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Small RNA Structure and Dicer/Argonaute Dependence During RISC Assembly in *Drosophila*

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ABSTRACT

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Short interfering RNAs (siRNAs) and microRNAs (miRNAs) are both processed from longer, double-stranded RNA (dsRNA) presursors by a member of the Dicer (Dcr) family of proteins. siRNAs generally arise from perfectly base-paired dsRNAs, whereas miRNAs are excised from 60-70 nt pre-miRNA hairpins containing multiple bulges and mismatches within the duplexed region. In animals, siRNAs usually induce target mRNA endonucleolytic cleavage, whereas miRNAs usually direct translation inhibition or exonucleolytic degradation. The Argonaute (Ago) proteins associate directly with mature small RNAs within RNA-induced silencing complexes (RISCs). When RISC directs silencing via endonucleolytic mRNA cleavage, the small-RNA-directed "slicer" activity resides within the Argonaute protein itself. The *Drosophila* siRNA and miRNA pathways involve functionally specialized isoforms of Dicer and Argonaute proteins. Previous studies showed that Dcr-1 and Ago-1 are primarily involved in miRNA biogenesis and silencing, whereas Dcr-2 and Ago-2 are mostly devoted to the siRNA pathway. A more recent model suggested Ago-2 is involved in the functioning of some miRNAs, and Dcr-2 acts as a gatekeeper for the assembly of Ago-2 mediated RNA-induced silencing complexes (RISCs) by promoting the incorporation of siRNAs and disfavoring miRNAs as loading substrates for Ago-2. Our study used the differential Dicer-2 dependence exhibited by siRNA and miRNA duplexes *in vitro* to define RNA features that dictate Dcr-2 specificity. We found

that the degree of base pairing at the 5' end of the guide strand plays a predominant role in specifying siRISC assembly. We also found unanticipated variety in the Dcr-2 and Ago-2 dependence displayed by a series of miRNAs with different structural features, some of which disagree with current models. These data, combined with other studies, suggest that miRNAs may possess less uniformity than previously envisioned in their functional characteristics.

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1-1. The discovery of double-stranded RNA induced silencing and the main players

The discovery of double-stranded RNA induced silencing

In 1990, Napoli and Jorgensen conducted a study to generate violet petunias by overexpressing chalcone synthase (CHS), an enzyme in the anthocyanin biosynthesis pathway that is responsible for the deep violet coloration in petunias. Instead of the deep violet color that they had expected, the transgene resulted in white petals. The levels of both endogenous and introduced CHS were 50-fold lower than in wild type petunias, which led them to hypothesize that the introduced transgene was "co-suppressing" the endogenous CHS gene (Napoli et al., 1990). In 1992, Romano and Macino reported a similar phenomenon in *Neurospora crassa*, noting that introduction of homologous RNA sequences caused "quelling" of the endogenous gene (Romano and Macino, 1992). In 1995, Guo and Kemphues observed that the introduction of sense or antisense RNA to par-1 mRNA resulted in degradation of the par-1 mRNA in *C. elegans* (Guo and Kemphues, 1995). At that time, antisense RNA was thought to function by hybridization with endogenous mRNAs resulting in double-stranded RNA (dsRNA), which either inhibited translation or was targeted for destruction by cellular ribonucleases. Surprisingly, Guo and Kemphues found that sense and antisense par-1 RNA preparations are each sufficient to target the par-1 message for degradation. This finding could not be explained by the current dogma because the sense par-1 RNA would not hybridize with the endogenous par-1 transcript.

In 1998, Fire and Mello provided an explanation for the previously reported silencing of endogenous genes by "cosuppression, quelling and sense mRNA". They tested the requirement for structure of the interfering RNA, and surprisingly found that double-stranded RNA (dsRNA) was substantially more effective at producing interference than was either purified sense or antisense RNA alone. They indicated that the seemingly paradoxical finding of Guo and Kemphues showing that introduction of sense RNA leads to gene silencing was due to the contamination of preparations of ssRNA by dsRNA resulting from the activity of bacteriophage RNA polymerases. Thus, Fire and Mello provided the first explanation for previous observations, implicating integrated transgenes in the production of dsRNA in plants and fungi, and contamination of sense RNA by dsRNA in worms. This work established an entirely new conceptual framework for the effects of RNA on gene silencing by highlighting a role for dsRNA. This inhibition, which was later seen in flies (Kennerdell and Carthew, 1998) and many other eukaryotic organisms, came to be known as RNA interference (RNAi).

Discovery of a silencing intermediate

Even when minute quantities of dsRNA was injected into one region of a worm or plant, the interference spread throughout the organism, which led to the hypothesis that the RNAi effect was mediated by some derivative of the dsRNA (Fire et al., 1998; Grishok et al., 2000; Voinnet and Baulcombe, 1997). This hypothesis was further supported by the observation that gene silencing could be passed from parent to progeny in C. elegans. The first clue in the search for such “derivatives” came from study of silencing in plants (Hamilton and Baulcombe, 1999). Although the full-length antisense strand was never detected, Hamilton and Baulcombe detected

~25 nucleotides (nt) antisense RNA, and they suggested that this length was necessary for RNAi specificity. Soon, similar small RNAs were found in extracts made from *Drosophila* embryo and S2 cells by two independent groups of biochemists. Both groups found that 21–23 nt RNA always co-fractionated with RNAi activity, suggesting that dsRNA was converted to shorter intermediates, small interfering RNAs (siRNAs), guiding mRNA cleavage (Hammond et al., 2000; Zamore et al., 2000). To determine that the 21–23 nt dsRNAs are indeed the effector molecules of the RNAi pathway, Tuschl and colleagues incubated Drosophila cell extracts with chemically synthesized 21–22 nt dsRNAs targeting a firefly luciferase transcript (Elbashir et al., 2001b). The siRNAs were able to act as guides to mediate cleavage of the target mRNA. siRNAs with 2–3 nt overhangs on their 3' ends were more efficient in reducing the amount of target mRNA than siRNAs with blunt ends. The target mRNA was found to be cleaved near the center of the region encompassed by the 21–22 nt RNAs, 11 or 12 nt downstream of the first base pair between the siRNA and target mRNA. In addition to Drosophila, they also showed that these chemically synthesized 21–22 nt siRNAs duplexes specifically suppressed expression of endogenous and heterologous genes in different mammalian cell lines (Elbashir et al., 2001a).

Identification of Dicer

Using Drosophila S2 cell extracts, Bernstein et al. (2001) determined that the enzymes responsible for the cleavage of the dsRNA into siRNAs were different from those involved in the cleavage of the target mRNA. They found that by high-speed centrifugation the activity that cleaved the target mRNA, which they coined RNA-induced silencing complex (RISC), could be separated from the activity that cleaved the dsRNA into siRNAs. These two distinct phases of the

RNAi pathway were designated the initiator (dsRNA converted to siRNAs) and effector (RISC-mediated cleavage of target mRNA) phases. Using a candidate gene approach, they identified the initiator enzyme: Dicer (Dcr), an RNase III family member (Bernstein et al., 2001).

RNase III family members are among the few nucleases that show specificity for dsRNA. There are three types of enzymes in this family. First is the canonical RNase III, which contains a single RNase III signature motif and a dsRNA-binding domain (dsRBD). Second is a class represented by Drosha, an enzyme that contains two RNase III motifs and a dsRBD. The third class, represented by Dicer, contains an amino-terminal helicase domain in addition to two RNase III motifs and one dsRBD. Dicer homologues have been found in all organisms in which RNAi activity has been reported. They cleave long dsRNAs into 20-23 nt dsRNAs that contain 3' di-nucleotide overhangs and bear 5'-monophosphate and 3'-hydroxyl termini.

Identification of "Slicer"

To purify RISC, Martinez et al. pulled down affinity-tagged siRNAs in human HeLa cell extracts, and analyzed the associated proteins. They demonstrated that a single-stranded siRNA resides in the RISC together with Argonaute 1 (Ago-1) and/or Argonaute 2 (Ago-2) proteins (Martinez et al., 2002). Argonaute proteins are a highly conserved family whose members contain two characteristic domains: PAZ and PIWI. Argonaute proteins have been found to be associated with RNAi in various organisms in screens for RNAi-deficient mutants. These include C. elegans rde-1, Arabidopsis AGO1, and Neurospora QDE2 (Cogoni and Macino, 1997; Fagard

et al., 2000; Tabara et al., 1999). There are eight human Argonaute proteins, of which four belong to the Argonaute subcategory (Ago1–4) and four to the Piwi subcategory (hPiwi 1–4).

Although Argonaute proteins had been identified as key mediators for RNAi, the lack of canonical ribonuclease domains in the amino acid sequence of Ago1 and Ago2 made them poor candidates for the "slicer" enzyme that is responsible for cleaving target mRNAs. In 2003 and 2004, it was revealed that the PAZ domain adopts the structure of a nucleic-acid-binding module, termed the oligonucleotide/oligosaccharide-binding fold (OB-fold), for the 3' di- nucleotide overhang of a small RNA (Lingel et al., 2003; Ma et al., 2004; Song et al., 2003; Yan et al., 2003). Shortly after, the first structures incorporating the PIWI domain showed that a portion of PIWI resembles RNase H (Parker et al., 2004; Song et al., 2004), leading to the discovery that PIWI – or Argonaute – is the "slicer" (Liu et al., 2004; Meister et al., 2004; Rivas et al., 2005; Yuan et al., 2005).

The active site of the RNase H domain is comprised of the DDE motif, which consists of three amino acids with the side chain carboxylates positioned to catalyze the cleavage reaction. The reaction is dependent on divalent cations such as Mg^{2+} or Mn^{2+}. Unlike most ribonucleases, the RNase H enzymes resemble deoxyribonucleases, which leave 3' OH and 5' phosphate termini. Slicer activity, like RNase H enzyme activity, is dependent on divalent cations and leaves 3' OH and 5' phosphate termini (Martinez and Tuschl, 2004). Having solved the crystal structure of the Argonaute protein from *Pyrococcus furiosus* (PfAgo), Song et al. modeled the structure of human Ago-1. Accordingly, the N-terminal, PIWI, and middle domains of Argonaute lie in a crescent to support the fourth domain, PAZ. The 3' end of the single-stranded siRNA sits in the

groove of the PAZ domain, which allows the rest of the siRNA to bind to target mRNA. The target mRNA then sits on top of the PIWI domain, which causes cleavage of the message (Song et al., 2004). By solving the crystal structure of PfAgo with Mn^{2+} ion, Rivas et al. identified a DDH motif that comprises the active site of Argonaute, instead of the DDE motif in RNase H enzymes (Rivas et al., 2005). This DDH motif is conserved in both human Ago-2 (hAgo2) and human Ago-3, whereas the histidine is an arginine in human Ago-1 and human Ago-4. Mutation studies showed all three catalytic residues are required for slicer function, which provided an explanation to the catalytic defect in hAgo1 and hAgo4. But why hAgo3 is incapable of cleaving mRNA remains to be answered. Recently, Patel and colleagues solved the crystal structure of *Thermus thermophilus* argonaute bound to a 5'-phosphorylated 21-base DNA guide strand. They showed that amino acid residues at the active site and the 5'-phosphate-binding pocket of the Mid domain are critical for cleavage activity, whereas the ones at the 3'-end-binding pocket in the PAZ domain are not (Wang et al., 2008).

dsRNA-binding proteins

RNAi is initiated by the Dicer-mediated processing of long dsRNA to siRNA duplexes. SiRNA is then assembled on to the effector RISC complex to direct the sequence specific cleavage of target mRNA. However, it was unclear as to how the initiation and effector steps were connected. In 2003, Liu et al. discovered a novel component of the RNAi pathway during chromatographic purification of siRNA-generating activity to homogeneity from *Drosophila* S2 cell extract (Liu et al., 2003). Mass spectrometry revealed Dicer-2 and the previously uncharacterized protein CG7138. This protein was named R2D2 because of its two dsRNA-

binding domains (R2) and association with Dicer-2 (D2). Dicer-2 and R2D2 form a stable complex, and either protein alone is unstable. The role of R2D2 was revealed in a partially reconstituted RISC system. Recombinant Dicer-2, Dicer-2/R2D2 or Dicer-2/mutant R2D2 was added to partially purified RISC fractions to reconstitute dsRNA- or siRNA-initiated RISC. Recombinant Dicer-2/R2D2 produced much higher RISC activity than Dicer-2 alone or Dicer-2/mutant R2D2. These findings were supported by genetic studies employing extract from *r2d2* null flies that were fully competent for siRNA production but markedly defective for RISC activity. Recombinant R2D2 fully rescued this defect, possibly by bridging the initiation and effector complexes (Liu et al., 2006).

The discovery of a dsRNA-binding protein (dsRBP) partner for Drosophila Dicer-2 raised the possibility of such a cofactor for human Dicer. Domain homology search revealed two candidates as tandem partners for human Dicer. Trans-activating response RNA-binding protein (TRBP) and protein activator of protein kinase R (PACT) each harbors three annotated dsRNA binding domains and was shown to interact with Dicer (Chendrimada et al., 2005; Haase et al., 2005; Lee et al., 2006). siRNA knockdown of TRBP resulted in diminished miRNA production and loss of reporter gene silencing in HeLa cells. Similar results were observed in similar experiments targeting PACT. However, the precise role of TRBP and PACT in mediating human RISC activity remains poorly understood.

1-2. Drosophila siRISC pathway mechanism

Drosophila RISC assembly

Using native polyacrylamide gel analysis to characterize RISC assembly in Drosophila embryo extracts, Pham et al. revealed a Dcr-2-dependent siRNA-protein complex initially known as R1 (Pham et al., 2004). The factors that form this complex cofractionate with dsRNA-processing activity and consist solely of Dcr2 and R2D2 (Pham and Sontheimer, 2005a). A commitment/chase experiment showed that the Dcr2–R2D2–siRNA complex is a precursor to active RISC. The complex was thus named R2D2/Dcr-2 initiator (RDI) complex. The siRNA within RDI is double-stranded, and formation of the complex does not require ATP. However, the 5' phosphates on the siRNA are important for the RDI formation.

siRNA incorporation into RISC can be asymmetric. That is, one siRNA strand is preferentially loaded on to RISC. The rules for RISC entry for synthetic siRNAs that do not undergo dsRNA processing seem to be largely dictated by the relative thermal stability of base pairing at the two ends of the siRNA duplex (Khvorova et al., 2003; Schwarz et al., 2003). If one end of the siRNA duplex is less stably base-paired than the other, then the strand that has its 5' terminus at that end is favored for RISC incorporation and becomes the 'guide' strand, and the other strand (the 'passenger' strand) is discarded.

Tomari *et al*. conducted crosslinking experiments to elucidate the interaction of Dcr-2 and R2D2 with siRNA. The results indicated a possible role for R2D2 in sensing thermodynamic siRNA structure. R2D2 bound the more stable end of the siRNA duplex, thereby orienting the Dcr-

2/R2D2 heterodimer on the siRNA. The strand whose 5' terminus was bound to R2D2 (the passenger strand) was thus excluded from being incorporated into RISC (Tomari et al., 2004b). Subsequent studies revealed that this potential asymmetric sensing mechanism was also possible for siRNA processed from long dsRNA precursors (Preall et al., 2006).

Drosophila RISC activation

The bi-phasic model for RISC function was: Dicer and its dsRBP partner generate duplex siRNA. Ago and single-stranded RNA direct the silencing of target mRNAs. However, it remained unknown how duplex siRNA are converted to single-strand guide RNA during RISC assembly.

In 2001, Nykanen et al. observed the dissociation of radiolabeled duplex siRNA strands in *Drosophila* embryo extracts. The unwinding activity was ATP dependent and eluted as a peak during gel filtration (Nykanen et al., 2001). This siRNA strand separation model was supported by observations that purified human RISC complex contained only one siRNA strand (Martinez et al., 2002). These findings gave rise to a model of RISC activation in which duplex siRNA was unwound by a helicase. Subsequent co-immunoprecipitation studies and genetic analyses revealed several candidate helicases associated with known RISC components, including Dcr-2, Dmp68 and Armitage (Ishizuka et al., 2002; Meister et al., 2005; Meister and Tuschl, 2004; Tomari et al., 2004a). However, no helicase has been demonstrated to function directly in the activation of RISC.

At the end of 2005, two groups overturned the long-held belief that duplex siRNAs were unwound by helicases, by demonstrating that fly Ago-2 is responsible for cleaving the passenger strand of the siRNA duplex, allowing the guide strand to be incorporated into RISC (Matranga et al., 2005; Rand et al., 2005). Both groups detected 9-nucleotide 5' cleavage and 12-nucleotide 3' cleavage products, indicating that the passenger strand of the siRNA is a substrate for RISC.

RISC size and components

Various groups have reported that RISC activity is associated with complexes with sizes ranging from 160 kDa, 500 kDa, to 80S. By fractionating Drosophila S2 cell extract, Hammond et al. purified a ribonucleoprotein complex of ~500 kDa that contains RISC activity (Hammond et al., 2001). However, size fractionation of a Drosophila embryo extract done by Nykanen et al. yielded RISC activity in a broad range of fractions, with peak RISC activity occurring in the ~200kDa fractions and almost no activity in the ~500kDa fractions (Nykanen et al., 2001). Drosophila embryo extracts that were fractionated under mild conditions showed RISC activity predominantly in an ~80S holo-RISC complex (Pham et al., 2004). A form of RISC (which is known as 'minimal RISC') that was purified from cultured human cells under high salt conditions is ~160 kDa (Martinez et al., 2002).

Protein components of various forms of RISC have been identified by mass spectrometry or western blot. Argonaute proteins were found in all forms of RISC. Dcr-2/R2D2 co-fractionate with the ~80S holo-RISC, which indicates that these proteins assemble into active RISC together with the siRNAs that they bind in RDI complex. Dcr-1, the enzyme that is involved in

microRNA biogenesis, was also found to co-fractionate with holo-RISC. Other proteins that have been found to associate with Drosophila RISC include: the vasa intronic gene (Vig) protein; the orthologue of the human fragile-X metal-retardation protein (FMRP), which is known as Fxr or Fmr-1; Tsn, a protein with multiple repeats of the staphylococcal nuclease domain; RNA helicase Dmp68; and components of the large ribosomal subunit.

Target mRNA cleavage

Once the functional RISC is assembled and activated, it contains a single siRNA strand (guide strand) that is available for base pairing with its mRNA target. Within the RISC, mRNA cleavage occurs between residues base paired to nucleotides 10 and 11 of the siRNA. This accuracy is not compromised when the pairing partners of the siRNA 5'-terminal 4 to 5 nts are mismatched or absent. The fact that the cleavage site is selected in the same manner even when the structure of the intervening RNA changes suggests that target-site selection apparently involves more than a simple measurement along an A-form RNA duplex, and the scissile phosphate is specified by a protein loaded onto the siRNA during RISC assembly, that is, before the encounter of the RISC with its target RNA. Although limited numbers of siRNA–mRNA mismatches generally do not affect cleavage accuracy, RISC with extensive mismatches between the siRNA and target is quite slow to cleave. Therefore, even though the remarkable tolerance of RISC for mismatches between the siRNA and its targets implies that a large number of off-target genes should be expected for many siRNA sequences, off-target effects may be minimized by keeping the amount of RISC as low as possible (Haley and Zamore, 2004; Sontheimer, 2005).

