northern blot analysis

The total RNA from a population of 50 000 animals was obtained by resuspending worm pellet in TRI Reagent

(Sigma) and lysed by flash-freezing in liquid nitrogen. Ten micrograms of purified total RNAs was then used for northern blot analysis following a protocol from the Bartel Lab (<http://web.wi.mit.edu/bartel/pub/protocols.html>) with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride chemical crosslinking (27). P<sup>32</sup>-radiolabeled probes to detect miRNAs were synthesized using the IDT StarFire kit (probes sequences can be found in the supplemental online section).

### ALG-1 and ALG-2 complexes immunoprecipitation

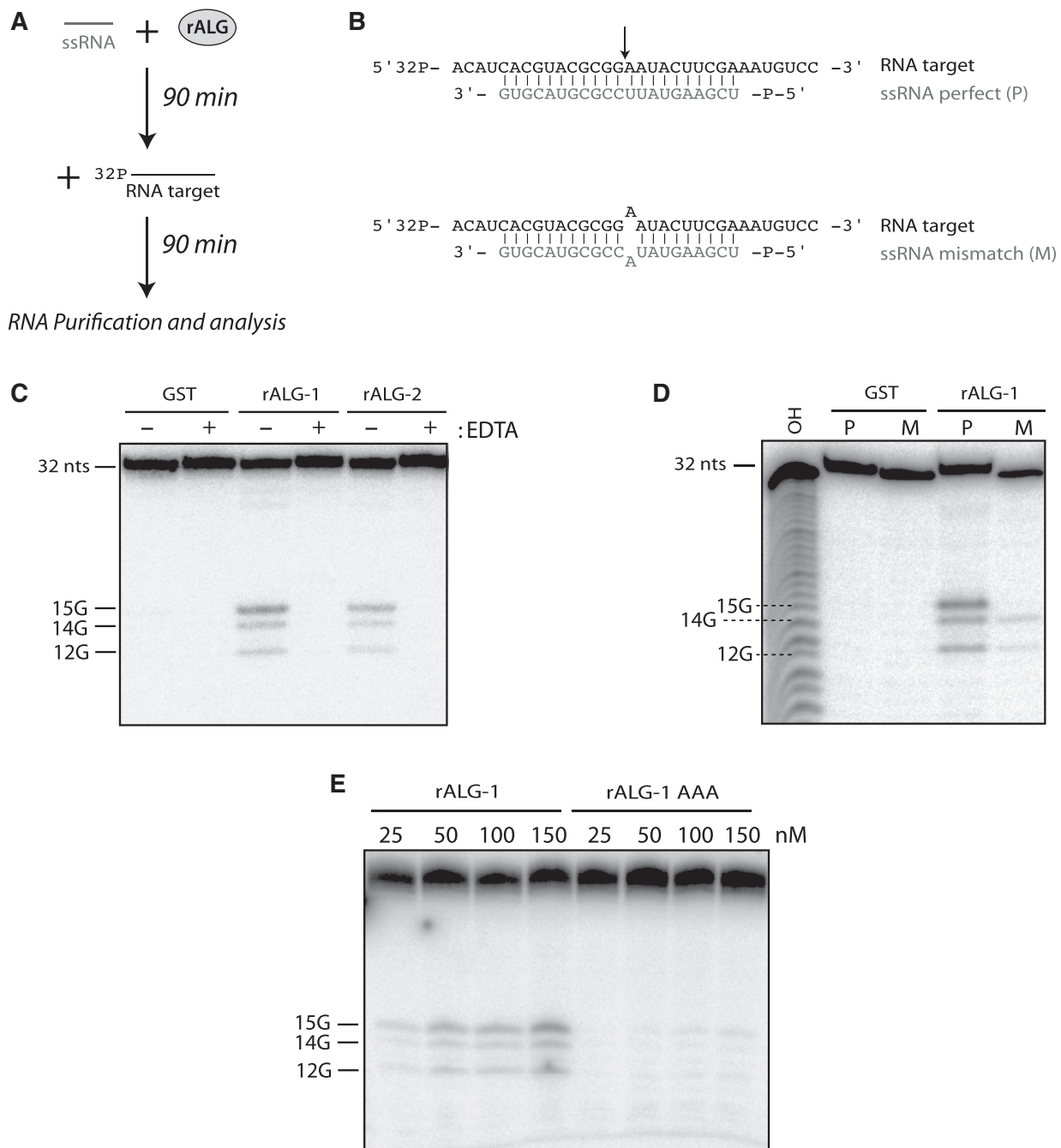
Whole worm extracts were prepared from either a population of transgenic *alg-1* animals carrying *alg-1 wt* or *alg-1 AAA* extrachromosomal transgene arrays exposed to *alg-2(RNAi)* or a population of transgenic *alg-2* animals carrying *gfp::alg-2 wt* or *gfp::alg-2 AAA* extrachromosomal transgene arrays exposed to *alg-1(RNAi)*. Total protein extracts were prepared as described in (28).

Immunoprecipitations were performed by first pre-clearing whole worm lysate with 20  $\mu$ l of protein G agarose beads (Roche) for 60 min at 4°C. The cleared extract was then incubated with beads coated with affinity-purified rabbit polyclonal anti-ALG-1 antibody or with beads coated with monoclonal anti-GFP (Chromotek) for 1 h at 4°C. Beads were then washed three times with ice-cold lysis buffer, a fraction kept to detect the presence of ALG-1 or GFP-ALG-2 proteins by western blotting, and RNA from the remaining beads was eluted and resolved to monitor *lin-4* mature and passenger strands by northern blot analysis.

