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Genes and Mechanisms Related to RNA Interference Regulate Expression of the Small Temporal RNAs that Control *C. elegans* Developmental Timing

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Summary

RNAi is a gene-silencing phenomenon triggered by double-stranded (ds) RNA and involves the generation of 21 to 26 nt RNA segments that guide mRNA destruction. In *Caenorhabditis elegans*, *lin-4* and *let-7* encode small temporal RNAs (stRNAs) of 22 nt that regulate stage-specific development. Here we show that inactivation of genes related to RNAi pathway genes, a homolog of *Drosophila Dicer* (*dcr-1*), and two homologs of *rde-1* (*alg-1* and *alg-2*), cause heterochronic phenotypes similar to *lin-4* and *let-7* mutations. Further we show that *dcr-1, alg-1,* and *alg-2* are necessary for the maturation and activity of the *lin-4* and *let-7* stRNAs. Our findings suggest that a common processing machinery generates guide RNAs that mediate both RNAi and endogenous gene regulation.

Introduction

In numerous organisms the introduction of dsRNA can induce the sequence specific posttranscriptional silencing of a corresponding gene (reviewed in Cogoni and Macino, 2000). The experimental application of dsRNA to induce gene silencing has been termed RNA interference or RNAi. Genetic studies have linked RNAi to transposon silencing in *C. elegans* (Ketting et al., 1999; Tabara et al., 1999), while a related posttranscriptional gene silencing (PTGS) mechanism, called cosuppression, has been linked to viral resistance in plants (reviewed by Baulcombe, 1999), raising the possibility that these phenomena represent a form of sequence-directed immunity.

A striking paradigm to emerge from the study of PTGS mechanisms in plants and animals is that of the small RNA guide that can direct an RNA-protein complex to a complementary target sequence. Several studies of PTGS have identified the guide molecule as a species of small RNA of approximately 22 nt, recently termed "small *i*nterfering RNAs" (siRNAs) (Hamilton and Baulcombe, 1999; Hammond et al., 2000; Zamore et al., 2000; Elbashir et al., 2001a). For example, in *Drosophila* cell culture, small RNAs of approximately 22 nt copurify with and provide sequence specificity to an RNase complex that degrades the target mRNA (Hammond et al., 2000). Furthermore, small synthetic dsRNAs of 22–26 nt are sufficient to direct destruction of complementary RNAs both *in vitro* and *in vivo* (Elbashir et al., 2001a; Parrish et al., 2000), and duplexes of small 21 nt RNAs have recently been shown to suppress gene expression in cultured mammalian cells (Elbashir et al., 2001b).

It is tantalizing that the size of the siRNAs implicated in RNAi is similar to the approximately 22 nt size of the *lin-4* and *let-7* small temporal RNAs (stRNAs) that regulate *C. elegans* developmental timing (Lee et al., 1993; Reinhart et al., 2000). The *lin-4* stRNA triggers the transition from larval stage one to larval stage two, whereas the *let-7* stRNA controls a later larval to adult transition. stRNAs induce developmental progression by negatively regulating the expression of proteins encoded by mRNAs whose 3′ untranslated regions (3′UTRs) contain sites complementary to the stRNAs (Lee et al., 1993; Wightman et al., 1993; Moss et al., 1997; Reinhart et al., 2000; Slack et al., 2000). For example, LIN-14 protein level decreases late in larval stage one when *lin-4* stRNA is expressed (Feinbaum and Ambros, 1999; Olsen and Ambros, 1999), and LIN-41 diminishes at later larval stages as *let-7* stRNA appears (Slack et al., 2000). Probable orthologs of both *let-7* and its target gene, *lin-41*, have been found in numerous metazoans, including humans (Pasquinelli et al., 2000; Slack et al., 2000); because the temporal regulation of the *let-7* orthologs is also conserved, temporal control of development by this 22 nt stRNA may be ancient (Pasquinelli et al., 2000).

Although the 22 nt forms of the *let-7* and *lin-4* RNAs are more abundant, low levels of larger transcripts of approximately 70 nt can also be detected for each gene (Lee et al., 1993; this paper). These larger forms are predicted to fold into similar stem-loop structures. Human and *Drosophila let-7* orthologs are also expressed as larger forms that, likewise, have the potential to fold into stable stem-loop structures (A.E.P. and G.R., unpublished observations), suggesting that this potential secondary structure may have functional importance (Pasquinelli et al., 2000). The existence of possible dsRNA precursors and the 22 nt size of the *lin-4* and *let-7* stRNAs has fueled speculation about possible

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mechanistic similarities with RNAi (Pasquinelli et al., 2000; Sharp, 2001).