The 5'- and 3'- terminal cleavage products carry 3'-hydroxyl and 5'-phosphomonoester termini, respectively (Martinez and Tuschl, 2004; Schwarz et al., 2004). The cleavage itself does not require ATP. The guide siRNA remains associated with the complex, allowing it to carry out multiple rounds of RNA cleavage (Haley and Zamore, 2004; Hutvagner and Zamore, 2002; Martinez et al., 2002). Although ATP is not essential for cleavage, in the absence of ATP, the rate of multiple rounds of catalysis is limited by release of the cleaved products from the enzyme, suggesting an ATP-dependent RNA helicase facilitates product release (Haley and Zamore, 2004).

1-3. microRNAs

The discovery of microRNAs

The founding member of the microRNA (miRNA) family, lin-4, was identified in *C. elegans* through a genetic screen for defects in the temporal control of post-embryonic development. In *C. elegans*, cell lineages have distinct characteristics during 4 different larval stages (L1–L4). Mutations in *lin-4* caused L1-specific cell-division patterns to reiterate at later developmental stages. lin-4 encodes a 22-nucleotide non-coding RNA that is partially complementary to 7 conserved sites located in the 3'-untranslated region (UTR) of the lin-14 gene. lin-14 is a protein coding gene, and its loss-of-function mutations result in premature appearance of later developmental stages (Ambros and Horvitz, 1984). Lin-14 protein is normally down-regulated from the late L1 to adult stages, and this down-regulation is critical to the transition from the L1 to the L2 stage. An intact 3'UTR of lin-14 mRNA, as well as a functional lin-4 gene are required for the negative regulation of Lin-14 protein expression (Lee et al., 1993; Ruvkun and Giusto,

1989; Wightman et al., 1991). Based on these genetic interactions, molecular and biochemical studies were conducted, and they discovered that the direct, but imprecise, base pairing between lin-4 and the lin-14 3'UTR was essential for the ability of lin-4 to control Lin-14 expression through the regulation of protein synthesis (Ha et al., 1996; Olsen and Ambros, 1999; Wightman et al., 1993). Through an analogous mechanism, lin-4 was also found to negatively regulate the expression of lin-28, a protein that initiates the developmental transition from the L2 to the L3 stage (Moss et al., 1997).

In 2000, almost 7 years after the initial identification of lin-4, the second miRNA, let-7, was discovered, also through a genetics screen in worms. Let-7 encodes a temporally regulated 21-nt small RNA that is required for the transition from the L4 stage to the adult stage. Similar to lin-4, let-7 negatively regulates its target mRNAs by binding to their 3'UTR, thereby inhibiting their translation (Abrahante et al., 2003; Lin et al., 2003; Reinhart et al., 2000; Slack et al., 2000; Vella et al., 2004). Unlike lin-4, whose orthologues in flies and mammals were only recognized later, both let-7 orthologues were detected in a wide range of animal species, including vertebrate, asidian, hemichordate, mollusc, annelid and anthropod (Pasquinelli et al., 2000). This extensive conservation strongly indicated a more general role of small RNAs in developmental regulation, as supported by the later characterization of miRNA functions in many metazoan animals and plants.

Today, numerous miRNA genes have been uncovered from genomes of muticellular organisms. Although only 100–200 miRNAs are expressed in lower metazoa, 1000 or more are predicted to function in humans, possibly regulating ~30% of human genes. Target mRNAs and biological

function have been assigned to only a few dozen miRNAs, but it is becoming apparent that miRNAs participate in the regulation of almost every process investigated. The expression of many miRNAs is specific to particular tissues or developmental stages, and miRNA profiles are altered in several human diseases (Bartel, 2004).

miRNA genomic loci

Most miRNA genes come from regions of the genome quite distant from previously annotated genes, implying that they derive from independent transcription units. However, a sizable minority are in the introns of pre-mRNAs. These are preferentially in the same orientation as the predicted mRNAs, suggesting that most of these miRNAs are not transcribed from their own promoters but are instead processed from the introns. Other miRNA genes are clustered in the genome with an arrangement and expression pattern implying transcription as a multi-cistronic primary transcript. Although the majority of worm and human miRNA genes are isolated and not clustered, over half of the known Drosophila miRNAs are clustered. The miRNAs within a genomic cluster are often, though not always, related to each other; and related miRNAs are sometimes but not always clustered (Aravin et al., 2003; Lagos-Quintana et al., 2001; Lai et al., 2003; Lau et al., 2001; Lee and Ambros, 2001; Lim et al., 2003a; Lim et al., 2003b).

Nearly all of the first cloned miRNAs are conserved in closely related animals, such as human and mouse, or C. elegans and C. briggsae. Many are also conserved more broadly among the animal lineages. For instance, more than a third of the C. elegans miRNAs have easily recognized homologs among the human miRNAs (Lim et al., 2003b). In more recent years,

researchers, using integrative approaches combining bioinformatic predictions with microarray analysis and sequence-directed cloning, have discovered that a certain number of the miRNAs are not evolutionally conserved and might have species-specific function(s) (Bentwich et al., 2005; Berezikov et al., 2006).

miRNA biogenesis

Transcription of most miRNA genes is mediated by RNA polymerase II (pol II) (Cai et al., 2004; Lee et al., 2004a). A number of mammalian miRNAs are transcribed by pol III (Borchert et al., 2006). Transcription of miRNA genes yields primary transcripts, pri-miRNAs, that are usually several kilobases long and contain a local hairpin structure. The stem-loop structure is cleaved by the nuclear type-II RNase III Drosha to release the precursor of miRNA (pre-miRNA) (Lee et al., 2003). It remains to be seen whether the 5' and 3' fragments that surround the stem-loop have their own functions.

Drosha is a large protein of ~160 kDa, and is conserved in animals. It contains two tandem RNase III domains and a double-stranded RNA-binding domain (dsRBD) that are crucial for catalysis. Drosha forms a large complex of ~500 kDa in D. melanogaster, or ~650 kDa in humans. In this complex, which is known as the Microprocessor complex, Drosha interacts with its cofactor, the DiGeorge syndrome critical region gene 8 (DGCR8) protein in humans (also known as Pasha in D. melanogaster and C. elegans). DGCR8/Pasha is a ~120 kDa protein that contains two dsRBDs (Denli et al., 2004; Gregory et al., 2004; Han et al., 2004; Landthaler et al., 2004). The Drosha-DGCR8 complex initiates miRNA maturation by precise cleavage of the

stem loops that are embedded in pri-miRNAs. A typical metazoan pri-miRNA consists of a stem of ~33 bp, with a terminal loop and flanking segments. The terminal loop is unessential, whereas the flanking ssRNA segments are critical for processing. The cleavage site is determined mainly by the distance (~11 bp) from the stem-ssRNA junction. Purified DGCR8, but not Drosha, interacts with pri-miRNAs both directly and specifically, and the flanking ssRNA segments are vital for this binding to occur. Thus, DGCR8 may function as the molecular anchor that measures the distance from the dsRNA-ssRNA junction (Han et al., 2006).

Following nuclear processing by Drosha, pre-miRNAs are exported to the cytoplasm. Once there, they are subjected to the second processing step by Dicer to generate the final ~22nt product. Due to compartmentalization of the two processing events, nuclear export of pre-miRNAs is a crucial step in miRNA biogenesis. Export of pre-miRNA is mediated by one of the nuclear transport receptors, exportin-5 (Bohnsack et al., 2004; Lund et al., 2004; Yi et al., 2003). When the cells were depleted of exportin-5, the pre-miRNA level and the mature miRNA level were reduced in the cytoplasm. Notably, pre-miRNA does not accumulate in the nucleus subsequent to the depletion of exportin-5. This indicates that pre-miRNA might be relatively unstable and also that pre-miRNA might be stabilized through its interaction with exportin-5. Exportin-5 can also export adenoviral RNA VA1, a 160-nucleotide ncRNA. Analysis of cis-acting elements for nuclear export in VA1 revealed a structural motif known as the 'minihelix motif', which consists of a >14-bp stem and a 3–8 nucleotide 3' overhang. A similar structural motif can be found in pre-miRNA stem-loops, which typically comprise a stem of ~22 bp, a terminal loop and a 3' overhang of ~2 nucleotides. By introducing mutations in the pre-miR-30a,

Cullen and colleagues confirmed that an RNA stem of >16 bp and a short 3' overhang are significant structural requirements for pre-miRNA export (Zeng and Cullen, 2004).

Following their export from the nucleus, pre-miRNAs are subsequently processed into ~22-nucleotide miRNA duplexes by the cytoplasmic RNase III Dicer (Grishok et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001). The multiple steps in miRNA biogenesis seem to be very well coordinated. Drosha initiates miRNA processing by the specific cropping of the stem-loop precursor in the nucleus, creating a short stem with a ~2-nucleotide 3' overhang, which seems to be a signature motif for all dsRNAs that are involved in small-RNA pathways. Exportin-5 recognizes this signature motif to export pre-miRNA to the cytoplasm. Following export, pre-miRNA is handed over to Dicer, which has a preference for the terminus of dsRNAs containing the short 3' overhang (Zhang et al., 2004). In addition, Drosha pre-determines mature miRNA sequences by precisely generating one end of the mature miRNA. The other end is created by Dicer and measures ~22 nucleotides from the pre-existing terminus of the pre-miRNA.

Bartel and colleagues recently identified an alternative pathway for miRNA biogenesis, in which certain debranched introns mimic the structural features of pre-miRNAs to enter the miRNA processing pathway bypassing Drosha-mediated cleavage (Ruby et al., 2007).

1-4. Compare siRNA and miRNA

Similarity between siRNA and miRNA

Although siRNA and miRNA were initially discovered in unrelated studies, both types of small RNA are closely related in their biogenesis, assembly into RNA–protein complexes and ability to regulate gene transcripts negatively in diverse eukaryotes.

Both siRNAs and miRNAs are generated by Dicer. Like siRNAs, nascent miRNAs generated by Dicer are double-stranded duplexes. Lim et al. cloned small RNAs corresponding to the non-miRNA side of the pre-miRNA's stem from C. elegans (named miRNA*). Although these miRNA* sequences were recovered at about 100 times lower frequency than the miRNAs themselves, they could always be paired with the corresponding miRNA to give miRNA duplexes with ~2nt overhanging 3'ends (Lim et al., 2003b).

These siRNA and miRNA duplexes need to be unwound before they can be assembled into RISC (siRISC for siRNA, and miRISC for miRNA). The assembly of both siRISC and miRISC is an asymmetric process. The selection of the guide strand of an siRNA is based on thermodynamic stability of the two ends (see above). Computational analyses revealed that most miRNA from human, mouse, fly and worm exhibited a common thermodynamic signature: the 5' terminus of the mature miRNA is at the less stably base-paired end, indicating the same rule for strand selection applies to both siRNA and miRNA (Khvorova et al., 2003; Schwarz et al., 2003).

In vitro and in vivo biochemical studies have shown that depending on the complementarity

between the small RNA and its target mRNA, a siRISC can function as a miRISC to repress translation of the target mRNA; similarly, a miRISC can function as a siRISC to cleave the target mRNA (Doench et al., 2003; Hutvagner and Zamore, 2002; Zeng et al., 2003). This functional interchangeability between a siRISC and a miRISC argues that siRISCs and miRISCs are highly similar. Much evidence suggests, however, that siRISCs and miRISCs are distinct types of complex (see below).

Differentiate between siRNA and miRNA

Structure

Generally speaking, although siRNA and miRNA are both 21-23 nt dsRNAs that contain 3' di-nucleotide overhangs and bear 5'-monophosphate and 3'-hydroxyl termini, siRNAs arise from perfectly base-paired dsRNAs, whereas miRNAs are excised from 60-70 nt pre-miRNA hairpins containing multiple bulges and mismatches within the duplexed region. Both small RNAs can specify multiple modes of silencing, but in animals, siRNAs usually induce target mRNA endonucleolytic cleavage, whereas miRNAs usually direct translation inhibition or exonucleolytic degradation.

Dicer and its dsRBD partner

Unlike human and C. elegans, both of which have only one Dicer that is required for both siRNA and miRNA pathways, several organisms contain more than one Dicer gene, with each Dicer preferentially processing dsRNAs that come from a specific source. Drosophila melanogaster has two paralogues: Dicer-1 (Dcr-1) and Dicer-2 (Dcr-2). Dcr-2 does not play a role in miRNA

biogenesis, but is required to cleave long dsRNAs into siRNAs. Dcr2/R2D2 also play a role in binding siRNA duplexes to form a complex that initiates the assembly of siRISC (see above). Dcr-1 processes pre-miRNAs into miRNAs, and it is required for the assembly of miRISC. Dicer-1 also seems to be required downstream of siRNA-production in siRISC assembly, though its precise role has not been defined (Lee et al., 2004b).

The discovery of dsRNA-binding protein (dsRBP) partner for Dicer-2 and Drosha raised the possibility of such a cofactor for Dicer-1. Sequence alignment and biochemical purification of miRNA generating activity revealed a novel protein, a paralogue of Drosophila R2D2 featuring three dsRBDs. It was named Loquacious (Loqs) or R3D1 (Forstemann et al., 2005; Jiang et al., 2005; Saito et al., 2005). Genetic mutation and RNAi knockdown of Loqs resulted in defective pre-miRNA processing in vitro and accumulation of endogenous pre-miRNAs. The same phenotype is observed when cells are depleted of Dicer-1, but not Dicer-2 or R2D2. Saito et al. suggested that Loqs confers substrate specificity for pre-miRNAs to Dicer-1. They found that Dicer-1 processes long dsRNA as well as pre-miRNA substrates when Loqs is removed from the complex. Re-addition of Loqs inhibited dsRNA processing and enhanced pre-miRNA processing (Saito et al., 2005). However, in a more recent report, Liu et al. showed that the lack of Loqs had differential effects on mature miRNAs: some are diminished, whereas others maintain wild-type levels. They also showed that Dcr-1, but not Loqs, is critical for assembly of miRISCs by analyzing *dcr-1* or *loqs* null egg extract (Liu et al., 2007). These data indicate that Loqs and R2D2 represent two distinct functional modes for dsRBPs in the RNAi pathways.

Duplex unwinding

Unlike siRNAs that are assembled into siRISC by a passenger strand cleavage-assisted mechanism mediated by Ago proteins, miRNAs, when paired to their natural passenger strands — the miRNA* strands — are loaded into the Ago RISC without cleavage of the miRNA*. They seem to use a 'bypass' mechanism to be unwound. This may be because of the multiple mismatches typical of miRNA/miRNA* duplexes, which presumably inhibit the cleavage step of the cleavage-assisted mechanism (Matranga et al., 2005). It was suggested that siRNA, due to the tight binding between its two strands, has to go through the passenger strand cleavage mechanism, whereas the disruption of base-pairing between the miRNA and miRNA* enables the bypass mechanism to begin to play a substantial role.

Sorting into different Ago-RISCs

Members of the Argonaute proteins lie at the core of all known RNA silencing effector complexes. The Drosophila genome encodes five Argonaute proteins, which form two subclades. The Ago subclade comprises Ago1 and Ago2. Piwi, Aub, and Ago3 form the Piwi subclade of The Piwi proteins bind repeat-associated siRNAs (rasiRNAs; also called piRNAs), which direct silencing of selfish genetic elements such as transposons (Brennecke et al., 2007; Gunawardane et al., 2007; Saito et al., 2006; Vagin et al., 2006).

In 2004, Okamura et al. showed that distinct Argonaute proteins act at different steps of the small RNA silencing mechanism and suggest that there are inherent differences between siRISC and miRISC in Drosophila. They demonstrated that Ago-2 is an essential component for siRNA-directed silencing response, and it is required for the unwinding of siRNA duplex and in

subsequent assembly of siRNA into RISC in Drosophila embryos. But Ago-2 is not required for miRNA-directed target RNA cleavage. On the other hand, Ago-1, which is dispensable for siRNA-directed target RNA cleavage, was found to associate with Dcr-1 and pre-miRNA, and is required for mature miRNA production (Okamura et al., 2004). The same group later proved that in Drosophila, in addition to Ago-2, Ago-1 is also capable of showing slicer activity, by showing that slicer activity was reconstituted with recombinant full-length Ago-1 or its PIWI domain alone (Miyoshi et al., 2005). Thus, a model of Drosophila siRNA and miRNA pathways was generated (Figure 1-1A). miRNAs are cleaved from pre-miRNA by Dcr-1, acting with its dsRNA-binding protein partner Loqs. siRNAs are produced from long dsRNA by Dcr-2, which partners with the dsRNA-binding protein R2D2. The different origins of miRNAs and siRNAs direct them to distinct Argonaute proteins, with Dcr-1/Loqs recruiting Ago1 to miRNAs and Dcr-2/R2D2 directing siRNAs to Ago2.

In 2007, Zamore and collegues reported findings that indicate the previously reported division of labor between siRNAs and miRNAs, Ago1 and Ago2 is not absolute, and that partitioning of effector complexes is uncoupled from small-RNA processing (Figure 1-1B). They showed that the specific pathway that produces a miRNA or siRNA does not predestine that small RNA to associate with a particular Argonaute protein. They found a miRNA produced by Dcr-1 and Loqs can nonetheless be loaded by Dcr-2 and R2D2 into an Ago2-containing RISC. Instead of the distinct biogenesis machineries, it was the specific structural differences between a miRNA/miRNA* duplex and an siRNA duplex that promote their sorting into Ago1- and Ago2-containing RISC. In particular, a central unpaired region serving as both an anti-determinant for the Ago2-loading pathway and a preferred binding substrate for the Ago1 pathway. Supporting

this view, miRNAs that contain central mismatches, such as let-7 and bantam, assemble primarily into Ago1-RISC, whereas miR-277, whose central region is base paired, partitions between Ago1 and Ago2 in vivo. They also report that the Dcr-2/R2D2 heterodimer acts as a gatekeeper for the assembly of Ago2-RISC. Dcr-2/R2D2 binds well to highly paired small-RNA duplexes but poorly to duplexes bearing central mismatches, thus promoting the incorporation of siRNAs and disfavoring the use of miRNAs as loading substrates for Ago-2. An independent mechanism acts in parallel to favor assembly of miRNA/miRNA* duplexes into Ago1-RISC and to exclude siRNAs from incorporation into Ago-1. These two pathways compete for loading small-RNA duplexes with structures intermediate between that of a siRNA and a typical miRNA/miRNA* duplex.

In addition, they disagreed with Miyoshi's claim that both Ago-1 and Ago-2 function as slicer, arguing that Ago1-RISC and Ago2-RISC are functionally distinct: Ago1, but not Ago2, can repress an mRNA containing multiple, partially complementary miRNA-binding sites in its 3'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 their finding that while Ago2 is a robust, multiple-turnover RNA-directed RNA endonuclease, yet Ago1 is not (Forstemann et al., 2007; Tomari et al., 2007).

