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A MicroRNA in a Multiple-Turnover RNAi Enzyme Complex

György Hutvágner and Phillip D. Zamore*

Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Lazare Research Building, Room 825, 364 Plantation Street, Worcester, MA 01605, USA.
*To whom correspondence should be addressed. E-mail: phillip.zamore@umassmed.edu

In animals, the double-stranded RNA-specific endonuclease Dicer produces two classes of functionally distinct, tiny RNAs: microRNAs (miRNAs) and small interfering RNAs (siRNAs). miRNAs regulate mRNA translation, whereas siRNAs direct RNA destruction via the RNA interference (RNAi) pathway. Here we show that, in human cell extracts, the miRNA *let-7* naturally enters the RNAi pathway, suggesting that only the degree of complementarity between a miRNA and its RNA target determines its function. Human *let-7* is a component of the miRNP, which we show is an RNAi enzyme complex. Each *let-7*-containing complex directs multiple rounds of RNA cleavage, explaining the remarkable efficiency of the RNAi pathway in human cells.

Two types of 21-23 nt RNAs are produced by the multi-domain, RNase III-enzyme Dicer: small interfering RNAs (siRNAs) from long double-stranded RNA (*1*, *2*) and microRNAs (miRNAs) from ~70 nt hairpin precursor RNAs whose expression is often developmentally regulated (*3*–*8*). siRNAs direct the cleavage of complementary mRNA targets, a process known as RNA interference (RNAi) (*9*). Target RNA cleavage is catalyzed by the RNA-induced silencing complex (RISC) which acts as an siRNA-directed endonuclease, cleaving the target RNA across from the center of the complementary siRNA strand (*10*, *11*). Assembly of the RISC is ATP-dependent and precedes target recognition (*10*, *12*). Unlike siRNAs, miRNAs are single-stranded and pair with target mRNAs that contain sequences only partially complementary to the miRNA and represses mRNA translation without altering mRNA stability (*13*–*19*). Although at least 135 miRNAs have been identified collectively from *Caenorhabditis elegans*, *Drosophila melanogaster*, and humans, none is fully complementary to any mRNA sequence in these organisms, suggesting that miRNAs do not function in the RNAi pathway, since RNAi requires extensive complementarity between the siRNA and its mRNA target (*20*).

siRNAs and miRNAs have been proposed to act in distinct biochemical pathways, in part because distinct PPD ('PAZ' and 'Piwi' domain) proteins are required in *C. elegans* for RNAi (*21*) and miRNA function (*22*). In this model, the specific PPD protein associated with an siRNA or miRNA, or miRNA precursor determines the pathway in which a small RNA functions. Unique features of miRNAs or their precursors might lead them to associate with miRNA-specific PPD proteins. Thus, the sequence or structure of a miRNA or its precursor would ensure that it functions as a translational repressor and not as a trigger of RNAi.

We asked if an siRNA duplex in which one strand corresponded exactly to the miRNA *let-7* (*let-7* siRNA) could function in the RNAi pathway (Fig. 1A). We incubated the *let-7* siRNA duplex with *Drosophila* embryo lysate in an in vitro RNAi reaction containing a 5′ ^{32}P-radiolabled target mRNA. This target RNA contained sequence fully complementary to *let-7* as well as the sequence from the 3′ untranslated region of the *C. elegans lin-41* mRNA that mediates *let-7*-directed translational repression (Fig. 1B) (*23*). The *let-7* siRNA directed cleavage of the target RNA only within the sequence that was fully complementary to *let-7* (Fig. 1C). Thus, the intrinsic sequence of *let-7* does not preclude its functioning in RNAi. No cleavage was observed at any other site, including within the *lin-41* sequences. The *lin-41* sequence differs from the *let-7*-complementary sequence at only four of the 19 positions that determine siRNA-specificity (Fig. 1B). The *lin-41* sequence was not refractory to RNAi, since an appropriately complementary siRNA directs its cleavage (Fig. 1D) (*lin-41* siRNA).