The Dicer protein has been implicated in RNAi in *Drosophila* where it appears to function in the processing of longer dsRNAs into the siRNAs which subsequently guide mRNA destruction (Bernstein et al., 2001). Dicer belongs to a conserved family of proteins, whose members contain a helicase domain, one or two dsRNA binding domains, and two RNase type III domains (Bass, 2000; Cerutti et al., 2000; Bernstein et al., 2001). Also present in Dicer family members is a PAZ domain (Cerutti et al., 2000), which was identified in the Piwi/Argonaute/Zwille/RDE-1 family of proteins introduced below. The *Arabidopsis* ortholog of Dicer, Carpel Factory (caf 1), is required for normal plant development (Jacobsen et al., 1999), but has not yet been shown to play a role in PTGS mechanisms.

Genetic studies in *C. elegans* have identified several genes essential for RNA interference (Tabara et al., 1999; Ketting et al., 1999). Probable null mutations in *rde-1* (for *R*NAi *de*fective) cause a complete lack of RNAi but no other discernible phenotypes (Tabara et al., 1999). *rde-1* encodes a 1020 amino acid protein that is a member of a large family of proteins found in a wide range of eukaryotes. Members of the RDE-1 family have two conserved domains of unknown biochemical function. The 300 amino acid PIWI domain located in the C-terminal region of these homologs shows the highest degree of sequence conservation (Cox et al., 1998; Cerutti et al., 2000). The 110 amino acid PAZ domain is located N-terminal to the PIWI domain and is also found in the Dicer family of proteins. RDE-1 homologs in the fungus, *Neurospora*, and the plant, *Arabidopsis*, have also been implicated in PTGS mechanisms (Catalanotto et al., 2000; Fagard et al., 2000) suggesting that RDE-1 family members not only share conserved structures but also have conserved functions in gene silencing in three kingdoms of eukaryotic organisms.

Mutations in *rde-1* homologs have also been shown to have developmental consequences. For example, in *Drosophila*, the *ago1* gene is required for embryogenesis (Kataoka et al., 2001), the *piwi* gene is required for the maintenance of the germline stem cell population (Cox et al., 1998), and *aubergine* is required for the proper expression of the germline determinant Oskar (Wilson et al., 1996). Additionally, *aubergine* (also known as *Sting*) has been implicated in the PTGS-like suppression of the repetitive *Stellate* locus in the *Drosophila* germline (Schmidt et al., 1999). In *Arabidopsis* two very similar genes, *argonaute (ago1)* and *pinhead/zwille*, are required for stem cell patterning of the plant meristem (Bohmert et al., 1998; Moussian et al., 1998; Lynn et al., 1999). *argonaute* is also necessary for PTGS in *Arabidopsis* (Fagard et al., 2000). The *C. elegans* genome contains 23 homologs of *rde-1* including orthologs of both *piwi* and *ago1*. Previous studies have shown that the *C. elegans piwi* and *ago1* orthologs have germline and possibly additional developmental functions (Cox et al., 1998; Cikaluk et al., 1999). The pleiotropic nature of the defects associated with loss-of-function mutations in members of this family could reflect discrete regulatory functions in numerous developmental events or alternatively might reflect a more general misregulation of silencing mechanisms that are necessary to insure proper stem cell maintenance and differentiation.

In this paper we provide evidence for the involvement of RNAi related genes and mechanisms in the expression and activity of the stRNA genes, *lin-4* and *let-7*. We show that the activities of two *C. elegans* homologs of *rde-1*, *alg-1* and *alg-2*, are essential for the proper function of the *lin-4* and *let-7* stRNA pathway. *alg-1* and *alg-2* activities are necessary for efficient processing of the *lin-4* stRNA but may be less important for *let-7* stRNA expression. Further, we demonstrate that the *C. elegans* ortholog of *Drosophila* Dicer, *dcr-1*, is an essential gene and is required for both RNAi and for processing of the *lin-4* and *let-7* precursor RNAs. Inhibition of *dcr-1* or *alg-1* and *alg-2* causes heterochronic phenotypes that are consistent with their effects on *lin-4* and *let-7* RNA processing. These findings suggest that natural dsRNAs are processed into small regulatory RNAs via a mechanism analogous to that involved in processing the double-stranded RNAs that trigger RNAi. Thus, a common processing machinery may produce natural small guide RNAs that regulate the activities of endogenous mRNA targets as well as the small interfering RNAs implicated in RNA interference and viral surveillance.

