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Drosophila microRNAs are sorted into functionally distinct Argonaute protein complexes after their production by Dicer-1

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SUMMARY

Both small interfering RNAs (siRNAs) and microRNAs (miRNAs) serve as guides for distinct classes of RNA-induced silencing complexes (RISCs) that repress mRNA expression in diverse biological processes, ranging from development to antiviral defense. In *Drosophila*, separate but conceptually similar endonucleolytic processing pathways produce siRNAs and miRNAs. Here, we show that despite their distinct biogenesis, double-stranded miRNAs and siRNAs participate in a common sorting step that partitions them into Ago1- or Ago2-containing effector complexes that silence their target RNAs by different mechanisms. As a consequence, miRNAs are not restricted only to Ago1, as previously proposed, but can assemble into Ago2-RISC, the small RNA directed complex typically associated with siRNA-mediated RNAi. We find that Ago2-RISC is unable to repress mRNA target in which the miRNA-binding sites contain central mismatches. Conversely, Ago1 cannot mediate RNAi because it is an inefficient nuclease whose catalytic rate is limited by the dissociation of its reaction products. Our data suggest that in *Drosophila*, the two members of the Ago sub-clade of Argonaute proteins are functionally specialized, but specific classes of small RNAs are not restricted to associate with Ago1 or Ago2.

INTRODUCTION

microRNAs (miRNAs) are ~22 nt RNA guides that control gene expression in both plants and animals (Bartel, 2004; Du and Zamore, 2005). miRNAs regulate genes required for a wide range of cellular functions such as differentiation and development (Kanellopoulou et al., 2005; Lee et al., 1993; Reinhart et al., 2000; Lee et al., 2004; Bernstein et al., 2003; Ketting et al., 2001; Grishok et al., 2001; Harfe et al., 2005; Li and Carthew, 2005), metabolic homeostasis (Poy et al., 2004; Teleman et al., 2006), and memory (Schratt et al., 2006; Ashraf et al., 2006). In animals, miRNAs typically reduce the stability or repress translation of the mRNAs they regulate. miRNAs can regulate mRNAs with which they are only partially complementary, because they bind their target RNAs largely through a small region at the 5′-end of the miRNA (positions 2 through 7 or 8), the 'seed' (Lewis et al., 2003; Lai, 2002; Brennecke et al., 2005). Consequently, half or more of the protein coding genes in *Drosophila* and humans are

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predicted to be regulated by miRNAs (Stark et al., 2003; Lewis et al., 2005; Lewis et al., 2003; Krek et al., 2005; Rajewsky and Socci, 2004).

Animal miRNAs are produced by the sequential action of two distinct RNase III endonucleases. Drosha converts primary miRNAs, most of which are full-length RNA polymerase II transcripts, into pre-miRNAs, ~70 nt RNAs with partial self-complementarity such that they fold into a stem-loop or hairpin structure. Dicer then excises the mature miRNA, bound to its miRNA* strand, from the pre-miRNA (Hutvágner et al., 2001; Grishok et al., 2001; Ketting et al., 2001; Lee et al., 2003). In *Drosophila*, distinct Dicer enzymes produce siRNA and miRNA. Dicer-1 (Dcr-1) acts with a double-stranded RNA (dsRNA) binding protein partner, Loquacious (Loqs), to convert pre-miRNA to a miRNA/miRNA* duplex, whereas Dicer-2 (Dcr-2) produces siRNA duplexes by cleaving long dsRNA (Bernstein et al., 2001; Forstemann et al., 2005; Saito et al., 2005; Jiang et al., 2005; Lee et al., 2004). Dcr-2 also acts with its dsRNA-binding partner protein, R2D2, to load an siRNA duplex into Ago2 (Tomari et al., 2004b; Tomari et al., 2004a; Matranga et al., 2005; Liu et al., 2003; Liu et al., 2006), a function that is separable from its role in siRNA production (Lee et al., 2004; Pham et al., 2004).

Both siRNAs and miRNAs act as components of RNA-induced silencing complexes (RISCs); the core protein component of all RISCs is a member of the Argonaute family of small RNA-guided RNA-binding proteins (Tabara et al., 1999; Hammond et al., 2001; Song et al., 2003; Meister et al., 2004b; Rand et al., 2004; Liu et al., 2004; Song et al., 2004; Rivas et al., 2005; Baumberger and Baulcombe, 2005; Qi et al., 2005). The *Drosophila* genome encodes five Argonaute proteins, which form two sub-clades. The Ago sub-clade comprises Ago1 and Ago2, which have been reported to bind miRNAs and siRNAs respectively (Hammond et al., 2001; Okamura et al., 2004). Piwi, Aub, and Ago3 form the Piwi sub-clade of Argonaute proteins and are believed to bind repeat-associated siRNAs (rasiRNAs), which direct silencing of selfish genetic elements such as transposons (Saito et al., 2006; Vagin et al., 2006).

Argonaute proteins are readily identified by their characteristic single-stranded RNA-binding PAZ domain and their Piwi domain, a structural homolog of the DNA-directed RNA endonuclease, RNase H. The Piwi domain is thought to bind a small RNA guide both by coordinating its 5′ phosphate and through contacts with the phosphate backbone, arraying the small RNA so as to create the seed sequence. Only a subset of Argonaute proteins contain Piwi domains that retain their RNA-directed RNA endonuclease activity: e.g. Ago1 in plants, Ago2 in mammals, and both Ago1 and Ago2 in flies. *Drosophila* Ago1 and Ago2 have been proposed to be restricted to the miRNA and siRNA pathways respectively (Okamura et al., 2004; Saito et al., 2005). Such restriction of each class of small RNA to a distinct Argonaute complex could occur because miRNAs and siRNAs are produced by different Dicer pathways in flies (Figure 1A).

In this manuscript, we show that the specific pathway that produces a miRNA or siRNA does not predestine that small RNA to associate with a particular Argonaute protein (Figure 1B). Rather, we find that a miRNA produced by Dcr-1 and Loqs can nonetheless be loaded by Dcr-2 and R2D2 into an Ago2-containing RISC. Our data from both cultured *Drosophila* cells and adult flies suggest that small RNA production and small RNA loading into Argonaute protein complexes are separate steps in vivo. In the accompanying manuscript (Tomari and Zamore), we describe the molecular basis for sorting of small RNA duplexes, explaining why some miRNAs associate predominantly with Ago1 while others associate mainly with Ago2. Here, we show that the sorting of miRNAs into Ago1- and Ago2-RISCs has unexpected consequences for the mechanism of target mRNA regulation: Ago1, but not Ago2, can repress an mRNA containing multiple, partially complementary miRNA-binding sites in its 3~ untranslated region (UTR), whereas Ago2, but not Ago1, can silence an mRNA containing fully complementary miRNA-binding sites. The different regulatory capacities of Ago1 and

Ago2 can be explained, in part, by our finding that while Ago2 is a robust, multiple-turnover RNA-directed RNA endonuclease, Ago1 is not.

RESULTS

miR-277 is produced by Dcr-1, but loaded into Ago2

Like all known *Drosophila* miRNAs, miR-277 is produced by cleavage of its precursor by Dcr-1, acting together with Loquacious (Loqs) (Forstemann et al., 2005), rather than Dcr-2, which generates siRNAs (Forstemann et al., 2005; Saito et al., 2005; Jiang et al., 2005; Lee et al., 2004). Both siRNAs and miRNAs are proposed to be loaded into Argonaute-containing effector complexes from double-stranded intermediates: guide/passenger strand duplexes for siRNAs and miRNA/miRNA* duplexes for miRNAs (Hutvágner and Zamore, 2002; Matranga et al., 2005; Rand et al., 2005; Leuschner et al., 2006; Miyoshi et al., 2005). The miR-277/miR-277* duplex is predicted to have more double-stranded character than typical miRNA/miRNA* duplexes, which are interrupted by mismatches and internal loops (Khvorova et al., 2003; Han et al., 2006). Thus, miR-277, an authentic miRNA generated by Dcr-1 cleavage of pre-miR-277, has a miRNA/miRNA* duplex that resembles an siRNA. We asked if the resemblance of the miR-277/miR-277* duplex to an siRNA led to its being loaded into Ago2, rather than Ago1, in *Drosophila* cells. That is, is the biogenesis of a miRNA tightly coupled to its loading into Ago1? Or are miRNAs, and perhaps siRNAs, sorted into distinct Ago proteins by a step unlinked to the Dicer that produced them?