Nor does the structure or sequence of the *let-7* precursor preclude entry of *let-7* into the RNAi pathway. We added synthetic *Drosophila melanogaster* pre-*let-7* RNA (Fig. 1A) to an in vitro RNAi reaction containing the *let-7* complementary target RNA. Again, the target RNA was cleaved within the *let-7* complementary sequences, but not at any other site (Fig. 1C). Although pre-*let-7* RNA promoted a lower level of RNAi than a *let-7* siRNA duplex, it was surprising that RNAi occurred at all, since Dicer cleavage of pre-*let-7* generates single-stranded *let-7* in vivo and in vitro (*3*, *22*, *7*). It is not known how Dicer produces single-stranded miRNAs but double-stranded siRNAs. One model is that Dicer initially generates an siRNA-like, double-stranded intermediate whose non-miRNA strand is then selectively destroyed. In this model, Dicer would produce the "pre-*let-7* siRNA" duplex shown in Fig. 1A. In an in vitro RNAi reaction, this siRNA duplex produced approximately the same amount of target cleavage as pre-*let-7* itself (Fig. 1C). Only a small fraction (~5%) of the input pre-*let-7* RNA (100 nM) is converted to mature *let-7* in vitro (*3*). Thus, when produced from pre-*let-7*, ~5 nM mature *let-7* entered the RNAi pathway as efficiently 100 nM of the pre-*let-7* siRNA duplex, suggesting that production by Dicer cleavage enhances entry of *let-7* into the pathway. Dicer action may therefore be coupled to RISC formation, consistent with the interaction of Dicer with the RISC components Ago-2 in *Drosophila* (*31*) and Rde-1 in *C. elegans* (*24*).

These experiments suggest that the degree of complementarity between a miRNA and its target RNA is the sole determinant of its function, since single-stranded *let-7* can clearly act as an siRNA in vitro. Nonetheless, *let-7* might be precluded from entering the RNAi pathway in vivo. We therefore tested if the endogenous *let-7* produced by cultured human HeLa cells (*3*) enters the RNAi pathway.

We tested both HeLa cytoplasmic (S100) and nuclear extracts for their ability to direct cleavage of the *let-7*-complementary RNA target (Fig. 1B) (*25*). In *Drosophila*

embryo lysate, which contains no *let-7*, this target RNA is cleaved at the *let-7*-complementary site only upon addition of exogenous *let-7* siRNA duplex. In contrast, HeLa cytoplasmic S100—but not nuclear—extract (Fig. 2A) directed target RNA cleavage within the *let-7*-complementary sequences, in the absence of any exogenous siRNA. Target cleavage in HeLa S100 occurred between nucleotides 541 and 542, exactly the same cleavage site directed by the exogenous *let-7* siRNA in *Drosophila* embryo lysate (Fig. 2B). No target cleavage occurred within the *lin-41* sequences contained in the target RNA. These sequences do not pair with *let-7* at position 9 and 10 (Fig. 1B); mispairing between an siRNA and its target at these positions blocks RNAi (*20*) (Dianne Schwarz and PDZ, unpublished). These results suggest that the endogenous *let-7* in the HeLa cytoplasmic extract is associated with the RNA-induced silencing complex (RISC), the enzyme complex that mediates endonucleolytic cleavage in the RNAi pathway.

To test directly if *let-7* was associated with the RISC, we asked if the cleavage activity co-purified with the protein eIF2C2, a member of the PAZ-and-Piwi domain (PPD) family of proteins. PPD proteins are required for RNAi and PTGS in animals (*21*, *26*, *27*), plants (*28*, *29*), and fungi (*30*). In flies, the PPD protein, Ago-2, is a component of the RISC complex (*31*), and in *Neurospora* (Qde-2) (*32*) and *C. elegans* (Rde-1) (*24*) PPD proteins are associated with siRNAs in multi-protein complexes likely to correspond to the RISC. A human RISC has not yet been characterized, but human eIF2C2 has been is associated with an RNA-protein complex (miRNP) that contains miRNAs and is nearly the same size as the *Drosophila* RISC (*33*). The *let-7* paralog, miR-98, which differs from *let-7* at two positions, was previously shown to be in the miRNP (*33*). We immunoprecipitated eIF2C2 from HeLa S100 with either monoclonal (8C7) or polyclonal (411-1) antibodies and tested the immunoprecipitates for their ability to cleave the target RNA at the *let-7*-complementary sequence (Fig. 2C) (*34*). Both *let-7* (Fig. 2D) and the nuclease activity specific for *let-7* complementary target sequences (Fig. 2C) co-purified with eIF2C2. We therefore refer to this activity as a *let-7*-programmed RISC.

