minimum of 400 cells were counted, and all experiments were performed in duplicate.
22. Apoptosis was assessed by staining with annexin V–FITC/PI (Calbiochem). Stained cells were analyzed in a Beckton-Dickinson flow cytometer. For DNA fragmentation analysis, DNA from 2×10^6 cells was isolated by phenol extraction and analyzed on a 1% agarose gel (*19*).
23. For construction of a stable cell line expressing 24p3, the 24p3 cDNA was amplified by polymerase chain reaction and cloned into the Eco RI and Bam HI sites of pcDNA3 vector (Invitrogen). COS-7 cells were transfected with pcDNA3/24p3 and selected with G418 at 600 μg/ml. G418-resistant colonies were isolated and screened for 24p3 expression by Northern blotting and immunoblotting.
24. For establishment of a 24p3 ecdysone-inducible cell line, the 24p3 cDNA containing a hemagglutinin (HA) tag at the COOH-terminus was cloned into the ecdysone-inducible vector pIND (Invitrogen). FL5.12 cells were transfected with Superfect (Qiagen). FL5.12 cells were first transfected with pVgRXR expressing the subunits of the receptor and selected with Zeocin (600 μg/ml) (Invitrogen). The resulting clones were then transfected with pIND/24p3-HA and selected with G418 (800 μg/ml) (Gibco-BRL). 24p3 expression was induced by addition of 10 μM ponasterone A (Invitrogen).
25. Comparable expression of a 24p3 derivative lacking the NH2-terminal signal sequence failed to induce cell death (*44*), suggesting that secretion of 24p3 is required for apoptosis.
26. Recombinant 24p3 was synthesized as a glutathione *S*-transferase (GST) fusion protein (pGST-2TK-24p3) and isolated to >90% purity. The GST moiety was removed by thrombin digestion. Purified 24p3 was added to cells to a final concentration of 0.8 to 10 μg/ml.
27. Phosphorothioate oligonucleotides were purchased from Genosys. Sense and antisense 1 oligonucleotides span −12 to −5 and antisense 2 oligonucleotide spans +585 to +593 of 24p3 mRNA (+1, translation start site). FL5.12 cells were transfected with 2 μM of each oligonucleotide using lipofectamine (Gibco-BRL). After 24 hours, cells were washed with RPMI medium plus 10% fetal calf serum (FCS) and again transfected with 2 μM of oligonucleotide. Transfected cells were deprived of IL-3 24 hours after the second transfection.
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35. For analysis of Bad phosphorylation, FL5.12 cells were treated with conditioned medium from COS-7 cells transfected with pcDNA3 or the 24p3 expression vector. Cells were lysed in 1% NP-40 lysis buffer containing 20 mM tris-HCl (pH 7.4), 150 mM NaCl, 1.5 mM MgCl2, 1 mM EDTA, 10 mM NaF, 0.2 mM Na_3VO_4, 1 mM Na_3MoO_4, and protease inhibitor tablets from Boehringer-Mannheim. Cell lysates were incubated with 2 μg of Bad antibody (Transduction Labs, Lexington, KY). Immune complexes were resolved by 12% SDS-PAGE and transferred onto a polyvinylidene diflouride membrane (Millipore). Blots were incubated with either a phospho-specific antibody to Bad (New England Biolabs) or an antibody to Bad and were developed with an ECL kit (Amersham).
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45. We thank C. Thompson and S. Durum for cell lines; M. Stevenson and P. Newburger for primary cells; M. Kress for the 24p3 cDNA; Y.-H. Chen for 24p3 protein and antibody; K. Rock, M. Czech, S. Corvera, M. Kelliher, M. Zapp, and members of our laboratory for helpful discussions and comments on this manuscript; and S. Persengiev for advice on Scatchard plot analysis. Supported in part by grants from NIH and Cap Cure (M.R.G.) and by the Leukemia and Lymphoma Society (L.R.D.).

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A Cellular Function for the RNA-Interference Enzyme Dicer in the Maturation of the *let-7* Small Temporal RNA

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The 21-nucleotide small temporal RNA (stRNA) *let-7* regulates developmental timing in *Caenorhabditis elegans* and probably in other bilateral animals. We present in vivo and in vitro evidence that in *Drosophila melanogaster* a developmentally regulated precursor RNA is cleaved by an RNA interference-like mechanism to produce mature *let-7* stRNA. Targeted destruction in cultured human cells of the messenger RNA encoding the enzyme Dicer, which acts in the RNA interference pathway, leads to accumulation of the *let-7* precursor. Thus, the RNA interference and stRNA pathways intersect. Both pathways require the RNA-processing enzyme Dicer to produce the active small-RNA component that represses gene expression.