Figure 1-1. (A) Small RNA biogenesis and RISC assembly are tightly coupled. miRNAs are exclusively loaded into Ago-1 and siRNAs into Ago-2. (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 solely according to their structures: Dcr-2/R2D2 bind well to highly paired small- RNA duplexes but poorly to duplexes bearing central mismatches; such duplexes are therefore disfavored for loading into Ago2. Ago1 favors small RNAs with central mismatches, but no Ago1-loading proteins have yet been identified.

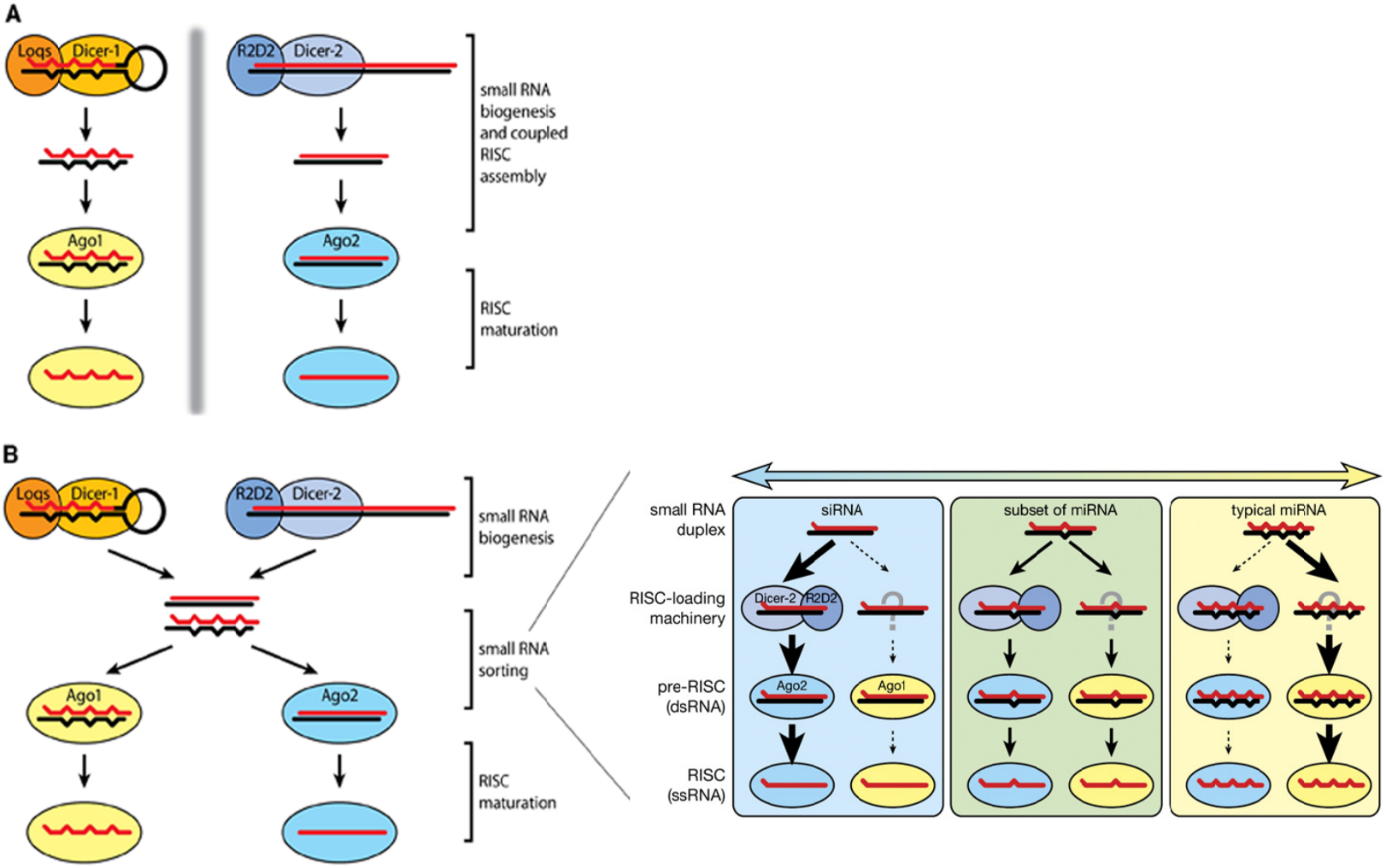


Figure 1-1. miRNA and siRNA sorting into Ago1- and Ago2-RISCs in Drosophila (Forstemann et al., 2007; Tomari et al., 2007)

1-5. Mechanisms of miRNA-mediated gene repression

In plants, miRNAs generally show nearly perfect complementarity to target sequences positioned in either coding or 3'UTR regions of mRNAs. The perfect base pairing triggers mRNA degradation through a mechanism similar to siRNAs. In animals, with very few exceptions, miRNAs regulate gene expression by base pairing imperfectly to the 3'UTR of target mRNAs and inhibiting protein synthesis or causing mRNA degradation. Even when miRNAs induce considerable degradation of their targets, the degradation usually does not fully account for the reduction in protein synthesis, further supporting an independent role for translational repression. Two major features of animal miRNA–mRNA interactions are the contiguous Watson–Crick pairing in the miRNA 5' proximal seed region (usually positions 2–8) and a lack of complementarity in the central part of the miRNA (usually positions 10 and 11) that precludes the RNAi-like endonucleolytic cleavage of the target mRNA in the middle of the duplex. Although one perfectly complementary site is sufficient for the siRNA- or miRNA-induced cleavage of mRNA, studies using reporter mRNAs have indicated that effective translational repression usually requires multiple imperfect sites recognized by the same or several different miRNAs. The molecular basis of this apparent miRNP cooperativity remains unknown.

Translational repression

The events during translation can be broadly divided into three stages: initiation, elongation and termination. During m7G cap-dependent translation, interaction of the cap-binding factor eIF4E (part of the eIF4F complex) with the cap is a starting event in the assembly of the initiation complex. Another initiation factor, eIF4G, functions as a bridge by simultaneously interacting

with eIF4E and the initiation factor eIF3, finally to recruit the 40S subunit to the mRNA. Subsequent events include the movement of 40S along the mRNA 5'UTR and joining of the 60S subunit at the AUG codon to begin the elongation phase. Once the ribosome reaches the termination codon, translation release factors mediate the termination process. Translation of many viral and some cellular mRNAs does not require cap recognition by eIF4E. This type of initiation is independent of the 5'cap and is driven by special RNA structures called internal ribosome entry site (IRES) present in the 5'UTR or at the beginning of the coding region of the mRNA. During the cap-independent translation, ribosomes are recruited to the initiating AUG, frequently positioned far away from the mRNA 5'end, by IRES (Pillai et al., 2007).

Support for miRNAs inhibiting translation at the initiation step comes from experiments performed in mammalian cell cultures using either reporters or endogenous mRNAs that had multiple binding miRNA binding sites in their 3'UTR. These experiment revealed that the m7G cap is essential for translation repression— the translation of mRNAs containing IRES elements or a non-functional ApppN cap was not is not repressed by miRNAs (Humphreys et al., 2005; Mathonnet et al., 2007; Pillai et al., 2005; Thermann and Hentze, 2007; Wang et al., 2006). In addition, polysome profile analysis of reporter mRNAs, repressed by either endogenous let-7 miRNP or miRNA-independent tethering of Ago proteins, showed a marked shift of the repressed mRNA towards the lighter fractions of a sucrose gradient, indicating reduced ribosome loading on the mRNA (Pillai et al., 2005). Likewise, de-repression of the miRNA-regulated mRNA, cat-1, caused a fraction of the mRNA to shift from the lighter part of the gradient to the heavy polysomal region (Bhattacharyya et al., 2006).

A series of recent investigations gave rise to a model explaining how miRNAs might inhibit translation initiation. During mRNA translation, eIF4G not only provides a bridge between eIF4E and eIF3, but also interacts with the polyadenylate-binding protein 1 (PABP1), which brings the two ends of the mRNA together and increase the efficiency of translation initiation (Derry et al., 2006; Wells et al., 1998). Recent data suggest that miRNAs might disrupt this synergy between the 5'cap and 3'poly(A) tail by targeting one of the two terminal mRNA structures (Filipowicz et al., 2008). Kiriakidou et al. reported that the Mid domain of Ago proteins bears significant sequence similarity to eIF4E. They showed that human Ago2 can compete with eIF4E for m7G binding and thus prevent m7G cap-dependent translation (Kiriakidou et al., 2007). In vitro experiments using extracts made from rabbit reticulocytes and human cells showed that in addition to the m7G cap, poly(A) tail is also required for miRNA-mediated translational repression. Studies in human cell extracts also showed that miRNA let-7 directs the deadenylation of its target mRNAs (Wakiyama et al., 2007).

An alternative mechanism of miRNA translational repression was proposed by Chendrimada et al. They found that human RISC associates with a multiprotein complex containing the anti-association factor eIF6, a protein known to prevent productive assembly of the 80S ribosome. They showed that depletion of eIF6 in human cells and in C. elegans diminishes miRNA-mediated regulation of target protein and mRNA levels, possibly by reducing the 60S ribosomal subunit joining to the 40S initiation complex.

Evidence supporting a post-initiation, rather than initiation, mechanism of repression has been obtained in both C. elegans and mammalian cell cultures. Early studies in C. elegans showed that

the lin-4 target mRNAs are successfully loaded with ribosomes (Olsen and Ambros, 1999; Seggerson et al., 2002). Similar results were for mammalian cells with mRNAs bearing sites partially complementary to synthetic or endogenous miRNAs. These studies showed that the repressed mRNAs were associated with actively translating polysomes that are sensitive to different conditions that inhibit translation (Maroney et al., 2006; Nottrott et al., 2006; Petersen et al., 2006). Petersen et al. also found that translation of both the cap-dependent and IRES-dependent cistrons present in bi-cistronic reporters is repressed by synthetic miRNA, consistent with a post-initiation effect. The authors proposed a ribosome drop-off model, in which the ribosomes engaged in translation of miRNA-associated mRNAs are prone to terminate translation prematurely.

It was also suggested that association of repressed mRNAs with actively translating polysomes could be explained by the ability of miRNPs to recruit proteolytic enzymes that would degrade nascent polypeptides emerging from the actively translating ribosomes (Petersen et al., 2006). However, there is no experimental support for this mechanism. Nascent polypeptides could not be detected when the reporter mRNA underwent repression. Proteasome inhibitors had no effect on the repression. Last, repression was not prevented when reporter proteins were targeted to the endoplasmic reticulum, which should protect them from proteolysis (Petersen et al., 2006; Pillai et al., 2005).

Repression through P-bodies

P-bodies or GW-bodies are discrete cytoplasmic foci that are enriched in translationally inactive mRNAs as well as factors involved in mRNA decay and translational repression. The decay

factors include enzymes catalyzing partial deadenylation of mRNA (by deadenylases such as the Ccr4:Not1 complex), mRNA decapping (by the decapping enzyme composed of two proteins, Dcp1 and Dcp2) and the 5' to 3' exonucleolytic degradation of mRNA (by exonuclease Xrn1) (Parker and Song, 2004). Ago proteins, miRNAs and mRNAs repressed by miRNAs have all been found to accumulate in P-bodies, and there is a good correlation between miRNA-mediated translational repression and accumulation of mRNAs in P-bodies (Behm-Ansmant et al., 2006; Bhattacharyya et al., 2006; Jakymiw et al., 2005; Liu et al., 2005b; Meister et al., 2005; Pillai et al., 2005). Mutated Ago2 proteins that are defective in mediating miRNA-induced repression did not accumulate in P-bodies (Liu et al., 2005a; Liu et al., 2005b). Knockdowns of P-body components such as Dhh1p, Rck/p54, eIF4E-T, Pat1p, decapping enzymes and GW182 inhibit miRNA-mediated repression (Behm-Ansmant et al., 2006; Eulalio et al., 2007; Jakymiw et al., 2005; Liu et al., 2005a; Meister et al., 2005; Rehwinkel et al., 2005). However, depletion of some P-body components (such as LSM1 or LSM3) that disrupts the integrity of microscopically visible P-bodies had no effect on miRNA function (Chu and Rana, 2006).

P-bodies are dynamic aggregates of RNA and proteins, and mRNAs accumulating in P-bodies are not necessarily destined for degradation. Under certain conditions, or in specific cells, mRNA can exit P-bodies and re-enter translation (Bhattacharyya et al., 2006; Brengues et al., 2005; Schratt et al., 2006). Thus, P-bodies can function as temporary storage sites for repressed mRNAs.

mRNA degradation

Early studies indicated that the levels of miRNA-repressed mRNAs remain mostly unchanged. However, many recent studies showed that miRNA-mediated repression is also frequently associated with a substantial degradation of target mRNAs (Bagga et al., 2005; Giraldez et al., 2006; Krutzfeldt et al., 2005; Lim et al., 2005; Rehwinkel et al., 2006; Schmitter et al., 2006; Wu et al., 2006).

In animals, miRNA–mRNA hybrids do not form a perfect A-form helix at the center of the duplex region, therefore the mRNA degradation cannot occur through the RNAi mechanism. Indeed, no cleavage fragment expected from a slicer mechanism was ever found in human cells or in worms. Instead, 5' to 3' exonuclease activity was suggested, as studies mapped many cleavage sites to mRNA positions upstream of the miRNA-binding sites (Bagga et al., 2005; Schmitter et al., 2006; Wu et al., 2006).

Depletion experiments conducted in Drosophila S2 cells implicated GW182, the decapping-complex proteins, and the Ccr4:Not1 deadenylase complex in the mRNA degradation process (Behm-Ansmant et al., 2006; Eulalio et al., 2007). Transcriptome analysis showed that GW182 and dAgo1 regulate a common set of mRNAs that are enriched in predicted and/or validated miRNA targets. Combined with other experimental results, a model was proposed. miRNA-bound dAgo1 recruits GW182, which in turn recruits the deadenylase. The deadenylation would then be followed by removal of the m7G cap by decapping enzymes Dcp1:Dcp2 and the 5' to 3' degradation of mRNA catalyzed by exonuclease Xrn1 (Bagga et al., 2005; Behm-Ansmant et al., 2006; Liu et al., 2005b; Rehwinkel et al., 2005).

An important question is whether the deadenylation and the degradation are primary or secondary to the translational repression. The translational status of an mRNA can directly affect mRNA stability, so it is possible that target mRNA degradation is a consequence of translational repression. Pillai et al. proposed a simple two-step model by which miRNAs repress gene expression. The first step would involve miRNA repressing translation, followed by the miRNA–mRNA complex aggregating into a P-body. This scenario helps to rationalize miRNA effects on the degradation of the mRNA because P-bodies are enriched in components of the mRNA decay pathway. Accumulation of repressed mRNAs in P-bodies also provides an elegant way to repress gene expression reversibly because mRNAs are not necessarily degraded in P-bodies but can exit them and re-enter translation in response to environmental or developmental cues (Pillai et al., 2007).

However, whether miRNAs inhibit protein synthesis by a primary single mechanism or by different mechanisms still remains unknown. It is possible that miRNAs can direct many different routes to modulate protein synthesis, including repression at both the initiation and post-initiation steps of translation. Yet it is also conceivable that the different experimental systems and methodologies significantly contributed to the differences in these results.

1-6. Other small silencing RNAs

Small RNA - siRNA and miRNA - mediated silencing pathways had been found to function in many aspects, including modulating the translation of mRNA into protein, establishing

chromosomal architecture, regulating stem cell renewal, and providing defense against invasive nucleic acids and selfish mobile genetic elements (transposons) that could cause deleterious mutations. In the past two years, two additional RNA silencing pathways, Piwi-interacting RNAs (piRNAs) and endogenous siRNAs (endo-siRNA), were discovered, making the world of small RNA silencing phenomena even larger.

piRNAs are generated by a dicer-independent mechanism that relies on the slicer function of the PIWI clade of argonaute proteins. These proteins use antisense transcripts (complementary to mRNA) encoded by piRNA clusters in the genome that harbor transposable element fragments, and target the destruction of transposon sense transcripts in germ line (Aravin et al., 2007).

The siRNA pathway in Drosophila had not been thought to play any roles in regulating endogenous gene expression, because RNAi mutants seem to be normal and fertile (Lee et al., 2004b; Okamura et al., 2004). On the other hand, these mutants are hypersensitive to viruses, suggesting RNAi is responsible for defending against exogenous invading dsRNAs (Ding and Voinnet, 2007). Until recently, endogenous siRNA (Endo-siRNA) pathways had been restricted to organisms that possess RNA-dependent RNA polymerases (RDRPs) (an enzyme that generates abundant secondary siRNAs corresponding to endogenous mRNAs): plants, C. elegans and fission yeast.

In 2008, several studies reported sequencing of extensive pools of small RNAs from various somatic and germline sources in flies and mice — two organisms lacking RdRP activity, and identified endo-siRNAs that are homologous to endogenous genomic sequences. In Drosophila

gonadal and somatic tissues, endo-siRNAs were found to correspond to transposons, cis-natural antisense transcripts, and hairpin RNAs (hpRNAs), and target both protein-coding genes and transposons (Czech et al., 2008; Ghildiyal et al., 2008; Kawamura et al., 2008; Okamura et al., 2008). They are predominantly associated with Ago-2. In mouse germline cells, endo-siRNAs were similarly mapped to transposons, hpRNAs and overlapping transcription units. Surprisingly, a subset of the mouse endo-siRNAs arises from pseudogenes. These small RNAs are often processed from dsRNAs formed by spliced mRNAs from protein coding genes and antisense mRNAs from homologous pseudogenes, indicating that pseudogenes, previously thought to be nonfunctional, may actually regulate the expression of their founder gene (Tam et al., 2008; Watanabe et al., 2008).

A striking discovery from these studies was that although Drosophila Ago-2 and Dcr-2 is responsible for function and production of endo-siRNAs, Dcr-2's partner R2D2 is not required in this process. Instead, mutants in Loqs are highly depleted in endo-siRNAs. At least some endo-siRNAs from each of these origins — transposons, cis-natural antisense transcripts, and hpRNAs — are dependent on Loqs. Proteomic analysis of Dcr-2 complexes revealed that Loqs interacts with Dcr-2 (Czech et al., 2008; Okamura et al., 2008).

From these reports, it seems clear that endo-siRNAs regulate specific protein-coding transcripts and transposons. However, biological relevance of these regulations is unknown. An intriguing question remains to be answered: why do RNAi mutants in flies, such as *ago-2* and *dcr-2* mutants, show very little phenotype? It has been proposed that some genes might evolve by very week selection, which means they will not show obvious phenotype when knocked out in normal

laboratory experiments. Rather, understanding the full function of such genes might require experiments on an evolutionary scale (Tautz, 2000). Would experiments involving an effective population of flies reveal phenotypes of RNAi mutants? If the main function of endo-siRNAs is to suppress gene disruptions caused by transposons, why haven't we seen any mutant phenotypes in our dcr-2 null fly populations that have been kept as homozygotes for years? As always, these new discoveries have given rise to many more interesting questions to be answered.