Next, we tested if other components of the miRNP—Gemin4 and the putative DEAD-box RNA helicase, Gemin3—were also components of the *let-7*-programmed RISC complex (*33*). Monoclonal antibodies to Gemin3 and Gemin4, but not the Survival of Motor Neurons (SMN) protein, immunoprecipitated *let-7*-programmed RISC activity (Fig. 2C). SMN is a component of a Gemin3/Gemin4-containing complex that restructures nuclear RNPs; this complex does not contain eIF2C2 (*33*). Consistent with the idea that *let-7* was pre-assembled into the miRNP before the HeLa cells were lysed, target cleavage in S100 was not enhanced by addition of exogenous *let-7* siRNA (data not shown).

Human Dicer protein has previously been shown to be localized to the cytoplasm (*35*). The experiments in Fig. 2 suggest that the rest of the human RNAi pathway is likewise cytoplasmic, since no RISC-associated *let-7* was detected in the nuclear extract, nor could the nuclear extract be programmed with a *let-7*-containing siRNA to direct target cleavage. In contrast, an exogenous siRNA duplex complementary to firefly luciferase sequences successfully programmed the HeLa S100 to cleave the target (D. Schwarz, G.H., and P.D.Z., submitted). Although both the endogenous human *let-7* and the exogenous luciferase siRNA triggered target cleavage, the two triggers differ in at least one respect: endogenous human *let-7* is single-stranded (*7*, *11*, *19*), whereas the siRNA was double-stranded. Double-stranded siRNAs must be unwound in order to direct RNAi; this unwinding requires ATP (*12*). Once unwound, RISC-associated siRNAs can cleave their targets in the absence of high energy cofactors (*12*). Since *let-7* is single-stranded, target cleavage by HeLa *let-7* should not require ATP. To test this hypothesis, we depleted ATP (*12*) from the HeLa S100, then added the *let-7*-complementary target RNA. The *let-7*-programmed RISC cleaved the target in the absence of ATP (Fig. 3A).

Therefore, as in *Drosophila* embryo lysates (*27*), RNAi in human HeLa cytoplasmic extracts does not require ATP for target cleavage. Accordingly, models for the RNAi pathway that invoke the synthesis of new RNA as a prerequisite for target RNA destruction (*36*) do not accurately describe the mechanism of RNAi in human cells. New RNA synthesis is thought to be an important step for RNAi in *C. elegans* and *Dictyostelium discoideum*, post-transcriptional gene silencing in plants, and quelling in *Neurospora crassa* (*9*). In each of these organisms, a member of a family of RNA-dependent RNA polymerases (RdRPs) is required for RNA silencing. In contrast, no such RdRP is encoded by the current release of either the *Drosophila* or human genome.

Why then is RNAi so efficient in flies and cultured mammalian cells? The concentration of *let-7* in HeLa S100 is ≤900 pM (Figs. 3C and D) (*37*), and therefore the concentration of *let-7*-programmed RISC in our reactions is ≤450 pM. Since the target RNA concentration in these experiments was ≥6 nM and 70% of the target was destroyed in 2 hours (Fig. 2A), each *let-7*-programmed RISC must catalyze the cleavage of ≥10 target molecules. (*let-7* produced de novo during the reaction is negligible, because the concentration of pre-*let-7* in the HeLa S100 is 10-to-20-fold lower than that of *let-7* itself.) Thus, the *let-7*-programmed RISC is a true enzyme, catalyzing multiple rounds of RNA cleavage. It seems highly likely that all RISC's are multiple-turnover enzyme complexes.

