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Precursor microRNA Programmed Silencing Complex Assembly Pathways in Mammals

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Summary

Assembly of microRNA Ribonucleoproteins (miRNPs) or RNA-Induced Silencing Complexes (RISCs) is essential for the function of miRNAs and initiates from processing of precursor miRNAs (pre-miRNAs) by Dicer or by Ago2. Here, we report an *in-vitro* miRNP/RISC assembly assay programmed by pre-miRNAs from mammalian cell lysates. Combining *in-vivo* studies in *Dicer Knock-Out* cells reconstituted with wild type or catalytically inactive Dicer, we find that the miRNA Loading Complex (miRLC) is the primary machinery linking pre-miRNA processing to miRNA loading. We show that a miRNA Precursor Deposit Complex (miPDC) plays a crucial role in Dicer-independent miRNA biogenesis and promotes miRNP assembly of certain Dicer-dependent miRNAs. Furthermore, we find that 5'-uridine, 3'-mid base pairing and 5'-mid mismatches within pre-miRNAs promote their assembly into miPDC. Our studies provide a comprehensive view of miRNP/RISC assembly pathways in mammals and our assay provides a versatile platform for further mechanistic dissection of such pathways in mammals.

Introduction

miRNAs are a class of ~22 nucleotide (nt) RNAs that play important roles in almost every biological process through post-transcriptional gene regulation. Proper miRNA functioning requires its assembly into an RNA-induced Silencing Complex (RISC) or microRNP (miRNP), where the miRNA serves as the specificity guide for target RNA recognition (Hammond et al., 2000; Hutvagner and Zamore, 2002; Martinez et al., 2002; Mourelatos et al., 2002; Nykanen et al., 2001). At the core of every miRNP/RISC is an Argonaute (Ago) protein, which directly binds to a single-stranded miRNA and upon target RNA recognition, orchestrates translational repression and degradation of the targeted mRNA (Djuranovic et

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al., 2011; Hutvagner et al., 2001; Martinez et al., 2002; Mourelatos et al., 2002; Siomi and Siomi, 2009). Most miRNAs are excised from the stems of stem-loop precursors (pre-miRNAs) by Dicer to generate transient, double-stranded miRNA duplexes (Grishok et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001; Knight and Bass, 2001; Zhang et al., 2004). Loading of miRNA duplexes to Ago proteins is assisted by Hsp70 and Hsp90 chaperones (Iwasaki et al., 2010; Maniataki and Mourelatos, 2005; Yoda et al., 2010), and is followed by unwinding of the duplex and retention of the single stranded miRNA by Ago. The miRNA duplex is structurally asymmetric and the strand with a thermodynamically less stable 5' end becomes the miRNA (guide strand) that is retained by Ago, while the other strand (passenger) is degraded (Khvorova et al., 2003; Schwarz et al., 2003). Recent studies have found that unwinding is facilitated by mismatches present in the seed (guide positions 2-8) or 3'-mid (guide positions 12-16) of miRNA duplexes (Kawamata et al., 2009; Matranga et al., 2005; Yoda et al., 2010).

Of the four mammalian Ago proteins, Ago2 is the only one with robust endonucleolytic activity towards RNA targets with extensive complementarity to the Ago2-bound miRNAs (Liu et al., 2004; Meister et al., 2004; Rivas et al., 2005). The nucleolytic activity of Ago2 is critical for cleavage of the passenger strand of small interfering RNA (siRNA) duplexes and assembly of the guide strand into RISC; it is also essential for the maturation of miR-451, a Dicer-independent miRNA, through cleavage of the 3' arm of pre-miR-451 (Cheloufi et al., 2010; Cifuentes et al., 2010; Leuschner et al., 2006; Matranga et al., 2005; Miyoshi et al., 2005; Rand et al., 2005; Yang et al., 2010). However, in terms of miRNA-directed mRNA translational repression and mRNA decay, all four Ago proteins function in a similar manner (Pillai et al., 2004; Wu et al., 2008). Not surprisingly, mammalian Ago proteins share a similar repertoire of miRNAs (Azuma-Mukai et al., 2008), which are enriched in 5'-U (Hu et al., 2009; Seitz et al., 2011), and have similar structural preference (i.e. mismatches at guide positions 9-11) for binding to miRNA duplexes (Yoda et al., 2010). The structural basis of the 5'-U preference was revealed in the crystallographic structure of human Ago2 in complex with the 5' nucleotide mimics, where a nucleotide specificity loop, conserved among all mammalian Ago proteins, was identified in the MID domain (Frank et al., 2010).

We and other groups have previously identified a miRNA loading complex (miRLC), which displays both pre-miRNA processing and miRNP/RISC cleavage activity when it is exposed sequentially to a miRNA precursor and its target (Maniataki and Mourelatos, 2005; Gregory et al., 2005; MacRae et al., 2008). This result, together with the finding that the miRLC is composed of Dicer, miRNA-free Ago, Hsp90 and TRBP, led to the conclusion that miRLC is the miRNA loading complex in mammals (Gregory et al., 2005; Maniataki and Mourelatos, 2005). Structural studies lend further support for the formation of an Ago-Dicer-TRBP miRLC tripartite complex (Wang et al., 2009). On the contrary, a recent study showed that mammalian RISC can be assembled with a miRNA duplex using cell lysates *in vitro*, raising the question of whether the miRLC previously identified represented the main or a bypass mechanism of miRNP/RISC assembly (Yoda et al., 2010).

RISC assembly assays from cell lysates have provided illuminating insights into the mechanisms of siRNA and miRNA biogenesis in *Drosophila melanogaster* and mammals (Kawamata et al., 2009; Miyoshi et al., 2009; Pham et al., 2004; Tomari et al., 2004a; Yoda et al., 2010). The majority of these studies have employed the Dicer processing product, siRNA or miRNA duplexes, as the substrate for RISC assembly. In *Drosophila*, free miRNA duplexes are released from the Dicer-1 and Loquacious complex before they are sorted by Dicer-2 and R2D2 into distinct Agos (Kawamata and Tomari, 2010). However, because mammalian Dicer binds directly to Agos (Tahbaz et al., 2004) through an Ago-binding site that is conserved only within vertebrate Dicers (Wakiyama et al., 2007), it remains a subject of debate whether there are free miRNA duplexes in mammals under physiological

conditions. More importantly, *in vivo*, all miRNA duplexes are born from pre-miRNAs, yet so far reconstitution of miRNP assembly in mammalian lysates with pre-miRNAs, the authentic miRNA precursors, has not been achieved.

Recently, the Haber lab found that a subset of pre-miRNAs can be cleaved by Ago2 (Diederichs and Haber, 2007). The Kiriakidou lab showed that pre-miRNAs remain associated with Ago proteins, even in the absence of Dicer, *in vivo*, implying that Ago2 may play additional roles in steps upstream of miRNPs (Tan et al., 2009). Yet within which context Ago2 binds to these pre-miRNAs remains unknown. More recently, it was found that the precursor of miR-451 (pre-miR-451), which has an unusually short stem with and extensive complementarity, is not processed by Dicer, but rather first cleaved by Ago2 and then trimmed into the mature miR-451. The mechanistic details of the Dicer-independent miRNA biogenesis pathway remain unclear. As these questions are related to pre-miRNAs rather than miRNA duplexes, establishment of an *in-vitro* assembly assay from mammalian lysates with pre-miRNAs will be needed to address these questions.