Results

alg-1 and *alg-2* Function in Embryogenesis and Larval Development

There are 23 *C. elegans* homologs of *rde-1* (Figure 1). cDNA clones for 14 *rde-1* homologs, indicated by asterisks in Figure 1, were tested for developmental functions by RNAi (see Experimental Procedures). dsRNAs derived from two closely related genes, F48F7.1 and T07D3.7, which we have named *alg-1* and *alg-2* (for *arg*onaute *l*ike *g*enes), induced developmental phenotypes in the progeny of injected animals, including a tendency to burst at the vulva (Figure 2A), and a lack of the adult specific alae, longitudinal stripes that run the length of the cuticle on both sides of the adult animal (Figure 2E). In addition these dsRNAs induced incompletely penetrant slow growth and germline abnormalities (Figure 2D and data not shown). The other 12 genes assayed did not exhibit discernable developmental phenotypes.

The *alg-1* and *alg-2* DNA sequences are 80% identical at the nucleotide level, suggesting a recent duplication of these genes, although they map to distinct chromosomes. This level of similarity is within the range where partial cross-interference is expected in RNAi assays (Parrish et al., 2000; Schubert et al., 2000). To target only *alg-1* or *alg-2*, we prepared dsRNAs from short 5′ unique segments of each gene (see Experimental Procedures). The dsRNA prepared from the unique segment of *alg-1* produced the same vulval bursting phenotype, although at a reduced frequency relative to that observed with longer dsRNAs (data not shown). No RNAi phenotype was observed after injections of the unique segment of *alg-2*.

We obtained a deletion allele of *alg-2* from the *C. elegans* gene knockout consortium. This allele, *alg-2(ok304)*, is an out-of-frame deletion that removes the nucleotides encoding amino acids 34–374, including the

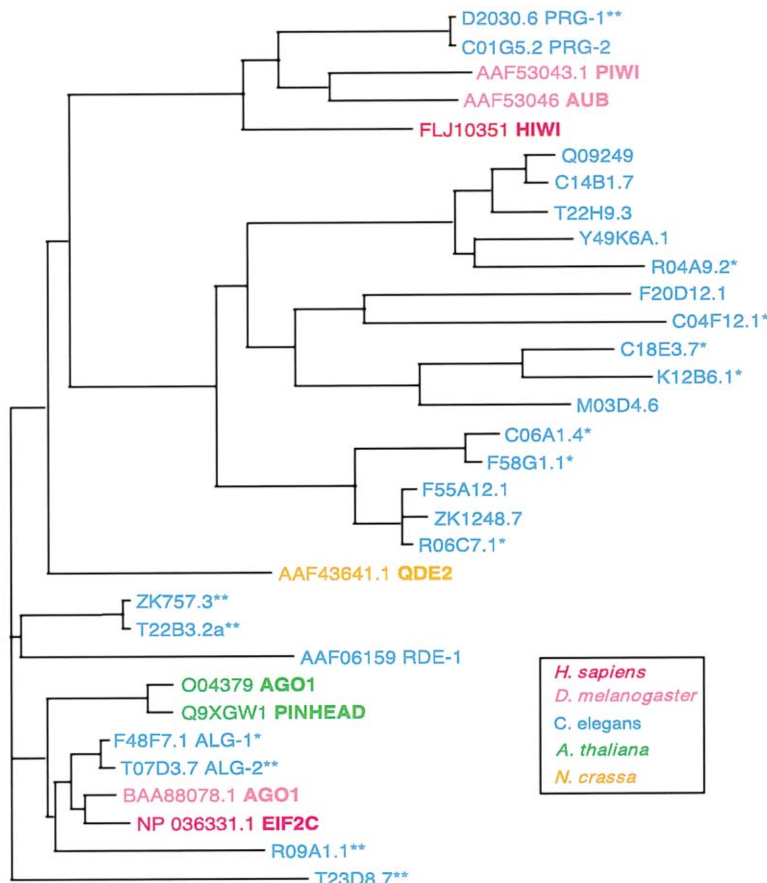


Figure 1. Phylogenetic Tree Grouping the RDE-1/AGO1/PIWI Protein Family Members

The PIWI domain defined by Cerutti et al., 2000 was used for alignment by CLUSTAL W (Thompson et al., 1994) and tree building using GrowTree in the Genetics Computer Group (GCG) Program. The asterisk indicates that RNAi was performed on this gene and the double asterisk indicates that, in addition, dsRNA was coinjected with *alg-1* dsRNA (see Results).