Chapter 2: Detection of microRNPs in Drosophila

Introduction

miRNAs do not function as naked RNAs but, instead, as components of ribonucleoprotein complexes (RNPs). Because miRNA-mediated repression seems to be accomplished by several mechanisms, it is conceivable there are specific interactions between distinct mRNPs (mRNA-containing RNPs) and the regulatory machinery. Such specificity could result from features of the mRNP itself (e.g. secondary structure characteristics or the presence of bound proteins), from the composition of the miRNA-containing RNPs (miRNP) itself (e.g. the presence of distinct auxiliary proteins), or from a combination of both (e.g. the composition of an mRNP could dictate which distinct miRNP can associate) (Nilsen, 2007).

A common constituent of all miRNPs is a member of the Argonaute protein family. Similarly, it is generally agreed that GW182 (or GW182-like proteins) have an essential role in miRNA-mediated regulation regardless of the mechanism (Behm-Ansmant et al., 2006; Ding et al., 2005; Liu et al., 2005a; Meister et al., 2005; Rehwinkel et al., 2005). In addition, Dicer has been shown to interact with Argonaute in human and Drosophila. So these proteins could be considered to be the 'core' of the miRNA machinery, and there are many candidates for additional factors. Several groups have used fractionation or co-immunoprecipitation to identify proteins associated with Argonaute or miRNA.

In human cells, Mourelatos et al. purified an approximately 15S RNP that contains at least 40 miRNAs. In addition to human Ago-2, Gemin3 (a member of the DEAD-box family of putative ATP-dependent RNA helicases) and Gemin4 were also found in this complex. These two proteins are also part of a multi-protein complex containing the Survival of Motor Neurons (SMN) protein, Gemin2, Gemin5 and Gemin6 (Mourelatos et al., 2002). Anti-Gemin3 and anti-Gemin4 immunoprecipitates is able to function as RISC to cleave target mRNA (Hutvagner and Zamore, 2002). Meister et al. found that the putative RNA helicase MOV10 and the RNA recognition motif (RRM)-containing protein TNRC6B/KIAA1093 localize to cytoplasmic P bodies, and they are functionally required to mediate miRNA-guided mRNA cleavage in human cells (Meister et al., 2005). Another human DEAD box helicase, RCK/p54, was found to interact with Ago1 and Ago2. It is required for the formation of P-bodies, Ago-2 localization to P-body, and miRNA-induced translational repression (Chu and Rana, 2006).

In Drosophila S2 cells, miRNA miR2b co-fractionates with Ago-1, and it co-immunoprecipitates with dFXR (the Drosophila homolog of the Fragile X Mental Retardation Protein (FMRP)) and VIG (vasa intronic gene) (Caudy et al., 2002). Tudor-SN (tudor staphylococcal nuclease)—a protein containing five staphylococcal/micrococcal nuclease domains and a tudor domain—was found to associate with several miRNAs in C. elegans, Drosophila and mammals (Caudy et al., 2003).

Although an increasing number of proteins have been found to associate with miRNAs, none of them, or their corresponding miRNPs, has been functionally characterized at the molecular level, mostly due to the complexity of miRNA-mediated gene repression mechanisms. On the other

hand, the siRISC assembly pathway had been well characterized by means of biochemical analyses, such as native electrophoresis and cell free extract fractionation. These studies not only identified the components of different siRNPs, they were also able to demonstrate the dynamics and relations between the complexes (see introduction). Therefore, we set out in an attempt to study the miRISC assembly pathway in a similar way our lab had used in the previous work to analyze the siRISC assembly.

Methods

The preparation of embryo lysates, standard RNAi reaction, native gel electrophoresis and UV crosslinking were all performed as previously described (Pham et al., 2004).

For the native gel assay, the RNAs were incubated with embryo lysates in a 10μl standard RNAi reaction mixture for 30min unless otherwise noted. The oligonucleotides for making the siRNAs, miRNAs and all the hybrid RNAs were provided by IDT. The RNAs were 5'-end labeled by γ-^{32}P –ATP on the mature miRNA strand or the siRNA guide strand, unless noted otherwise. The RNA strands were annealed in 1X annealing buffer (100mM KOAc, 30mM HEPES-KOH at pH 7.4, 2mM $Mg(OAc)_2$) at 25ºC for 3 hours after a 1 min incubation at 95ºC.

For UV crosslinking, ^{32}P-labeled siRNA duplexes were incubated for 30 min at 25˚ in a 10μl standard RNAi reaction mixture. The reactions were placed onto a parafilm-covered, pre-chilled, metal block in ice and exposed to 254 nm light (at full power) for 30 min in a Stratalinker 2400 (Stratagene). The reactions were then analyzed by 5% SDS-PAGE.

Results

We used miRNA/miRNA* duplexes of three *Drosophila* miRNAs (bantam, mir-8 and let-7) (Figure 2-1) in native gel electrophoresis assays to analyze the complexes that they form after incubation in embryo lysates.

For bantam, four complexes could be visualized on a native gel after incubation with wild type (WT) lysate (Figure 2-2A). They were named M1, M2, M3 and M4. Their mobilities relative to siRNA complexes (Pham et al., 2004) is shown in Figure 2-3. Only M1 seemed to appear consistently. The other three complexes could not be seen in every experiment due to unknown reasons. M1, M2 and M3 formed very fast (within 1 min) and then deceased over the course of incubation, whereas M4 seemed to accumulate. M1 and M4 were strand-specific since they appeared only when the bantam strand was labeled, whereas M3 appeared no matter which strand was labeled (Figure 2-2B). Some of these complexes seemed to form with other miRNAs as well (see below), and all of them need to be further examined. For instance, competition experiments could be performed to determine whether the complex formation is specific.

Because Dcr-2 is not involved in miRNA-mediated mRNA silencing *in vivo* (Lee et al., 2004b), we would not expect miRNA complexes to be dependent on Dcr-2. In our dcr-2 null mutant embryo lysate, however, M2 and M3 disappeared, and M4 levels decreased. On the other hand, M1 level increased and it accumulated over time in *dcr-2* (Figure 2-3A).

The Dcr-2 dependence of M2, M3 and M4 suggests these complexes may not be in miRNA pathway. However, it is possible that in embryo lysate, which is a different experimental system

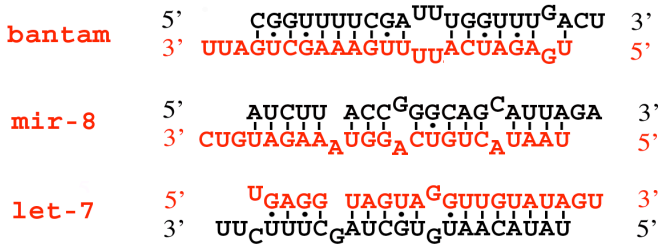


Figure 2-1. Structures of the miRNA duplexes used in experiments. Red: mature miRNA strand; black: miRNA* strand.

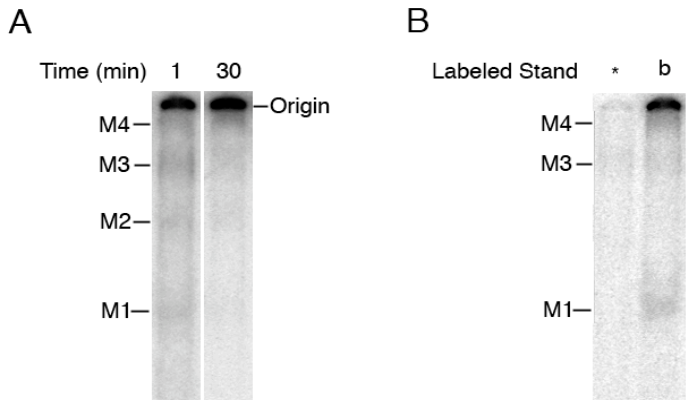


Figure 2-2. Bantam miRNA forms 4 complexes on native gel. (A) Bantam was radiolabled at the 5' end of the mature bantam strand. The duplex was incubated in wild type embryo lysate for 1 or 30 minutes, treated with heparin and analyzed by native gel. (B) Bantam was radiolabeled at the 5' end of either the * strand or the bantam strand. The duplex was incubated in lysate for 15 min and treated as in (A).

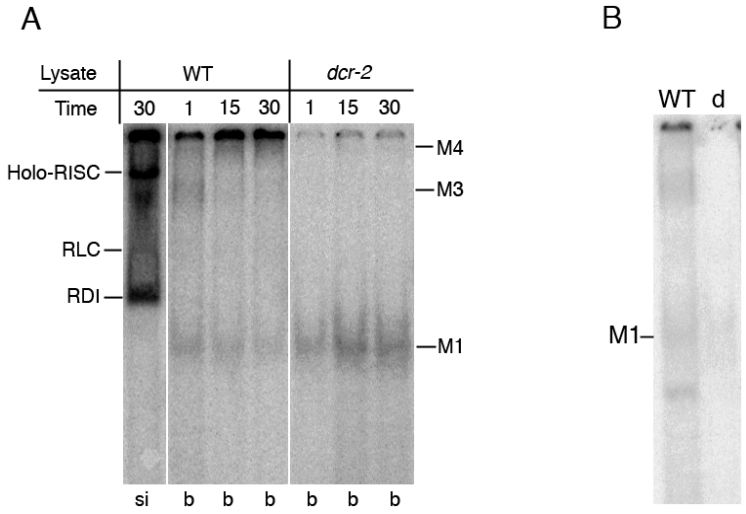


Figure 2-3. (A) Time course of bantam complexes done in wild type (WT) or Dcr-2 null mutant (*dcr-2*) lysate. Incubation time (in minutes) is shown at the top. The radiolabeled small RNA used in each reaction is shown at the bottom (si: siRNA; b: bantam). siRNA complexes are labeled on the left, and bantam complexes are labeled on the right. (B) Bantam was incubated in WT and Dcr-1 null mutant (d) lysates for 1 minute.

from the previous *in vivo* study, Dcr-2 is required for miRNA mediated mRNA cleavage. Therefore, we performed *in vitro* mRNA cleavage experiments using bantam miRNA as trigger. The result showed that the cleavage reaction is more efficient in *dcr-2* lysate than in WT (see chapter 3), which correlates with M1 whose level was higher in *dcr-2* than in WT. Therefore, M1 is likely to be a complex in the miRNA silencing pathway. Interestingly, M1 seemed to form in the lysate made from our Dcr-1 null mutant, though its intensity decreased compared to WT (Figure 2-3B). The Dcr-1 dependence of M1 should certainly be examined more closely since the experiment was only done once due to our inability to obtain enough *dcr-1* lysate. A fast-migrating band that looks similar to M5 (see below) appeared in WT, making this particular result suspicious, because this was the only time this band appeared with bantam. Nonetheless, It would also be interesting to test if M1 is dependent on Loqs and Ago-1 or Ago-2, and if possible, to analyze its components.

Let-7 miRNA formed much a higher level of M1 than bantam (Figure 2-4A). Similar to bantam, the M1 formed on let-7 also decreased over time in WT lysate (Figure 2-4C), and equal or greater level of M1 could be seen in *dcr-2* lysate than in WT (Figure 2-4A). No other complexes could be seen with let-7, except that a complex that looked like M2 occasionally appeared and its level seemed to increase in *dcr-2* lysate (Figure 2-4A). Mir-8 formed M4 and a faster-migrating complex that we called M5 (Figure 2-4B). M2 and M3 could also be seen occasionally. M5 level decreased over time, and M4 accumulated as in the bantam case. The formation of M5 was not strand specific, nor was it affected by absence of Dcr-2.

Figure 2-4. Complex formation of miRNAs on native gel electrophoresis. All RNAs were radiolabeled at the 5' end of the mature miRNA strand. (A) miRNA duplexes were incubated in WT or dcr-2 lysate for 30 minutes. Lane 1: bantam; lane 2 and 3: let-7. (B) mir-8 complex formation time course in WT and dcr-2 lysate. (C) Complex formation of miRNAs as labeled. Each miRNA was incubated in WT lysate for 5 and 30 min. (D) Structures of the miRNA duplexes used in (C). Red: mature miRNA strand; black: miRNA* strand.

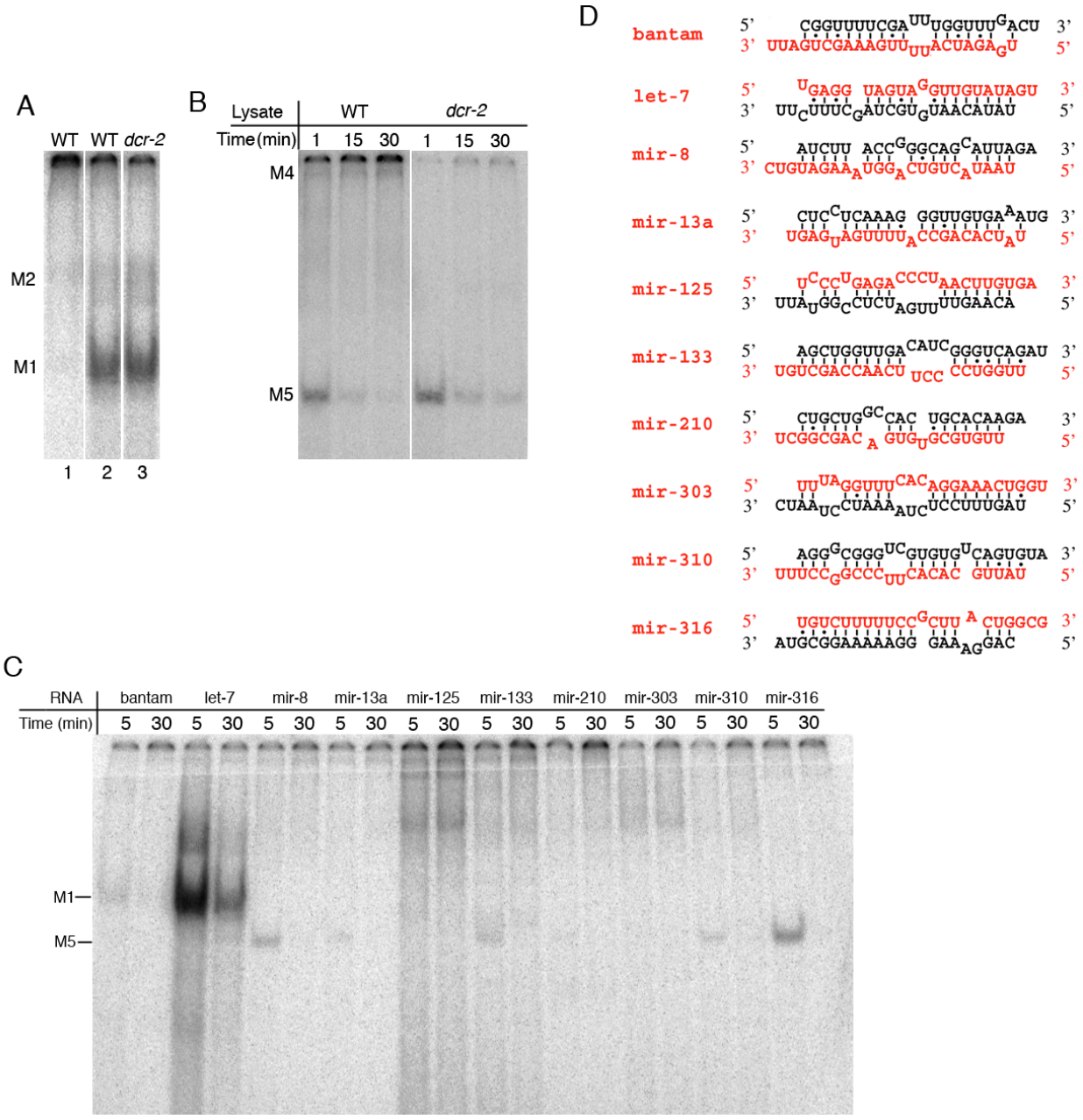

A
WT WT dcr-2
M2
M1
1 2 3
B
Lysate
WT
dcr-2
Time (min) 1 15 30 1 15 30
M4
M5
C
RNA
bantam let-7 mir-8 mir-13a mir-125 mir-133 mir-210 mir-303 mir-310 mir-316
Time (min) 5 30 5 30 5 30 5 30 5 30 5 30 5 30 5 30 5 30 5 30
M1
M5
D
bantam
let-7
mir-8
mir-13a
mir-125
mir-133
mir-210
mir-303
mir-310
mir-316

Figure 2-5.

We also examined complex formation on other miRNAs duplexes: mir-13a, mir-125, mir-133, mir-210, mir-303, mir-310 and mir-316. Mir-13a, mir-133, mir-310 and mir-316 formed M5 (Figure 2-4C and 2-4D). This experiment was only done once and it was very difficult to tell whether other complexes formed with these miRNAs.

In order to directly detect proteins that interact with the miRNAs in embryo lysates, UV-crosslinking experiments were performed. Radiolabeled bantam, mir-8 and Pp-luciferase siRNA duplexes were incubated in standard RNAi reactions with wild type embryo lysates and UV crosslinked by 254 nM light. The proteins that were crosslinked to the RNAs were separated and visualized on SDS-PAGE (Figure 2-5).

The two bands corresponding to Dcr-1 and Dcr-2 have been verified in a previous study by immunoprecipitation (Pham et al., 2004). The siRNA strongly crosslinked to Dcr-2. The two miRNAs crosslinked to Dcr-2 as well, although its level decreased over the time course. Dcr-1 seemed to crosslink to all three RNAs, but its level decreased over the time course in bantam. The bands corresponding to Ago-1 and Ago-2 have not been verified yet, but their apparent sizes are consistent with those presumed identities, and similar crosslinks have been reported and configured with different miRNAs (Forstemann et al., 2007). Ago-2 only crosslinked to the siRNA and it accumulated over time. Ago-1 crosslinked to all three RNAs and accumulated over time as well (Figure 2-5A). Interestingly, the apparent Ago-1 band disappeared in the dcr-2 null mutant lysate in all the RNAs, and the apparent Ago-2 band became much darker in the mutant (Figure 2-5B).