Here, we report a miRNP/RISC assembly assay using mammalian lysates programmed by pre-miRNAs instead of miRNA duplexes, which allows us to define the molecular steps of miRNP assembly of both Dicer-dependent and -independent miRNAs. Parallel studies in the *Dicer Knock-Out* cells and those reconstituted with either wild type or catalytically inactive Dicer, confirm the operation of the reported pathways *in vivo*. We reaffirm that the miRLC is the primary machinery responsible for processing of Dicer-dependent miRNA precursors and for loading miRNAs to Ago. In addition, we show that a miRNA Precursor Deposit Complex (miPDC), whose assembly is promoted by 5'-uridine, 3'-mid base pairing and 5'-mid mismatches, enhances the expression of certain Dicer-dependent miRNAs and plays a crucial role in the maturation of the Dicer-independent miR-451.

Results

An miRNP/RISC Assembly Assay Programmed by pre-miRNAs from Mammalian Cell Lysates

To further dissect the miRNA biogenesis pathways in mammals, we decided to establish a pre-miRNA programmed miRNP/RISC assembly assay. To distinguish RNA-protein complexes that are related to the miRNP assembly from those that are unrelated, we assembled miRNP with the precursor of a Dicer-dependent miRNA in lysates from inducible *Dicer Knock-Out* (*Dicer^{fl/fl}*) or *-heterozygous* (*Dicer^{+fl}*) mouse embryonic fibroblasts (MEFs) after deletion of the floxed allele(s) with Hydroxytamoxifen (4-OHT) (Figure 1A) (Harfe et al., 2005; Tan et al., 2009).

We first incubated 5' radiolabeled pre-miR-28 (Figure 1B) with equal mixture of cytoplasmic S100 lysates from *Dicer^{+/-}* and *Dicer^{-/-}* MEFs at 37 °C for 60 min and resolved the assembled complexes by native agarose gel electrophoresis (NAGE). One prominent complex, which we term complex D was detected (Figure 1C, reaction 1). We then sectioned the gel into five equal slices from the top and eluted RNAs from each slice (Figure 1C). Native polyacrylamide gel electrophoresis (PAGE) of the eluted RNAs showed that slice IV (complex D) contained predominantly pre-miR-28, with some miR-28 duplex but little single-stranded miR-28 (Figure 1D, reaction 1). This implies that pre-miR-28 is processed by Dicer within complex D. Interestingly, slices III and II above complex D contained traces of pre-miR-28, some miR-28 duplex and little single-stranded miR-28, with slice II containing more single-stranded miR-28 and less miR-28 duplex than slice III (Figure 1D, reaction 1). This finding is reminiscent of the structural change of an siRNA duplex during the RLC to RISC transition in the RISC assembly assay in *Drosophila* (Tomari et al, 2004b). Thus, we reasoned that a similar transition also occurred in our assay

and hypothesized that complex D contains miRLC while the region above complex D contains miRNP, even though its assembly was so inefficient that it was only faintly detected.

Further attempts to enhance the miRNP assembly efficiency led us to find that we could supplement ectopic Ago2 to stimulate miRNP assembly by replacing the *Dicer*^{-/-} lysate with the lysate from a *Dicer*^{-/-} MEF line that we engineered to overexpress myc-Ago2 from a stably transduced lentivirus (*Ago2+*) (Figure 1A). Now, another complex (E), whose assembly strictly depended on Dicer (Figure 1C, D reaction 3), was clearly detected in slices II and III (Figure 1C, reaction 2). Complex E contained single-stranded miR-28 and miR-28 duplex with very little pre-miR-28 (Figure 1D, reaction 2), suggesting that complex E is the miRNP. As every miRNP/RISC is composed of an Ago protein with a miRNA, stimulation of the miRNP assembly by supplementation of Ago2 also suggests that Ago2 is the core constituent of complex E.

A signature function of miRNP/RISC is its ability to directly bind to cognate RNA targets, which leads to either mRNA cleavage or translational repression and mRNA degradation (Djuranovic et al., 2011; Siomi and Siomi, 2009). As shown in Figure 1F, miR-28 directs the miRNP in complex E (Figure 1A) to bind specifically to a “cold” RNA target with miRNA recognition elements (MREs) for miR-28 but not miR-16. In addition, miR-28 can guide Ago2 to cleave a cognate target when a short perfectly complementary target is added to the immunoprecipitated miRNP after pre-incubation of the assembly reaction with 5′ phosphorylated, “cold” pre-miR-28 (Figure 1B). Taken together, these findings indicate that complex E is indeed a functional miRNP/RISC.

We next tested whether our assay was capable of assembling miRNP/RISC from pre-miR-27a, whose miRNA originates from the 3′-arm instead of the 5′-arm (Figure 2A). When 5′ (5′L) or 3′ (3′L) radiolabeled pre-miR-27a was used to program assembly reactions, only miR-27a emanating from the 3′ arm assembled into miRNP (Figure 2B, C and Figure 2A, B). This confirms that our assay is capable of recapitulating the asymmetric incorporation of miRNAs in miRNP/RISC (Figure 2B).

Kinetic analysis of RNA-protein complex formation with pre-miR-27a shows that the amount of complex E is in reverse proportion to that of complex D, suggesting that complex D is the precursor to complex E (Figure 2D). Furthermore, complex D can be “chased” into complex E (Figure 2E). Thus, complex D is the precursor to miRNP (complex E). Complex D is composed of Dicer, Ago and TRBP as antibodies against Dicer, Ago and TRBP, but not KSRP, an RNA binding protein not involved in miR-28 biogenesis (Trabucchi et al., 2009), could supershift complex D (Figure 1E and Figure 2B). Thus, complex D is the miRLC complex identified previously (Gregory et al., 2005; MacRae et al., 2008; Maniataki and Mourelatos, 2005).

miRLC is the Primary miRNA Loading Machinery

miRLC contains not only pre-miRNAs and miRNA duplexes but also single-stranded miRNAs (Figure 1D), indicating that both pre-miRNA processing and miRNA loading occur within miRLC. miRNA duplexes are loaded into Agos and their unwinding by Agos is facilitated by mismatches and G/U wobble base pairs present in the seed and the 3′ mid of miRNAs (Kawamata et al., 2009; Yoda et al., 2010). If miRNA loading occurs within miRLC, the miRNA duplex should remain in the miRLC when its unwinding is blocked. To test this hypothesis, we programmed assembly reactions with a defective unwinding mutant of pre-miR-28 (pre-miR-28-dum) in the *Dicer*^{fl/fl}; *myc-Ago2* lysate (Figure 1C, D). pre-miR-28-dum was processed by Dicer into the miRNA duplex within the miRLC, but miRNP did not assemble as evidenced by the absence of single-stranded miR-28 (Figure 1E).

Instead, a complex that contained pre-miR-28-dum and a faster migrating RNA whose mobility corresponded to Ago2-cleaved pre-miR-28-dum (ac-pre-miR-28-dum), appeared at the same position as miRNP (complex E) (SFigure 1D). As ac-pre-miR-28-dum disappeared upon incubation of pre-miR-28-dum in the *Dicer^{fl/fl}* lysate that overexpresses the catalytically-inactive myc-Ago2 (SFigure 1D, E), this suggested that Ago2 bound to and cleaved pre-miR-28-dum within complex E. Thus, the complex E formed by pre-miR-28-dum does not correspond to miRNP but to an Ago2-pre-miRNA containing complex and will be discussed further below. Notably, the miRNA duplex processed from pre-miR-28-dum resides mainly in the miRLC, confirming that miRNA loading primarily occurs within the miRLC (SFigure 1D, E).

miRLC is the Primary Machinery of Pre-miRNA Processing

The presence of pre-miRNAs, miRNA duplexes and single-stranded miRNAs in the miRLC strongly indicates that pre-miRNA processing and miRNA loading initiate in and are streamlined by the miRLC. The assembly of complex D strictly depends on Dicer (Figure 1C and SFigure 2A), indicating that Dicer is a core component of complex D. In that case, one would expect that the miRLC would accumulate when the catalytic activity of Dicer is inactivated as catalytically-inactive Dicer would trap the protein and RNA (pre-miRNA) components of miRLC. To test this hypothesis, we generated inducible *Dicer Knock-Out* MEFs that express HA-tagged *wildtype* (WT) or catalytically inactive mutant (*D2A*) *Dicer* transgene in a Doxycycline (Dox) responsive manner (SFigure 3A). We confirmed that only the introduction of the WT, but not the *D2A* mutant, could rescue the miRNA biogenesis defect in the *Dicer^{-/-}* MEFs (SFigure 3). Consistent with our hypothesis, we observed a dramatic accumulation of miRLC in assembly reactions with the *D2A* lysate (Figure 3A), indicating that miRLC is the primary machinery of pre-miRNA processing *in vitro*.