PAZ domain, and terminates after encoding 8 additional amino acids from reading frame two (Figure 2H). It is therefore likely to be a null allele of *alg-2*. The RNAi experiments above suggest that *alg-2* may be a nonessential gene, and consistent with this finding the *alg-2(ok304)* homozygotes are viable and show, at most, subtle defects in fertility and development (data not shown).

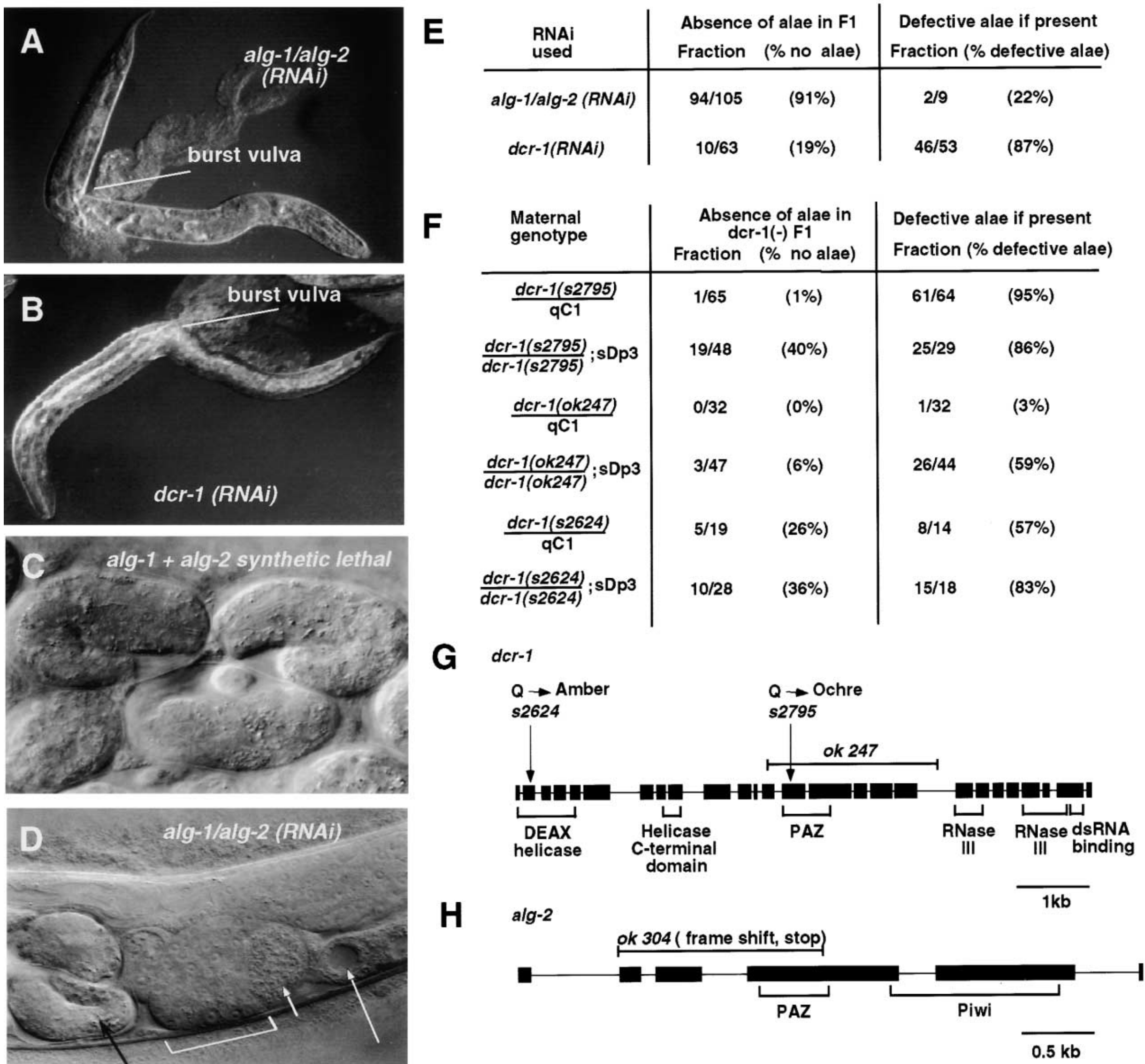
We next asked if *alg-1* and *alg-2* might have overlapping functions by coinjecting dsRNAs prepared from both genes and by injecting *alg-1* dsRNA into *alg-2(ok304)* homozygotes. Consistent with a shared function, coinjection of *alg-1* and *alg-2* dsRNAs caused enhanced larval lethality and also induced an embryonic lethal phenotype (Figure 2C, and data not shown). Injection of *alg-1* dsRNA into *alg-2(ok304)* homozygous animals resulted in a fully penetrant embryonic lethal phenotype identical to that observed in the double RNAi experiment (Figure 2C, and data not shown). No such synergy was observed when *alg-1* dsRNA was injected with dsRNAs prepared from other *rde-1* family members (Figure 1, double asterisks, see Experimental Procedures). These findings indicate that *alg-1* and *alg-2* have overlapping functions in both embryogenesis and larval development. Efficient induction of the larval developmental phenotypes described below required the injection of full-length *alg-1* dsRNA, a procedure that appears to partially inhibit *alg-2*. Therefore, we refer to animals produced in such experiments as "*alg-1/alg-2*" RNAi animals.