To this end, we established stable lines of Schneider S2 cells expressing GFP mRNA, GFP mRNA whose 3′ untranslated region (UTR) contained one or two sites fully complementary to miR-277, or GFP mRNA containing four 3′ UTR sites complementary to miR-277 but bearing mismatches with miR-277 nucleotides 9, 10, and 11 (Figure 2A, S1, and S2). GFP expression was quantified by flow cytometry. To determine if endogenous miR-277-programmed RISCs repressed each reporter, we transfected each stable S2 line with either a control or a miR-277 complementary 2′-*O*-methyl antisense oligonucleotide (ASO; Figure S1). In cultured cells and in vivo, ASOs inhibit the function of miRNAs to which they are complementary, relieving repression of their mRNA targets (Berger et al., 2005; Krutzfeldt et al., 2005; Meister et al., 2004a; Hutvagner et al., 2004). Figure 2A shows representative FACS traces from a single experiment; the arithmetic means for four trials are presented in Figure 2B. The presence of two miR-277-complementary sites in the 3′ UTR of the GFP reporter mRNA repressed its expression: transfecting the cells with the miR-277-specific but not with an unrelated luciferase-specific ASO increased GFP expression more than 5-fold (Figure 2B); a reporter bearing a single miR-277-complementary site behaved similarly (Figure S2). In contrast, GFP expression was indistinguishable for the cells expressing the GFP reporter mRNA that lacked miR-277-binding sites when they were transfected with the control or the miR-277-specific ASO.

Repression of the reporter bearing two perfectly complementary miR-277-binding sites required Ago2 but not Ago1 (Figure 3). Treating the S2 cells with dsRNA to deplete Ago2 by RNAi increased GFP expression ~6-fold (Figure 3A and 3B). This agrees well with the extent of derepression observed with the miR-277-specific ASO, suggesting that in the absence of Ago2, the reporter is no longer repressed. Surprisingly, *ago1(RNAi)* increased repression of the GFP reporter containing two fully complementary miR-277-binding sites. Essentially identical data were obtained for a GFP reporter containing a single miR-277-binding site (Figure S2). These observations are consistent with in vitro data that the Ago1 and Ago2 loading pathways compete for small RNA duplexes whose structures are intermediate between a fully complementary siRNA and a prototypical miRNA/miRNA* duplex (Tomari and Zamore, accompanying manuscript).

Expression of the miR-277-regulated reporter also increased when the S2 cells were treated with dsRNA to deplete Drosha, the enzyme that excises premiRNAs from their primary transcripts, or with dsRNA to deplete Dcr-1 or Loqs, which together convert pre-miRNA to miRNA/miRNA* duplexes (Figure 3A). RNAi directed against *ago1*, *ago2*, or *drosha* had no detectable effect on the expression of the GFP reporter lacking miR-277-binding sites, although dsRNA corresponding to GFP itself significantly decreased GFP expression. We note that the dsRNA used as a control is not inert with respect to Ago2-dependent silencing (i.e., RNAi), likely because the control dsRNA can compete with miR-277 for Ago2 loading. The idea that non-specific dsRNA can compete for Ago2 and other components of the Ago2-loading machinery is consistent with earlier reports that RNAi is a saturable process (Haley et al., 2004). Thus, the most straightforward method to assess the significance of the effect of different dsRNAs on miR-277-directed repression of the perfect reporter in this experiment is not to compare the individual specific knock-down experiments to the control dsRNA, but rather to compare the change in GFP expression for the unregulated reporter to that observed for the perfect reporter for each RNAi knock-down. Analyzed this way, depletion of components of the miRNA biogenesis pathway clearly has a significant effect on miR-277-directed repression of the reporter: *dcr-1(RNAi)*, $p < 0.005$; *drosha(RNAi)*, $p < 0.007$; *loqs(RNAi)*, $p < 0.028$.

Together with previously published results (Forstemann et al., 2005), our data therefore suggest that miR-277 is produced by the standard miRNA pathway, but directs repression of the perfectly matched GFP reporter through Ago2.

Ago1 but not Ago2 mediates repression of target mRNAs bearing bulged miR-277-binding sites

mRNAs containing miRNA-binding sites with perfect complementarity to specific miRNAs occur in animals, but are rare (Yekta et al., 2004; Mansfield et al., 2004; Davis et al., 2005). Instead, most miRNA are incompletely complementary to the mRNAs whose expression they repress. Typically, these miRNAs bind to multiple sites in the 3′ UTR of their mRNA targets. This mode of miRNA-directed repression can be recapitulated by engineering a reporter mRNA bearing in its 3′ UTR four, partially mismatched, miRNA-binding sites, each of which forms a central bulge when paired to its cognate miRNA (Doench et al., 2003; Zeng et al., 2002).

We established stable lines of S2 cells expressing a GFP mRNA bearing four such sites in its 3′ UTR (Figure 2A). Repression of the reporter was modest, but required miR-277: transfection of a miR-277-specific, but not a control, 2′-*O*-methyl antisense oligonucleotide caused a small but statistically significant ($p < 0.003$) increase in GFP fluorescence (Figure 2B). miR-277 is relatively abundant in S2 cells, which contain ~2,200 miR-277 molecules per cell (MH and PDZ, unpublished). Nonetheless, we wondered if the free pool of endogenous Ago1-loaded miR-277 was insufficient to repress expression of the bulged reporter. If so, additional miR-277 might increase the amount of miR-277-programmed Ago1 available to repress the partially complementary reporter mRNA. Our idea builds on earlier observations that small RNAs in the Ago2 pathway act catalytically, guiding repeated cycles of target recognition and cleavage (Haley and Zamore, 2004; Hutvágner and Zamore, 2002) and the idea that when small RNAs accelerate mRNA decay or direct translational repression of imperfectly complementary target RNAs, they bind stoichiometrically.

We increased the expression of miR-277 by engineering stable S2 lines expressing both the GFP reporter and a ‘mini’ pri-miR-277 driven by the ubiquitin promoter. The resulting doubling of miR-277 expression (Figure 5C) caused a dramatic increase in the repression of the bulged GFP reporter, as evidenced by the more than 3-fold increase in GFP fluorescence observed when a miR-277-specific ASO was transfected into the cells (Figure 4A). Compared to the repression of this reporter by endogenous miR-277, the exogenous miR-277 increased repression of the bulged reporter by 230 percent (Figure 2A and 4A). Repression was also

enhanced, but to a smaller extent, for the reporter bearing two perfectly complementary miR-277-binding sites.

For both the reporter bearing perfectly complementary miR-277-binding sites and the reporter with four bulged miR-277-binding sites, miR-277 reduced GFP expression by reducing the stability of the reporter mRNA, rather than by repressing GFP translation. We used qRT-PCR to measure the steady-state reporter mRNA abundance (Figure S3A) and FACS to measure GFP protein abundance (Figure S3B) for the stable cell lines expressing the GFP control reporter and the reporters bearing two perfect or four bulged miR-277-binding sites. For each stable cell line, we measured reporter mRNA and protein expression after transfection with a control ASO or a miR-277-specific ASO. Even when miR-277 was over-expressed, nearly all of the increased GFP protein expression observed when miR-277 was specifically blocked with an ASO could be accounted for by a corresponding increase in GFP mRNA expression. This can be seen most clearly in Figure S3C, which reports the relative GFP protein expression normalized to the relative GFP mRNA expression. In all cases, the ratio of relative protein expression to relative mRNA expression when miR-277 was inhibited was close to one, indicating that most of the miR-277-directed reporter repression was caused by mRNA destablilization rather than translational repression. Alternatively, for the bulged reporter, mRNA degradation might be tightly couple to translational repression and therefore be a consequence, rather than a cause, of the decrease in protein.

Silencing of the bulged reporter required Ago1 but not Ago2: *ago1(RNAi)* increased reporter expression, whereas *ago2(RNAi)* (Figure 3A) caused a small but statistically significant decrease in reporter expression ($p < 0.008$) (Figure 4B). While RNAi directed against *drosha*, *dcr-1*, or *loqs*—all genes required for miRNA biogenesis—increased expression of the bulged reporter, *dcr-2(RNAi)* and *r2d2(RNAi)*—both genes required to load small RNAs into Ago2, but not for loading Ago1—caused a small but statistically significant ($p < 0.001$ and $p < 0.003$, respectively) decrease in reporter expression. These data suggest that (1) the Ago1 and Ago2 pathways compete for miR-277 and (2) Ago1 and Ago2 are functionally distinct and non-redundant, with Ago2 alone mediating small RNA-directed silencing of perfectly complementary target mRNAs and Ago1 mediating silencing by a pathway not requiring base pairing between the center of the small RNA guide and the corresponding region of its RNA target.

miR-277 accumulation requires Ago2

Our experiments in stable S2 reporter cell lines suggest that miR-277 is loaded predominantly into an Ago2-containing RISC and that Ago1 and Ago2 compete for miR-277 in cultured *Drosophila* cells. Moreover, they suggest that miR-277 repressed the reporter to which it was fully complementary as a component of an Ago2-RISC, but repressed the bulged reporter as a component of an Ago1-RISC. Supporting this view, the cellular concentration of miR-277 decreased when Ago2 was depleted by RNAi, but not when Ago1 was depleted (Figures 5A). The concentration of *bantam*, a miRNA shown previously to associate exclusively or predominantly with Ago1 (Okamura et al., 2004), was reduced by *ago1(RNAi)*, but unaffected by *ago2(RNAi)*. Pre-*bantam* RNA was unaltered by either treatment (Figure 5A), supporting the idea that the loss of *bantam* in *ago1(RNAi)* S2 cells reflects a failure to load the miRNA into its Ago1-RISC, rather than a defect in pre-miRNA processing, which would cause pre-*bantam* to accumulate.