Our results suggest that human *let-7* is in the enzyme complex that mediates RNAi, yet human cells do not contain mRNAs that could function as *let-7* RNAi targets. Perhaps *let-7* enters two separate complexes, one for RNAi and one for translational control. Such a model implies that a portion of *let-7* enters a complex that serves no function in human cells. More likely is that the recently identified miRNP (*33*) is the human RISC (*10*, *31*), and that this one complex carries out both target cleavage in the RNAi pathway and translational control in the miRNA pathway (Fig. 4). Such a view does not preclude miRNAs or siRNAs from also being associated with smaller complexes that contain only a subset of RISC components, but that are nonetheless capable of target RNA cleavage (*12*).

At least one plant miRNA (miR171) is perfectly complementary to a potential regulatory target mRNA, raising the possibility that miRNAs may naturally be used in plants for RNAi-based regulation of gene expression (*8*, *38*). We anticipate that in plants artificial miRNAs will direct translational control when they do not pair with their mRNA targets at the site of cleavage in the RNAi pathway. Conversely, we predict that in animals, synthetic siRNA duplexes with only partial complementarity to their corresponding mRNA targets will repress translation of the mRNA without triggering RNA degradation.

References and Notes

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23. The transcription template for the target RNA was prepared by PCR using synthetic deoxyoligonucleotide primers: 5′-GCG TAA TAC GAC TCA CTA TAG GAG ATA CGC CCT GGT TCC TG-3′ and 5′-CCC ATT TAG GTG ACA CTA TAG ATT TAC ATC GCG TTG AGT GTA GAA CGG TTG TAT AAA AGG TTG AGG TAG TAG GTT GTA TAG TGA AGA GAG GAG TTC ATG ATC AGT G-3′. Target transcription, purification, radiolabeling, siRNA and pre-*let-7* RNA preparation, and RNAi reactions were as described (*3*, *12*). *lin-41* siRNA comprised the synthetic RNAs: 5′-UGA GUG UAG AAC GGU UGU AUA-3′ and 5′-UAC AAC CGU UCU ACA CUC AAC-3′.
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34. 25 µl Protein G agarose (Invitrogen) bound with 40 µg α–eIF2C2 monoclonal antibody (mAb) or 7 µl polyclonal serum, 5 µl α–Gemin3 mAb, 5 µl α–Gemin4 mAb antibody, or 5 µl α–SMN mAb was incubated with 90 µl HeLa S100 at 4°C for 30 min, washed thrice with lysis buffer (*12*) containing 0.1% NP-40. 2.5 µl of washed immune-agarose beads were used in a standard RNAi reaction at 37°C.
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37. Synthetic *let-7* (Dharmacon) served as concentration standards; 5′ ^{32}P-radiolabeled anti-sense *let-7* RNA as probe. Concentration of twice gel-purified target RNA was determined by UV absorbance at 260 nm and confirmed by estimating the yield of ^{32}P-RNA after the second gel purification step.
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39. We acknowledge for discussions; David Bartel and members of the Zamore lab for comments on the manuscript; Melissa Moore for a generous gift of HeLa extracts; and Gideon Dreyfuss for kindly providing antibodies. P.D.Z. is a Pew Scholar in the Biomedical Sciences and a W.M. Keck Foundation Young Scholar in Medical Research. Supported in part by a grant to P.D.Z. from the National Institutes of Health (GM62862-01).