Two small temporal RNAs (stRNAs), *lin-4* and *let-7*, regulate the timing of development in *Caenorhabditis elegans* (*1–3*). stRNAs encode no protein, but instead appear to block the productive translation of mRNA by binding sequences in the 3′-untranslated region of their target mRNAs (*1*, *2*, *4–11*). *let-7* is present in most if not all bilaterally symmetric animals, including *Drosophila melanogaster* and humans (*12*). In *Drosophila*, *let-7* first appears at the end of the third larval instar, accumulates to high levels in pupae, and persists in adult flies (*12*).

The mechanism by which stRNAs are synthesized is unknown. The ~21-nucleotide (nt) *let-7* RNA has been proposed to be cleaved from a larger precursor transcript (*12*). The generation of small RNAs from a longer, structured precursor—double-stranded RNA (dsRNA)—is an essential feature of the RNA interference (RNAi) pathway, raising the possibility that stRNAs are generated by mechanisms similar to the initial steps in RNAi and suggesting that enzymes such as the *Drosophila* protein Dicer might play a role in generating stRNAs (*13–18*).

A candidate RNA for the *Drosophila let-7* precursor. Examination of the developmental expression of *let-7* in *Drosophila* revealed a candidate for a *let-7* precursor RNA, *let-7L* (*19*). *let-7L* was detected at the end of the third larval instar and at the beginning of pupation, the same developmental stages where *let-7* itself is first expressed (Fig. 1A) (*12*). Consistent with the transcript being a *let-7* precursor, the amount of *let-7L* RNA declined as *let-7* accumulated. *let-7L* RNA was slightly shorter than a 76-nt RNA standard. Previous analysis of the genomic sequence flanking *Drosophila let-7* led to the proposal that a 72-nt RNA hairpin might be a *let-7* precursor (*12*) (Fig. 1B).

let-7 is also expressed in human tissues (*12*) and in cultured human HeLa cells, but not in *Drosophila* embryos or cultured *Drosophila* S2 cells (Fig. 1C). Primer extension analyses confirmed that the mature *let-7* RNA detected by Northern hybridization was bona fide *let-7* (*20*). Primer extension products corresponding to the 5′ ends of mature *let-7* RNAs were detected in total RNA from early and unstaged *Drosophila* pupae and from human HeLa cells (Fig. 1D). Primer extension analysis of total RNA from un-

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staged worms (Fig. 1D), as well as Northern hybridization experiments (Fig. 1A), indicated that worm *let-7* is 1 nt longer than that in flies and humans. In early pupae, primer extension analysis also detected three longer extension products. The major, middle product and the less abundant, lower product comigrate with primer extension products templated by a synthetic 72-nt RNA corresponding to putative pre-*let-7* (Fig. 1, B and D). This longer transcript from early pupae (Fig. 1A) has the same 5′ end as the 72-nt *let-7* precursor proposed by Ruvkun and coworkers (Fig. 1B) and is therefore a good candidate for a *let-7* precursor RNA (*12*).

***let-7* maturation in vitro.** To determine if the *let-7L* RNA detected in vivo was, in fact, the direct precursor of mature *let-7*, we tested processing of the proposed pre-*let-7* stem-loop RNA into *let-7* in *Drosophila* embryo lysates, which contain no detectable *let-7* RNA (Fig. 1C) (*12*). These lysates recapitulate RNAi in vitro (*21*), allowing us to ask if the proposed precursor RNA was cleaved into mature *let-7* by an RNAi-like mechanism. The 72-nt RNA (Fig. 1B) was incubated with *Drosophila* embryo lysate for various times, then assayed for the production of *let-7* by primer extension (Fig. 1E). As seen in vivo, mature *let-7* RNA accumulated in the cell-free reaction. Thus, an RNA corresponding to the proposed *let-7* precursor was converted to an RNA with precisely the same 5′ ends as authentic *let-7* by one or more factors in the *Drosophila* embryo lysate.

Only *let-7* RNA, not its complement, has been detected in vivo in worms, flies, and human tissues (*22*, *12*). Thus, we expected that bona fide *let-7* maturation in vitro would be asymmetric, yielding only *let-7* and not small RNAs complementary to *let-7*, such as antisense *let-7*. In contrast, processing of long, dsRNA by the RNAi pathway is symmetric, yielding double-stranded 21- to 22-nt RNAs. Therefore, we asked if processing of the proposed pre-*let-7* RNA in vitro was symmetric or asymmetric, yielding *let-7* but not its complement. We prepared four pre-*let-7* RNAs by in vitro transcription, each uniformly labeled with a different α-^{32}P–nucleotide—adenosine 5′-triphosphate (ATP), cytidine 5′-triphosphate, guanosine 5′-triphosphate, or uridine 5′-triphosphate—and incubated them separately in an in vitro reaction (*23*). Since *let-7* contains no cytosine, accurate in vitro processing of pre-*let-7* should produce a 21- to 22-nt product for RNAs labeled at A, G, or U but not at C. A product of the appropriate size for *let-7* was produced for pre-*let-7* transcripts labeled at A, G, and U (Fig. 2A). No ^{32}P-labeled product accumulated from the ^{32}P-C-labeled pre-*let-7* RNA; the faint ~22-nt band visible in the ^{32}P-C lanes does not correspond to a processing product because it was present before the reaction was initiated (0 min lane) and disappeared upon incubation in the *Drosophila* embryo lysate. Although pre-*let-7* RNA continued to disappear with incubation in the lysate, mature-*let-7* production rapidly reached a plateau. Because single-stranded