Moreover, most *bantam* but only a minority of miR-277 is physically associated with Ago1 (Figure 5, B and C). We immunoprecipitated Ago1 using a monoclonal antibody bound to agarose beads. Western blotting with the same antibody demonstrated that the overwhelming majority of Ago1, but little or no Ago2, was depleted from the supernatant and recovered with

the beads (Figure 5B). Northern blotting showed that more than half of *bantam*, but less than a third of miR-277, was recovered with the beads (Figure 5C).

In vivo, miR-277 is produced by Dcr-1, then loaded by Dcr-2 into Ago2

Both *dcr-1(RNAi)* and *dcr-2(RNAi)* increased GFP expression for the reporter mRNA bearing two fully complementary miR-277-binding sites (Figure 3A). While the effect of *dcr-1* dsRNA was anticipated, current models for the miRNA pathway in *Drosophila* do not predict a role for Dcr-2 in miRNA function. Moreover, *dcr-2(RNAi)* did not detectably alter the expression of the known components of the miRNA pathway, Dcr-1, Loqs, Drosha, or Ago1 (Figure 3B). We can imagine two explanations for the reduction in miR-277 function when Dcr-2 was depleted. Dcr-1 and Dcr-2 might both act in the production of miR-277, with each contributing to the conversion of pre-miR-277 to miR-277/miR-277* duplex. Alternatively, Dcr-1 alone might excise miR-277 from pre-miR-277, remanding the resulting miR-277/miR-277* duplex to the RISC-loading complex (RLC), whose core constituent is the Dcr-2/R2D2 heterodimer and which is required to load siRNA duplexes into Ago2.

To distinguish between these two explanations, we examined in adult flies the expression of the same GFP reporter bearing two fully complementary miR-277-binding sites that we used in our S2 cell experiments. To separate the role of Dcr-2 in small RNA production from its function in loading Ago2, we used the *dcr-2*^{G31R} allele (Lee et al., 2004). The G31R mutant Dcr-2 protein cannot dice long dsRNA into siRNAs, but retains the ability to load small RNA duplexes into Ago2. The GFP reporter was expressed from a single-copy transgene in flies heterozygous and homozygous for the *dcr*^{G31R} mutation. For comparison, we also examined GFP reporter expression in *dcr2*^{L811fsX} mutant flies, which produce no Dcr-2 protein and can neither produce siRNA duplexes nor load siRNAs into Ago2. We prepared protein extracts from adult flies and measured GFP expression by Western blotting and by fluorescence per μg of total protein (Figure 6 and data not shown).

By both measures, GFP expression of the reporter bearing two perfectly complementary miR-277-binding sites increased significantly in homozygous *dcr2*^{L811fsX} mutant flies, relative to that measured in extracts from their heterozygous siblings (Figure 6), corroborating our observation that expression of this reporter was increased in S2 cells treated with dsRNA targeting *dcr-2* (Figure 3A). However, reporter expression was unaltered in homozygous *dcr-2*^{G31R} mutant flies, relative to their heterozygous siblings (Figure 6). Reporter expression similarly increased in flies lacking R2D2 (Figure 6); R2D2 acts together with Dcr-2 to load Ago2, but is not required for siRNA production (Liu et al., 2003). We conclude that the requirement for Dcr-2 in miR-277-directed silencing of the GFP reporter bearing two fully complementary miR-277-binding sites reflects a role for Dcr-2 in loading miR-277 into Ago2, rather than in the conversion of pre-miR-277 into mature miR-277.

In contrast to the perfectly matched reporter, the GFP reporter bearing four bulged miR-277-binding sites was unaltered in flies homozygous for either the *dcr-2*^{L811fsX} null allele or the *dcr-2*^{G31R} separation-of-function allele. Thus, repression of this reporter in vivo does not require Ago2 loading, strong support for our conclusion that the bulged reporter is regulated by miR-277-programmed Ago1-RISC. In fact, we observed a small but statistically significant increase in the repression of the bulged reporter in flies homozygous for the *r2d2*^{1} allele (Figure 6B), which expresses normal amounts of Dcr-2 but lacks detectable R2D2 in adults (Tingting Du and PDZ, unpublished). These data suggest that as in vitro (Tomari and Zamore, accompanying manuscript) and in cultured cells (see above), Ago1 and Ago2 in vivo compete for loading with miR-277, and that in the absence of the Ago2-loading machinery, more miR-277-programmed Ago1-RISC is produced.

Ago1 cleaves target RNAs with low efficiency

Drosophila Ago1 retains the ability to catalyze endonucleolytic cleavage of a perfectly matched target RNA (Miyoshi et al., 2005; Okamura et al., 2004). Thus, it is surprising that for the GFP reporter bearing two perfectly complementary miR-277-binding sites in its 3′ UTR, the sub-population of miR-277 associated with Ago1 did not detectably rescue the loss of silencing caused by depletion from S2 cells of Ago2 or the loss in adult flies of Dcr-2—a core component of the Ago2-loading machinery. Instead, our data suggest that only Ago2 can catalyze RNAi in flies. To assess the molecular basis for the distinct functional capacities of Ago1 and Ago2, we analyzed in vitro the kinetics of target cleavage by each protein (Figure 7).

In *Drosophila*, the structure of a small RNA duplex governs into which Argonaute protein—Ago1 versus Ago2—it is loaded (Tomari and Zamore, accompanying manuscript). For the *let-7* miRNA sequence, an siRNA duplex containing *let-7* as its guide strand loads Ago2 almost exclusively, whereas the *let-7*/*let-7** duplex loads only Ago1. By adjusting the time allowed for RISC assembly in *Drosophila* embryo lysate programmed with either a *let-7* siRNA or the *let-7*/*let-7** duplex, we generated approximately equal concentrations (4.6 to 4.7 nM) of Ago1- and Ago2-associated *let-7*, as determined by the amount of ^{32}Pradiolabeled *let-7* that could be captured on an immobilized 2′-*O*-methyl oligonucleotide antisense to *let-7*. For each *let-7*-programmed RISC, we measured the rate of cleavage of an RNA target containing a single site with complete complementarity to *let-7* (Figure 7A).

Our data reveal two differences between *Drosophila* Ago1 and Ago2. First, Ago2 is a faster enzyme than Ago1: the initial rate of target cleavage for Ago2 was at least 12-fold greater than that of Ago1 (Figure 7A). Second, Ago1, unlike Ago2, failed to efficiently catalyze multiple rounds of target cleavage in vitro, even in the presence of ATP. That is, for Ago2-RISC, the rate of target cleavage was the same throughout the steady-state phase of the reaction (when less than 20 percent of the substrate had been converted to product), while the rate of target cleavage for Ago1-RISC was biphasic (Figures 7A). Such biphasic behavior was reported previously for both *Drosophila* and human Ago2-mediated target cleavage in the absence of ATP and has been interpreted to suggest that product release is rate-determining for Ago2 under these conditions (Haley and Zamore, 2004; Rivas et al., 2005). By analogy to Ago2 catalysis in the absence of ATP, the first phase of the Ago1 reaction in the presence of ATP likely corresponds to a pre-steady state period in which most Ago1 proteins have not yet released the product of their first target cleavage event. The second phase may correspond to a steady-state period in which the products of target cleavage are slowly released from Ago1. Supporting this view, the second phase fit well to a line whose *y*-intercept, ~3.2 nM, was similar to the amount of Ago1-RISC measured by affinity purification using an immobilized 2′-*O*-methyl *let-7* ASO, ~4.7 nM.