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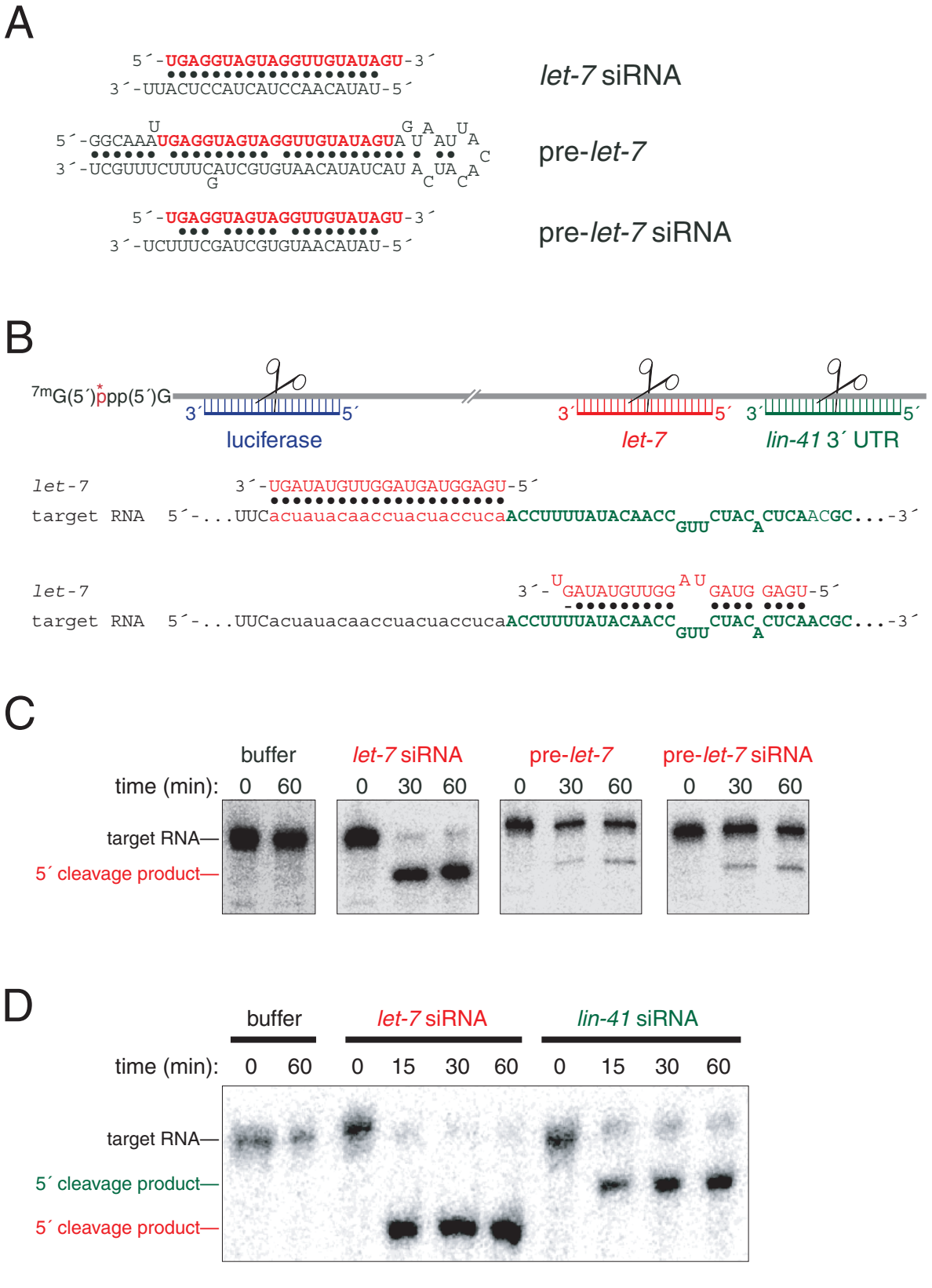
Fig. 1. Neither the sequence of *let-7* nor that of its precursor precludes *let-7* entering the RNAi pathway in vitro. (**A**) Sequences of RNAi triggers. *let-7* is in red. (**B**) Structure of the target RNA and its pairing with *let-7*. Red, *let-7* or *let-7* complementary sequence; green, *C. elegans lin-41* sequence. The 5′ ^{32}P radiolabel is indicated by an asterisk. (**C**) In vitro RNAi reactions with *Drosophila* embryo lysate using the triggers in (A) and the target in (B). (**D**) In vitro RNAi reactions as in (C) using *let-7*- and *lin-41*-specific siRNAs.