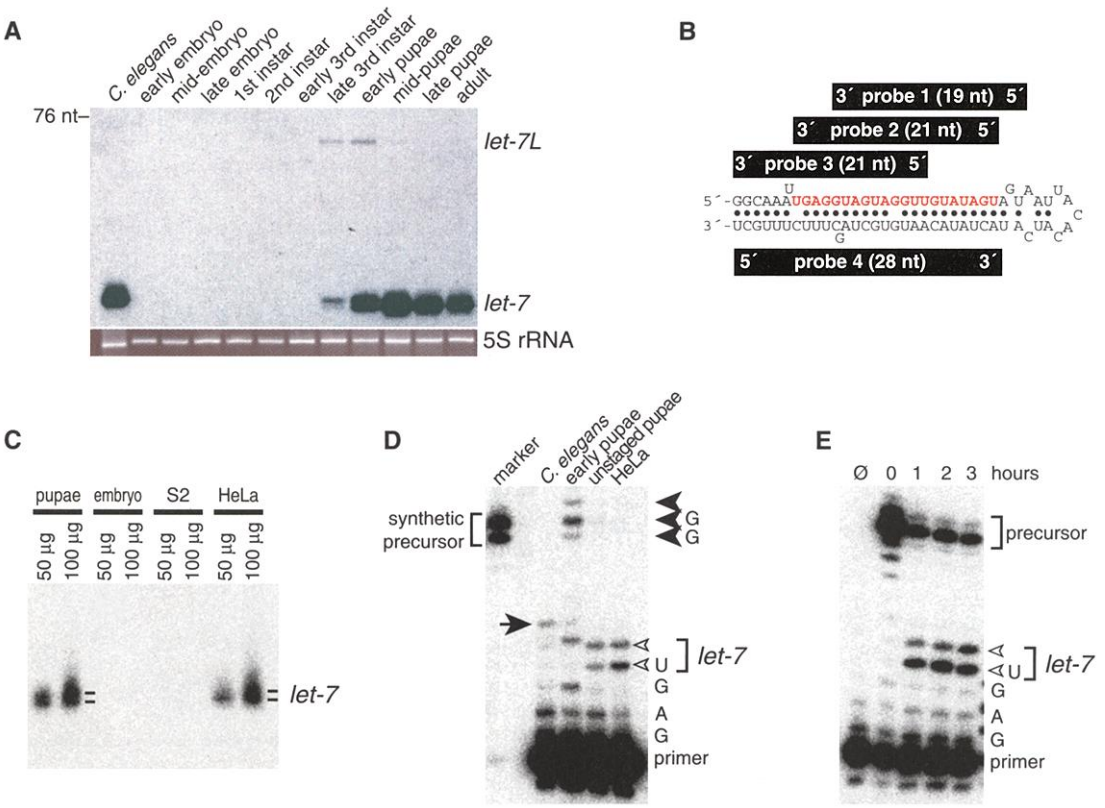

Fig. 1. Pre-*let-7* in vivo and in vitro. (**A**) Northern hybridization analysis of *let-7* expression during the *Drosophila* life cycle. The hybridization probe was complementary to both *let-7* and the putative *let-7* precursor RNA indicated in (B). Ethidium bromide staining of 5S ribosomal RNA demonstrates that approximately equal amounts of total RNA were loaded in each lane. *let-7* detected in *C. elegans* total RNA has an apparent mobility 1 nt longer than that detected in flies. (**B**) Schematic of the proposed *Drosophila* pre-*let-7* RNA (*12*), indicating the regions of complementarity to the primer extension probes used in this study. The sequence of mature *let-7* within the precursor is indicated in red. (**C**) Northern hybridization analysis of *let-7* RNA in *Drosophila* pupae, embryos, and cultured S2 cells, as well as in cultured human HeLa cells. Two dashes indicate the positions of the *let-7* doublet. The amount of RNA loaded in each lane is indicated at the top. (**D**) Primer extension with probe 1 to analyze total RNA from *C. elegans*, *Drosophila* early and unstaged pupae, and cultured human HeLa cells grown in suspension. Primer extension of a synthetic RNA with the sequence indicated in (B) (left lane) and primer extension in the presence of 2′,3′-terminal dideoxynucleotides (indicated at right) provide markers. Solid arrowheads indicate primer extension products diagnostic of pre-*let-7*, and open arrowheads indicate primer extension products diagnostic of *Drosophila* and human *let-7*. An arrow indicates the primer extension product diagnostic of *C. elegans let-7*. Two primer extension products were detected for *Drosophila* and human *let-7*, consistent with previous S1 nuclease mapping (*12*) that suggests that both *Drosophila* and human *let-7* exist in 21- and 22-nt forms that differ at their 5′ ends. Alternatively, the longer extension product may represent addition of one untemplated nucleotide by reverse transcriptase. (**E**) Primer extension analysis of an in vitro pre-*let-7* processing reaction. *Drosophila* embryo lysate was incubated in a standard cell-free RNAi reaction with or without (Ø) pre-*let-7* for the time indicated. The RNA was then deproteinized and analyzed by primer extension with probe 1. The identity of the 5′ ends of the extension products (indicated at right) was determined by reverse transcription in the presence of dideoxynucleotides.