## RESULTS

### ALG-1 and ALG-2 are slicer Argonautes

To test whether ALG-1 and ALG-2 Argonaute proteins possess the slicing activity, we produced recombinant GST-tagged ALG-1 and ALG-2 proteins (rALG-1 and rALG-2; Supplementary Figure S1A). Once pre-loaded with single-stranded 21-nt guide RNA (ssRNA; Figure 1A and B), both purified rALG-1 and rALG-2 were able to induce cleavage of a fully complementary 32-nt RNA target only in presence of magnesium (Figure 1C and Supplementary Figure S1C, respectively). Although we detected a major 15-nt long cleavage product corresponding to cleavage at the predicted site on the target RNA (16,29), two minor products were also produced (Figure 1C). Introducing a single mismatch at position 10 of the guide RNA abrogated the cleavage of the scissile bond between position 15 and 16 of the RNA target (Figure 1D) confirming that base pairing at the cleavage site is essential for precise target cleavage. When the DDH residues found in the catalytic center of the PIWI domain were mutated to three alanines (AAA), the slicing activity of both Argonautes was abrogated (Figure 1E and Supplementary Figure S1D, respectively) without significantly affecting RNA-binding ability (Supplementary Figure S1B). Thus, we conclude that ALG-1 and ALG-2 possess the slicing activity able to induce target cleavage at a precise site. In addition, ALG-1 and ALG-2 display some capacity to cleave at sites adjacent to the main target site,



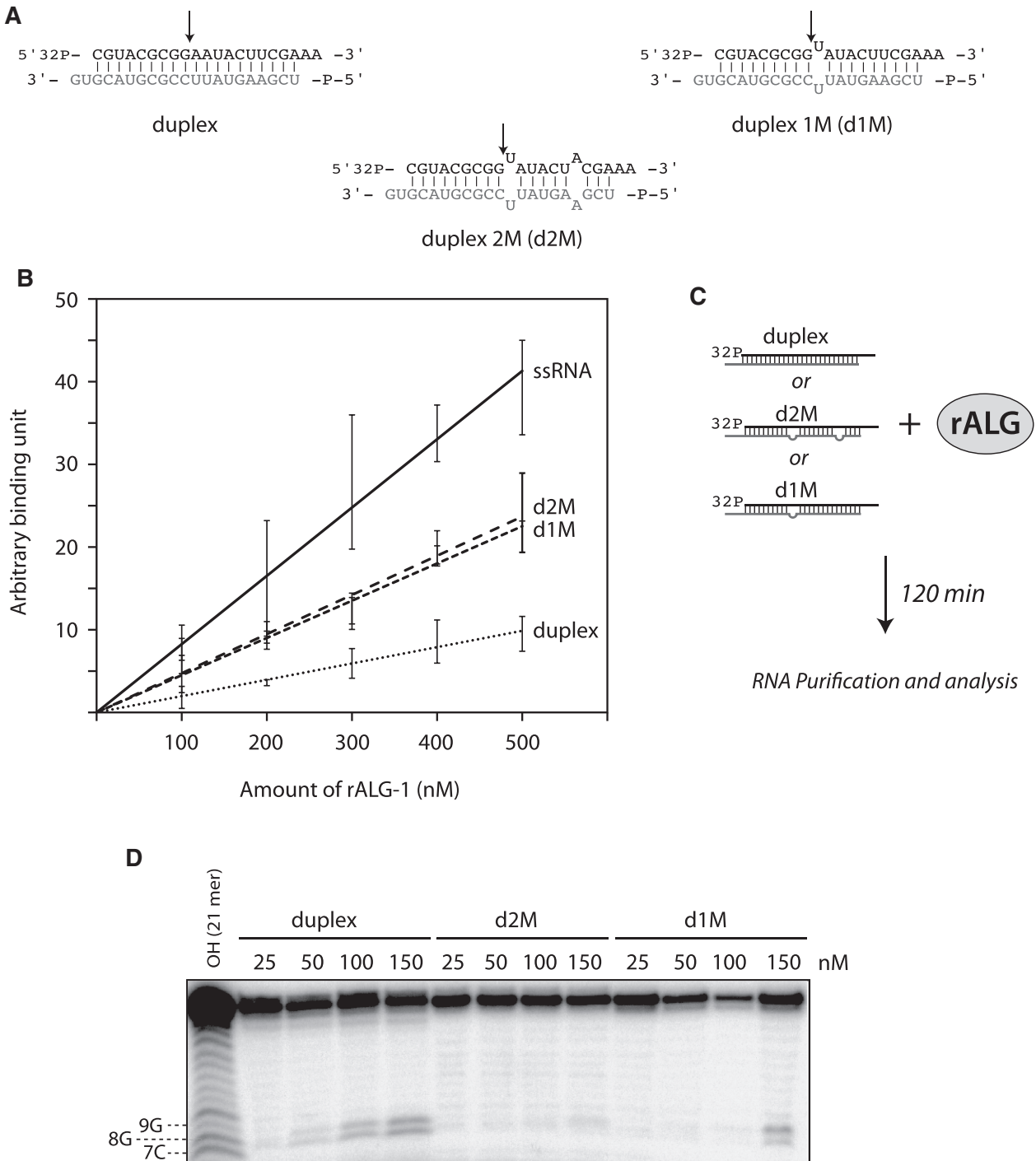
**Figure 1.** Purified ALG-1 and ALG-2 Argonautes have the slicing activity. (A) Schematic of the assay. (B) A representation of the RNA molecules used for the enzymatic assay. A 32 nucleotide (nt) 5'-end radiolabeled (<sup>32</sup>P) RNA target (in black) and fully complementary (P) or bulged next to the cleavage site (M) 21 nt ssRNA are shown in gray. (C) The radiolabeled RNA target is incubated with GST, GST-ALG-1 or GST-ALG-2 proteins with (+) the fully complementary 21-nt ssRNAs. Reaction performed with (+) or without (-) 10 mM EDTA to sequester magnesium ions essential for the enzymatic reaction are indicated. (D) Alteration of base pairing at the cleavage site (ssRNA M) abrogates target specific cleavage. The radiolabeled RNA target is incubated with either GST or GST-ALG-1 proteins. Hydroxylation of the RNA target (OH) is used as size marker (the 3'-end nucleotides of the radiolabeled RNA fragment are indicated). (E) Point mutations of the DDH motif in the PIWI domain of GST-ALG-1 abrogate cleavage activity. Amounts (nM) of recombinant proteins used in the assay are indicated.