Pre-miRNA is also processed by miRLC *in vivo*. As shown in Figure 3B, C, the amount of Dicer that associates with Agos and TRBP is increased in the *D2A* MEFs. In addition, increased amounts of pre-miRNAs were immunoprecipitated with Dicer from the *D2A* MEFs (Figure 3D), suggesting that pre-miRNAs are also processed *in vivo* by the miRLC. Collectively, all the results presented above indicate that miRLC is the primary machinery of pre-miRNA processing.

miRNP assembly with the precursor of a Dicer-independent miRNA via an Ago-containing, miRNA Precursor Deposit Complex (miPDC)

The biogenesis of miR-451 is independent of Dicer but is dependent upon the endonuclease activity of Ago2 (Cheloufi et al., 2010; Cifuentes et al., 2010; Yang et al., 2010). Though the primary transcript of miR-451 (pri-miR-451) is not expressed in MEFs, the biogenesis of miR-451 can be recapitulated when pri-miR-451 is ectopically expressed in MEFs (Yang et al., 2010). To test whether our assembly assay is capable of assembling miRNP with a Dicer-independent miRNA precursor, we incubated 5' radiolabeled pre-miR-451 (Figure 4A) with equal mixture of *Dicer^{+/-}* lysates with lysates from *Dicer^{-/-}* MEFs that were overexpressing *Ago2* (*Ago2⁺*). Four complexes assembled (Figure 4B), yet only complex E contained miR-451 (Figure 4C), suggesting complex E contains miRNP. In addition, complex E contained Ago2-cleaved pre-miR-451 (ac-pre-miR-451) and pre-miR-451 (Figure 4C), indicating that pre-miR-451 is directly bound and cleaved by Ago2 within complex E. The assembly of complex E is stimulated by supplementation of Ago2 but is independent of Dicer (Figure 4B and SFigure 4A), consistent with the independent role of Dicer in the maturation of miR-451 *in vivo* (Cheloufi et al., 2010; Cifuentes et al., 2010; Yang et al., 2010). However, "pulsing" of complex E failed to "chase" it into any other complex (SFigure 4B) and kinetic analysis of the assembled RNA-protein complexes with pre-miR-451 also failed to identify any precursor complex to complex E (SFigure 4C),

implying that the mature miR-451 resides in the miRNP while pre-miR-451 and ac-pre-miR-451 are probably retained in another complex that we name “miRNA Precursor Deposit Complex” (miPDC).

To test this hypothesis, assembly reactions were set up in lysates from *Dicer*^{-/-} MEFs overexpressing a catalytically inactive *Ago2* mutant (*D669A+*) instead of the *wild-type Ago2*. Interestingly, assembly in the *D669A+* lysate retarded the migration of complex E (Figure 4D). This is likely the result of blocking the pre-miR-451 cleavage, as a similar retardation is also observed when a pre-miR-451 mutant that is resistant to *Ago2* cleavage (m10) is assembled in the *Ago2+* lysate (SFigure 4D). Next, we asked which portion of complex E contained miPDC and which contained miRNP by sectioning the gel region corresponding to complex E into three slices (Figure 4D). Slice I, enriched in the reaction with the *D669A+* lysate (Figure 4D), contains predominantly pre-miR-451, (Figure 4E) and thus corresponds to miPDC. Slice II, common to both reactions assembled with the *Ago2+* and the *D669A+* lysate (Figure 4D), contains a mixture of pre-miR-451, ac-pre-miR-451 and mature miR-451 (Figure 4E) and thus is composed of both miPDC and miRNP. Slice III, enriched in the reaction with the *Ago2+* lysate (Figure 4D), contains primarily mature miR-451 and ac-pre-miR-451, (Figure 4E) and thus consists of miRNP. Taken together, we conclude that complex E is composed of both miPDC and miRNP.

The Hsp70 inhibitor 2-phenylethanesulfonamide (PES) inhibited the assembly of complex E (SFigure 4E), suggesting that pre-miR-451 is loaded into *Ago2* by a similar PES-sensitive machinery that loads the miRNA duplex into *Ago* proteins (Iki et al., 2010; Iwasaki et al., 2010; Miyoshi et al., 2010). Thus the loading of pre-miR-451 into *Ago2* is crucial for miPDC and miRNP assembly.

Assembly of certain *Dicer*-dependent pre-miRNAs into miRLC and miPDC

The assembly of miPDC with pre-miR-451 and with the defective unwinding mutant of pre-miR-28 pre-miR-28-dum (SFigure 1C, D, E) prompted us to test other pre-miRNAs for their ability to assemble miPDC. One such pre-miRNA is pre-miR-16 (Figure 5A). Similar to pre-miR-28 and pre-miR-27a, the miRLC for pre-miR-16 resides in complex D (Figure 5B, C, D, E) and the assembly of miRNP from pre-miR-16 is strongly stimulated by supplementation of *Ago2* (Figure 5B, C). However, in assembly reactions with pre-miR-16, complex E contains in addition to miRNPs, significant amounts of pre-miR-16 (Figure 5B, C, reaction 1), in contrast to the E complexes formed with pre-miR-28 and pre-miR-27a, which are composed primarily of miRNPs. In addition, complex E containing pre-miR-16 still formed in the absence of *Dicer* (Figure 5B, C, reaction 3). The assembly of pre-miR16 into complex E is not an artifact due to the incorporation of “unstructured” single-stranded pre-miR-16 into *Ago2*, as a pre-miR-16 mutant with a 2-nt 5′ overhang instead of the native 3′ overhang, does not assemble complex E (SFigure 5A). Single-stranded miR-16 also resides in complex E (Figure 5B, C, reaction 1), suggesting that similar to pre-miR-451, the complex E formed in assembly reactions with pre-miR-16 is also composed of two co-migrating complexes-- miRNP with single-stranded miR-16 and another complex with pre-miR-16. Three lines of evidence support that the complex that co-migrates with miRNP is miPDC. First, this complex is supershifted by an anti-Myc antibody, suggesting that pre-miR-16 is bound by myc-*Ago2* (SFigure 5E). In addition, an *Ago2*-cleavable pre-miR-16 mutant (cm), which has complementary mutations introduced into the 3′-mid region surrounding the *Ago2*-cleavage site (SFigure 5B), is cleaved by *Ago2* (SFigure 5D), indicating that pre-miR-16 is bound to *Ago2* by anchoring its 5′ phosphate in the MID domain of *Ago2* and uses the 5′ arm as a guide to cleave the 3′ arm. Interestingly, assembly of miPDCs with pre-miR-28-dum and pre-miR-16-cm in the *D669A+* lysate does not cause retardation of complex E (SFigure 1D and SFigure 5C), which is unlike the retardation seen when pre-miR-451's miPDC is formed in the *D669A+* lysate. This suggests that the

retardation observed by blockage of Ago2 cleavage is specific to pre-miR-451, which is probably resulting from the unusually short length of pre-miR-451 and the extensive complementarity of its stem. Finally, the assembly of pre-miR-16 into complex E is also strongly inhibited by the Hsp70 inhibitor PES (SFigure 5F, G). Collectively, these results suggest that complex E formed in pre-miR-16 programmed assembly reactions is composed of both miRNP and miPDC.