21-nt RNAs are generally unstable in the embryo lysate (*24*, *25*), this likely reflects degradation of *let-7* in the lysate, which may lack factors required for *let-7* stabilization and function. Nonetheless, it is remarkable that *let-7* RNA accumulates at all, because exogenous, single-stranded, 21-nt RNAs are degraded by the lysate within minutes.

Next, we analyzed the products of an in vitro reaction by Northern hybridization (Fig. 2B) using three different deoxyoligonucleotide probes (Fig. 1B). Probe 2 was entirely complementary to mature *let-7*. Probe 3 was complementary to the first 21 nt of the precursor and therefore only partially complementary to mature *let-7*. Control experiments showed that probe 3 detected mature *let-7* substantially less well than probe 2, whereas probe 3 detected as well or better than probe 2 products derived from the precursor sequence that is 5′ to the region encoding *let-7* (*23, 26*). Finally, probe 4 was complementary to the side of the stem of the precursor opposite the portion encoding *let-7* (Fig. 1B). Thus, probe 4 should detect the products of symmetric processing of the precursor RNA. Control experiments demonstrated that probe 4 readily detected synthetic antisense *let-7* RNA, but not *let-7* itself (*23, 26*). Northern hybridization experiments were quantified by determining the amount of each probe that hybridized to the region of the blot corresponding to the ~21-nt reaction product and, as a control for hybridization efficiency, the amount of hybridization of each probe to the unreacted precursor remaining at 3 hours, because the full-length precursor is perfectly complementary to all three probes (Fig. 2C). Probe 2, which is complementary to *let-7*, readily detected an RNA that accumulated with time. In contrast, probe 3 detected only weakly an RNA that accumulated over the course of the reaction, consistent with it detecting by partial hybridization mature *let-7* but not reaction products derived from the region of the precursor 5′ to the *let-7* sequence. Most important, probe 4, which was designed to detect reaction products like antisense *let-7*, did not detect products that accumulated upon incubation of pre-*let-7* in the lysate (Fig. 2, B and C). These data strongly imply that symmetric processing products such as antisense *let-7* are either not generated at all or are far less stable than *let-7* in the in vitro reaction. Thus, the in vitro reaction displays the same specificity and asymmetry that characterize *let-7* biogenesis in vivo.

It remained possible that the mechanisms of cleavage in vitro and in vivo differ. To assess the type of ribonuclease (RNase) that might be responsible for pre-*let-7* processing, both in vitro and in vivo, we analyzed the 5′ and 3′ ends of both the *let-7* generated by the in vitro processing reaction and the *let-7* from pupae (Fig. 3) (*27*). Treatment with periodate, followed by β-elimination, of either RNA from the in vitro processing reaction or total pupal RNA increased the apparent mobility of *let-7* by nearly 2 nt, a change diagnostic of RNAs bearing 2′,3′-terminal hydroxyl groups (Fig. 3, A and C). Treatment with calf intestinal phosphatase (CIP) of in vitro–generated *let-7* or pupal RNA decreased the apparent mobility of *let-7* by 1 nt, consistent with the removal of a charged phosphate group (Fig. 3, B and C). Furthermore, treatment of the CIP-treated RNA with polynucleotide kinase and ATP restored its original mobility, demonstrating that *let-7* contains a monophosphate (*28*). Because *let-7* contains 2′- and 3′-terminal hydroxyls, this single phosphate must be at its 5′ end. Thus, *let-7* produced by in vitro processing and *let-7* isolated from pupae have the same terminal structure: a 5′ monophosphate and 2′- and 3′-terminal hydroxyls. Notably, such termini are characteristic of the products of cleavage of dsRNA by RNase III (*29*).