which may reflect a requirement for accessory factor(s) to 'proofread' during ALG-1/2 slicing activity.

#### ALG-1 and ALG-2 bind and cleave the passenger strand of Dicer-like products

In flies and human cells, Argonautes have been involved in the loading, cleaving and unwinding of Dicer-processed

products (14,16,30). We therefore tested whether ALG-1 and ALG-2 share these functions. Taking into consideration the Dicer enzymatic signature (5' phosphate and 2-nt 3' overhang) along with the thermodynamic stability of the 5'-end base pairing of the guide strand as defined by the asymmetry rule (31), we designed three different small RNA duplexes (Figure 2A). These duplexes were as



**Figure 2.** Recombinant ALG-1 can bind and cleave small RNA duplexes. (A) Representation of the small RNA duplexes used for the assay. Strands complementary to the target RNA are shown in gray. (B) Determination of the affinity of ALG-1 for different types of small RNAs. Filter binding assays were performed with increasing amounts of GST-ALG-1 AAA (nM) incubated with different 5'-radiolabeled (<sup>32</sup>P) 21-nt RNAs illustrated in A (ssRNA is shown in Figure 1). The error bars represent a 95% confidence interval from three independent experiments. (C) Schematic of the slicing assay. (D) ALG-1 is able to cleave a perfectly paired small RNA duplex. Increasing amounts of GST-ALG-1 (nM) are incubated with different 5'-radiolabeled (<sup>32</sup>P) small RNA duplexes (as shown in A). Hydroxylation of the 21-nt RNA (OH) is used as size marker (the 3' end nucleotides of the radiolabeled RNA fragment are indicated). 8G and 7C represent other cleavage products.

follows: (i) fully complementary (duplex), (ii) possessing a single central mismatch at position 10 of the guide strand (duplex 1 M) and (iii) possessing two mismatches at positions 4 and 10 (duplex 2 M) that mimic a miRNA duplex predicted to be produced by Dicer from a precursor

miRNA. We first compared the capacity of ALG-1 and ALG-2 to bind these different small RNA duplexes and observed that both Argonautes have a greater affinity for a ssRNA than small RNA duplexes. Furthermore, the binding affinity of ALGs to double-stranded small

RNAs increases with the presence of mismatches in the duplex (Figure 2B and Supplementary Figure S2A).

We next determined if ALG-1 and ALG-2 could cleave these small RNA duplexes (Figure 2C). As previously observed for target cleavage with ssRNA, increasing the concentration of Argonaute proteins led to the cleavage of the radiolabeled strand from perfectly paired duplex (Figure 2D and Supplementary Figure S2B). The introduction of a mismatch altered the cleavage between positions 10 and 11, and the miRNA-like duplex was not efficiently cleaved (Figure 2C and Supplementary Figure S2B). Notably, it has been recently observed that small RNA molecules remain associated with purified recombinant Argonautes despite extensive purification methods (21,22). It is therefore important to consider that the RNA affinity measurement as well as the efficiency of duplex cleavage might be affected by the presence of bacterial RNA molecules associated with recombinant ALG-1 and ALG-2 proteins. Taken together, our data indicate that ALG-1 and ALG-2 proteins can bind Dicer-processed dsRNA products and induce the cleavage of a perfectly paired duplex, but cannot cleave a miRNA-like duplex.

#### **ALG-1 and ALG-2 can use a miRNA-like duplex to induce target cleavage**

Recently, it has been reported that both recombinant and tagged purified human Argonautes can unwind small RNA duplexes containing mismatches (30,32). We therefore decided to test whether *C. elegans* ALG-1 and ALG-2 can use miRNA-like duplexes to cleave a target RNA. To achieve this, we first pre-incubated recombinant Argonautes with various small RNA duplexes (Figure 2A) followed by the addition of a radiolabeled target RNA complementary to the guide strand (Figure 3A). Hence, if ALG-1 and ALG-2 were capable of releasing the passenger strand of the duplex, cleavage of the target RNA would be detected. Although the perfectly paired duplex and the duplex containing a central mismatch could barely induce target cleavage, the miRNA-like duplex efficiently triggered cleavage of the RNA target (Figure 3B). However, the miRNA-like duplex was still less effective than ssRNA at inducing ALG-mediated cleavage of the target RNA as at least twice the amount of proteins required to produce similar level of target cleavage when the miRNA-like duplex was used (Figure 3C and Supplementary Figure S3). These results indicate that *C. elegans* Argonautes can load miRNA-like duplexes and favor strand release to form a complex that is able to induce target cleavage.