We next tested whether the complexes that we identified in our *in vitro* assembly assays are also found *in vivo*. Ago is the core component of both miRLC and miPDC. In the *Dicer*^{-/-} MEFs, assembly of miRLC is greatly reduced while that of miPDC is not affected (Figure 5B, reaction 3). In contrast, in the *D2A* MEFs, miRNP is reduced but neither miRLC nor miPDC assembly is affected (Figure 5E, SFigure 5H). If a pre-miRNA can remain associated with Agos in the *Dicer*^{-/-} MEFs, where miRLC assembly does not take place, this implies that the pre-miRNA interacts with Ago through miPDC. Indeed, Figure 5F and SFigure 5J show that pre-miR-16 and pre-miR-21, which assemble both miRLC and miPDC (Figure 5B and SFigure 5I), co-immunoprecipitate with Agos in both the *Dicer*^{-/-} and the *D2A* MEFs. In contrast, pre-miR-28 and pre-miR-27a, which assemble only miRLC, co-immunoprecipitate with Agos only in the *D2A* but not the *Dicer*^{-/-} MEFs (Figure 5F and SFigure 5J). Notably, the interaction of pre-miR-16 and pre-miR-21 with Agos is observed not only when miRNA biogenesis is blocked in the *Dicer*^{-/-} and the *D2A* MEFs, but also in the *WT* MEFs (Figure 5F and SFigure 5J), arguing that their interaction with Ago is not a simple result of the availability of more pre-miRNAs in the *Dicer*^{-/-} and the *D2A* MEFs but is physiologically relevant. Taken together, these results suggest that some pre-miRNAs (e.g. pre-miR-16 and pre-miR-21) assemble both miRLC and miPDC whereas others (e.g. pre-miR-28 and pre-miR27a) assemble only miRLC *in vivo*.

Processing of pre-miR-16 by Dicer occurs in the miRLC as photocrosslinking experiment with pre-miR-16 containing a single 4-thiouridine adjacent to Dicer's cleavage site (pre-miR-16-s21U) showed that pre-miR-16 was crosslinked to Dicer only in the miRLC but not in the miPDC (SFigure 5K, L, M). A previous study found that certain Dicer-dependent miRNA precursors, such as the pre-let-7 family members, were cleaved by Ago2 before Dicer-processing to facilitate removal of the passenger strand during unwinding (Diederichs and Haber, 2007). Our results suggest that Dicer-dependent miRNA precursors are cleaved by Ago2 primarily in the miPDC before they are processed by Dicer in the miRLC (SFigure 5D). Indeed, a small amount of ac-pre-miR-16-cm, which was produced by Ago2 cleavage within the miPDC, was also recovered in the miRLC (SFigure 5D), suggesting that miPDC may be a precursor to miRLC. In that scenario, one would expect accumulation of miPDC when the assembly of miRLC and miRNP is blocked in the *Dicer*^{-/-} lysate. As shown in Figure 5B, reaction 3 and SFigure 5I, that is exactly what occurs. On the contrary, miPDC does not accumulate as miRLC accumulated in the *D2A* lysate (Figure 5E and SFigure 5H) suggesting that the accumulation of miPDC in the *Dicer*^{-/-} lysate is not simply due to availability of more "free" pre-miRNAs in the *Dicer*^{-/-} lysate. Hence, Ago stimulates miRNP assembly not only by serving as the acceptor of miRNAs but also by promoting the assembly of certain pre-miRNAs into the miPDC.

5' Nucleotide Identity and Structural Features Important for miPDC Assembly

We have demonstrated that pre-miRNAs containing extensive base pairing around the 10th nucleotide from the 5' end are cleaved by Ago2 within the miPDC in a similar way that miRNAs cleave their targets. This indicates that the 5' ends of such pre-miRNAs are anchored in the MID domain of Ago2, which was shown to mediate the interaction of Ago2 with the 5' phosphate of RNA guides (Ma et al., 2005; Parker et al., 2005). A recent structural study identified a nucleotide specificity loop in human Ago2 that plays a critical role in determining the nucleotide binding preference (U > A >> G ≈ C) at the 5' end of

miRNAs (Frank et al., 2010). We notice that both pre-miR-27a and pre-miR-28, which do not assemble miPDC (Figure 1B, C and Figure 2A, B), begin with an A. In contrast, pre-miR-16 and pre-miR-21, which assemble miPDC (Figure 5B and SFigure 5I), begin with a U. However, the first nucleotide is not the only determinant for miPDC assembly because pre-miR-451, which assembles miPDC, begins with an A. In addition, the defective unwinding mutant of pre-miR-28 (pre-miR-28-dum), which has two complementary mutations introduced into both the seed and the 3'-mid, can also assemble miPDC although it begins with an A (SFigure 1C, D, E), indicating that the secondary structure of a pre-miRNA also plays a role in the miPDC assembly.

To identify the 5' nucleotide and structural features of pre-miR-16 important for miPDC assembly, we first changed its first nucleotide into an A (U1A) or a G (U1G). Mutation to a C was not attempted as such a mutation would allow the first nucleotide to base pair with the 3' penultimate nucleotide, which could affect guide strand (mature miRNA) selection. However, changing the first nucleotide of pre-miR-451 to a C (A1C) led to marked inhibition of miPDC assembly and as a result miRNP formation, verifying that miPDC assembly is critical for generation of miR-451 miRNP (Figure 6A, B). Mutation of the first nucleotide of pre-miR-16 to an A or a G resulted in 2- and 4-fold reduction in the efficiency of miPDC assembly, respectively (Figure 6D, E), indicating that the identity of the first nucleotide of pre-miR-16 plays a major role in guiding miPDC assembly. In addition, the amount of miR-16 was reduced when the first nucleotide of pre-miR-16 was mutated to a G (Figure 6F). Next, we introduced point mutations that alter base pairing properties of nucleotide 4 (m4) or 13 (c13) of pre-miR-16 (Figure 6C). In addition, we introduced these secondary structure mutations into the 5' nucleotide mutants (U1A or U1G) to assess their relative contribution to the miPDC assembly. As shown in Figure 6D, E, F and SFigure 6A, mismatch in the 4th position and base pairing in the 13th position promoted both miPDC and miRNP assembly. However, structural mutants with 5'-G and 5'-A did not assemble more miPDC than their 5'-A and 5'-U counterparts with the native structure respectively (Figure 6D, E), suggesting that the secondary structure plays an accessory role in the miPDC assembly. When a pre-miR-28 point mutant with base pairing at the 13th nucleotide (pre-miR-28-c13) assembled more miPDC and miRNP than its native counterpart (SFigure 6B, C, D).

Next, we introduced single mismatch mutations, which disrupt base pairing of the seed (m2-m8) or 3' mid (m12-m16) region of miR-451 without changing the sequence of mature miR-451, into the 3' arm of pre-miR-451 (SFigure 6E). Consistent with the findings with pre-miR-16, mismatches at the 3' mid (12th-16th) region attenuated miPDC assembly whereas mismatches at the 5'-mid (5th-7th) positions enhanced miPDC assembly (SFigure 6F, G). The inhibition of miPDC assembly by mismatches at the 2nd and 3rd positions (SFigure 6F, G) are specific to pre-miR-451 probably because of its extremely short stem, as we did not observe inhibition of miPDC assembly when a similar mismatch was introduced into the 3rd position of pre-miR-16 (our unpublished data).

Taken together, the findings presented above indicate that the 5' nucleotide identity and the secondary structure of a pre-miRNA play major roles in miPDC assembly: 5'-U and base pairing at the 3' mid region promotes miPDC assembly while base pairing at the 5' mid region attenuates miPDC assembly.