The small interfering RNAs (siRNAs) that mediate RNAi also bear a 5′ monophosphate and 2′- and 3′-terminal hydroxyls (*25*). In *Drosophila*, siRNA duplexes are produced by the cleavage of long dsRNA by the enzyme Dicer (*18*). Cleavage by Dicer is thought to be catalyzed by its tandem RNase III domains. Only two types of RNase III enzymes are predicted to occur in *Drosophila*: Drosha (*30*) and Dicer. Dicer is the only RNase III domain protein in the publicly available sequence of the *Drosophila* genome that contains an ATP-binding motif, the DEAD-box RNA helicase domain (*18*). Cleavage of dsRNA by Dicer is strictly ATP-dependent (*18*). Figure 4A shows that cleavage of pre-*let-7* into mature *let-7* in *Drosophila* embryo lysates also required ATP. Taken together, the chemical structure of mature *let-7* RNA in vitro and in vivo and the ATP dependence of pre-*let-7* processing in vitro strongly implicate Dicer in *let-7* maturation. However, we note that expression of Dicer protein in *Drosophila* larvae or pupae has not yet been demonstrated, although the RNAi pathway, which requires Dicer, functions in larvae and pupae (*31*).

A more stringent test for a role for Dicer in pre-*let-7* processing would be to assay *let-7* production in flies lacking Dicer protein. However, mutant alleles of Dicer have yet to be identified in *Drosophila*. As an

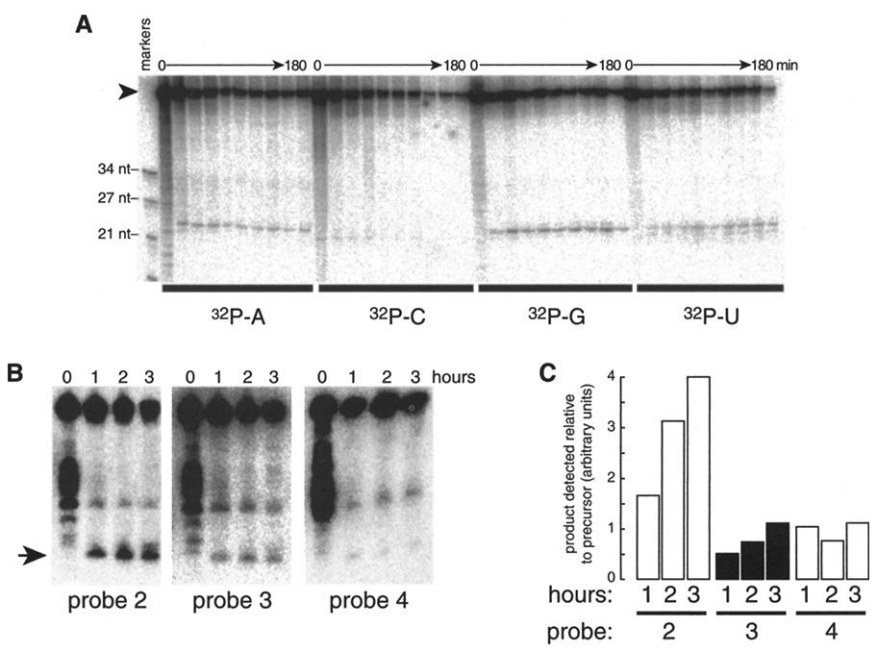

Fig. 2. In vitro processing of *Drosophila* pre-*let-7*. **(A)** Time course of in vitro processing for in vitro–transcribed pre-*let-7* RNAs labeled with the α-^{32}P nucleotide indicated at the bottom of the figure. Molecular size markers are from a complete T1 digestion of a uniformly labeled RNA. Because the electrophoretic mobility for small RNAs is a function of sequence and terminal structure as well as of length, both synthetic 21-nt *let-7* RNA (not shown) and in vitro–processed *let-7* migrate at ~23 nt relative to these markers. **(B)** Northern hybridization analysis with probes 2, 3, and 4 (see Fig. 1B) of the products of an in vitro pre-*let-7* processing reaction. The arrow indicates the position of mature *let-7*. The same filter was stripped and reprobed for the experiments in each panel. **(C)** Quantitation of the data in (B). The amount of hybridization of the probe to the 21- to 22-nt RNA products was quantified and was normalized to the amount of hybridization of the probe to the unreacted precursor remaining at 3 hours to correct for differences in hybridization efficiencies among the three probes.

alternative approach, we used a recently reported sequence-specific method in which cultured mammalian cells are transfected with synthetic 21-nt siRNA duplexes to suppress gene expression (*32*). Because they are <30 base pairs long, the siRNA duplexes do not trigger the sequence-non-specific responses that complicate standard dsRNA-induced interference in mammalian cells.