In theory, the difference in efficiency between Ago1- and Ago2-catalyzed target cleavage might reflect a difference in the rate of catalysis or in the affinity of the Argonaute-*let-7* complex for the perfectly complementary site on the target RNA. To distinguish between these two explanations, we performed a kinetic analysis of Ago1- and Ago2-RISC programmed with *let-7* in *Drosophila* embryo lysate. We estimated the amount of active *let-7* programmed RNAi enzyme complex from the size of the burst for Ago1-RISC in the presence of ATP and for Ago2-RISC by depleting ATP after RISC assembly. In the presence of ATP, Ago2-RISC conforms to a simple Michaelis-Menten scheme (Haley and Zamore, 2004):

$$\text{Ago2}-\textit{let}-7+\text{target RNA}<->[\text{Ago2}-\textit{let}-7\bullet\text{target RNA}]->\text{Ago2}-\textit{let}-7+\text{cleaved target RNA}$$

In contrast, Ago1-RISC appears to follow a more complex kinetic scheme, even in the presence of ATP:

$$\text{Ago1} - \textit{let} - 7 + \text{target RNA} < - > [\text{Ago1} - \textit{let} - 7 \bullet \text{target RNA}] - >$$
$$[\text{Ago1} - \textit{let} - 7 \bullet \text{cleaved target RNA}] < - > \text{Ago1} - \textit{let} - 7 + \text{cleaved target RNA}$$

Because Michaelis-Menten parameters are determined from the initial velocity of the enzyme observed at different concentrations of substrate (target RNA), we analyzed Ago1 as if it followed the same kinetic scheme as Ago2. This pseudo-Michaelis-Menten analysis allows the Ago1 K_M to retain the same meaning—an approximation of the affinity of the enzyme for its substrate—as that determined for Ago2. Our data (Figure 7B and Table 1) suggest that *let-7*-programmed Ago1 and Ago2 bind the *let-7* complementary sequence in the target RNA with nearly the same affinity: the K_M for Ago1 was 13.3 ± 3.2 nM; for Ago2, 8.4 ± 1.0 nM. In contrast, k_{cat} for Ago1 was 43-fold less than that determined for Ago2 (0.005 ± 0.0013 s^{-1} versus 0.215 ± 0.025 s^{-1}). In vitro, Ago1 binds its target RNAs as well as Ago2, but cleaves them much more slowly than Ago2. These in vitro data suggest that in vivo, Ago1 is too inefficient to silence a perfectly matched target by endonucleolytic cleavage, the hallmark of RNAi in flies and mammals.

DISCUSSION

The experiments presented here show that in lysates from *Drosophila* embryos, in cultured *Drosophila* S2 cells, and in adult flies, miRNAs are loaded into both Ago1 and Ago2. Our data suggest that sorting miRNAs into Ago1- and Ago2-RISC generates silencing complexes with distinct functional capacities: Ago1-RISC represses expression of targets with which its guide miRNA matches only partially, whereas Ago2 silences fully matched target RNAs. These differences result, in part, from the surprisingly different catalytic efficiencies of Ago1 and Ago2: only Ago2 catalyzes robust, multiple-turnover target cleavage.

Why does *Drosophila* Ago1 retain its endonuclease activity?

In mammals, only Ago2 retains the ability to catalyze guide RNA-directed endonucleolytic cleavage of RNA; the three other mammalian Argonaute proteins, Ago1, Ago3, and Ago4, lack a functional active site that is presumed to have been present in the evolutionarily ancestral Argonaute protein. Why then has *Drosophila* Ago1 retained any endonuclease activity at all, if it is so inefficient at target cleavage that it cannot measurably contribute to small RNA-directed RNAi? One potential explanation is that the primary role of the Ago1 endonuclease activity is to facilitate loading of Ago1-RISC. That is, the predominant substrate for the Ago1 endonuclease is not target RNA, but rather miRNA* strands, and perhaps the occasional siRNA passenger strand. Because miRNA* strand cleavage would occur only in *cis* and only once per loaded Ago1-RISC, efficient, multiple-turnover cleavage of target RNA would not be required if the endonuclease activity of Ago1 acted predominantly or exclusively during RISC assembly, rather than the effector step of RNA silencing.

Our data reveal an important biochemical difference between Ago2 and Ago1, but they do not explain the molecular basis for the inefficiency of Ago1-directed cleavage of target RNA. We can envision two explanations for the more than 40-fold lower k_{cat} of Ago1 compared to Ago2. First, the active site of Ago1 might be less well suited to catalyzing phosphodiester bond cleavage. Alternatively, Ago1 might be slow to assume a catalytically active conformation. In this second model, the rate of breaking a phosphodiester bond would not limit the rate of target RNA cleavage. Rather, the rate of a conformational rearrangement would limit the speed of target RNA cleavage by Ago1. Such a conformational rearrangement of the siRNA guide has been proposed previously for Ago2 (Tomari and Zamore, 2005; Filipowicz, 2005).

Implications for the mechanism of guide strand choice

Neither the current genome sequence of *Drosophila melanogaster* nor Genbank in its entirety contains an mRNA with complete complementarity to miR-277. Why then do flies load miR-277 into Ago2-RISC? Perhaps there are—yet unknown— viral RNAs targeted by Ago2-loaded miR-277. Such an innate immune response function has previously been proposed for miRNAs in mammals (Lecellier et al., 2005). Regardless of the biological purpose for loading miR-277 into Ago2, miR-277 provides an important in vivo test of the controversial proposal that the production of small RNA duplexes by Dicer is uncoupled from the loading of one strand of the duplex into Argonaute proteins and the concomitant destruction of the other strand (Aza-Blanc et al., 2003; Schwarz et al., 2003; Khvorova et al., 2003). That Dcr-2 and R2D2 act in vivo to load Ago2 with miR-277, a miRNA produced by Dcr-1 and Loqs, confirms previous in vitro data suggesting that both ends of a small RNA duplex are available for examination by the Ago2 loading machinery (Schwarz et al., 2003; Tomari et al., 2004a; Preall et al., 2006). Our results suggest that the miR-277/miR-277* duplex dissociates from Dcr-1 after the dicing of pre-miR-277 and is then bound by the Dcr-2/R2D2 heterodimer, which loads it into Ago2; Sontheimer and colleagues reached similar conclusions about small RNA loading from in vitro experiments that asked if dicer processing and Ago2-loading were coupled (Preall et al., 2006).

We reason that Ago1 loading is also uncoupled from dicing. In all animals, some miRNAs are found on the 5′ and other on the 3′ arm of their pre-miRNA stem-loops. In contrast, the geometry of Dcr-1 with respect to the two arms of the pre-miRNA stem is essentially the same for all miRNAs: Dcr-1 always makes staggered cuts that separate the pre-miRNA loop from the miRNA/miRNA* duplex. If Dcr-1 were to load miRNAs directly into Ago1, without first releasing the miRNA/miRNA* duplex, we would expect that all miRNAs would reside on the same arm of the pre-miRNA stem. The simplest explanation, and one most consistent with the partitioning of miR-277 into both Ago1- and Ago2-RISCs, is that miRNA/miRNA* duplexes are released from Dicer immediately after their production, then rebound by the Ago1- and Ago2-loading machineries. Such a model allows both the terminal thermodynamics of the miRNA/miRNA* duplex to determine the mature miRNA strand (rather than its position within the premiRNA) and the pattern of mismatches within the duplex to determine how the miRNA partitions between Ago1 and Ago2.

Why are Ago1 and Ago2 functionally specialized?

In mammals, siRNAs produce off-target effects largely by acting like miRNAs (Jackson et al., 2006; Lim et al., 2005; Jackson et al., 2003). In flies, siRNAs loaded into Ago2 are believed to defend against viral infection (Wang et al., 2006; Galiana-Arnoux et al., 2006). Virus-derived siRNAs might therefore trigger widespread, off-target silencing of host genes as flies mount an anti-viral RNAi response. The partitioning of siRNAs into Ago2-RISC appears to circumvent this problem, because silencing by *Drosophila* Ago2 requires greater complementarity between the siRNA and its target than silencing by Ago1. It is tempting to speculate that a similar functional specialization among Argonaute proteins—with silencing by Ago2 requiring greater complementarity to its targets—has gone undetected in mammals.