Fig. 2. Endogenous human *let-7* enters the RNAi pathway. (**A**) In vitro RNA reactions with *Drosophila* embryo lysate, human HeLa nuclear or S100 extract, using the target RNA described in Fig. 1B, with or without exogenous the *let-7* siRNA duplex described in Fig. 1A. (**B**) Denaturing gel analysis of the cleavage products from the RNAi reactions in (A). (**C**) Immunoprecipitation of the *let-7*-programmed RISC activity by antibodies to components of the miRNP (eIF2C2, Gem3, Gem4) or to the SMN protein. (**D**) Relative amount of *let-7* RNA recovered, as determined by Northern hybridization, in the immunoprecipitates in (C).

Fig. 3. (**A**) In vitro RNAi reactions using the target RNA and HeLa S100. ATP-depleted reactions (–) contained ~100-fold less ATP than non-depleted reactions (+). (**B**) Northern hybridization analysis to measure the concentration of *let-7* in HeLa S100. (**C**) Quantification of (B). Hybridization signal are plotted for the synthetic *let-7* standards (closed circles), for 3.6 µl (open circle) or 7.2 µl (open square) HeLa S100.

Fig. 4. A model for a common pathway in which miRNAs direct translational repression and siRNAs direct target RNA destruction (RNAi). The RNA duplex in brackets is proposed to be a short-lived intermediate. We propose that Dicer cleavage of both miRNA precursors and double-stranded