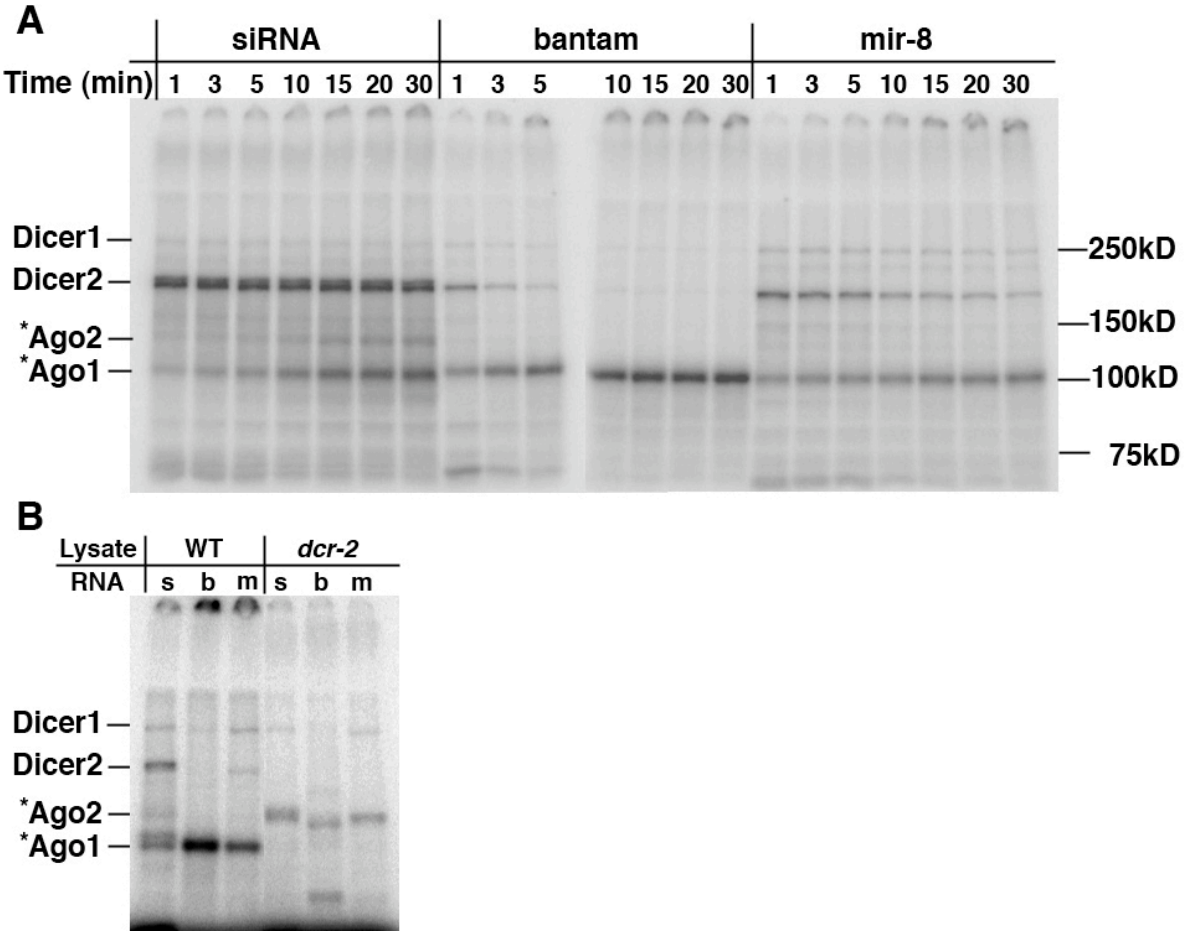


Figure 2-5. UV crosslinking of Bantam, mir-8 and Pp-luciferase siRNA. (A) The RNA duplexes were radiolabeled at the 5'-end of the mature miRNA strand or siRNA guide strand, and incubated in standard RNAi reactions with wild type embryo lysates for the time indicated. The reaction mixtures were then UV crosslinked at 254 nM, and run on 5% SDS-PAGE. * The bands labeled as Ago-1 and Ago-2 have not been verified yet. (B) The crosslinking was done the same way as in (A), except all reactions were incubated for 30 min, and dcr-2 mutant embryo lysate was used in comparison with wild type. s: Pp-luciferase siRNA; b: bantam; m: mir-8.

Discussion

From the results above, we can conclude that different miRNAs probably interact with different proteins and form different complexes in our *in vitro* system, unlike the siRNA complexes that uniformly formed on different siRNAs regardless of their sequences. However, whether these miRNA complexes are functional is still to be determined.

In the UV crosslinking experiments, Dcr-2 and Ago-2 crosslinked to the siRNA more strongly than to the miRNAs, which is consistent with their dedicated roles in the siRNA pathway. The two miRNAs crosslinked to Dcr-2 very fast (in 1 min), but they seemed to gradually dissociate from it, with bantam dissociating faster than mir-8. This behavior is reminiscent of the bantam complex M1, and the mir-8 complex M5. However, these two complexes do not seem to contain Dcr-2 because their level remains the same (or even increases) in the absence of Dcr-2. Ago-1 and Ago-2 have been suggested to compete for loading small RNA duplexes (Tomari et al., 2007), and it is possible that Dcr-1 and Dcr-2 also compete for small RNAs. Therefore, the lack of Dcr-2 might cause an increase in the interaction between Dcr-1 and RNA. Nonetheless, the M1 complex was not dependent on Dcr-1 though this result needs to be confirmed, and the crosslinking result showed the crosslinking between the RNAs and Dcr-1 did not increase in dcr-2 mutant lysate. Instead, it was the Ago-2 level that went up in dcr-2 mutant in all three RNAs, which is quite surprising given our current understanding of the relations between Dcr-2 and Ago-2 (see chapter 3). It is not surprising though, that in WT lysate Ago-2 only crosslinked to the siRNA, although Ago-1 crosslinked to all the RNAs.

However, caution must be taken when interpreting these UV crosslinking data, because crosslinking efficiency not only depends on the distance between an RNA and a protein, it also depends on the sequence of the RNA and amino acid content of the protein. For a better comparison between miRNA and siRNA, the Pp-luciferase siRNA should be replaced with si bantam or si mir-8 (see chapter 3). Furthermore, the Ago-1 and Ago-2 bands should be verified with specific antibodies, or with mutant lysates.

The difference in complex formation among miRNAs to some extent was not unexpected. Besides the difference in sequence, miRNAs differ significantly in their structures. The position and number of mismatches and bulges of a dsRNA affect the stability of the duplex and are likely to determine how the RNA interact with proteins once it is added into the embryo lysates. To this day, there has not been an efficient assay to test miRNA-mediated translational repression *in vitro*. Although Thermann and Hentze reported a cell-free system from *Drosophila* embryo that recapitulates endogenous miRNA mediated translational repression (Thermann and Hentze, 2007), this method requires large quantities of material that are difficult to obtain, especially for mutant lysates. In addition, it is unknown whether this system would recapitulate translational repression with exogenous miRNAs. Without a good functional assay, it will be very difficult to identify complexes involved in translational repression. On the other hand, miRNAs are capable of mediating RNA cleavage when the target RNA contains a sequence that is perfectly complimentary to the miRNA. Therefore, we should be able to identify miRNA complexes that are involved in RNA cleavage. The question then becomes whether these complexes represent miRNA complexes involved in translational repression. A recent study suggested that miRNAs, after being generated by Dcr-1, are sorted into functionally distinct

complexes containing either Ago-1 or Ago-2, which silences RNA by either translational repression or RNA cleavage, respectively (Forstemann et al., 2007). This suggests that miRNAs would form different complexes for different functional pathways. It would be interesting if we could detect intermediate complexes that form between Dcr-1 processing and effector Ago complexes, which would then help to explain how miRNAs are sorted.

Chapter 3: The Dicer/Argonaute Dependence of small RNAs During RISC Assembly

Introduction

In flies, distinct Dicer complexes produce siRNAs and miRNAs. miRNAs are cleaved from pre-miRNA by Dcr-1, acting with its dsRNA-binding protein partner, Loqs. siRNAs are produced from long dsRNA by Dcr-2, which partners with the dsRNA-binding protein R2D2.

Dcr-2 and R2D2 also play roles after dsRNA processing, in loading siRNAs into RISC. Native gel electrophoresis has shown that when siRNAs are added to embryo lysates, they rapidly bind one or more Dcr2–R2D2-containing complexes that function as precursors to RISC. These include the RDI complex that contains only Dcr-2/R2D2 and double stranded siRNA (Pham et al., 2004; Pham and Sontheimer, 2005b), and the RISC-loading complex (RLC) that may contain other factors in addition to Dcr-2/R2D2 and double stranded siRNA (Pham et al., 2004; Pham and Sontheimer, 2005a; Tomari et al., 2004a). Dcr-1's function also extends beyond pre-miRNA processing. As shown by Lee et al., both Dcr-1 and Dcr-2 are required for assembly of siRNA into RISC (Lee et al., 2004b).

RISC variants could be distinguished by their Argonaute protein. For years, it was thought that siRNAs are incorporated into Ago2-RISC with the help of Dcr-2/R2D2, and miRNAs are incorporated into Ago1-RISC (whether this Ago1-RISC incorporation is facilitated by Dcr-1/Loqs was unknown). Both Ago1-RISC and Ago2-RISC are capable of cleaving mRNA.

However, two studies published in 2007 by Zamore and colleagues (Forstemann et al., 2007; Tomari et al., 2007) showed that at least some Drosophila miRNAs partition between Ago1- and Ago2-RISC, whereas siRNAs associate almost exclusively with Ago2-RISC. They also claimed that Ago1- and Ago2-RISC are functionally distinct, silencing different types of target RNAs by different mechanisms. Specifically, only Ago1-RISC can repress an mRNA containing multiple, partially complementary miRNA-binding sites in its 3'UTR, and only Ago2-RISC can silence an mRNA containing fully complementary miRNA-binding sites. These claims were based on enzyme kinetic studies done on Ago1- and Ago2-RISCs, which showed that: the initial rate of target cleavage for Ago-2 was at least 12-fold greater than that of Ago-1, and Ago-1, unlike Ago-2, failed to efficiently catalyze multiple rounds of target cleavage in vitro, even in the presence of ATP.

Furthermore, they demonstrated that specific structural differences in small RNA duplexes could determine their RISC destinations (Ago1 or Ago2). They synthesized ten small RNA duplexes: an authentic let-7/let-7* duplex; a let-7 siRNA in which the guide and passenger strands were fully paired except at the guide position 1; and eight let-7 siRNA duplexes derivatives incorporating one additional mismatch between the guide and passenger strands along the length of let-7 siRNA. They found that while the let-7/let-7* crosslinked only to Ago-1, the let-7 siRNA crosslinked predominantly to Ago-2. For the eight siRNA derivatives, the closer the mismatch was to the center of the duplex region, the more the RNA duplex was directed into Ago-1 rather than Ago-2. They also showed that the Dcr-2/R2D2 heterodimer acts as a gatekeeper for the assembly of Ago2-RISC. Dcr-2/R2D2 binds well to highly paired small RNA duplexes but

poorly to duplexes bearing central mismatches, thus promoting the incorporation of siRNAs and disfavoring the use of miRNAs as loading substrates for Ago-2. An independent mechanism acts in parallel to favor assembly of miRNA/miRNA* duplexes into Ago1-RISC and to exclude siRNAs from incorporation into Ago-1. These two pathways compete for loading small-RNA duplexes with structures intermediate between that of a siRNA and a typical miRNA/miRNA* duplex.

In order to see if this finding of central mismatch being a sorting determinant holds in vivo, they examined three endogenous miRNAs in fly S2 cells: let-7 and bantam (each of which contains central mismatches), and miR-277, whose central region is base paired. Immunopricipitation experiments showed let-7 and bantam assemble primarily into Ago1-RISC, whereas miR-277 partitions between Ago1 and Ago2, which supports the sorting determining role of central mismatches. Recent Ago1 and Ago2 small RNA profiling experiments, however, have not been consistent with Ago2/mir-277 association in vivo (Kawamura et al., 2008).

In order to discern the molecular mechanisms of miRNA and siRNA pathways in Drosophila, we have further explored the relative roles that Dicers and Argonautes play in the two pathways.

Methods

The RNAi (target cleavage) reactions were performed as previously described (Pham et al., 2004) except the incubation lasted for 3 hours after the mRNA target was added.

The oligonucleotides for making the siRNAs, miRNAs and all the hybrid RNAs were provided by IDT. The RNA strands were annealed at 20μM in 1X annealing buffer (100mM KOAc, 30mM HEPES-KOH at pH 7.4, 2mM $Mg(OAc)_2$) at 25ºC for 3 hours after a 1 min incubation at 95 ºC.

The pre-miRNAs were made by *in vitro* transcription using the MEGAshortscript™ T7 kit (Ambion). In order to make precise 5' and 3' ends, each of the DNA templates contains a T7 promoter followed by a hammerhead ribozyme upstream of the pre-miRNA sequence; the two nucleotides at the 5' end of the antisense DNA template are 2'-OMe, in order for the RNA polymerase to generate precise end. The precursor RNAs were gel purified after transcription.

The recombinant Dcr-2, R2D2, Dcr-1 and Loqs proteins were expressed in insect cells by the BAC-to-BAC baculovirus expression system from Invitrogen. All the virus strains were gifts from Qinghua Liu (Liu et al., 2003).

For recombinant Dcr-2/R2D2, Sf9 insect cells were co-infected with His-tagged Dcr-2 and His-tagged R2D2 viruses. Cells were harvested after 96 hours and the proteins purified by Ni-NTA beads (Liu et al., 2003). The eluate was then purified by Superdex-200 HR 10/30 column and HiTrap Q column where the RDI fractions were collected (Pham et al., 2004). The Dcr-2 concentration was determined by quantitative Western blot.

For Dcr-1, Sf9 cells were infected with His-tagged Dcr-1 virus. Cells were harvested and treated as above. The protein was purified by Talon Metal Affinity Resin (Clontech) using the same

buffer conditions as for the Ni-NTA beads (above). The Dcr-1 concentration was determined by Bradford protein assay.

For the competitive binding assay, a standard gel shift reaction was performed with either the purified Dcr-2/R2D2 (3-30 nM) and ~5nM radiolabeled si let-7 RNA, or the purified Dcr-1 (~150 nM) and ~0.5nM radiolabeled mi let-7 RNA, in the presence of various concentrations of an unlabeled competitor (the si let-7, mi let-7, H4 let-7 or H5 let-7). Reactions were incubated for 5 hours (for Dcr-2/R2D2) or 1 hour (for Dcr-1) at 25 ºC, heparin was added to the Dcr-2/R2D2 reactions, but not to the Dcr-1 reactions, before loading onto the gel. The complex bands were quantified using ImageQuant. Kaleidagraph was used to fit the curve to the equation: $Y= Nonspecific+(Total-Nonspecific)/(1+[D]/IC_{50})$, where Y is the total binding, and [D] is the concentration of the competitor. Nonspecific is the binding in the presence of a saturating concentration of D, and Total is the binding in the absence of competitor. The IC_{50} that was determined by this equation was then used to calculate the K_d (of si let-7) or K_i (of the competitor) using equation: $K_i=IC_{50}/(1+[radioligand]/K_d)$ (Cheng and Prusoff, 1973).

The bantam sensor reporter transgenic flies and control sensor reporter transgenic flies were gifts from Stephen M. Cohen (Brennecke et al., 2003). These flies were crossed into *ago-2*^{414} background. Wing discs were dissected and fixed in 4% formaldehyde, and the EGFP signal from the sensor reporter was analyzed by fluorescence microscope.

Results

It had been thought that Dcr-2/R2D2 generate siRNAs and incorporate them into Ago2-RISC, and Dcr-1/Loqs generate miRNAs that are then incorporated into Ago1-RISC (by unknown factors) that is capable of cleaving mRNA. Studies have shown that the lack of Dcr-2 has no impact on miRNA mediated mRNA silencing *in vivo* (Lee et al., 2004b), and lack of Ago-2 has no impact on miRNA mediated RNA cleavage *in vitro* (Okamura et al., 2004). However, when we performed *in vitro* mRNA cleavage assays using several different miRNAs as triggers, the results showed that absence of Dcr-2 or Ago-2 did affect the cleavage efficiency of some of the miRNAs (Figure 3-1).

Dcr-2 Dependence

The effect of miRNA structure on target cleavage

My results showed there were two categories of miRNAs: one including bantam and mir-303 whose activity did not change or increased in the absence of Dcr-2; and another including mir-8, let-7, mir-125, mir-210, and mir-316 whose activity decreased in *dcr-2* mutant lysate (Figure 3-1). A recent report by Forstemann et al. showed that in S2 cells Dcr-2 plays a role in loading mir-277 into miRISC that degrades a reporter mRNA containing sites fully complimentary to mir-277 (Forstemann et al., 2007), which agrees with my result and indicates that Dcr-2 function is not exclusive to siRNAs.

We focused on examining one miRNA from each category: bantam and let-7. For these experiments, miRNA/miRNA* duplexes were used as trigger for mRNA cleavage in WT or *dcr-2* null mutant embryo lysate. For each miRNA, we included a siRNA duplex in which the guide

strand (the mature miRNA strand) is perfectly paired to the passenger strand. Each of these duplexes was incubated in lysate, and then a target RNA containing a site perfectly complementary to the mature miRNA was added to the reaction. As expected, the siRNAs absolutely required Dcr-2 even though their miRNA counterparts were partially or completely independent of Dcr-2 (Figure 3-2).

In order to find the structural determinants in the miRNA duplexes that decrease the miRNA's requirement for Dcr-2 compared to the siRNA duplexes, we systematically altered the structures of the two miRNA (bantam and let-7) duplexes and tested their target RNA cleavage activity. The non-Watson-Crick base-pairings were changed to Watson-Crick base-pairings (repaired) based on the base-pairing type or their position in the duplex (Figure 3-3A). Hybrids 2 and 3 repair mismatches/bulges and G-U wobble pairs, respectively. Hybrids 4 and 5 repair all non-Watson-Crick base-parings at the 5'-half (regarding to the mature miRNA strand) and the 3'-half of the duplex, respectively. Hybrids 6 through 10 repair non-Watson-Crick base-pairings in the middle or at the end of the duplexes. When every non-Watson-Crick base-pairing is repaired, we obtain the siRNA. All the duplexes except the miRNAs and siRNAs are called hybrids because their structures represent features of both mi and si duplexes. In each miRNA set, only the passenger strand sequence was changed, so all the duplexes form the same active RISC and cleave the same target RNA.

The mutations introduced into the miRNA duplexes changed their overall ability to direct target cleavage in wild type embryo lysate (Figure 3-3B and 3-3C upper panels) as well as their requirement for Dcr-2 (Figure 3-3B and 3-3C lower panels). For example, Hybrid 3 of both

bantam and let-7 (all G-U wobble repaired) showed significant higher activity than the miRNAs in both WT and dcr-2 mutant, yet its Dcr-2 dependence remained at similar level as the miRNAs. On the other hand, hybrid 3, the duplex that repaired all mismatches and bulges while maintaining all G-U Wobbles, only slightly increased cleavage activity in WT (bantam and let-7), and it had different effects on Dcr-2 dependence for the two miRNAs, which is likely to be a result of the different positions of G-U wobbles in the two duplexes: G-Us spread through the whole length of bantam but they are absent from the 3'end of let-7. These results suggest that the cleavage activity of small RNA duplexes and how much it relies on Dcr-2 may be affected by the type of non-Watson-Crick base-pairings as well as their positions in the duplex. We realize that terminal repairs are made in a few duplexes, which stabilizes the 5' end of the mature miRNA and thus will possibly change the asymmetry of the duplex. However, asymmetry change is not a concern to this study since we only tested how much the absence of Dcr-2 affects the activity of the mature miRNA strand.