#### **The slicer activities of ALG-1 and ALG-2 are essential for *C. elegans* viability**

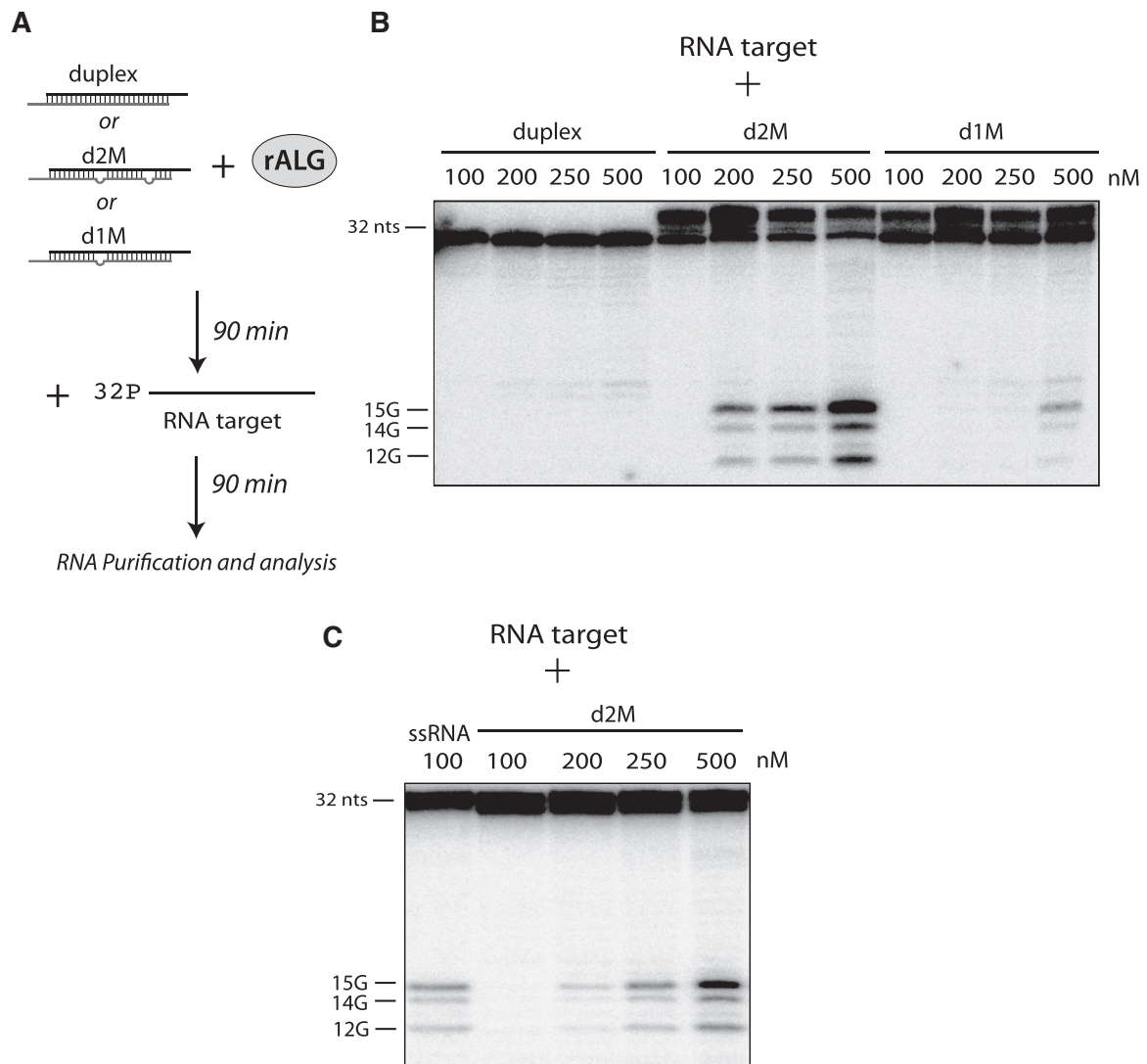
To determine the contribution of ALG-1/2 slicing activities *in vivo*, we performed several rescue experiments in animals with plasmid arrays containing either wild-type or catalytically inactive *alg-1* and *alg-2* genes. Although we observed that the *alg-1* wild-type transgene array rescued the *alg-1* mutant defects, the expression of catalytically inactive *alg-1* and *alg-2* genes in their respective

mutant backgrounds led to noticeable phenotypes in the transgenic animal population (Table 1). Particularly, we also observed that expression of the *alg-2* wild-type transgene in the *alg-2* mutant background caused lethality in 7% of the animal population (Table 1), indicating that a tight regulation of the ALG-2 protein level is important to maintain miRNA homeostasis.