EXPERIMENTAL PROCEDURES

Construction of reporter plasmids and RNAi trigger dsRNAs

To create an expression vector for both cultured cells and transgenic flies, we PCR amplified the 3′ UTR and SV40 poly-A signal from plasmid pEGFP-N1 (Clontech, Mountain View, CA, USA) with oligonucleotides 5′-ATC ACT CTC GGC ATG GAC GAG-3′ and 5′-GTG AAT TCA TAC ATT GAT GAG TTT GGA C-3′ and inserted the resulting PCR product into pUbi-Casper2 (a kind gift of Dr. Siu Ing The) using the *Not*I and *Eco*RI restriction sites, creating

vector pKF60. For the GFP-insert, we transferred a *Bam*HI-*Not*I fragment from pEGFP-N1 (Clontech) into pBluescript (Stratagene, La Jolla, CA, USA) cut with *Bam*HI/*Not*I, creating pKF20. Subsequently, we annealed the oligos 5′-CAT GGA ACA AAA ACT TAT TTC TGA AGA AGA CTT GGG-3′ and 5′-CAT GCC CAA GTC TTC TTC AGA AAT AAG TTT TTG TTC -3′, encoding a myc-tag, and ligated this DNA-fragment into *Nco*I-cut pKF20. After sequencing, one clone was selected that contained a triple insertion in the correct orientation (pKF30). From this plasmid, the myc_3-GFP-sequence was transferred as a *Bam*HI-*Not*I-fragment into pKF60, resulting in plasmid pKF62. To remove an *Xba*I-site from the pCASPER2 polylinker, pKF62 was cut with *Xba*I, the ends treated with Klenow polymerase (New England Biolabs, Ipswitch, MA, USA), and the vector was re-ligated, creating pKF63. This plasmid was transformed into *dam/dcm* negative bacteria (strain GM2163, New England Biolabs), which rendered a second *Xba*I-site in the 3′-UTR, adjacent to the *Not*I-site, cleavable. To insert the miR-277 target sites, we annealed oligos 5′-GGC CTG TCG TAC CAG ATA GTG CAT TTA CAG TGT CGT ACC AGA TAG TGC ATT TA-3′ and 5′-CTA GTA AAT GCA CTA TCT GGT ACG ACA CTG TAA ATG CAC TAT CTG GTA CAG CA-3′ for the two perfectly matched sites, and oligos 5′-GGC CTG TCG TAC CAG AGG ATG CAT TTA CAG TGT CGT ACC AGA GGA TGC ATT TAT GTC GTA CCA GAG GAT GCA TTT ACA GTG TCG TAC CAG AGG ATG CAT TTA -′ and 5′-CTA GTA AAT GCA TCC TCT GGT ACG ACA CTG TAA ATG CAT CCT CTG GTA CGA CAT AAA TGC ATC CTC TGG TAC GAC ACT GTA AAT GCA TCC TCT GGT ACG ACA-3′ for the four bulged sites, then ligated the DNA fragments into *Not*I-*Xba*I-cut pKF63, creating pKF67 and pKF68, respectively.

These pCASPER2-derived expression plasmids were used both for the generation of stable S2-cell lines and for the P-element-mediated genetic transformation *Drosophila melanogaster* (Rubin and Spradling, 1982).

Constructs to make dsRNA directed against GFP, *dcr-1*, *dcr-2*, *loqs* and *drosha* were described previously (Forstemann et al., 2005). Templates for the synthesis of dsRNA directed against *ago1* and *ago2* were generated by T/A-cloning PCR products generated using the oligonucleotides 5′-CGC ACC ATT GTG CAT CCT AAC GAG-3′and 5′-GGG GAC AAT CGT TCG CTT TGC GTA-3′ for *ago2* and 5′-ATT TGA TTT CTA TCT ATG CAG CCA-3′ and 5′-GCC CTG GCC ATG GCA CCT GGC GTA-3′ for *ago1* into the modified Litmus28i vector described previously (Forstemann et al., 2005). The template for producing dsRNA targeting *r2d2* was generated by PCR using oligonucleotides 5′-CGT AAT ACG ACT CAC TAT AGG CAT ACA CGG CTT GAT GAA GGA TTC-3′ and 5′-CGT AAT ACG ACT CAC TAT AGG TTG CTT GTG CTC GCT ACT TGC-3′. Templates for in vitro transcription were generated by PCR-amplification of each plasmid construct using a single primer corresponding to the T7 promotor (5′-CGT AAT ACG ACT CAC TAT AGG-3′) and dsRNA for knock-down was generated as described in (Haley et al., 2003).

Construction of cell lines with increased miR-277 expression

A 270 nt fragment of genomic DNA surrounding the miR-277 sequence was PCR-amplified from S2-cell genomic DNA with the oligonucleotides 5′-GCG GAT CCG GTA CCT ATA CAT ATA TAA CGA GGC CTA ACG-3′ and 5′-ATG CGG CCG CAA AAC AGT GTC TTA CAA ACA AGT GG-3′. The resulting DNA fragment was cloned *Bam*HI to *Not*I into pKF62, yielding mini-pri-miR-277 transgene under the control of the ubiquitin promotor. miR-277 over expression was quantified by comparing the amount of miR-277, relative to the amount of endogenous *bantam* miRNA, in extracts from cells expressing only endogenous miR-277 and in cells containing the mini-pri-miR-277 transgene (Figure 5C).

Cell culture and flow cytometry

Drosophila Schneider 2 cells were cultured at 28°C in Schneider's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum (Invitrogen). GFP expression plasmids were transfected with siLentfect (see below) at 1 μg of plasmid per well of a 24-well plate. For selection of stable transformants, 20 ng of phsNeo (Steller and Pirrotta, 1985) was co-transfected with 1 μg each GFP reporter plasmid. Five days after transfection, the cells were split 1:5 into medium supplemented with 1.2 mg/ml G418 (Invitrogen). The cells were diluted 1:5 every 7 days into G418-containing medium for three weeks, then serial dilutions were plated into a 96-well plate in growth medium supplemented with 1% (v/v) sterile-filtered medium used previously for S2-cell culture (conditioned medium). After two weeks of growth, wells with a single colony of cells were expanded and analyzed by flow cytometry. Cell clones that produced a single peak in the flow cytometer were retained for use as reporter cells.

For transfection with RNAi-triggers, the cells were seeded at a density of 1×10^6 cells/ml in 24 well plates (500 μl/well) using Schneider's medium without G418. Liposome/nucleic acid complexes were prepared with 1.5 ml siLentfect (BioRAD, Hercules/CA, USA) and either 1 μg of dsRNA (estimated by native agarose gel electrophoresis) or 10 pmol of cholesterol conjugated, 2′-*O*-methyl modified antisense-oligonucleotide (see Figure S1B) per well in a total volume of 100 μl Schneider's medium without serum, incubated at room temperature for 45 min and added to the cells (100 μl/well of a 24 well plate). After 6 days, the cells were analyzed by FACS (BD FACScan flow cytometer; Becton Dickinson, Franklin Lakes, NJ). GFP-expression was quantified by determining the arithmetic mean of the fluorescence using the CellQuest software package (Becton Dickinson).

Generation of anti-Dcr-1 and anti-Ago2 polyclonal antibodies

The KLH-conjugated peptide CQGLIAKKD was used to immunize rabbits to generate anti-Dcr-1 antibodies (Covance Research Products, Denver, PA, USA) as described (Bernstein et al., 2001). The antiserum was affinity-purified using the synthetic peptide coupled to a column matrix (Sulfolink, Pierce, Rockford, IL, USA) via an N-terminal cysteine according to the manufacturer's protocol. To generate anti-Ago2 antibodies, rabbits were immunized (ProSci, Poway, CA, USA) with the Ago2 PAZ-domain fused to glutathione-S-transferase expressed and purified as described (Lingel et al., 2003). The antiserum was purified using a NusA-Ago2-PAZ fusion protein (Lingel et al., 2003) immobilized on a column matrix (Aminolink plus, Pierce) according to the manufacturer's protocol. The KLH-conjugated peptide CSDEYESSKDKAMD was used to immunize chickens to generate anti-R2D2 antibodies (Gallus Immunotech, Cary, NC, USA).

Western Blotting

Proteins were extracted either from cultured S2 cells or from hand-dissected adult fly head and thorax with PBS containing 1% Triton X-100 (Sigma, St. Louis, MO, USA) and protease inhibitors (Complete without EDTA, Roche Molecular Biochemicals, Basel, Switzerland). For quantification of myc_3GFP-expression in transgenic flies, 20 μg of total protein was resolved by electrophoresis through a 12% polyacrylamide/SDS gel and transferred to PVDF-membrane (Immobilon-P, Millipore, Billerica, MA, USA) by semi-dry transfer (BioRAD, Hercules, CA, USA) in 25 mM Tris (pH 8.3), 250 mM glycine, 10% (v/v) methanol as anode buffer and 20 mM CAPS, pH 11.0, as cathode buffer at 20 V for 120 min. Purified monoclonal anti-myc 9E10 (Sigma #M4439) was used diluted 1:1000 in 25 mM Tris, 137.5 mM NaCl, 2.5 mM KCl, 0.02% (v/v) Tween-20 (Sigma) for 90 min at room temperature. HRP-conjugated goat anti-rabbit secondary antibody, used at 1:1,000 dilution, and chemiluminescent substrate were from the Pierce SuperSignal West Dura kit (Pierce). HRP-conjugated rabbit anti-chicken secondary antibody (Gallus Immunotech), diluted 1:15,000, was used to detect the affinity purified anti-

R2D2 chicken IgY. Western Blot images were acquired using a Fuji LAS-3000 (Fujifilm Life Sciences, Stamford, CT, USA) and quantified using ImageGauge (Fujifilm Life Sciences). As a loading control, **α**-tubulin was detected with anti-**α**-tubulin DM1A (Sigma #T6199) diluted 1:1000. A standard curve for myc_3-GFP detection was created by diluting extract from pKF63-transgenic flies into extract from yw flies, and initial Western blot signals corrected using this standard curve.