We noticed that for both bantam and let-7, the Hybrid 4, which repairs the 5' half (relative to the mature miRNA) of the duplex, made the RNA more dependent on Dcr-2 compared to the miRNA, and the level of dependence became very close to the siRNA. On the other hand, repairing the 3' half of the duplex (Hybrid 5) maintained miRNA's Dcr-2 independency.

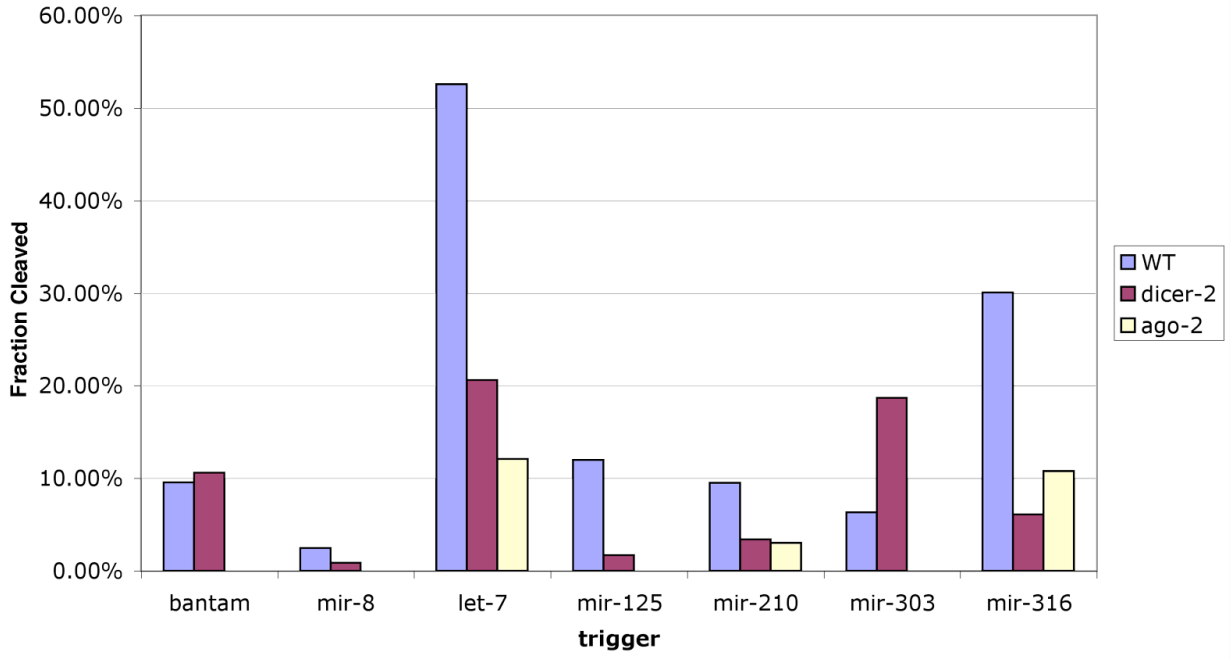


Figure 3-1. Target RNA cleavage reactions triggered by miRNA duplexes idicated at the bottom. Reactions lasted for 3 hours and the fraction of target RNA that was cleaved was quantified. Lysates used are shown on the right: WT: wild type; *dicer-2*: *dcr-2* null mutant; *ago-2: ago-2* null mutant.

The effect of miRNA structure on Dicer binding

One possible explanation for this is that the structural features in the 5' half of a miRNA duplex can be recognized by the Dcr-2/R2D2 initiator complex, which means Dcr-2/R2D2 has higher binding affinity for siRNA and Hybrid 4 than for miRNA and Hybrid 5. Thus, more siRNA and Hybrid 4 is loaded by the Dcr-2/R2D2 initiator complex than miRNA and Hybrid 5, which causes siRNA and Hybrid 4's greater dependency on Dcr-2. According to the thermodynamic features of the miRNA duplex, Dcr-2 should be the one that binds to the 5' half of the duplex (Tomari et al., 2004b). Therefore, it will suggest that Dcr-2 does not bind imperfect RNA duplexes so well as it binds to perfect duplex regions. An alternative explanation to these results is that Dcr-2/R2D2 binds all of the RNA duplexes equally, but an alternative loading machinery (possibly Dcr-1) prefers to load the ones with imperfect base pairing in the 5' (miRNA and Hybrid 5), making these two duplexes less dependent on Dcr-2.

To distinguish between these possibilities, we examined the binding affinity of Dcr-2/R2D2 for the different RNA duplexes. Previous studies have used native gel electrophoresis to view the R2D2/Dcr-2 initiator (RDI) complex, which contains only Dcr-2/R2D2 heterodimer and siRNA duplex and subsequently assembles into RISC (Pham and Sontheimer, 2005a). First, we incubated the bantam and let-7 RNA duplexes (mi, si, Hybrid 4 and Hybrid 5) with wild type embryo lysate and ran native gel to visualize the RDI (Figure 3-4A). The siRNA and Hybrid 4 formed the RDI complex, whereas the miRNA and the Hybrid 5 formed none or very little RDI. This is consistent with the idea that Dcr-2/R2D2 has different binding affinity for the si-like (siRNA and Hybrid 4) versus mi-like (miRNA and Hybrid 5) RNA duplexes.

To quantitatively compare the Dcr-2/R2D2 binding affinity for the different RNAs, we performed a competitive binding assay to measure the dissociation constants. We incubated radiolabeled si-let-7 with recombinant Dcr-2/R2D2 heterodimer in the presence of a range of concentrations of a serises of unlabeled competitors including si-let-7, mi-let-7, Hybrid 4 let-7 or Hybrid 5 let-7. The reactions were analyzed on native gel where the RDI complex was quantified and data plotted to generate competitive binding curves (Figure 3-4B). The K_{app}s (see below) obtained from this experiment showed that siRNA and Hybrid 4 have higher binding affinity than miRNA and Hybrid 5 for Dcr-2/R2D2 heterodimer. Therefore, we conclude that for the small RNAs we have tested, the imperfect base pairing in the 5' half of the RNA duplexes disrupts Dcr-2 binding to the RNA and thus decreases the Dcr-2/R2D2-facilitated RISC loading.

However, it is unknown whether this is the sole reason these small RNA duplexes show different dependency on Dcr-2, which could otherwise be caused by a combination of Dcr-2 favoring and Dcr-1 disfavoring perfect base pairing in the 5' half of the duplex in the dcr-2 mutant. Therefore, we measured the binding affinity of Dcr-1 for the small RNA duplexes using competitive binding assay. There are several reasons that the dissociation constants measured by these experiments may not be taken as real K_ds. The binding reactions contained the ATP regenerating system (creatine phosphate and creatine kinase) and 1mM free ATP that was also present in all the standard RNAi reactions. And the recombinant Dcr-2/R2D2 and recombinant Dcr-1 were not

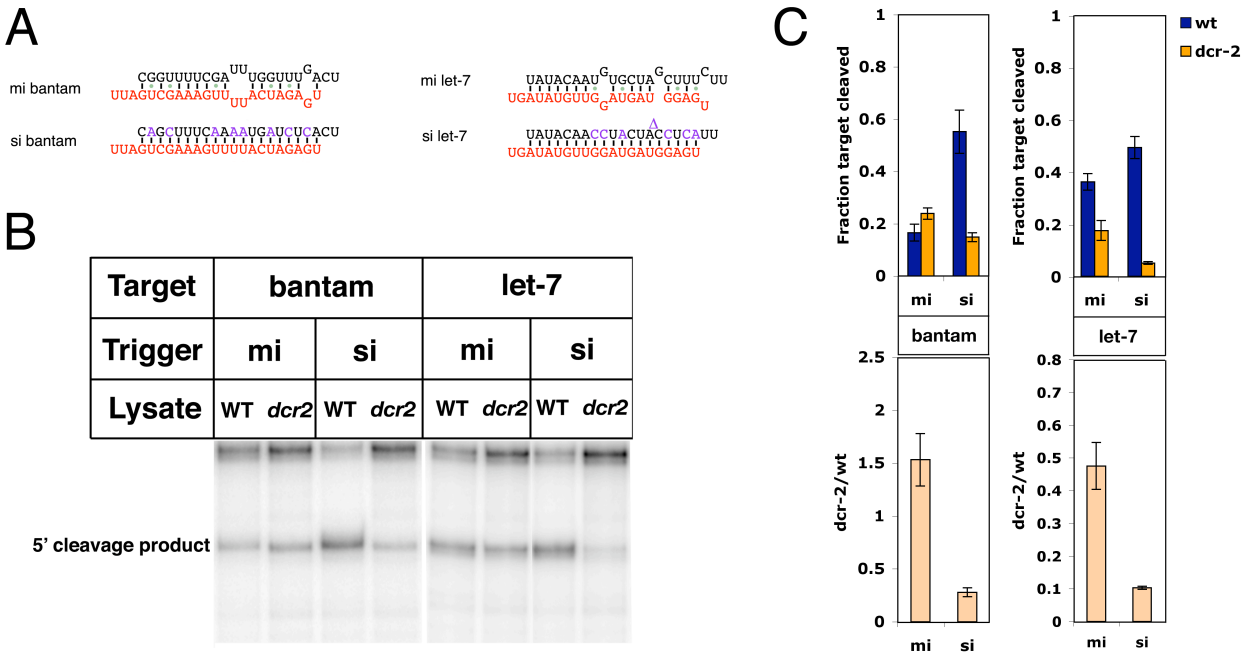


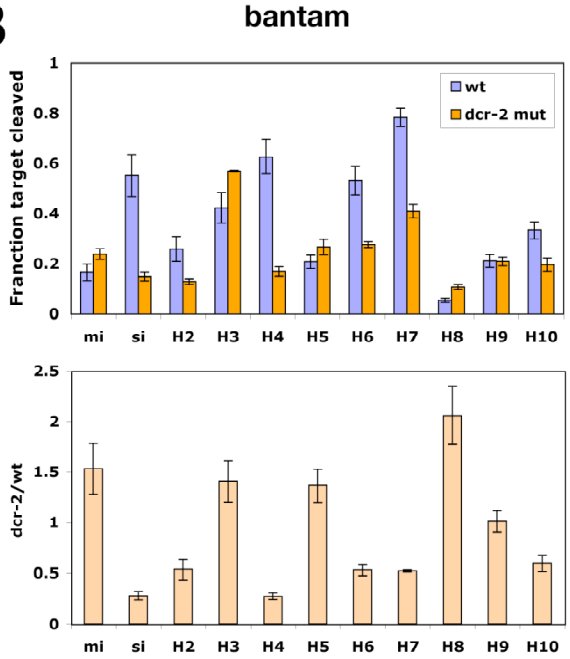
Figure 3-2. miRNA duplexes show greater dependence on Dcr-2 than siRNAs in target RNA cleavage reactions. (A) The structures of the small RNA duplexes used in (B). Red: mature miRNA strand; black: miRNA* or siRNA passenger strand; purple: nucleotides mutated from the miRNA* sequence. (B) bantam (mi-bantam and si-bantam) and let-7 (mi-let-7 and si-let-7) mediated target RNA cleavage reactions were performed in wild type (WT) and *dcr-2* null (*dcr-2*) embryo lysates. (C) Quantitation of (B). Top: percentage target cleaved in WT and *dcr-2* embryo lysates; bottom: the ratio between the cleavage activity in *dcr-2* lysate and the cleavage activity in WT lysate. The error bars represent standard error of the mean from 3 separate trials.

Figure 3-3. Small RNA duplex structure determines its dependence on Dcr-2 in target RNA cleavage reactions. (A) The structures of the bantam series and let-7 series of small RNAs. Red: mature miRNA strand; black: miRNA* or siRNA passenger strand; purple: nucleotides mutated from the miRNA* sequence. (B) Quantitation of the target RNA cleavage reactions mediated by the small RNAs listed in (A). Top: percentage target cleaved in WT and *dcr-2* embryo lysates; bottom: the ratio between the cleavage activity in *dcr-2* lysate and the cleavage activity in WT lysate. The error bars represent the standard error of the mean from 3 experiment trials.

A

	bantam	let-7	Repaired base pairs
mi RNA			
si RNA			All mismatches, bulges and G-U wobbles
Hybrid 2			All mismatches and bulges
Hybrid 3			All G-U wobbles
Hybrid 4			5' half of the mature strand
Hybrid 5			3' half of the mature strand
Hybrid 6			Middle third
Hybrid 7			Central mismatch(es)
Hybrid 8			Terminal mismatch
Hybrid 9			Non-terminal mismatch(es)
Hybrid 10			Non-terminal mismatch

B



C

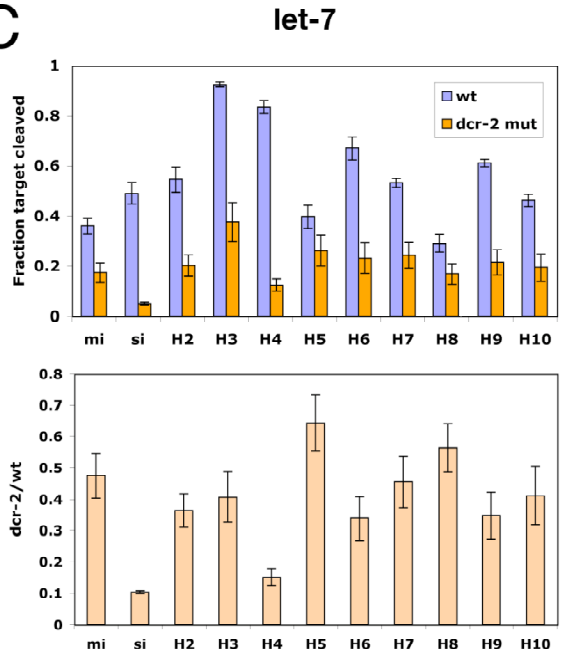


Figure 3-3.

extensively purified. Thus the reaction mixture probably contained factors that may affect the binding reactions. The impurity of the proteins may also have complicated our estimation of the protein concentration (see methods). Even if the proteins were adequately purified and the concentration measurements were accurate, the specific activity of these proteins is yet to be determined. Although the exact protein concentration is not a parameter of the equation that was used to calculate the dissociation constant, it is useful to determine whether the proper range of RNA concentration was used.

Nonetheless, our results (Figure 3-5) suggest that Dcr-1 does not preferentially bind to miRNA-like (let-7 and H5-let-7) duplexes. This does not support the hypothesis that Dcr-1 loads the miRNA-like duplexes favorably in the absence of Dcr-2 thus making the miRNA-like duplexes less dependent on Dcr-2. We did not do the binding assay using Dcr-1/Loqs heterodimer because a previous study showed that Loqs is not required for let-7/let-7* or siRNA-induced target mRNA cleavage *in vitro* (Liu et al., 2007). However, it is still possible that Loqs could affect Dcr-1's binding affinity to small RNAs, and it could become involved in let-7 RISC formation when Dcr-2 and R2D2 are absent. Therefore, it could be interesting to do the Dcr-1-RNA binding assay with Loqs present.

Figure 3-4. Dcr-2/R2D2 binds small RNAs with perfect base-pairing in their 5' half with higher affinity. (A) A native gel assay was done in wild type embryo lysate with different small RNA duplexes as labeled on the top. In each duplex, the mature miRNA strand is radiolabeled at the 5' end. (B) The competitive binding curves of the four non-radiolabeled competitors (mi-let-7, si-let-7, H4-let-7 and H5-let-7) competing with radiolabeled si-let-7 in competitive binding assays done using recombinant Dcr-2/R2D2. (C) The K_{app}s for the binding of Dcr-2/R2D2 with the let-7 RNA duplexes (mi-let-7, si-let-7, H4-let-7 and H5-let-7) were calculated using the IC_{50} values determined by the competitive binding curves in (B). The bars represent standard error of the mean from 4 experiment trials.

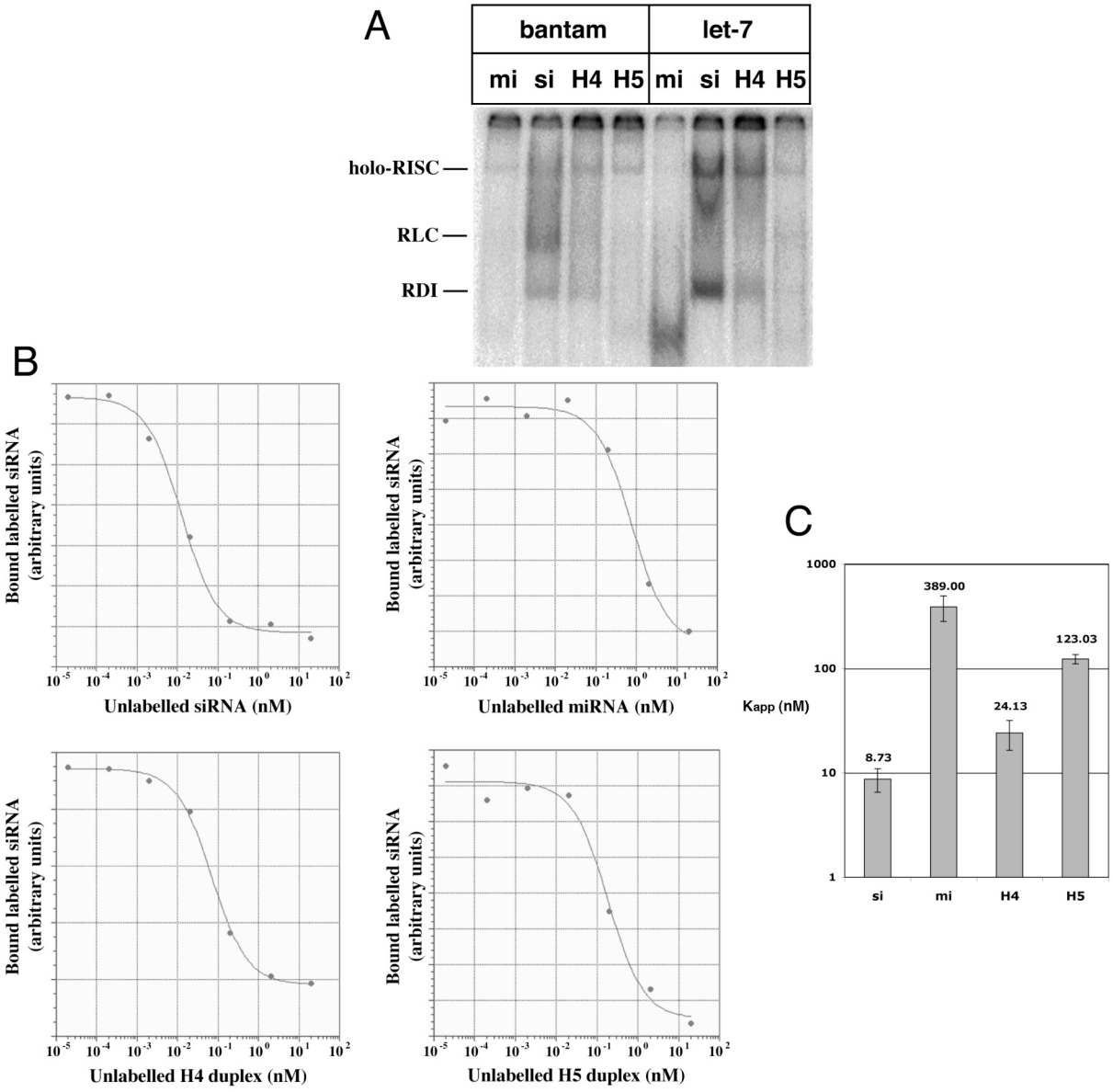

A
bantam
let-7
mi si H4 H5 mi si H4 H5
holo-RISC
RLC
RDI
B
Bound labelled siRNA (arbitrary units)
Unlabelled siRNA (nM)
Unlabelled miRNA (nM)
Unlabelled H4 duplex (nM)
Unlabelled H5 duplex (nM)
C
Kapp (nM)
1000
100
10
1
389.00
123.03
24.13
8.73
si
mi
H4
H5

Figure 3-4.