Discussion

The paucity of *in-vitro* systems that program assembly of miRNP/RISC with pre-miRNAs has been a stumbling block for dissection of mechanistic details of mammalian miRNP/RISC assembly pathways. With supplementation of Ago2 protein, we have established a

miRNP/RISC assembly assay in mammals, which can be programmed with both Dicer-dependent and Dicer-independent miRNA precursors and which faithfully recapitulates many key processes during miRNA biogenesis *in vivo*. Our studies reaffirm that miRLC is the primary machinery that carries both pre-miRNA processing and miRNA loading for Dicer-dependent miRNA precursors in mammals. Hence, contrary to a previous report that used miRNA duplexes instead of pre-miRNAs to study miRNP assembly (Yoda et al., 2010) but in agreement with a recent report (Noland et al., 2011), miRLC is the canonical miRNA loading complex in mammals and plays a key role in pre-miRNA processing, in loading of the Dicer products (miRNA duplexes) to Ago protein and in initiating miRNP formation.

We have found that miPDC, whose core is composed of a pre-miRNA directly binding to Ago2, is assembled with certain miRNA precursors beginning with 5'-U or 5'-A in the context of preferable secondary structure. Our studies confirm and extend the original discovery of Ago2-pre-miRNA complexes *in vivo* (Tan et al., 2009) and place them at the center of mammalian miRNP assembly pathways. Assembly of pre-miR-451 into miPDC is crucial for the maturation of miR-451 as miPDC couples Ago2 cleavage with the trimming of ac-pre-miR-451 to generate mature miR-45. The assembly of Dicer-dependent miRNA precursors into miPDC suggests that the Dicer-independent miR-451 maturation pathway probably evolved by using the pre-existing miPDC in the Dicer-dependent miRNA biogenesis pathway and diverting it into an Dicer-independent pathway.

The assembly of Dicer-dependent miRNA precursors into miPDC promotes their expression by escorting them to the miRLC for processing by Dicer. Notably, preferable features of pre-miRNAs that promote miPDC assembly resemble the characteristics of an authentic pre-miRNA and those of a productive miRNA duplex: a 5'-U and base pairing in the 3'-mid region for efficient miRNA loading (Kawamata et al., 2011; Seitz et al., 2011); and mismatches in the 5'-mid region for efficient unwinding (Kawamata et al., 2009; Yoda et al., 2010). However, although many of the features for miPDC assembly are shared with those for efficient loading or unwinding of Dicer products (miRNA duplexes), they are not identical. Importantly, some features present in natural mouse miRNA duplexes (Seitz et al., 2011), which cannot be explained by having a role in miRNA loading, make good sense when miPDC assembly is taken into account. For example, the Seitz lab has found that although miRNAs in mouse, *D. melanogaster* and *C. elegans* are enriched in 5'-U, regardless of their origin from the 5' or 3' arm of pre-miRNAs, the 5'-arm-derived miRNA*s (passenger strands) in mouse are enriched significantly in 5'-A compared to their 3'-arm-derived counterparts (Seitz et al., 2011). In theory, as the passenger (miRNA*) strand is not loaded into Ago, as long as it does not begin with a 5'-U to compete for guide (miRNA) strand selection, its first nucleotide does not need to be enriched in adenosine. Instead, we speculate that the preferential enrichment of 5'-A in the 5'-arm-derived miRNA*s, reflects their role in promoting miPDC assembly. It is also intriguing to note that the first nucleotide of pre-miR-451, which is strictly conserved in all organisms encoding miR-451, is an adenosine instead of a uridine. This does not seem to reflect a constraint for efficient Drosha processing, as neither A nor U base pairs with the opposite C. In addition, it is outside of the seed and thus should have no impact on mRNA targeting specificity. Instead, it is possible that the conservation of a 5'-A instead of a 5'-U in pre-miR-451 reflects a need for the cell to restrict excessive loading of pre-miR-451 to miPDC, which might interfere with the assembly of other pre-miRNAs into miPDC and lower the availability of Agos for miRLC assembly.

Many studies have revealed that processing of certain pri-miRNAs by Drosha is stimulated in response to various intrinsic and extrinsic stimuli (Krol et al., 2010). Interestingly, a recent study found that there is competition between Dicer mRNA and pre-miRNAs for export by Exportin-5 and expression of excessive pre-miRNAs results in

downregulation of Dicer (Bennasser et al., 2011). Thus, it is tempting to speculate that the miPDC may also serve as a temporary storage site of pre-miRNAs during fluctuation of Dicer's level.

In summary, the pre-miRNA programmed miRNP/RISC assembly pathways revealed by our studies have allowed us to build a coherent model of how such pathways operate in mammals to form miRNPs/RISCs, the functional units for miRNAs (Figure 7). Furthermore, our assay provides a platform for studying and manipulating every step of the miRNP/RISC assembly pathway *in vitro*, which will facilitate elucidation of mechanistic details of miRNP biogenesis and its regulation.

Experimental Procedures

Preparation of Immortalized MEF Cell Lines and Cell Culture

The immortalized *Dicer^{+/-fl}*, *Dicer^{fl/fl}* MEF cell lines and the induction of *loxP* recombination has been previously described (Tan et al., 2009). The expression of *HA-Dicer* or *myc-Ago* transgene was initiated by addition of 0.5-1 $\mu\text{g/ml}$ of Doxycycline (Dox) to the medium. All transfections were performed with Lipofectamine 2000 (Invitrogen).

miRNP/RISC Assembly Assay

40 μg of two cytoplasmic S100 lysates from the indicated genotyped MEFs was mixed at 1:1 ratio and incubated with 50-100 fmole of 5' ³²P-radiolabeled pre-miRNAs with or without miRNA targets in 40 mM KOAc (pH 7.4), 3 mM Mg(OAc)₂, 1 mM DTT, 1 mM ATP, 0.2 mM GTP and 1 U/ μl of recombinant RNasin (Promega) at 37 °C for the indicated time. RNA products were isolated with TRIzol (Invitrogen) and resolved through a 15% denaturing polyacrylamide gel. To detect assembled RNA-protein complexes, native agarose gel electrophoresis was performed.

Native Agarose Gel Electrophoresis

Native agarose gel electrophoresis (NAGE) is modified from the protocol described in (Tomari et al., 2004a). In brief, a native agarose gel made of 1.5% of MetaPhor agarose (Lonza), 0.5% of SeaKem Gold agarose (Lonza) and 0.5 \times TBE with 1.5 mM MgCl₂, was cast into a glass plate sandwich with a Gelbond film (Lonza) attached on one plate. After RISC assembly assay, Ficoll-400 was added to 3% and the RNA-protein complexes were resolved on a native agarose gel at 5 W for 3.5-4 hr at 4 °C.

Identification of RNA Components in RNA-Protein Complexes

To identify the RNA components in the RNA-Protein complexes, native agarose gels were sliced at 2-2.5 cm intervals from the top (excluding the well) to the last complex or gel slices containing individual complexes was recovered. The RNAs were eluted in PK buffer (100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 12.5 mM EDTA, 0.5% SDS), extracted with acid phenol-chloroform and precipitated with ethanol. For denaturing PAGE analysis, the RNA pellets were redissolved in Gel Loading Buffer II (Ambion) and resolved on a 15% denaturing PAGE gel. Native PAGE analysis is adapted from (Tomari et al., 2004a). Specifically, RNA pellets were redissolved in native loading buffer and resolved on a 10% native polyacrylamide (19:1) gel at 4 °C at 7 W, using 0.5 \times TBE with 1 mM MgCl₂ as the running buffer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Development of *in-vitro* miRNP/RISC assembly assay programmed by pre-miRNAs.
- miRLC is the primary machinery that links pre-miRNA processing to miRNA loading.
- Roles of miPDC in miRNA biogenesis.
- 5'-U, 3'-mid base pairs, 5'-mid mismatches in pre-miRNAs promote miPDC assembly.