The RNAi enzyme Dicer is required for maturation of human *let-7* RNA. We used this method to evaluate the role of the human ortholog of Dicer (Helicase-MOI) in *let-7* biogenesis. Human Dicer was identified by its unique domain structure, comprising an NH_2-terminal DEXH-box ATP-dependent RNA helicase domain, PAZ domain, tandem RNase III motifs, and COOH-terminal dsRNA-binding domain, and by its sequence homology to *Drosophila* Dicer (*18*, *33*, *34*). HeLa cells were transfected with a single, synthetic siRNA duplex containing 19 nt of the coding sequence of human Dicer mRNA, beginning at position 183 relative to the start of translation (*35*). Three days after transfection, total RNA was prepared from the cells and analyzed by reverse transcriptase–polymerase chain reaction (RT-PCR) for Dicer and actin mRNA levels and by primer extension for the presence of *let-7* (Fig. 4, B and C). The level of Dicer mRNA in the Dicer siRNA-treated cells was four- to sixfold lower than in the control samples, whereas actin mRNA levels were unchanged (Fig. 4B). Separate controls showed that ~70 to 80% of the cells were transfected. Thus, the observed decrease in Dicer mRNA levels demonstrates that the Dicer siRNA induced substantial degradation of Dicer mRNA in the fraction of the cells that were successfully transfected.

Transfection of HeLa cells with the siRNA duplex corresponding to human Dicer, but not the control siRNA duplex, led to the accumulation of a longer *let-7*–containing RNA, *let-7L*: Primer extension analysis of RNA from cells transfected with

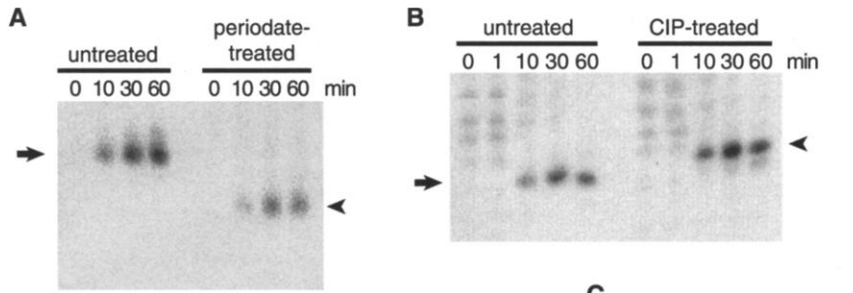

Fig. 3. Analysis of the ends of *let-7* produced in vitro and in vivo. *let-7* RNA was detected by Northern hybridization. (**A**) *Drosophila* pre-*let-7* RNA was processed in vitro for the times indicated and analyzed with and without periodate treatment followed by β-elimination. The apparent electrophoretic mobility of untreated *let-7* (arrow) is increased by ~2 nt (arrowhead) upon periodate treatment. (**B**) *Drosophila* pre-*let-7* was processed in vitro for the times indicated and analyzed with and without calf intestinal phosphatase (CIP) treatment. The apparent mobility of *let-7* (arrow) is reduced by ~1 nt (arrowhead) upon CIP treatment. (**C**) Analysis of mature *let-7* in total pupal RNA (Ø) or in total pupal RNA treated with CIP (CIP) or periodate followed by β-elimination (β). Control lanes correspond to a synthetic 21-nt *let-7* RNA bearing a 5′ monophosphate and 2′- and 3′-terminal hydroxyl groups (Ø) or the same RNA treated with CIP (CIP) or periodate followed by β-elimination (β). The apparent mobility of pupal *let-7* (arrows) is reduced by ~1 nt (open arrowheads) when treated with CIP and increased by ~2 nt (filled arrowheads) when treated with periodate.