Figure 3-5. Binding of Dcr-1 with let-7 RNA duplexes. (A) Native gel assay was done using recombinant Dcr-1 with mi-let-7 and si-let-7. In each duplex, the mature miRNA strand is radiolabeled at the 5' end. (B) The competitive binding curves of the four non-radiolabeled competitors (mi-let-7, si-let-7, H4-let-7 and H5-let-7) competing with radiolabeled mi let-7 in competitive binding assays done using recombinant Dcr-1. (C) The K_{app}s for the binding of Dcr-1 with the let-7 RNA duplexes (mi-let-7, si-let-7, H4-let-7 and-H5 let-7) were calculated using the IC_{50} values determined by the competitive binding curves in (B). The bars represent standard error of the mean from 3 experiment trials.

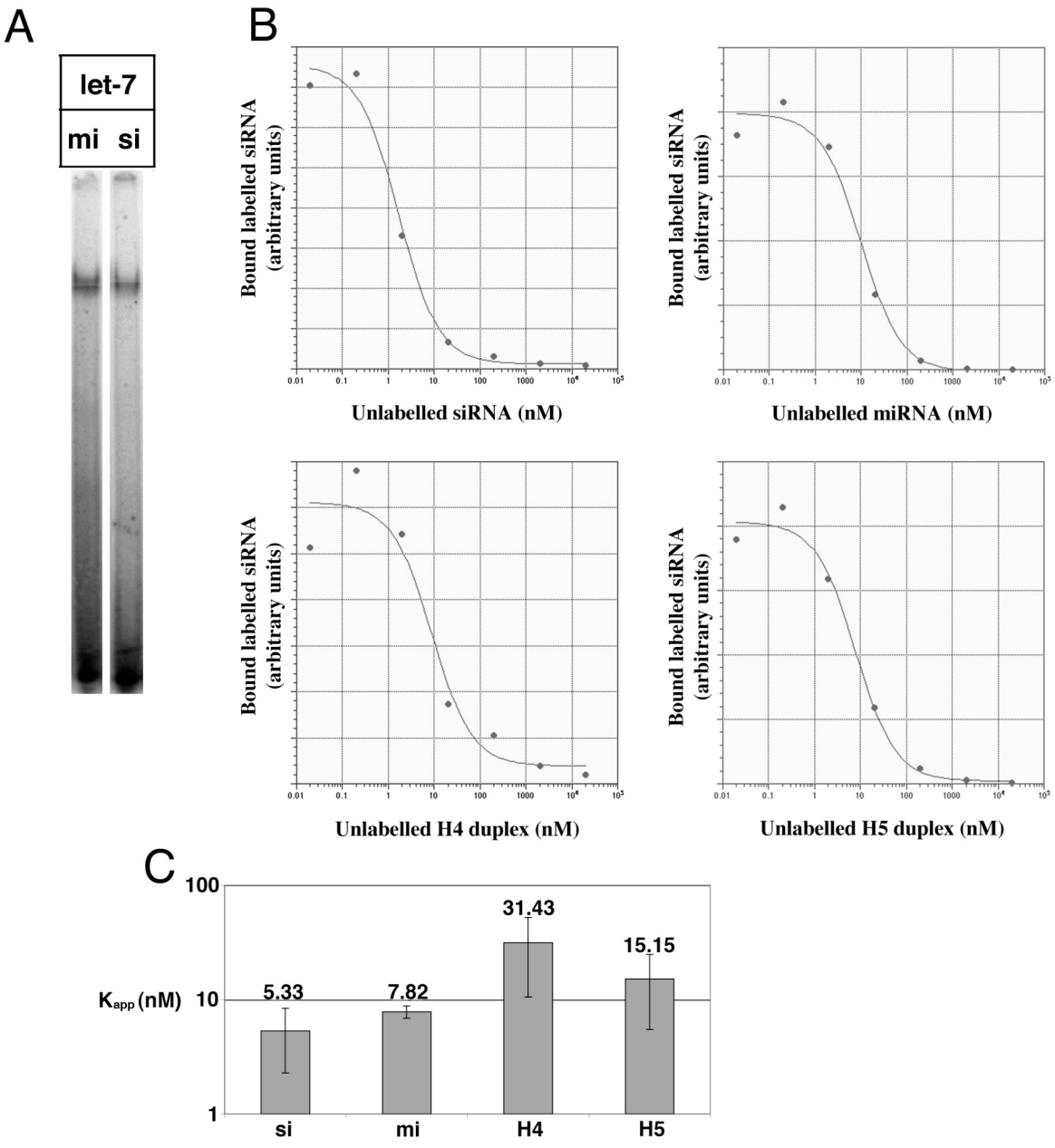

A
let-7
mi si
B
Bound labelled siRNA (arbitrary units)
Unlabelled siRNA (nM)
Unlabelled miRNA (nM)
Unlabelled H4 duplex (nM)
Unlabelled H5 duplex (nM)
C
K_{app} (nM)
100
10
1
5.33
7.82
31.43
15.15
si
mi
H4
H5

Figure 3-5.

The effect of miRNA structure on Dicer processing

In the cell, miRNA/miRNA* duplexes are the products of Dicer processing pre-miRNAs. We wondered if the same miRNA/miRNA* structural features recognized by Dcr2 during RISC assembly can be recognized when they are embedded in the miRNA precursor. The nuclear RNase III Drosha generates pre-miRNAs, leaving the characteristic 2nt 3' overhang ends, which is then recognized by Dicer in the cytoplasm. We made pre-let-7 RNA with precise 5' and 3' ends by *in vitro* transcription, and the same precursor form for the si let-7, Hybrid 4 and 5 substrates, based on the pre-let-7 sequence (Figure 3-6). The precursors were incubated in WT or dcr-2 null mutant embryo lysate and the processing products were quantified (Figure 3-6). The absence of Dcr-2 slightly decreased the processing of all four precursors. This result suggests Dcr-2 is partially involved in pre-miRNA processing *in vitro*, although indirect effects of Dcr-2 absence cannot be ruled out. Nonetheless, Dcr-1 seems to be the enzyme primarily responsible for this process, regardless of the structure of the stem part of the precursor hairpin.

Notably, the product generation curves were different for the miRNA-like (pre-mi-let-7 and pre-hybrid 5) and the siRNA-like (pre-si-let-7 and pre-hybrid 4) duplexes (Figure 3-6). The siRNA-like duplexes accumulated in an approximately linear fashion, whereas the miRNA-like duplexes followed distinctly non-linear kinetics. Several factors could contribute to the different behaviors exhibited by siRNA-like and miRNA-like duplexes, including changes in the processing reaction rates, differential turnover of the small RNA products, structural inhomogeneities in the precursors, or loss of signal due to dephosphorylation of the precursors or products in the lysates. Nonetheless, the precursor processing experiment showed that the H5-let-7 was indeed miRNA-

like and the H4-let-7 was siRNA-like, which is in agreement with the target RNA cleavage experiment and the Dcr-2/R2D2 binding experiment.

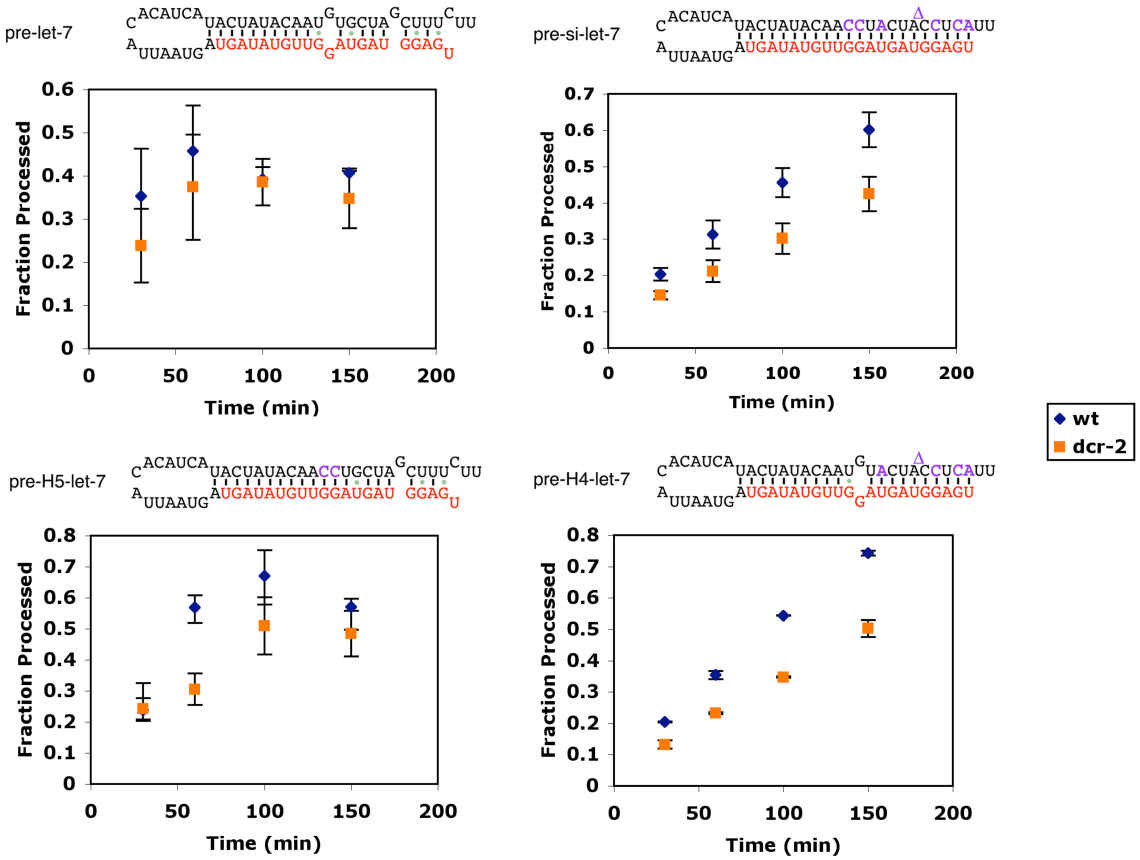


Figure 3-6. Dcr-1 is the major precursor processing enzyme regardless of the stem structure of the hairpin. For each reaction, 50nM non-radiolabeled precursor and a trace amount of the same precursor that was radiolabeled at the 5' phosphate was incubated in wild type (WT) or *dcr-2* null (dcr-2) embryo lysate.

Ago Dependence

Okamura et al. had previously shown that miRNAs-mediated mRNA cleavage did not require Ago-2, suggesting that Ago-1 was responsible for the slicer activity in miRISC (Okamura et al., 2004). However, Forstemann et al. reported an enzyme kinetic study that the k_{cat} of Ago-1 is more than 40-fold lower than that of Ago-2, and concluded that Ago-1 is too inefficient to silence a target RNA by endonucleolytic cleavage (Forstemann et al., 2007).

We performed target RNA cleavage experiment in *ago-2* null mutant lysate using si-let-7 and mi-let-7. In *ago-2,* si-let-7 had little activity, whereas mi-let-7 still had about 30% to 50% activity remained (Figure 3-1 and Figure 3-7). The slicer activity in the absence of Ago-2 was assigned to Ago-1, because Ago-1 and Ago-2 are the only proteins known to have the function of executing miRNA and siRNA-guided mRNA cleavage in fly. Our results suggested that si-let-7 was exclusively loaded to Ago-2 RISC, and mi-let-7 was loaded to both Ago-1 and Ago-2 RISCs. The fact that Ago-1 is more than 60 times less proficient than Ago-2 as a slicer, and mi let-7 only lost 50% activity in the absence of Ago-2 could suggest that the majority of mi-let-7 was loaded to Ago-1 RISC. In fact, Forstemann et al. reported that mi-let-7 was as active in *ago-2* as in WT lysate, suggesting all mi-let-7 was loaded to Ago-1 RISC. This result, however, disagrees with our result that mi-let-7 significantlty lost activity in *ago-2* mutant. It is not known whether the time difference could account for this inconsistency. Their RISC assembly lasted for only 3 to 5 minutes followed by a quenching reaction that stops the assembly process before the target mRNA was added. Our assembly lasted for 15 minutes and was allowed to continure in the lysate even after the target was added. It is possible that mi let-7 is loaded to Ago-1 RISC

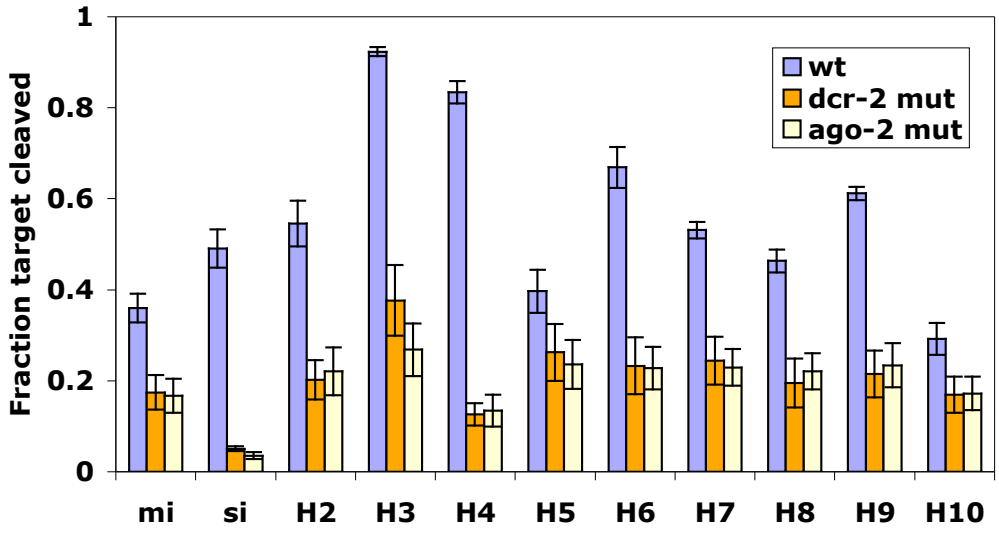


Figure 3-7. Quantitation of target RNA cleavage reactions mediated by let-7 small RNAs (structures shown in Figure 3-3A) in wild type (wt), *dcr-2* null mutant and *ago-2* null mutant lysates.

faster than to Ago-2 RISC, and so the Ago-2 effect appears only when the assembly reaction lasts long enough. One other miRNA that we have tested, mir-316, also showed more than 30% of its WT target cleavage activity in *ago-2* (Figure 3-1), indicating this miRNA was also loaded to both RISCs.

Except for let-7, mir-210 and mir-316, all the miRNAs that we tested completely lost their ability to mediate target RNA cleavage in the absence of Ago-2 (Figure 3-1). This is contradictory to previous studies that suggested miRNAs require Ago-1, but not Ago-2 to induce RNA cleavage. One explanation for the discrepancy is that we examined different miRNAs, and different miRNAs are loaded differently to Ago-1 or Ago-2. In fact, this is obvious from the group of miRNAs that we tested (Figure 3-5).

Tomari et al. suggested a gate-keeping role of Dcr-2/R2D2 during Ago-2 RISC assembly, where Dcr-2/R2D2 promotes the incorporation of siRNAs and disfavors miRNAs as loading substrates for Ago-2 RISC (Tomari et al., 2007). They showed that the affinities of a few variant miRNA duplexes (see Chapter 3 Introduction) for Dcr-2/R2D2 correlated well with their propensities for loading to Ago-2, and increasing the concentration of Dcr-2/R2D2 in lysates increased Ago-2 loading. In support of the gate-keeping model, Forstemann et al. showed that an endogenous miRNA, mir-277, required Dcr-2/R2D2 to form Ago-2 RISC *in vivo* (Forstemann et al., 2007).

However, my experiment on bantam generated results that are inconsistent with the gate-keeping model. Although bantam-mediated target RNA cleavage activity was completely dependent on Ago-2, it was not at all impaired in the absence of Dcr-2 (Figure 3-1), which would suggest that

something other than Dcr-2/R2D2 is responsible for loading bantam to Ago-2 RISC. It would be interesting to find out whether it is Dcr-1/Loqs that facilitates Ago-2 RISC loading. A similar yet more surprising behavior could be seen with mir-303 (Figure 3-1). mir-303 showed much higher activity in *dcr-2* mutant, although little activity is left in *ago-2* mutant. This suggests that Dcr-2 or Dcr-2/R2D2 directly or indirectly represses mir-303 mediated target RNA cleavage in WT lysate. It has been suggested that Ago-1 and Ago-2 pathways compete for loading small RNA duplexes with different structural features (Tomari et al., 2007). If we assume a similar competition exists between Dcr-1 and Dcr-2, and then the increased activity of mir-303 lysate could be due to an increase in mir-303/Dcr-1 binding in the absence of Dcr-2. This would mean that in WT situation, Dcr-2 or Dcr-2/R2D2 efficiently interacts with mir-303 and prevents the miRNA from being loaded to Ago-2 RISC. This is completely opposite to the current view in which Dcr-2/R2D2 facilitates Ago-2 loading.

In the *in vitro* assays that we have performed, not every miRNA showed a well-coupled dependence on Dcr-2 and Ago-2, with mir-303 as the most extreme example. On the other hand, miRNAs like let-7 showed partial dependency on both Dcr-2 and Ago-2. This feature did not change in any of the hybrid let-7 duplexes (Figure 3-7). Their dependence on Dcr-2 always correlated well with their dependence on Ago-2. On the contrary, all of the hybrid bantam duplexes showed absolute dependence on Ago-2, despite the obvious difference in the Dcr-2 dependence (Figure 3-3).

So far, all my experiments were done in the *in vitro* cell free system from fly embryos. In order to know how relevant these results are to real physiological situations in a animal, we examined

transgenic flies carrying a GFP sensor reporter with perfectly complementary binding sites for bantam (Brennecke et al., 2003). In a WT background, the GFP reporter was largely repressed in the wing disc. In *ago-2* mutant background, the reporter was still repressed in the wing disc as in WT (figure 3-8).