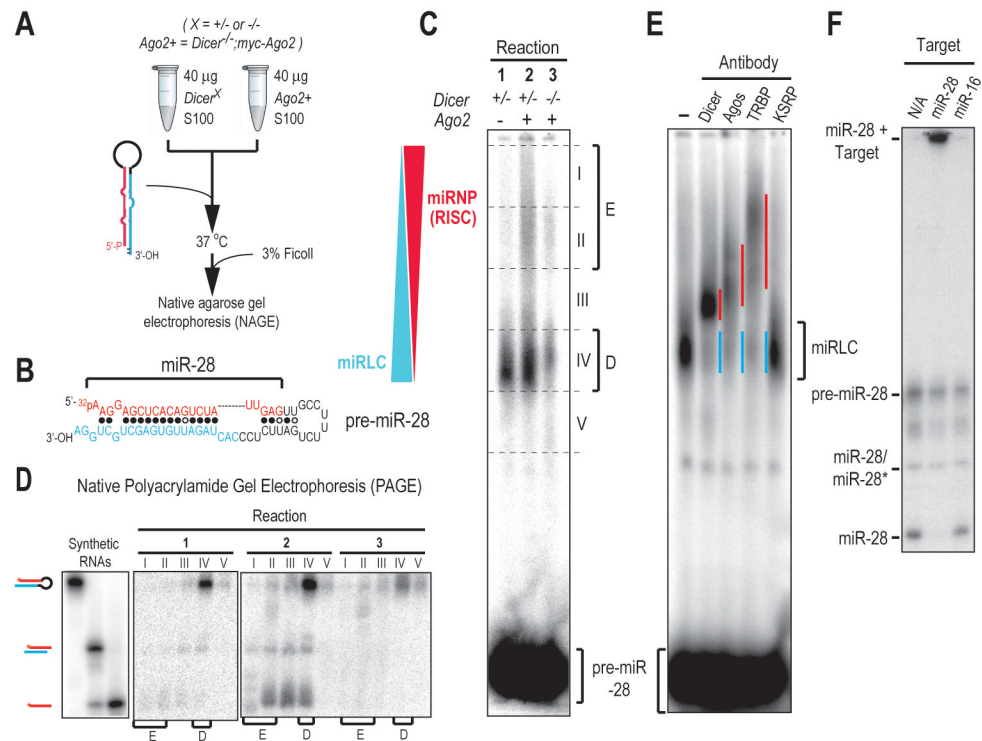


Figure 1. miRNP/RISC Assembly Assay Programmed by pre-miRNAs from Mammalian Cell Lysates

A. Schematic of assay. S100 lysates from MEFs heterozygous (+/-) or homozygous *Dicer* (-/-) knock-out are mixed with S100 lysates from *Dicer*^{-/-} MEFs overexpressing myc-Ago2 (*Ago2*⁺); end-radiolabeled synthetic pre-miRNAs are added and complexes are analyzed by native agarose gel electrophoresis (NAGE).

B. Secondary structure of pre-miR-28. Guide (miRNA) and passenger strand is depicted in red and blue respectively. The same notation applies to all figures.

C. NAGE of assembly reactions with pre-miR-28 in equal mixture of *Dicer*^{+/-} or *Dicer*^{-/-} lysates and lysates from *Dicer*^{-/-} MEFs overexpressing *Ago2* (*Ago2*⁺) or not (*Ago2*⁻). Dotted lines delineate the five sections that were sliced.

D. Native Polyacrylamide Gel Electrophoresis (PAGE) analysis of RNAs isolated from gel slices as delineated in (C).

E. Antibody supershift experiments. Reactions were assembled with pre-miR-28 in equal mixture of *Ago2*⁻ overexpressing *Dicer*^{-/-} MEF lysates with lysates from *Dicer*^{-/-} MEFs reconstituted with an HA-tagged *Dicer* transgene (*WT*). The red line marks the supershifted complex while the blue line denotes the disappeared complex. The same symbol representations apply to all figures.

F. Native PAGE of RNAs isolated from complex E (shown in SFigure 1A) in reactions programmed with 5'-radiolabeled pre-miR-28 without "cold" RNA target (N/A), or with "cold" RNA target with bulged miRNA binding sites of mir-28 or mir-16. Complex E, containing miR-28 miRNP that was assembled from pre-miR-28, recognizes specifically the miR-28 RNA target as evidenced by the shifting of labeled miR-28, bound to its cognate RNA target, towards the top of the gel.

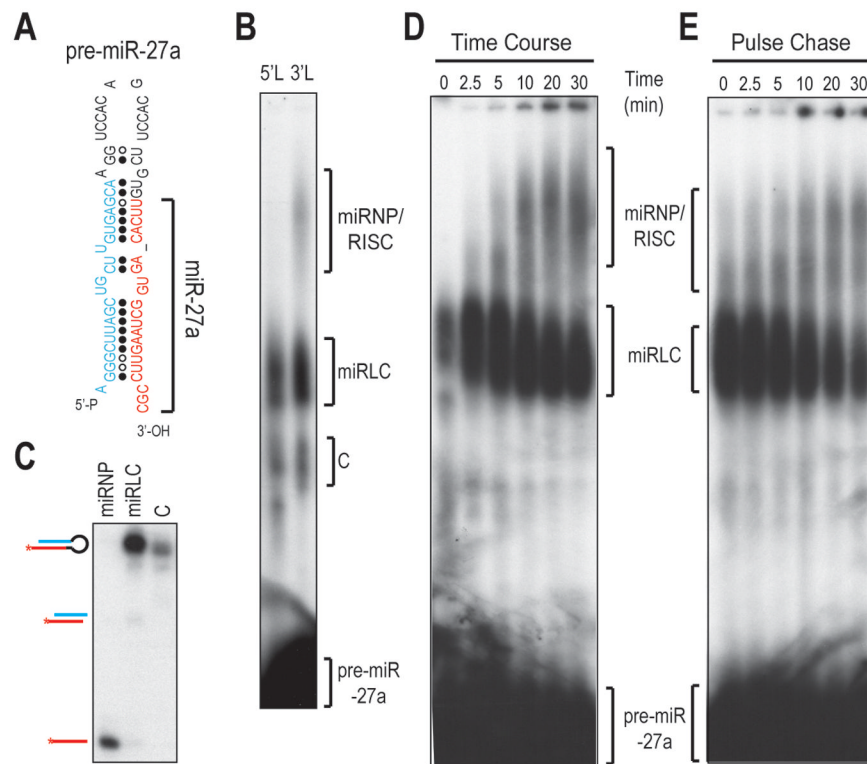


Figure 2. Asymmetric miRNA loading in miRNP/RISC is faithfully recapitulated with a pre-miRNA substrate

A. Schematic of pre-miR-27a.

B. NAGE of assembly reactions with 5' - (5'L) or 3' - radiolabeled (3'L) pre-miR-27a.

C. Native PAGE analysis of RNAs isolated from complexes assembled with pre-miR-27a-3'L in (B).

D. Kinetics of the miRNP/RISC assembly with pre-miR-27a-3'L.

E. Pulse-chase experiment. miRLC was pulsed in equal mixture of *Ago2+* lysates with *WT* lysates at 37 °C for 5 min with 3' -radiolabeled pre-miR-27a before 20-fold unlabeled pre-miR-27a was added; chase of complex miRLC into miRNP was monitored at 30°C.