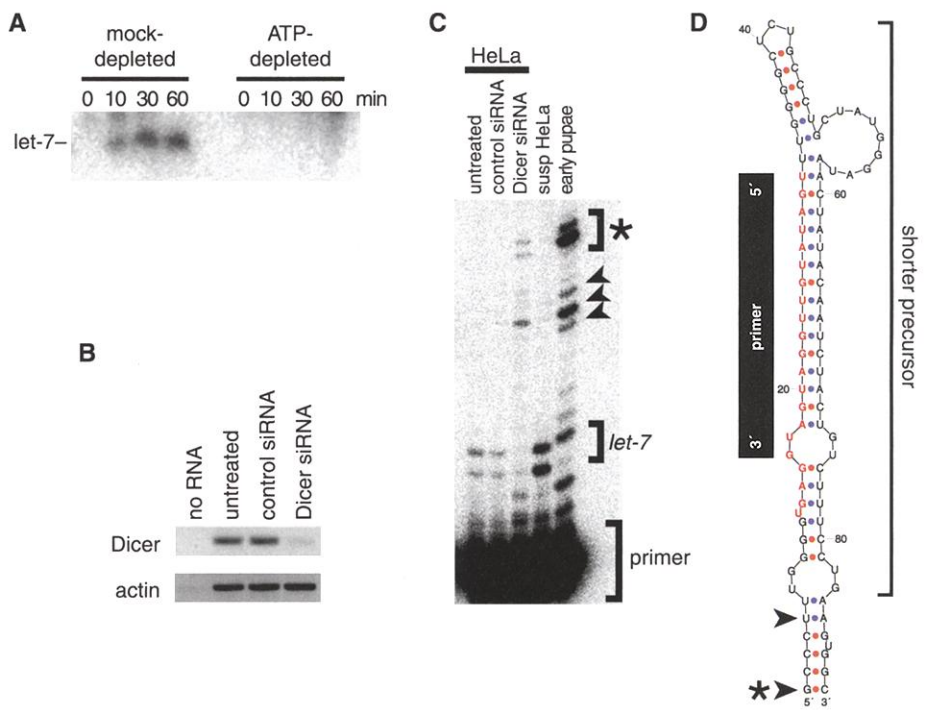

Fig. 4. Evidence for a role for Dicer protein in pre-*let-7* processing in vitro and in vivo. (**A**) In vitro processing of pre-*let-7* requires ATP. The predicted *Drosophila* pre-*let-7* RNA (50 nM) was incubated with *Drosophila* embryo lysate in vitro with (mock-depleted) and without (ATP-depleted) ATP for the times indicated. Production of *let-7* was monitored by Northern hybridization. (**B**) Semiquantitative RT-PCR assays for Dicer and actin mRNA levels in HeLa cells transfected with buffer (untreated), control siRNA, or human Dicer siRNA. The PCR primers span one (Dicer) or two (actin) introns. Therefore, the PCR products correspond to amplification of mRNA, not genomic, sequences. (**C**) siRNA-targeted degradation of human Dicer mRNA leads to the accumulation of *let-7L* in cultured human HeLa cells. Total RNA from untreated (no siRNA), control siRNA-treated, or human Dicer siRNA-treated HeLa cells, and from HeLa cells grown in suspension and from early *Drosophila* pupae was analyzed by primer extension to detect *let-7L* and mature *let-7*. Primer extension products diagnostic of *let-7*, the predicted human and *Drosophila* *let-7* precursors (arrowheads), and an extended stem form of the precursor (*) are indicated. (**D**) Proposed structure of the shorter and extended human *let-7L* RNAs detected in (C). The sequence of mature *let-7* is shown in red. The 5′ ends of the primer extension products corresponding to the shorter precursor (arrowhead) and the extended precursor (arrowhead with asterisk) are indicated at right. The structure was predicted with the RNA-folding algorithm mFold 3.1 (*42*, *43*). If the unpaired nucleotides at positions 7, 8, 16, and 17 are allowed to form G●U base pairs, then the stem may comprise as many as 32 consecutive base pairs interrupted only by the unpaired uracil residues at positions 12 (the 5′ end of mature *let-7*) and 87.

the Dicer siRNA detected an RNA with a 5′ end ~7 nt and ~11 to 12 nt upstream of the mature *let-7* product (Fig. 4C). These products are consistent with the accumulation of the predicted human *let-7* precursor RNA (*12*) and with a longer form of this precursor containing an extended stem (Fig. 4D). The mature human *let-7* RNA was readily detected in control cells, but not in the cells transfected with the Dicer siRNA duplex (Fig. 4C), providing additional evidence for a role for Dicer in *let-7* maturation. These findings, together with our in vitro data, provide strong evidence that Dicer protein function is required for the maturation of *let-7*. Thus, the RNAi and stRNA pathways intersect; both require the RNA-processing enzyme Dicer to produce the active small-RNA component that represses gene expression (Fig. 5). The two pathways must also diverge after the action of Dicer, because siRNA duplexes generated from long, dsRNA direct mRNA cleavage, whereas the single-stranded stRNA *let-7* represses mRNA translation.

Recently, Mello and co-workers have shown that the Dicer homolog Dcr-1 is required for both *lin-4* and *let-7* function in *C. elegans* (*36*). Thus, Dicer is likely to have a broad role in the biogenesis of stRNAs and perhaps other small regulatory RNAs. Furthermore, mutations in the *Arabidopsis* homolog of Dicer, *SIN-1/CARPEL FACTORY* (*SIN1/CAF*), have dramatic developmental consequences (*37–39*). Perhaps SIN1/CAF protein in plants, like Dicer in bilateral animals, processes structured RNA precursors into small RNAs that regulate development.

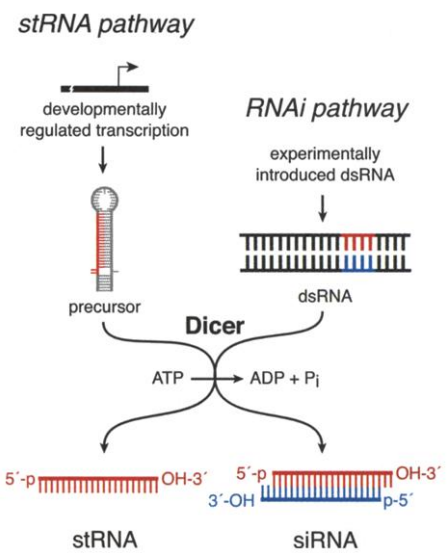

Fig. 5. The RNAi and stRNA pathways intersect. In this model, transcription of pre-*let-7* and other stRNA precursors is regulated developmentally. Dicer and perhaps other proteins act on these pre-stRNAs to yield mature, single-stranded stRNAs that repress mRNA translation. In RNAi, Dicer cleaves long, dsRNA to yield siRNA duplexes that mediate targeted mRNA destruction.