We then asked what is the contribution of Argonaute slicing activities when both endogenous *alg-1* and *alg-2* genes are absent. Because the genetic alteration of both *alg-1* and *alg-2* genes leads to embryonic lethality (33), we generated animals with reduced *alg-1* and *alg-2* post-embryonic expression by using RNAi to knockdown expression of either the *alg-1* or *alg-2* gene in *alg-2* and *alg-1* mutant animals, respectively. As previously described (20), we also observed that the simultaneous loss of both Argonautes resulted in lethality due to a variety of severe developmental defects (for instance, some animals die during larval stages, whereas others die from vulval bursting when they reach adulthood; Table 1). In addition, the phenotypes observed were similar to those observed when miRNAs themselves are lost or mutated (20,34–36). These results confirm the essential roles of ALG-1 and ALG-2 in the miRNA pathway. Although the *alg-2* wild-type transgene partially rescued the developmental defects of the *alg-1; alg-2* double mutant, the transgenic expression of the wild-type *alg-1* gene nearly abolished the lethality observed in this mutant population (Table 1). These data suggest a more important contribution of ALG-1 than ALG-2 to the miRNA pathway. Interestingly, when either an *alg-1* or *alg-2* catalytically inactive form was expressed in animals with both *alg-1; alg-2* reduced function, all animal populations displayed developmental defects (Table 1). Taken together, these data strongly support the notion that the slicing activity of at least one miRNA-specific Argonaute is essential for *C. elegans* viability.

#### **The slicer activities of ALG-1 and ALG-2 are critical for the production of miRNAs**

To gather insights about the molecular role(s) of ALG-1/2 slicing activity in an animal miRNA pathway, we monitored the processing of several different miRNAs. Using northern blots, we examined both the guide and passenger strands of four different types of miRNAs: *lin-4* and *let-7* (miRNAs located on the 5'-arm of their precursors); *miR-90* (located on the 3'-arm of the precursor) and *miR-50* (a miRNA encoded within an intron). When compared with wild-type animals, we first observed that animals carrying loss-of-function alleles of either *alg-1* or *alg-2* gene have generally less mature miRNAs (Figure 4 and Supplementary Figure S4; compared with SL1 RNA, as previously reported (20,28)). We also observed that the loss of the *alg-1* Argonaute leads to the accumulation of precursor forms for all miRNA tested, while only *miR-50* and *miR-90* precursors accumulate in *alg-2* mutant animals (Figure 4A and Supplementary Figure S4A). The introduction of a transgene carrying either wild-type or catalytically inactive *alg-1* and *alg-2* genes (*alg-1 AAA*



**Figure 3.** ALG-1 can use a miRNA-like duplex to induce target cleavage. (A) Schematic of the assay. (B) The radiolabeled (<sup>32</sup>P) 32 nt RNA target was incubated with different concentration of GST-ALG-1 protein (nM) along with RNA duplexes containing zero (duplex), one (d1M) or two mismatches (d2M). (C) Comparative analysis of the cleavage induced with either single stranded RNA (ssRNA) or miRNA small RNA duplex (d2M). Cleavage product sizes are indicated as Figure 1.

**Table 1.** Phenotypic analyses of ALG-1 and ALG-2 slicing defective animals

Genotype	Transgene	RNAi	Fertile adults	Sterility (less severe)	Lethality (most severe)	n
<i>alg-1 (gk214)</i>		Control	86	0	14	125
<i>alg-1 (gk214)</i>	<i>alg-1 wt</i>	Control	100	0	0	122
<i>alg-1 (gk214)</i>	<i>alg-1 AAA</i>	Control	90	0	10	157
<i>alg-1 (gk214)</i>		<i>alg-2</i>	0	0	100	151
<i>alg-1 (gk214)</i>	<i>alg-1 wt</i>	<i>alg-2</i>	86	0	14	235
<i>alg-1 (gk214)</i>	<i>alg-1 AAA</i>	<i>alg-2</i>	0	27	73	207
<i>alg-2 (ok304)</i>		Control	100	0	0	130
<i>alg-2 (ok304)</i>	<i>alg-2 wt</i>	Control	93	0	7	137
<i>alg-2 (ok304)</i>	<i>alg 2 AAA</i>	Control	86	0	14	111
<i>alg-2 (ok304)</i>		<i>alg-1</i>	0	0	100	153
<i>alg-2 (ok304)</i>	<i>alg-2 wt</i>	<i>alg-1</i>	28	22	50	176
<i>alg-2 (ok304)</i>	<i>alg-2 AAA</i>	<i>alg-1</i>	0	28	72	200

Effect of catalytically ineffective ALG-1 and ALG-2 on animals. Transgenes expressing either wild-type or catalytically inactive ALG-1 and ALG-2 proteins were introduced in animals carrying loss-of-function (*lf*) alleles of *alg-1* or *alg-2* genes as indicated. Transgenic animals were then exposed to dsRNA targeting the other miRNA-specific Argonaute and phenotypes that are similar to the loss of the miRNA pathway were scored as percentage of n number of animals. Control RNAi corresponds to animals fed with bacteria containing the empty dsRNA-expressing plasmid. The lethality observed in animals is a consequence of a variety of severe developmental defects (some animals die during larval stages, whereas others die from vulval bursting when they reach adulthood).