RNA is coupled to the formation of a miRNP/RISC complex. The RISC is envisioned to act stoichiometrically in repressing translation, but catalytically in RNA destruciton via RNAi.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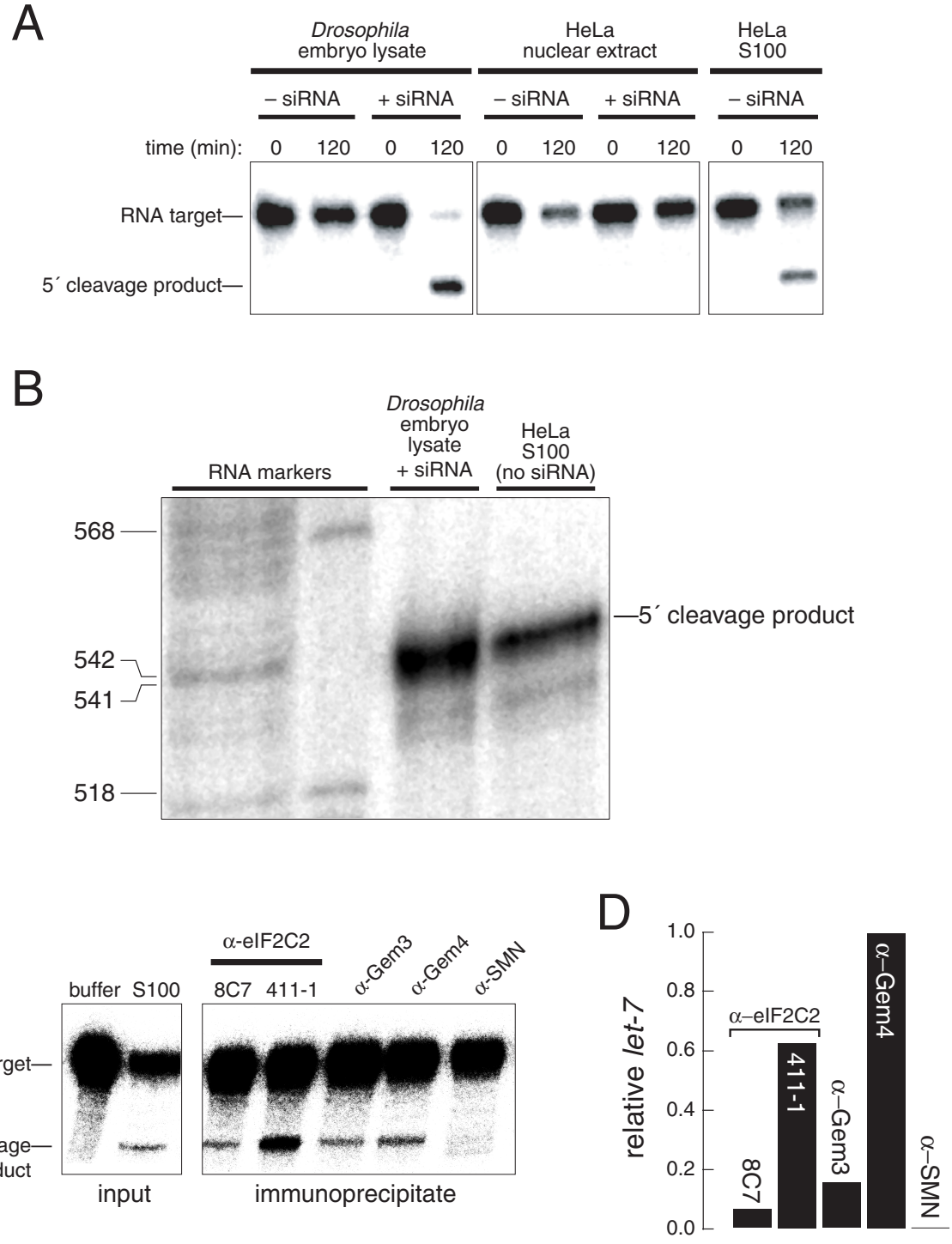