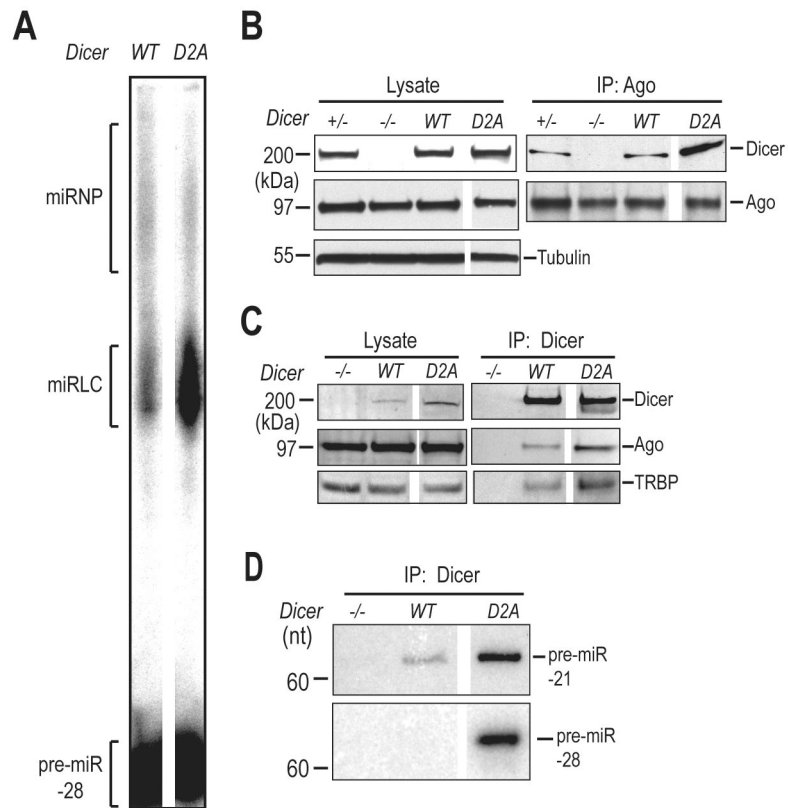


Figure 3. pre-miRNA processing and miRNA loading initiate in and are streamlined by the miRLC

A. NAGE of assembly reactions with pre-miR-28 in equal mixture of *Ago2*-overexpressing *Dicer*^{-/-} MEF, lysates and lysates from *Dicer*^{-/-} MEFs expressing *wild-type* (WT) or catalytically inactive (*D2A*) *Dicer* transgenes.

B,C. Western Blots of whole cell lysates and Ago (**B**) or Dicer (**C**) immunoprecipitates (IP). NMS: Nonimmune mouse serum.

D. Northern Blots of RNAs isolated from Dicer immunoprecipitates.

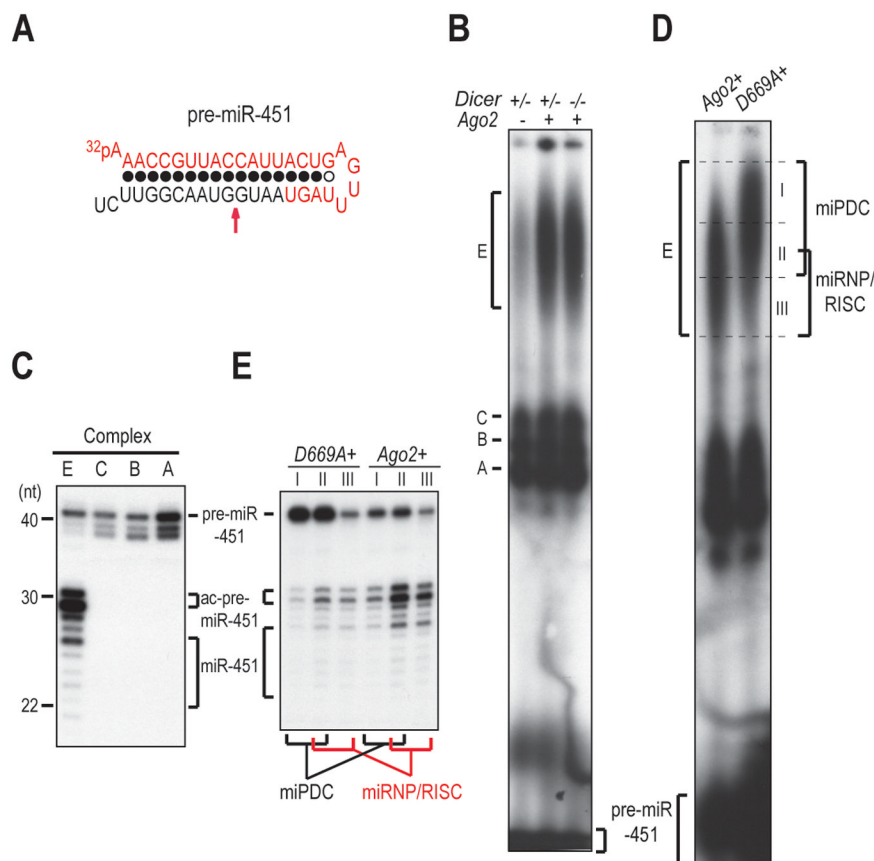


Figure 4. miRNP/RISC assembly with the precursor of a Dicer-independent miRNA via a miRNA Precursor Deposit Complex (miPDC)

A. Secondary structure of pre-miR-451. The red arrow denotes the Ago2 cleavage site.

B. NAGE of assembly reactions with pre-miR-451 in equal mixture of *Dicer*^{+/-} or *Dicer*^{-/-} lysates and lysates from *Dicer*^{-/-} MEFs overexpressing *Ago2* (*Ago2*⁺) or not (*Ago2*⁻).

C. Denaturing PAGE of RNAs isolated from indicated RNA-protein complexes assembled in equal mixture of *Dicer*^{+/-} lysates and *Ago2*⁺ lysates.

D. NAGE of assembly reactions with pre-miR-451 in equal mixture of *Dicer*^{+/-} lysates and lysates from *Dicer*^{-/-} MEFs overexpressing *wild-type* (*Ago2*⁺) or catalytically inactive (*D669A*⁺) *Ago2* transgenes.

E. Denaturing PAGE of RNAs isolated from the sliced sections of NAGE shown in (C).

Trace amounts of ac-pre-miR-451 in reactions assembled with catalytically-inactive *D669A* *Ago2*, are generated by small amount of endogenous *Ago2* in the lysate.