and *alg-2 AAA*) in their respective mutants restored the level of mature miRNAs to that which was detected in wild-type animals (Figure 4A and Supplementary Figure S4A). Notably, the levels of *let-7* and *miR-90* miRNAs were increased relative to wild-type levels upon the expression of the *alg-1/2* transgenes, suggesting that some miRNAs may be sensitive to the expression level of miRNA-specific Argonautes (Figure 4A and Supplementary Figure S4A). Similarly, the accumulation of *lin-4* and *let-7* miRNA precursors that were detected in *alg-1* mutant animals was also abrogated in animals carrying both transgenes (Figure 4A). In contrast, only those animals expressing wild-type ALG-2 Argonaute had the levels of pre-miR-50 and pre-miR-90 restored to the levels observed in wild-type animals (Supplementary Figure S4A). Thus, these data support that both ALG-1 and ALG-2 are important for the accumulation of miRNAs in *C. elegans* and that some specificity may exist between slicer ALG-1 and ALG-2 Argonautes for the processing of some miRNAs.

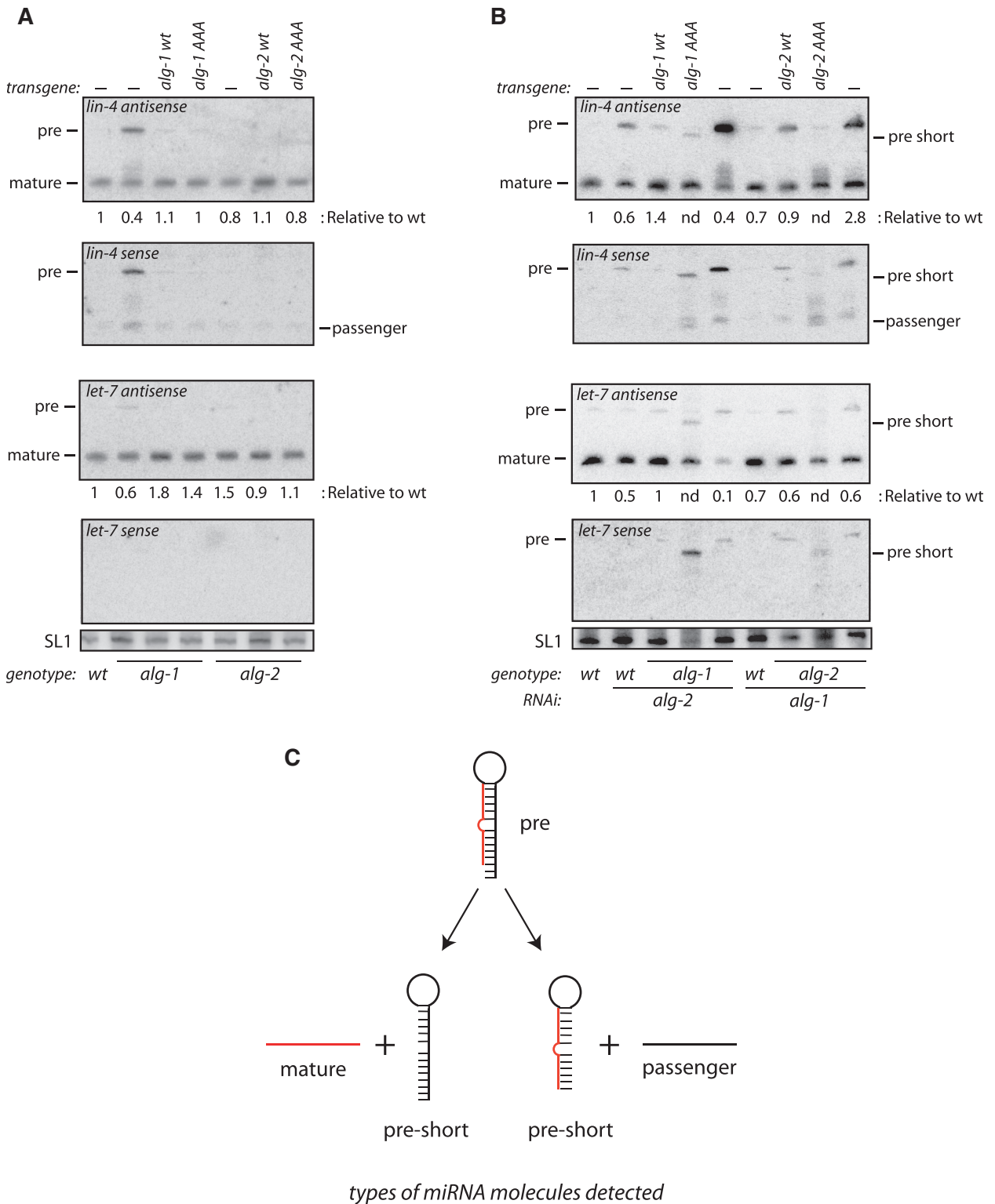
We then moved on to test the impact on various miRNA species when neither ALG-1 nor ALG-2 are slicing competent. We observed that, in the absence of both *alg-1* and *alg-2*, mature miRNA levels decreased and the precursor forms accumulated (Figure 4B and Supplementary Figure S4B). The introduction of transgenes carrying a wild-type copy of *alg-1* or *alg-2* re-established mature miRNAs to a level equivalent to that of wild-type animals, while the pre-miRNAs species was almost completely depleted (Figure 4B and Supplementary Figure S4B). When we expressed catalytically inactive forms of ALG-1/ALG-2 (*alg-1 AAA* and *alg-2 AAA*) in animals lacking both endogenous *alg-1* and *alg-2* genes, we observed an accumulation of miRNAs (Figure 4B and Supplementary Figure S4B). However, an additional RNA molecule that corresponds to the size of a truncated form of precursor miRNA (pre-short) was also observed in these conditions, as supported by the presence of a hybridization signal when either sense or antisense probes for each miRNA were used (Figure 4B and Supplementary Figure S4B). Several lines of evidence suggested that these truncated forms of the precursor miRNAs correspond to pre-miRNAs lacking the Dicer-produced mature miRNA (or passenger strand): (i) for all miRNAs tested, miRNAs accumulated to detectable levels (even the *lin-4* passenger strand is detected under these conditions; Figure 4B); (ii) the size of the truncated precursor was ~21 nt shorter than full-length precursor miRNAs (Supplementary Figure S5A) and (iii) sequencing of the cloned miR-50 truncated precursor indicated that >50% of the RNA species corresponded exactly to the precursor sequence minus the mature or passenger miRNA (Supplementary Figure S5B). We therefore conclude that both Argonautes are important for the production and the maintenance of the level of miRNAs in animals. The absence of catalytically competent ALG-1 and ALG-2 leads to the accumulation of a new truncated precursor miRNA species without significantly affecting the production of mature miRNAs.