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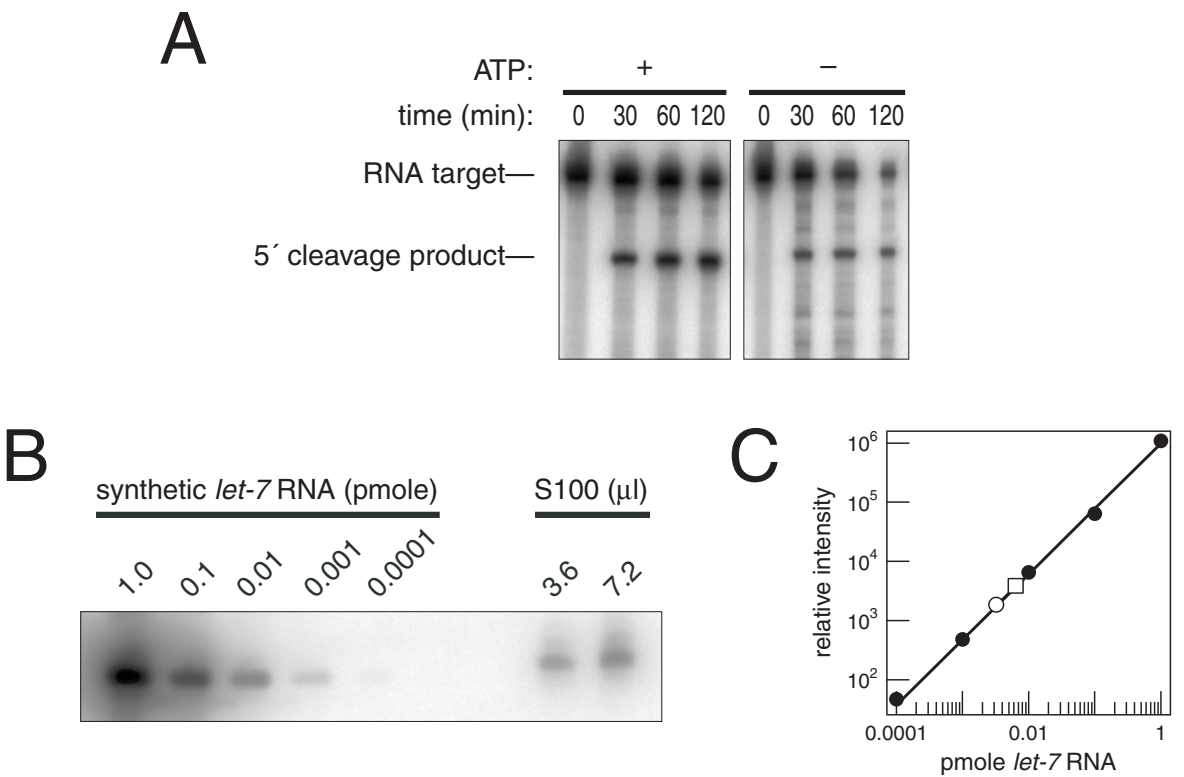
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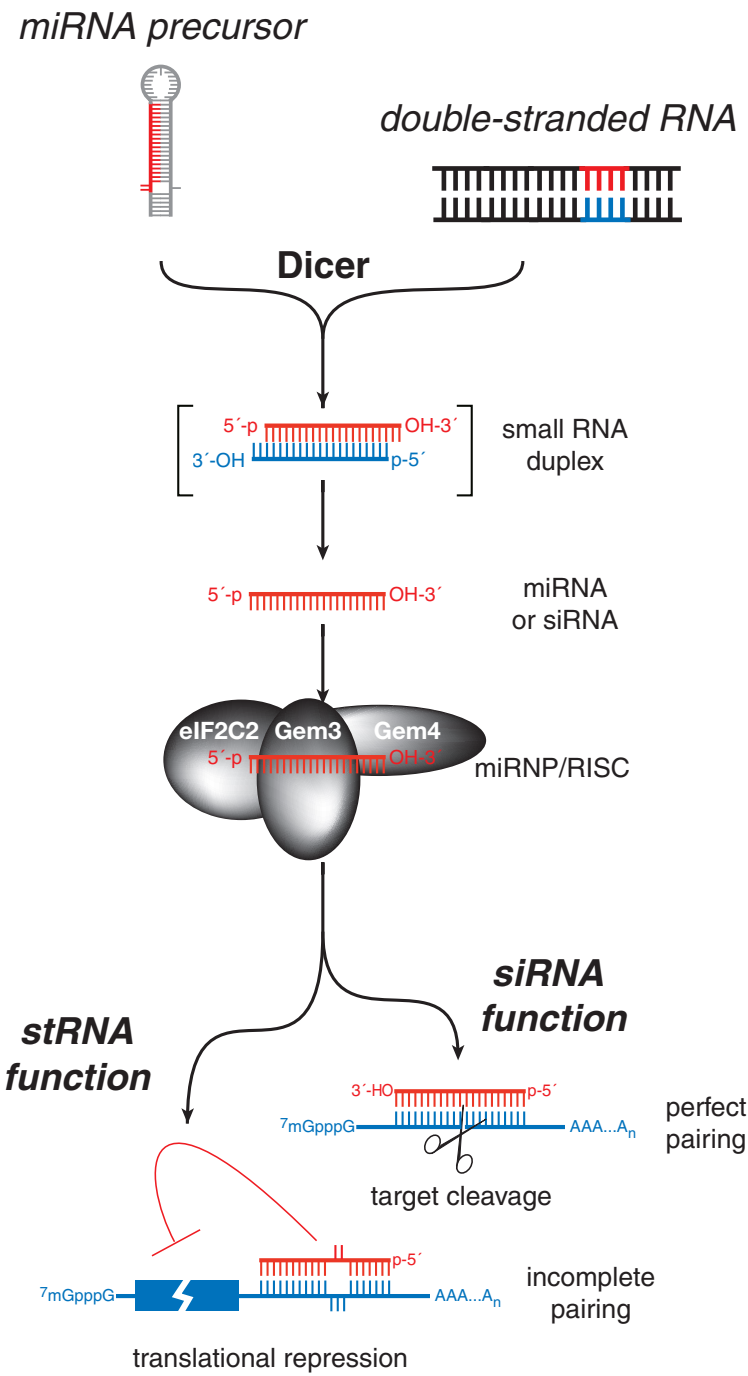
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