To detect S2-cell proteins, 50 μg total protein was resolved by electrophoresis through an 8% polyacrylamide/SDS gel and transferred to PVDF as above. Incubation with primary antibodies was overnight at 4°C; secondary antibodies were incubated with the membrane for 120 min at room temperature. The primary antibodies were diluted 1:2000. Ant-Dcr-2 antibody was the kind gift of Qinghua Liu (Liu et al., 2003).

Ago1 and Ago2 target cleavage kinetics

Target cleavage reactions were performed essentially as previously described (Haley and Zamore, 2004; Haley et al., 2003). In Figure 7A, 50 nM *let-7* siRNA or *let-7/let-7** duplex was incubated with *Drosophila* 0–2 h embryo lysate for 2 min or 5 min to program Ago2-RISC (~4.7 nM) or Ago1-RISC (~4.6 nM), respectively. In Figure 7B, 20 nM *let-7* siRNA or *let-7/let-7** duplex was incubated with lysate for 3 min or 8 min to program Ago2-RISC or Ago1-RISC, respectively. For Ago2 cleavage in Figure 7B, RISC was diluted 10-fold in *N*-ethyl maleimide (NEM) treated embryo lysate (Haley and Zamore, 2004; Nykanen et al., 2001). Control experiments demonstrated that in $ago2^{414}$ lysate the *let-7* siRNA assembled little or no active RISC, as assessed by target cleavage activity, whereas *let-7/let-7** was as active in $ago2^{414}$ as in wild-type lysate, indicating that the *let-7* siRNA and *let-7/let-7** duplex are almost exclusively loaded Ago2- and Ago1-RISC, respectively.

RISC assembly was stopped by treatment with NEM followed by DTT to quench unreacted NEM for both Ago1- (Figure S4A) and Ago2-RISC (Nykanen et al., 2001). Control experiments (Figure S4B) established that the biphasic kinetics of Ago1-RISC in the presence of ATP were not a consequence of treatment with NEM. RISC concentration was estimated by 2′-*O*-methyl ASO affinity purification in Figure 7A and by the size of the pre-steady-state burst in Figure 7B (Haley and Zamore, 2004; Schwarz et al., 2003). The concentration of RNA target was 100 nM in Figure 7A and 0.5 to 100 nM in Figure 7B. Data were analyzed using IGOR 5 (WaveMetrics) and *Visual*Enzymics 2005 (Softzymics) software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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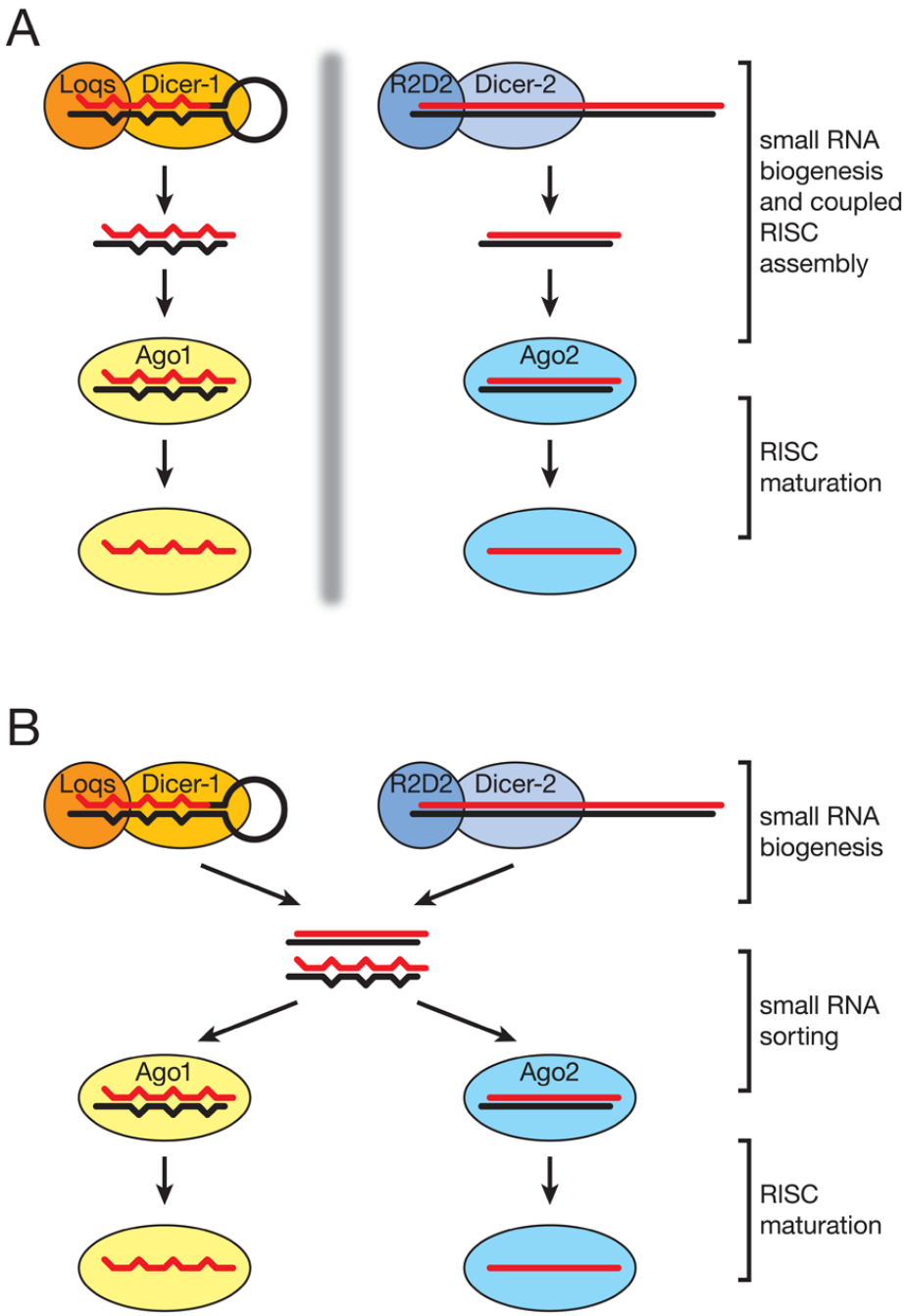

Figure 1. Two models for the miRNA and siRNA pathways in *Drosophila*

(A) Small RNA biogenesis and RISC assembly are tightly coupled. miRNAs are exclusively loaded into Ago1 and siRNAs into Ago2 . (B) Small RNA biogenesis and RISC assembly are independent. After their production, small RNA duplexes are proposed to be actively sorted into distinct Ago proteins according to their structures, not their precursor origins.

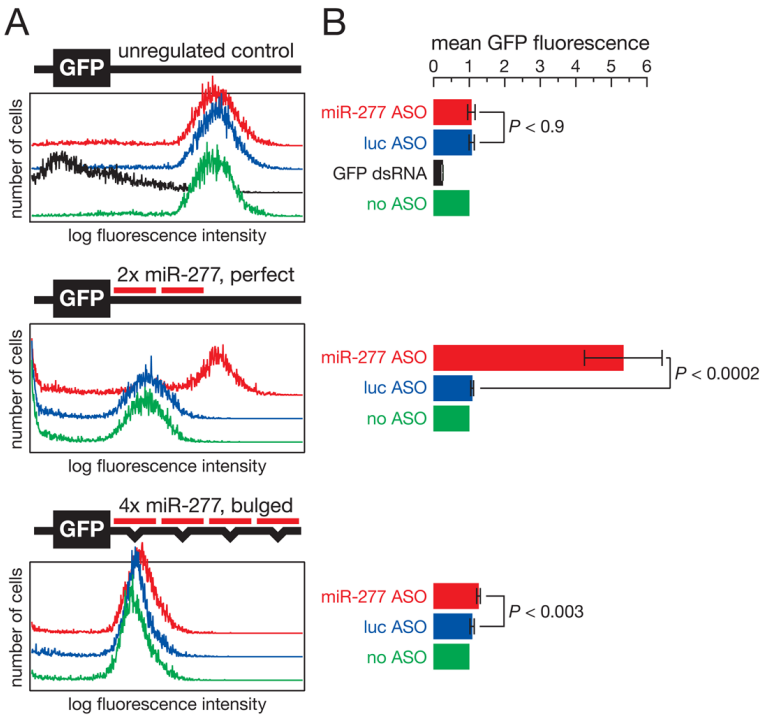

Figure 2. Regulation of GFP reporter expression in cultured *Drosophila* S2 cells by endogenous miR-277

Clonally derived stable cell lines were generated that expressed control GFP unregulated by miR-277, GFP bearing two miR-277-complementary sites in its 3′ untranslated region (UTR), and GFP bearing in its 3′ UTR four miR-277-complementary sites, each containing three central mismatches to miR-277, producing a 'bulge.' Each cell line was transfected with a cholesterol-conjugated, 2′-*O*-methyl modified, antisense oligonucleotide (ASO) complementary to miR-277 or to an unrelated luciferase sequence. As a control, the unregulated GFP reporter cell line was transfected with GFP dsRNA. (A) Representative FACS profiles from a single experiment. (B) The average of mean fluorescence recorded in three trials. Error bars indicate ± one standard deviation. *P*-values were calculated using a two-sample T-test assuming equal variances.