### ALG-1 and ALG-2 slicer activity is essential to generate miRISC

Despite the fact that the phenotypes of animals lacking catalytically active ALG-1 and ALG-2 were similar to phenotypes resulting from the loss of miRNAs, we still observed an accumulation of mature miRNAs in these animals. Therefore, other molecular defects should be considered to explain the alteration of miRNA-mediated gene regulation in these animals. One possibility is that in the absence of catalytically active ALG-1/ALG-2, a significant fraction of the miRNA pool may remain associated with the truncated precursor and thus decrease the level of functional miRISC. To address this, we examined *lin-4* miRNA in ALG-1 immunoprecipitated (IPed) complexes (miRISC) that were isolated from *alg-1; alg-2* animals expressing either the wild-type or catalytically inactive ALG-1 protein. Both wild-type and catalytically inactive ALG-1 proteins share similar affinities for ssRNAs (Supplementary Figure S1B). Although we could not detect truncated pre-miRNA in the catalytically inactive ALG-1 complexes (data not shown), we observed decreases in the level of mature miRNAs associated with this ALG-1 mutant (Figure 5). This decrease in miRISC-associated mature *lin-4* correlates with an increase of free *lin-4* miRNA when compared with the levels observed for the wild-type ALG-1 immunoprecipitation experiments (Figure 5). Similarly, less *lin-4* miRNA is associated with the catalytically inactive ALG-2 mutant complexes than with wild-type ALG-2 complexes isolated from *alg-1; alg-2* transgenic animals (Supplementary Figure S6). Therefore, our data indicate that the production of miRISC is severely altered in animals lacking the miRNA-specific slicing Argonautes.

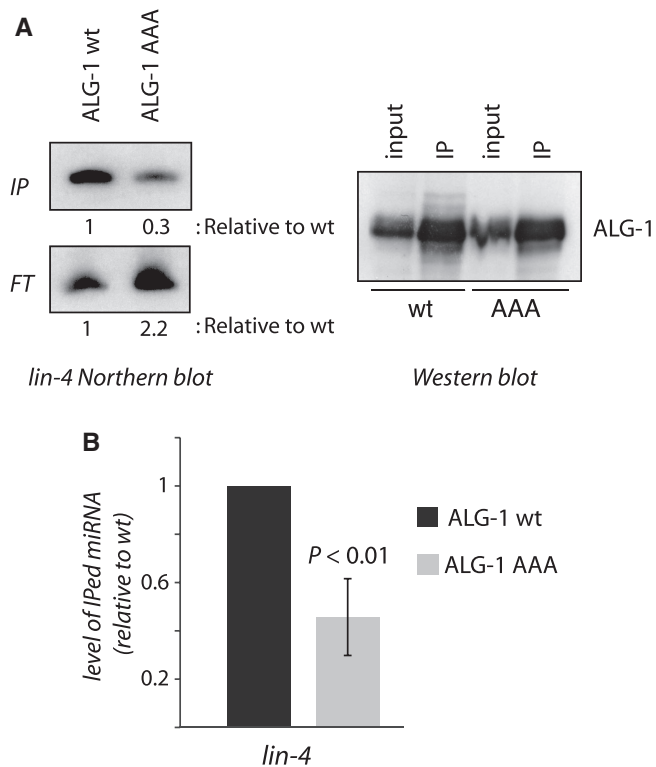
### DISCUSSION

The biochemical study of ALG-1 and ALG-2 confirms that these Argonautes have retained the molecular characteristics found in catalytically competent Argonautes of *Schizosaccharomyces pombe*, plants and other animals. The enzymatic activity of ALG-1/2 requires both magnesium (a divalent cation) (16) and the DDH motif within the PIWI domain of the proteins (37). We also observed that recombinant ALG-1/2 can bind small RNA duplexes and is capable of separating strands from miRNA-like molecules that contain mismatches. Similar data have been reported for human Argonautes (30,38). Recent studies performed with *Drosophila* embryo lysates demonstrated that the miRNA-specific Argonaute Ago1 protein also displays a preference for imperfectly paired duplexes and can only form functional miRISC complexes once loaded with these types of RNA molecules (39). In all these conditions, it is possible that the presence of a complementary target RNA to the guide strand contributes to the release of the passenger strand by Argonautes. Therefore, our results support the idea that miRNA-specific animal Argonautes have the capacity to release the miRNA passenger strand from a Dicer-processed miRNA duplex to form the functional miRISC.