Pre-*let-7* is processed asymmetrically to yield only *let-7*. We do not yet know what structural or sequence features of pre-*let-7* determine its asymmetric cleavage. RNase III enzymes cleave perfectly paired dsRNA on both strands, producing a pair of cuts, one on each strand, displaced by two nucleotides. For the R1.1 RNA hairpin of T7 bacteriophage, internal loops and bulges constrain the *Escherichia coli* RNase III dimer to cut only one strand of the stem (*40*). The proposed *let-7* precursor contains such an internal loop at the site of 5′ cleavage. It is possible that if the stem were uninterrupted by such distortions, a pair of 21- to 22-nt RNAs might be generated, rather than the single stRNA *let-7*. If so, it might be possible to design stem-loop RNA precursors that produce an siRNA duplex. The hope is that such an siRNA duplex, generated in vivo in a specific cell type or at a specific developmental stage, would be able to target an mRNA for destruction by the RNAi machinery, thereby extending the utility of RNAi to the study of mammalian development.

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19. RNA was isolated from in vitro processing reactions as described (*21*), and RNA from pupae and HeLa cells was isolated with TRIzol Reagent (Life Technologies) according to the manufacturer's instructions. RNAs were separated by electrophoresis on 15% denaturing polyacrylamide gels and electroblotted onto Hybond N+ (Amersham). DNA oligonucleotides were radioactively labeled with polynucleotide kinase and [γ-^{32}P]ATP (ICN). Membranes were hybridized with the probe at 42°C in 50% formamide, 5× saline sodium phosphate EDTA (pH 7.4), 5× Denhardt's reagent, 0.5% SDS, denatured herring sperm DNA (80 μg/ml) and washed at 42°C in 2× saline sodium citrate and 0.1% SDS. In Fig. 1A, the deoxyoligonucleotide probe was 5′-AACTATACAACCTACTACCTCACCGGATCC-3′.
20. Primer extension was performed with Superscript II reverse transcriptase (Life Technologies) at 37°C for 40 min followed by incubation at 50°C for 30 min with 5 μg of RNA with 1 pmol of the [γ-^{32}P]ATP-labeled deoxyoligonucleotide, 5′-CTACTATACAACCTACTAC-3′. Extension products were separated by electrophoresis in a 15% denaturing polyacrylamide gel.
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23. Pre-*let-7* RNA was transcribed from a synthetic deoxynucleotide template with T7 RNA polymerase in the presence of α-^{32}P–labeled nucleotide, gel isolated, and incubated at ≤50 nM in a standard RNAi reaction as described (*21*). Northern hybridization experiments and controls were performed as described (*19*). Synthetic RNAs (*let-7* in Fig. 1B) (5′-GGCAAAUUGAGGUAGUAGGUU-3′ and 5′-UAUACAAUGUGCUAGCUUUCU-3′; Dharmacon Research) were used to assess probe specificity in control hybridizations.
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35. Synthetic RNAs (Dharmacon) were deprotected according to the manufacturer's directions. The siRNA duplex targeting Dicer mRNA was prepared by annealing 5′-UGCUUGAAGCAGCUCUGGAdTdT-3′ and 5′-UCCAGAGCUGCUUCAAGCAdTdT-3′; the control siRNA duplex was formed by annealing 5′-CUUUAAGCUCCCUGAGCGUUU-3′ with 5′-ACGCUCAGGGAGCUUAAAGUG-3′. siRNAs were annealed as described (*25*). siRNA-mediated gene silencing was performed as described (*32*). Briefly, exponentially growing HeLa S3 cells were trypsinized and transferred to six-well plates. Cells (2 ml) were plated into each well (1×10^5 cells/ml). siRNAs were transfected at 18 nM concentration with LipofectAMINE 2000 reagent (Life Technologies) in media containing Dulbecco's modified Eagle's medium but lacking both serum and antibiotics. Untreated control cells received LipofectAMINE and buffer, but no siRNA. Two hours after transfection, the media were replaced with fresh media containing serum but no antibiotics. RNA was extracted 3 days after transfection. Transfection efficiency was determined by staining cells for β-galactosidase activity in a parallel transfection with a plasmid in which LacZ is driven by a cytomegalovirus promoter. Semiquantitative RT-PCR detection of human Dicer mRNA has been described previously (*34*). Actin was detected with the primers 5′-CGTGATGGTGGGCATGGGTCAG-3′ and 5′-CTTAATGTCACGCACGATTTCC-3′.
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