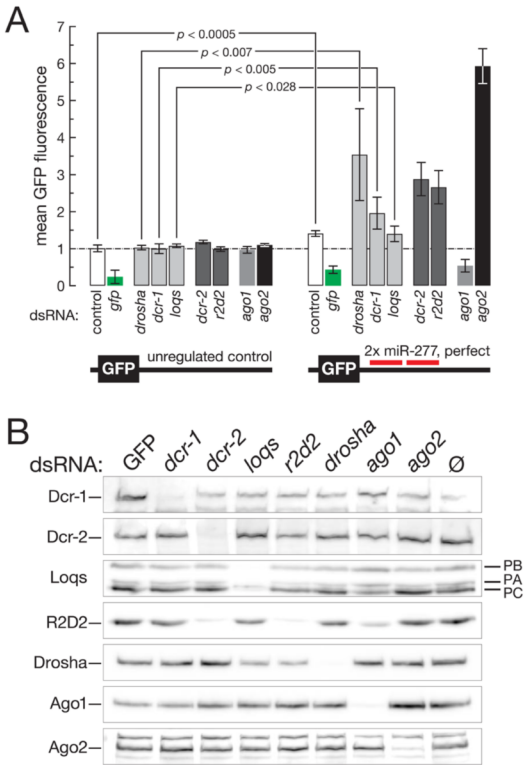

Figure 3. Silencing of the reporter bearing two perfectly matched miR-277 complementary sites required components of both the miRNA biogenesis and the RNAi pathways, including Ago2, but is potentiated by depletion of Ago1

(A) Mean GFP fluorescence (average ± standard deviation for three or four trials). DsRNA-triggered RNAi was used to deplete the cells of the indicated protein. (B) Western blotting confirmed the success and specificity of the RNAi-mediated depletion for each protein. *dcr-2 (RNAi)* reduced the abundance of both Dcr-2 and R2D2, as previously reported (Liu et al., 2003), but r2d2(RNAi) had no detectable effect on Dcr-2 abundance. The three isoforms of Loqs are indicated at the right of the Loqs panel. The bands above and below Ago2 correspond to cross-reacting proteins characteristically detected with this antibody.

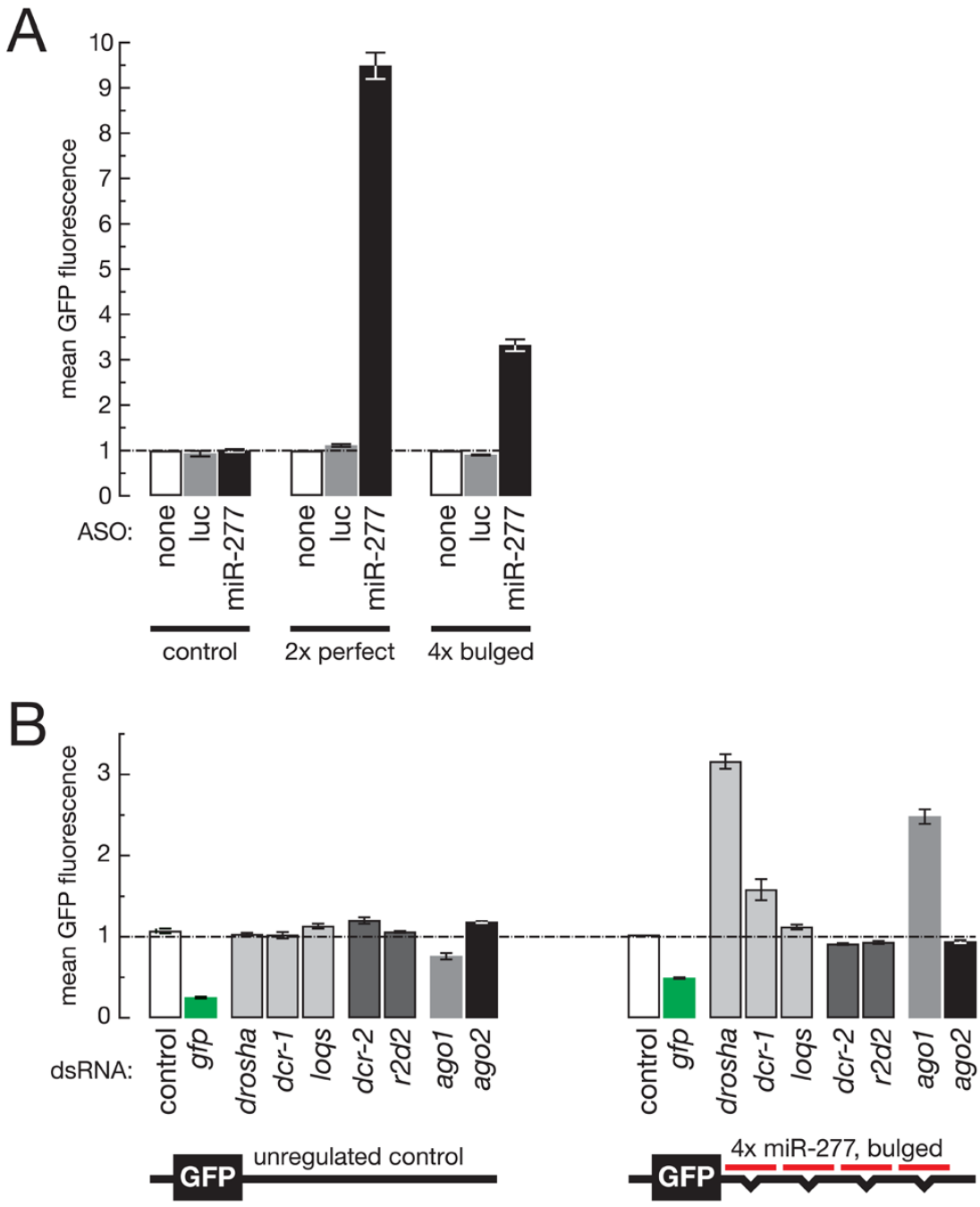

Figure 4. Silencing of the reporter bearing four imperfectly matched miR-277 complementary sites required components of both the miRNA biogenesis pathway and Ago1, but not Dcr-2, R2D2, or Ago2, components of the RNAi pathway

(A) Over-expression of miR-277 from a mini-pri-miRNA transgene increased repression of the miR-277-regulated perfectly matched and bulged reporters. (B) Mean GFP fluorescence (average ± standard deviation for three or four trials). DsRNA-triggered RNAi was used to deplete the cells of the indicated protein.

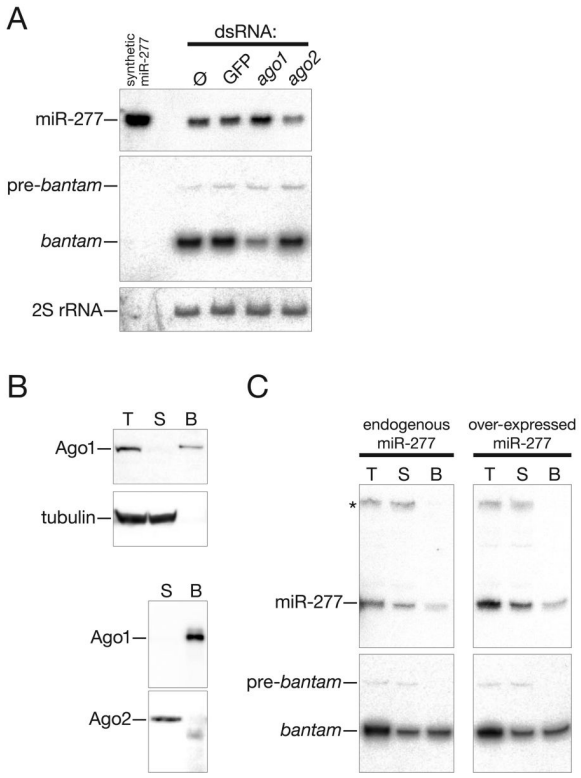

Figure 5. Most endogenous miR-277 is not associated with Ago1 in S2 cells

(A) Northern analysis reveals that *ago2(RNAi)* reduced the steady-state abundance of miR-277, but not *bantam*, whereas *ago1(RNAi)* decreased the abundance of *bantam*, but not pre-*bantam* or miR-277. (B) Western blotting shows that immunoprecipitation of Ago1 depleted nearly all Ago1, but little or no Ago2, from S2 cell cytoplasmic extract. (C) Northern analysis shows that the majority of *bantam*, but not pre-*bantam* co-immunoprecipitated with Ago1. In contrast, the majority of endogenous and of over-expressed miR-277 remained, in the supernatant, unbound by Ago1. The asterisk marks non-specific hybridization of the miR-277 probe with 5S RNA.