**Figure 4.** The presence of the slicing-competent ALG-1 or ALG-2 is essential for the production of *let-7* and *lin-4* miRNAs. (A and B) Northern blot detection of miRNAs in animals expressing ALG-1 and ALG-2 transgenes. Upon total RNA extraction from different transgenic animals carrying either wild-type (wt) or catalytically defective (AAA) *alg-1* and *alg-2* genes, *lin-4* and *let-7* were detected using probes complementary to mature miRNA (antisense) and to the complementary strand (sense). SL1 RNA was probed and used as loading control (the detection of SL1 is only shown for one blot because the same membrane was stripped and re-used to detect RNA species from both miRNA). Precursor (pre), truncated precursor (pre short) and mature miRNA forms are indicated. The genotype and RNAi (below) as well as transgene (above) found in each animal strain are indicated. (C) Representation of the RNA molecules detected by Northern blotting.





**Figure 5.** Slicing ALG-1 is required to generate functional miRISC. (A) Detection of *lin-4* in bound (IP) or unbound (FT) fractions after ALG-1 immunoprecipitations. Transgenically expressed ALG-1 wild-type (wt) or catalytically defective (AAA) protein were IPed from *alg-1(lf)*; *alg-2(RNAi)* animals. The presence of *lin-4* strands was detected by Northern blotting (left panel) and 10% of immunopurified complexes were used for detection of ALG-1 (right panel). (B) The quantification of three independent experiments is shown in the lower panel. Errors bars represent a 95% confidence interval and a Student's two-sided *t*-test was applied to obtain *P* values.

The first study of *C. elegans* *alg-1* and *alg-2* Argonautes by Grishok *et al.* demonstrated that the loss of both Argonautes led to a decrease of mature *let-7* and *lin-4* miRNAs and an accumulation of pre-*lin-4* molecules (20). Although this initial observation suggested a role for ALG-1 and ALG-2 in the stability and production of miRNAs in *C. elegans*, it was still unclear how these proteins could contribute to miRNA biogenesis. The data presented herein reveal the role of ALG-1 and ALG-2 in miRNA production and demonstrate, for the first time, the importance of ALG-1/2 slicing activity in processing multiple miRNAs. Furthermore, our studies unveil the functional differences of these miRNA-specific Argonautes in processing different miRNA species. Although the replacement of either ALG-1 or ALG-2 with their respective catalytically inactive forms had no observable effect in animals, the loss of slicing activity for both miRNA-specific Argonautes led to severe developmental defects that are associated with the loss of miRNAs in *C. elegans* (20,34–36). At the molecular level, we observed that the loss of this enzymatic activity in miRNA-specific Argonautes led to the accumulation of mature miRNAs along with the appearance of a shorter form of miRNA precursor molecules. These observations

suggest that in the absence of slicing-competent miRNA-specific Argonautes, mature miRNAs remain associated with the complementary strand found in precursor miRNA molecules, leading to a significant decrease of miRISC that is essential for animal viability. In addition, our data also support the idea that Dicer can cleave independently RNA strands from pre-miRNA substrates.

Several lines of evidence in our data support the idea that Dicer can cleave only one strand of the precursor miRNA: (i) the size of the aberrant precursor miRNA form is about 21 nt shorter than the pre-miRNA and (ii) the mature form for all miRNA tested was detectable by northern blot. Although many *in vitro* and *in vivo* studies demonstrate that the Dicer enzyme can cleave perfectly paired dsRNA on both strands (40–44), some experimental evidence also supports that this enzyme can separately cleave both strands of a miRNA precursor. *In vitro* analysis of *Drosophila* Dcr-1, which is required for the miRNA pathway, demonstrated that a single mutation in the active site of one of RNase III domains produced a 21-nt miRNA and a truncated miRNA precursor (45). Recently, it has been shown that cells carrying specific point mutations within each RNase III domain of human Dicer display defects in the production of either 5p or 3p miRNAs (46). Biochemical studies on recombinant human Dicer also demonstrate its enzymatic capacity to produce a truncated miRNA precursor and a mature miRNA *in vitro* (47–49). In agreement with those observations, our data indicate that at least one Argonaute competent slicer is essential to enable complete Dicer cleavage of pre-miRNAs. Therefore, in absence of the slicing Argonautes, Dicer may cleave only once and thus affects the formation of miRISC. Alternatively, we can also consider that after Dicer produces a single 21-nt molecule from the pre-miRNA, slicing Argonautes may be important to cleave within the complementary sequence to trigger the degradation of the truncated precursor.

Previous observations indicated that Argonaute proteins and Dicer are found in the same complex and form the RISC loading complex (50–54). It has recently been observed that human Ago2 can bind some pre-miRNA molecules even in absence of Dicer (55). Therefore, the presence of co-factors such as slicing competent Argonautes might be essential to coordinate Dicer cleavage in animals. Future analysis with purified RISC loading complex will help in gaining insight about the contribution of slicing Argonaute proteins to this process.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1–6 and Supplementary Methods.

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