Since the reporter contained perfectly complementary sites for bantam, it is unlikely that the repression was a result from any mechanism other than mRNA cleavage presumably executed by Ago-1. This would contradict the theory that Ago-1's endonucleolytic activity is too inefficient to silence RNA by cleavage. It is possible that under the in vivo experimental conditions, Ago-1 may not need to catalyze multiple turnover reactions because bantam-RISC could be in excess compared to the GFP reporter mRNA. However, Ago-1 exhibits very low efficiency even under single turnover conditions according to the kinetic studies. It is possible that Ago-1 is a much more efficient enzyme *in vivo* than *in vitro* where the enzyme kinetic experiments were done. In addition, pre-miRNA processing by Dcr-1 might help RISC be more effectively programmed in vivo, as in human, the Dcr cleavage activity was shown to be tightly coupled into the effector step of RNAi mediated by Ago-2 (Gregory et al., 2005).

This result is also contradictory to our *in vitro* result that showed Ago-2 is necessary to cleave RNA targeted by bantam. Endogenously generated bantam could be sorted differently than exogenously added bantam (miRNA/miRNA*), because the process of Drosha and Dcr-1 mediated miRNA generation could affect downstream sorting process. Forstemann et al. showed that in S2 cells, the majority of endogenous mir-277 was associated with Ago-2 and it required Ago-2 to repress a reporter with perfectly complimentary sites, whereas the majority of

endogenous bantam was associated with Ago-1, which supports that endogenous bantam is mostly loaded to Ago-1 RISC that silences mRNA.

We did not test reporter genes that carry imperfectly complementary sites to bantam to see how translational repression behaves, because the fly Ago-2 was reported to be only involved in RNA repression through endonucleolytic cleavage and presumably only Ago-1 would be responsible for translational repression (Tomari et al., 2007).

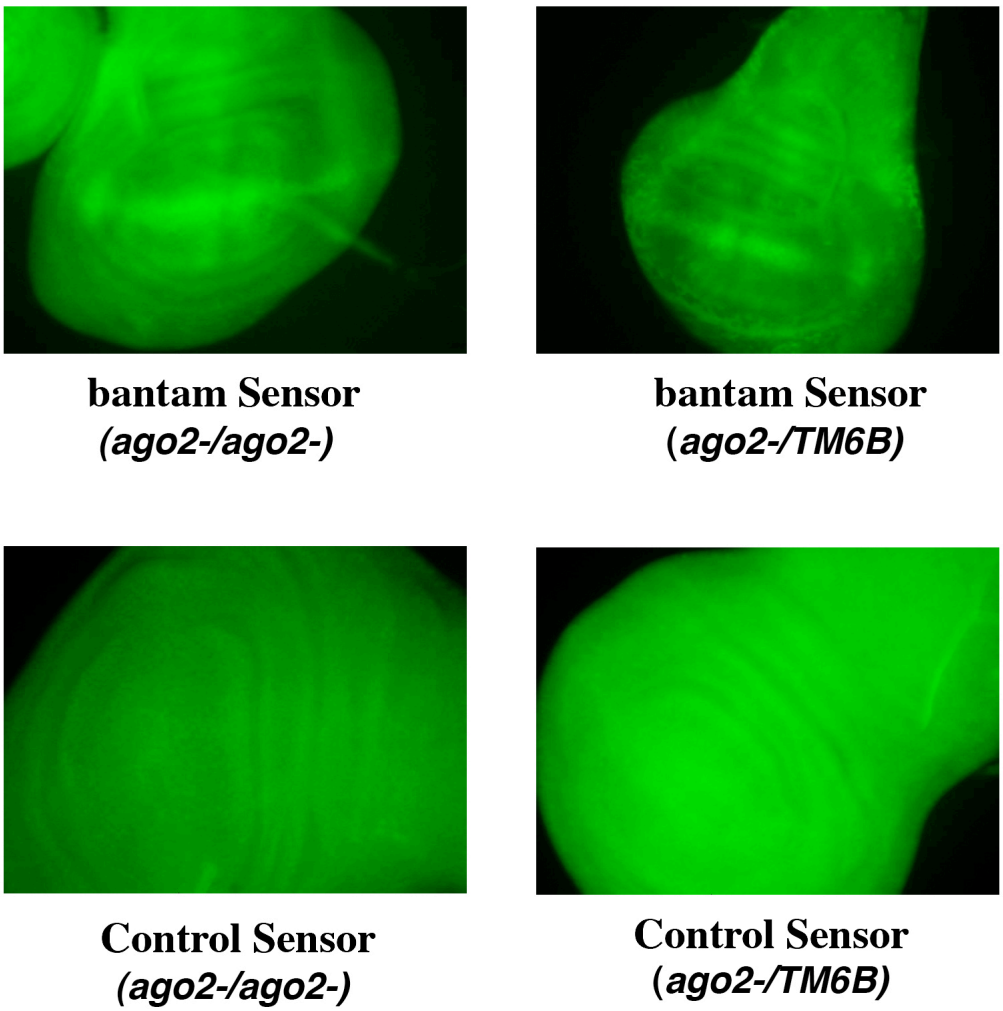


Figure 3-8. Wing discs expressing a tubulin-EGFP reporter transgene in *ago-2*$^{-}$ *(ago2-/ago2-)* or *ago-2*$^{+}$ *(ago2-/TM6B)* background. bantam sensor transgene contains two copies of a 31 nt sequence perfectly complementary to bantam. Control sensor transgene lacks bantam target sequences.

Disscusion

Thousands of miRNAs have been discovered in plants and animals. Bioinformatic studies have suggested that a substantial fraction of the mammalian genes are regulated by these small RNAs (Lewis et al., 2005). In *Drosophila*, the miRNA and siRNA pathways have common characteristics, yet are distinct from each other.

We started out to study the *Drosophila* miRNA pathway when our lab successfully characterized steps in the siRISC assembly pathway. The first thing we tried was to use the same tool - native gel electrophoresis - that had been used to study siRISC assembly. We tested several different miRNA/miRNA* duplexes, and to my surprise, they all showed different patterns of complex formation on native gel. This was unexpected because our lab had tested different siRNAs and they did not differ significantly in their complex formation. These complexes may not all be functionally relevant because this type of experiments often detects non-specific protein-RNA interaction. Controls such as RNA-DNA hybrids could be used to rule out some non-specific complexes, although having a functional assay will be necessary to determine which complexes are involved in miRNA pathway. So far, we have only been able to test the target mRNA cleavage function of a small RNA, which will only allow us to identify miRNA complexes that function in RNA cleavage rather than translational repression. The cell-free system described by Thermann and Hentze (Thermann and Hentze, 2007) will be promising for this purpose if it can be made to work with exogenously added miRNA/miRNA*.

Since miRNAs are naturally generated in a stepwise manner in the cell, it will be interesting to test complex formation with miRNA precursors in addition to miRNA/miRNA* duplexes. The miRNA biogenesis process might affect how mature miRNAs interact with other components and thus change its complex formation. In fact, this sort of phenomenon has been observed in humans, where RISC displays about 10-fold (in vitro) or up to 100-fold (in vivo) greater activity using pre-miRNA Dicer substrate than using duplex siRNA (Gregory et al., 2005; Kim et al., 2005).

When we tested the miRNA/miRNA* duplexes for their activity in target RNA cleavage, they again showed a variety of behaviors. Studies from our group and Siomi's group had indicated that Dcr-2 and Ago-2 are dedicated to the siRNA pathway, and that Dcr-1 and Ago-1 are required for miRNA pathway (although Dcr-1 was also implicated in siRNA functions). It was later shown that some miRNAs are associated with Ago-2 by Forstemann et al. and Kawamura et al. But Forstemann et al. suggested that Ago-1 only mediates translational repression and cannot efficiently repress gene expression through mRNA cleavage, and that Ago-2 is the only dedicated slicer that cleaves mRNAs. They also suggested Dcr-2/R2D2 acts as a gatekeeper for Ago-2 loading. In this model, Dcr-2/R2D2 selects the miRNAs to be loaded to Ago-2 and those to be rejected from Ago-2 RISC assembly.

Results from my experiments also showed miRNAs greatly depend on Ago-2 to induce target RNA cleavage. However for some miRNAs, significant target cleavage activity still could be seen in the absence of Ago-2, indicating that Ago-1 may be a more efficient slicer than suggested. A more surprising result came from my experiment with the GFP reporter transgenic

flies, in which the reporter was silenced (presumably through mRNA cleavage) as efficiently in *ago-2* null mutant as in WT. This suggests that Ago-1 could be a very efficient slicer *in vivo*.

The strange behavior of bantam and particularly mir-303 suggests that Dcr-2/R2D2 may not always act as a gatekeeper for Ago-2. It seems for these two miRNAs, Dcr-2 is dispensable for or even inhibits Ago-2 loading.

My results from the complex gel experiments and the target RNA cleavage experiments are far more complicated than we originally anticipated. We had expected to see more or less uniform behaviors among different miRNAs as with siRNA, yet the results showed a wide range of variety. We could argue these are largely artifacts and disregard them. As a matter of fact, the majority of the miRNAs tested by me are not naturally expressed in embryo. Nonetheless, the inconsistency seen in the behavior of these miRNAs is reminiscent of the greatly debated miRNA-mediated gene silencing mechanism. MiRNAs are a diverse group of small RNAs. They regulate gene expression at different developmental stages, at a variety of specific tissues and cells. Some of them may only function in response to certain stress conditions. An organism's cellular components are very different at different times and places. miRNAs may have adapted to their specific environment and specific tasks. Some of them degrade mRNAs, whereas others block translation. And they may achieve these tasks through different ways. We have also seen examples of different behavior in sorting and biogenesis: in S2 cells, bantam and let-7 partition differently to Ago-1 and Ago-2; Loqs is required for the production of some miRNAs but dispensable for the production of others (Liu et al., 2007).

What causes these differences? Other than the different cellular context, do the structural features of each miRNA cause intrinsic preferences for certain behaviors over others? Our results showed that under the same conditions in embryo lysate, miRNA duplexes with different structures do behave differently. The embryo is not their natural environment. Therefore, we do not know how these miRNAs behave under physiological conditions when other cellular components come into play. However, these kinds of *in vitro* studies that are done under well controlled conditions can offer insight to the intrinsic trends caused by distinct structures, though discerning such trends is not an easy task. Large-scale analysis is certainly necessary for this purpose. The two series of miRNA hybrid duplexes we examined, let-7 and bantam, showed that the 5'-half is important for determining the duplex's interaction with Dcr-2. The let-7 derivatives tested by Forstemann et al. showed a central mismatch is important for small RNAs loading to Ago-1. Yet these rules do not seem to apply to all the miRNAs that we tested. Large number of RNAs needs to be tested before general trends can become obvious. And it is impossible to accurately analyze the natural structural features without bioinformatics study.

Why are there mismatches, bulges and wobbles in the duplex region of all miRNAs? Are they under evolutionary pressure to maintain these features? Is their purpose only to distinguish themselves from other small RNAs, or it is to adapt to specific environment and functions? These are interesting questions that require further studies.

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Appendix

Co-authored papers

1. 'Distinct roles for Drosophila Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways.' Lee, Y.S., Nakahara, K., Pham, J.W., Kim, K., He, Z., Sontheimer, E.J., and Carthew, R.W. (2004). Cell *117*, 69-81. I contributed to figure 5D of this paper, which shows a synthetic siRNA cannot direct cleavage of a radiolabeled mRNA complementary to the siRNA in *dcr-2* mutant embryo extract.

2. 'Short interfering RNA strand selection is independent of dsRNA processing polarity during RNAi in Drosophila.' Preall, J.B., He, Z., Gorra, J.M., and Sontheimer, E.J. (2006). Curr Biol *16*, 530-535. I contributed to figure 4D of this paper, which shows that synthetic siRNAs are more potent than Dicer-substrate dsRNAs in Drosophila eggs. I performed embryo injection in this experiment.

3. "'siRNAs and miRNAs": A meeting report on RNA silencing.'He, Z. and Sontheimer, E.J. (2004). RNA *10*, 1165-1173.

4. 'Structural features of miRNA/miRNA* duplexes that specify Dicer-2-independent RISC assembly in *Drosophila*.' **He, Z.**, Behlke, M., Carthew, R.W., and Sontheimer, E.J. In preparation.

let-7 and mir-125 expression in vivo

The expression pattern and biological function of most miRNAs are unknown. In 2003, Brennecke et al. determined the expression pattern of endogenous bantam in flies by using an EGFP reporter transgene (bantam sensor) containing two sites perfectly complementary to bantam (see Figure 3-8).

To test the expression pattern of let-7 and mir-125, we made similar sensor reporters that contain two perfectly complementary sites to either let-7 or mir-125, and transformed them into flies. Flies transformed with an EGFP reporter that lacks any miRNA binding site were used as controls. All these reporters were expressed under the control of tubulin promoter, which acts as a uniform expression promoter. Different tissues – embryo at all stages, thorax muscle, ovaries and brains – from the transgenic flies were examined in order to see where the reporter expression was repressed. We were not able to find obvious repression of the EGFP signal in mir-125 sensor flies. The let-7 sensor seemed to be repressed in the brain (Figure A-1). It will be interesting to find out let-7's targets and its functions in the brain.

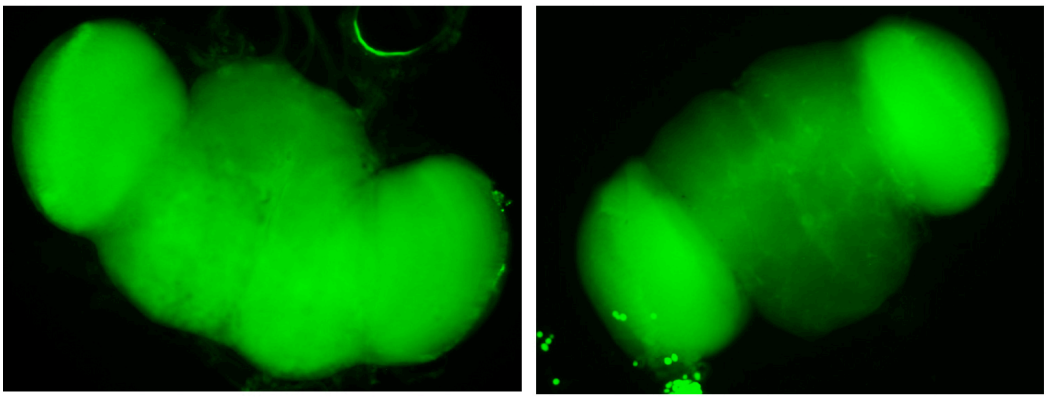

Figure A-1. let-7 sensor reporter is repressed in the brain. On the left: control sensor transgene without let-7 target sequences. On the right: let-7 sensor transgene with two sites perfectly complementary binding to let-7.

The role of Drosophila Gemin-3 in miRNA pathway

Human Gemin3 is a DEAD-box RNA helicase that binds to the Survival of Motor Neurons (SMN) protein and is a component of the SMN complex, which also comprises SMN, Gemin2, Gemin4, Gemin5, and Gemin6. Reduction in SMN protein results in Spinal muscular atrophy (SMA), a common neurodegenerative disease. The SMN complex has critical functions in the assembly/restructuring of diverse ribonucleoprotein (RNP) complexes. In 2002, Dreyfuss and colleagues reported that Gemin3 and Gemin4 are also components of a ~15S miRNP that also contains Ago-2. In particular, they performed immunoprecipitation with antibodies against Gemin3 from HeLa cell lysates, and the majority of the RNAs in the immunoprecipitates were ~22nt, which were identified as miRNAs (Mourelatos et al., 2002).

During their studies of Drosophila Gemin3's function in fly motor neurons, Dr. A. Gregory Matera and colleagues generated Gemin3 mutant flies, and they also made a rescue line with a Flag-tagged Gemin3 transgene in the mutant background. We wondered whether fly Gemin3 also interacts with miRNAs, as does its human homolog. Therefore, we performed immunoprecipitation with Flag antibody from head lysates made from the rescue transgene line and analyzed the RNAs in the immunoprecipitates. When the RNAs were 3' labeled with [5'-32P]-pCp and run on 15% denaturing polyacrylamide gels, instead of a ~22nt band that Dreyfuss and colleagues saw in their experiment that was done in the same way (Mourelatos et al., 2002), we saw a big smear in both the control Ago-1 IP and the Flag IP, suggesting this experiment might not have been done properly in my hands. When the Gemin3 associated RNAs were

probed with several miRNA probes in a Northern experiment, we did not see enrichment of any miRNAs although these miRNAs were clearly enriched in the control Ago-1 IP (Figure A-2).

However, we cannot conclude whether fly Gemin3 is involved in miRNA pathway simply based on these experiments. For example, Gemin3 might associate with miRNAs that we have not tested, or it might interact with miRNAs in tissues other than the head.

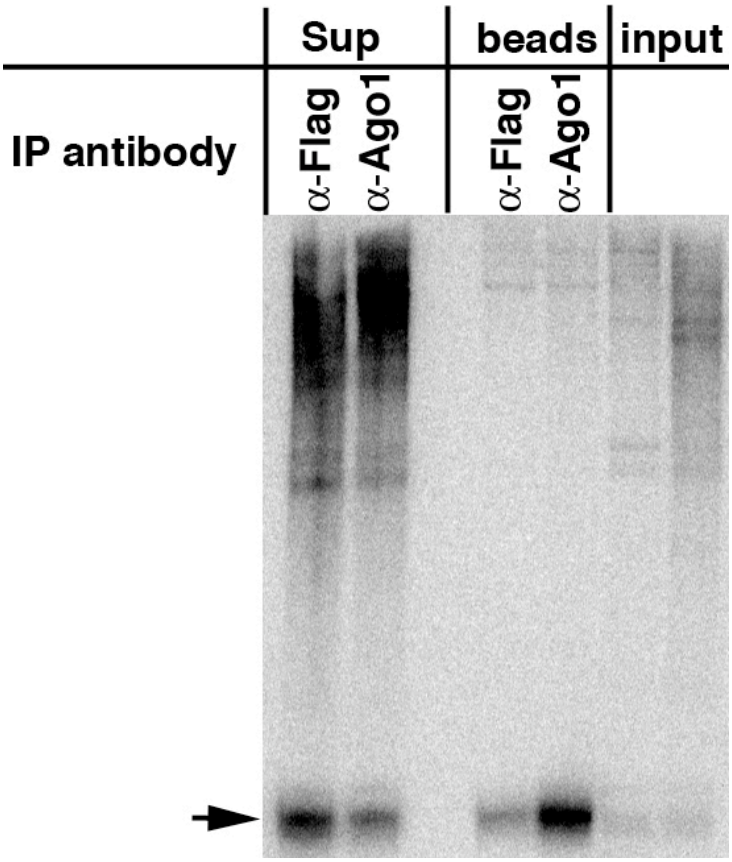

Figure A-2: Northern blot detecting miRNAs in head lysates made from Gemin3 transgene flies. The blot was hybridized with LAN probes against mir-8, mir-34 and mir-277. The arrow indicates the miRNAs.