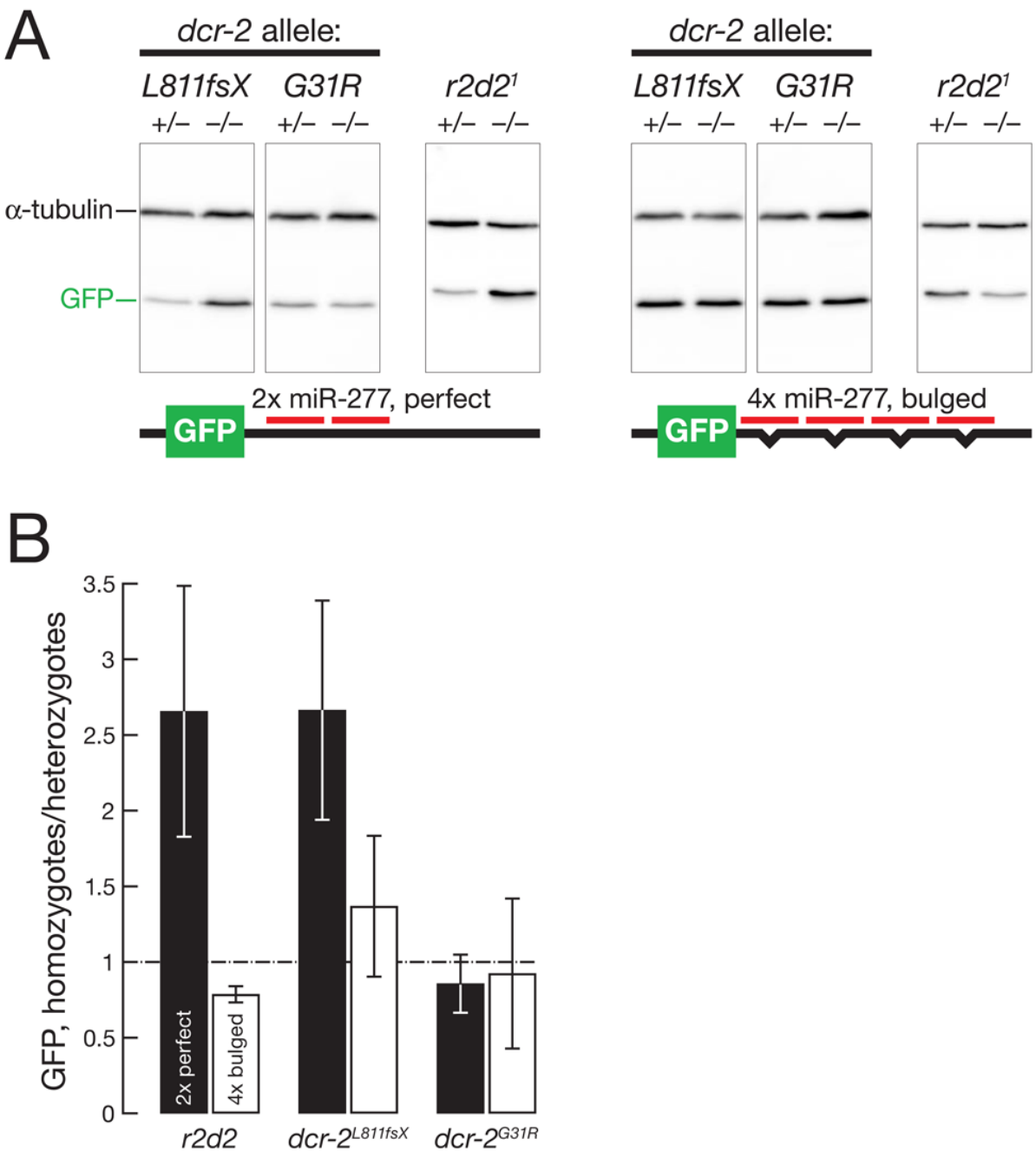

Figure 6. In vivo in adult flies, miR-277 repression of the GFP reporter via perfectly complementary sites requires the loading activity of Dcr-2 and R2D2, but repression via bulged sites does not

(A) Representative Western blotting data of **α**-tubulin and GFP reporter in total lysates from adult flies of the indicated heterozygous (+/−) and homozygous (−/−) mutant genotypes. (B) The average (± standard deviation) GFP expression in homozygous mutant flies, relative to heterozygotes, for three (*r2d2*) or four trials of the experiment in (A). The *dcr-2*^{L811fsX} mutant lacks detectable Dcr-2 protein, whereas the *dcr-2*^{G31R} point mutant produces a Dcr-2 protein that cannot dice long dsRNA, but can nonetheless load small RNA duplexes, such as siRNA and miRNA/miRNA* duplexes, into Ago2.

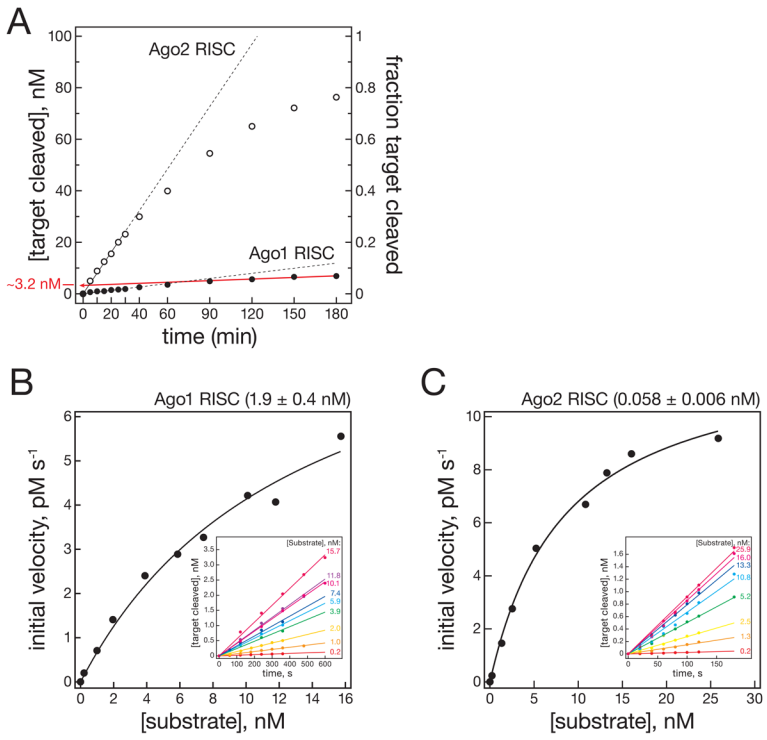

Figure 7. Ago1 is a poor endonuclease

(A) Distinct cleavage kinetics distinguish Ago1- and Ago2-RISC. At approximately equal enzyme concentrations, the initial velocity for Ago2-RISC was ~12-fold greater than that of Ago1-RISC. Cleavage by Ago2-RISC was linear throughout the reaction, as long as the substrate remained in vast excess, whereas cleavage by Ago1-RISC showed biphasic behavior, suggesting the product release step is rate-limiting. The RISC concentration estimated by burst analysis (~3.2 nM; red arrow) correlated well with that measured by 2′-*O*-methyl ASO affinity capture (~4.6 nM). (B) Pseudo-Michaelis-Menten and (C) Michaelis-Menten analyses of Ago1- and Ago2-RISC, respectively. Michaelis-Menten parameters are summarized in Table 1.

Table 1

Kinetic analysis of *Drosophila* Ago1- and Ago2-RISC.

	K_M (nM)	V_{max} (nM s^{-1})	[RISC] (nM)	k_{cat} (s^{-1})	k_{cat} (relative)	k_{cat}/K_M (nM^{-1} s^{-1})	k_{cat}/K_M (relative)
Ago1	13.3 ± 3.2	0.0096 ± 0.0013	1.9 ± 0.4	0.005 ± 0.0013	**1**	0.4 ± 0.1	**1**
Ago2	8.4 ± 1.0	0.0125 ± 0.006	0.058 ± 0.006	0.215 ± 0.025	**43**	25.5 ± 4.3	**64**