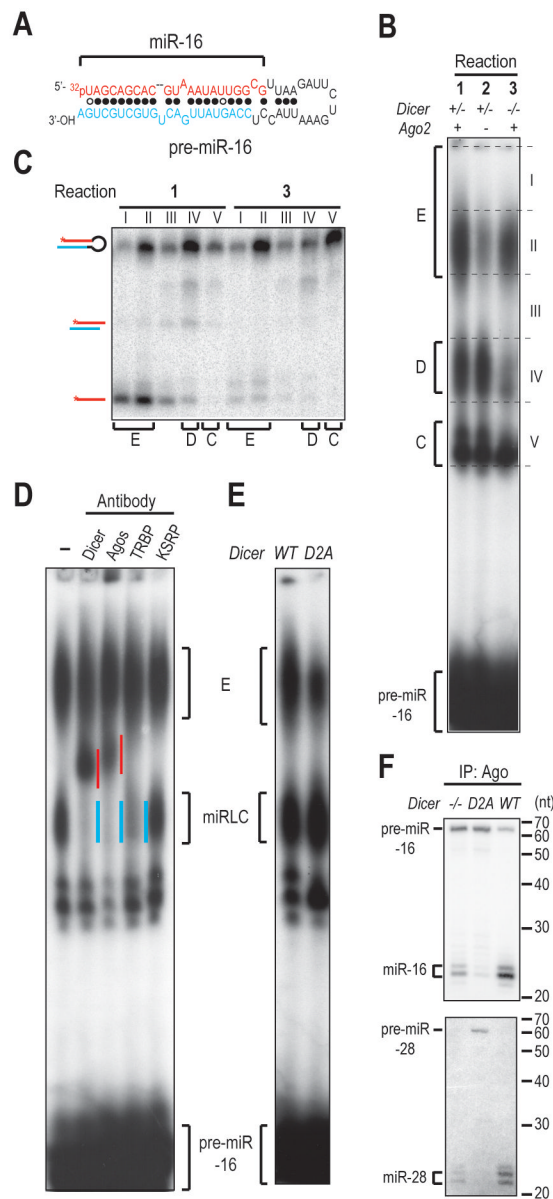


Figure 5. Assembly of the Precursor of a Dicer-dependent miRNA into miRLC and miPDC
A. Secondary structure of pre-miR-16-1., **B.** NAGE of assembly reactions with 5' - radiolabeled pre-miR-16 in equal mixture of *Dicer*^{+/-} or *Dicer*^{-/-} lysates and lysates from *Dicer*^{-/-} MEFs overexpressing *Ago2* (*Ago2*⁺) or not (*Ago2*⁻), **C.** Native PAGE of RNAs isolated from gel slices as delineated in (B), **D.** Antibody supershift experiments in equal mixture of *Ago2*-overexpressing *Dicer*^{-/-} lysates and lysates from *Dicer*^{-/-} MEFs reconstituted with HA-tagged *Dicer* transgene., **E.** NAGE of assembly reactions in equal mixture of *Ago2*-overexpressing *Dicer*^{-/-} lysates and lysates from *Dicer* Knock-Out MEFs expressing *wild-type* (WT) or catalytically inactive (*D2A*) *Dicer* transgene., **F.** Northern blots of RNAs isolated from Ago immunoprecipitates.

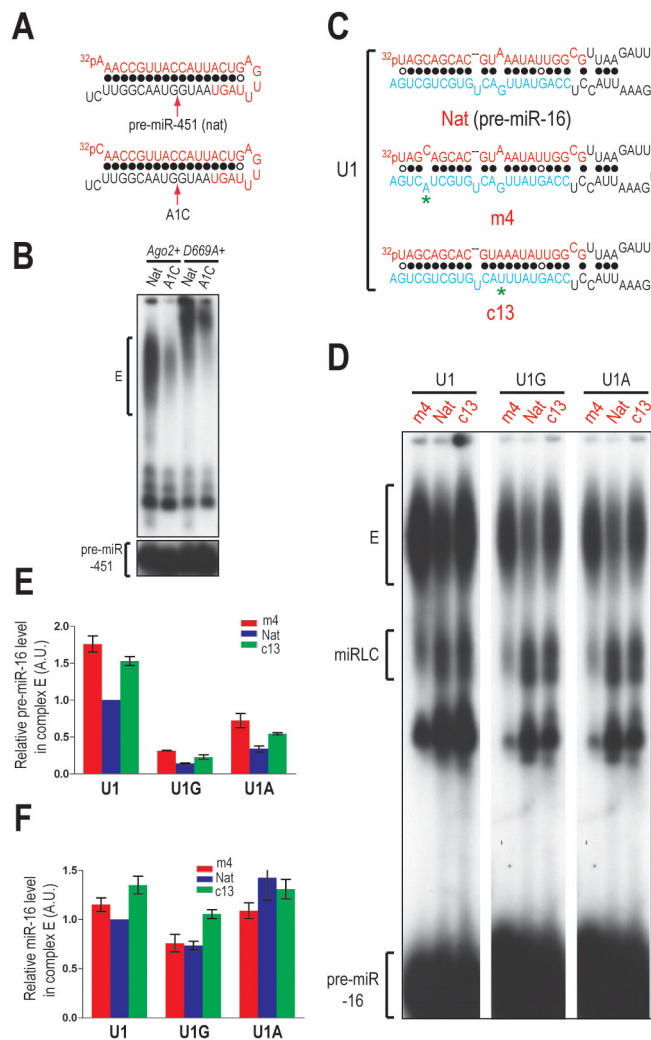


Figure 6. 5' Nucleotide Identity and Structural Features Important for miPDC Assembly
A. Secondary structures of the native (Nat) and the 5'-cytidine mutant (A1C) of pre-miR-451.

B. NAGE of assembly reactions in lysates from *Dicer^{fl/fl}* MEFs overexpressing *wild-type* (*Ago2+*) or catalytically inactive (*D669A+*) *Ago2* transgenes.

C. Secondary structures of pre-miR-16, m4 or c13 mutants. Mutated nucleotides are marked with asterisks.

D. NAGE of assembly reactions programmed with pre-miR-16 containing native (Nat) or altered secondary structures (m4 or c13) in the 5' wild-type (U1) or 5' mutant (U1G or U1A) background.

E, F. Relative amounts of pre-miR-16 (**E**) and miR-16 (**F**) in complex E. The standard error of the mean (SEM) is from two different experiments.

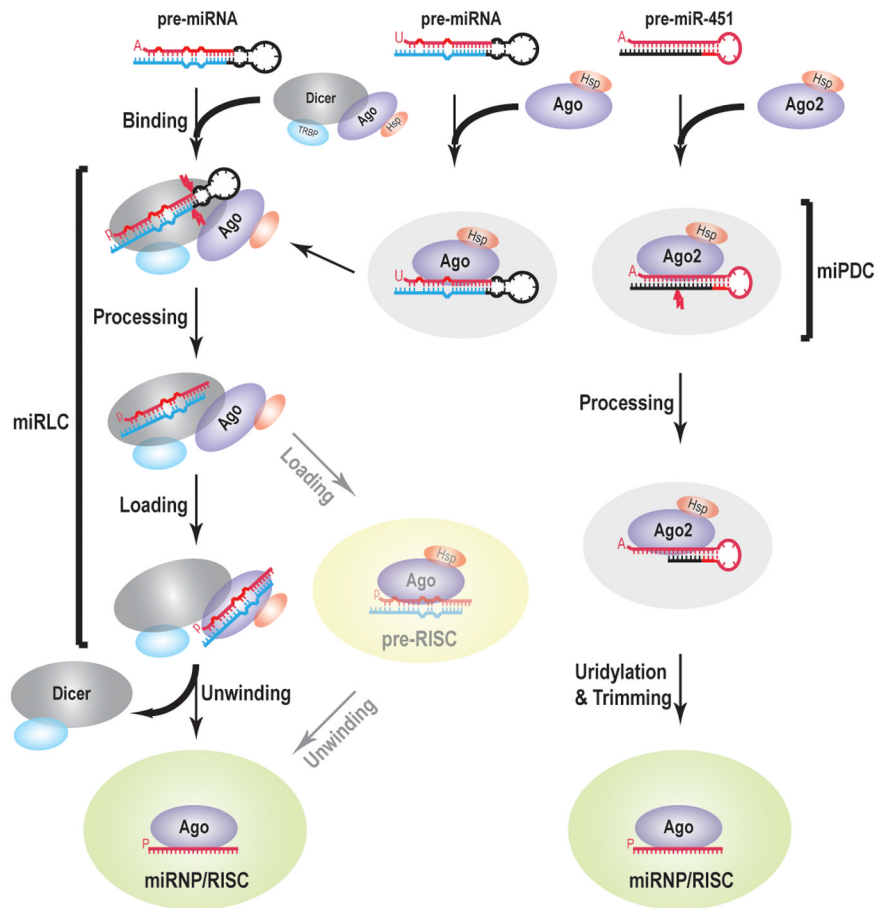


Figure 7. A model for pre-miRNA programmed silencing complex assembly pathways in mammals