Finally, we assayed *alg-1* and *alg-2* for possible roles in RNAi. The *alg-2(ok304)* homozygotes were fully sensitive to RNAi, and likewise the inhibition of *alg-1* or *alg-2* by RNAi did not suppress RNAi targeting a second gene (data not shown). These findings suggest that *alg-1* and *alg-2* are not necessary for RNAi. Nevertheless, it remains possible that these genes might have some redundant function in RNAi with *rde-1* or with other members of this gene family (see Discussion).

C. elegans dcr-1 Functions in Development and RNAi

The *C. elegans* gene K12H4.8, which we have named *dcr-1*, is predicted to encode a protein related to the *Drosophila* Dicer (Bernstein et al., 2001) and the *Arabidopsis* Carpel Factory (Jacobsen et al., 1999) proteins implicated in RNAi and regulation of development, respectively. A previous study has shown that RNA interference of *Drosophila Dicer* can induce a partial loss of RNAi (Bernstein et al., 2001). We used RNAi of *C. elegans dcr-1* to assess its role in developmental control and RNA interference. *dcr-1(RNAi)* induced developmental abnormalities during larval growth that were very similar to those induced by *alg-1/alg-2(RNAi).* These included a protruding and non-functional vulva, and a tendency to burst at the vulva shortly after the molt from the larval to the adult stage (Figure 2B). In addition, *dcr-1(RNAi)* animals frequently exhibited faint or missing adult-specific alae (Figure 2E).

Although the phenotypes induced by *dcr-1(RNAi)*



RNAi used	Absence of alae in F1 Fraction	(% no alae)	Defective alae if present Fraction	(% defective alae)
alg-1/alg-2 (RNAi)	94/105	(91%)	2/9	(22%)
dcr-1(RNAi)	10/63	(19%)	46/53	(87%)

Maternal genotype	Absence of alae in dcr-1(-) F1 Fraction	(% no alae)	Defective alae if present Fraction	(% defective alae)
dcr-1(s2795) / qC1	1/65	(1%)	61/64	(95%)
dcr-1(s2795) / dcr-1(s2795) ;sDp3	19/48	(40%)	25/29	(86%)
dcr-1(ok247) / qC1	0/32	(0%)	1/32	(3%)
dcr-1(ok247) / dcr-1(ok247) ;sDp3	3/47	(6%)	26/44	(59%)
dcr-1(s2624) / qC1	5/19	(26%)	8/14	(57%)
dcr-1(s2624) / dcr-1(s2624) ;sDp3	10/28	(36%)	15/18	(83%)

Figure 2. Genetic and RNAi Analysis of *dcr-1* and *alg-1/alg-2*

(A and B) Burst vulva phenotype among young adult animals after (A) *alg-1/alg-2 (RNAi)* induced by injection of dsRNA prepared from the partial *alg-1* cDNA yk403g7, that contains short regions of perfect nucleotide identity with *alg-2*, and (B) *dcr-1(RNAi)*.

(C) Nomarski image of three embryos arrested at the 2- to 3-fold stage of morphogenesis after simultaneous injection of both *alg-1* and *alg-2* dsRNAs (see Results).

(D) An adult animal with germline defects induced by *alg-1/alg-2(RNAi)*; undifferentiated germline cells (bracket) are observed more proximal to the uterus (black arrow) than are the sperm (short arrow) and oocytes (long arrow); approximately 50% of F1 RNAi animals exhibit this defect in one or both gonad arms.

(E and F) Cuticle defects among animals obtained from (E) RNAi targeting *dcr-1* and *alg-1/alg-2* and from (F) various *dcr-1* mutant strains.

(G and H) Schematic box and line diagrams indicating the exon-intron structure, conserved domains, and the lesions in (G) the predicted *dcr-1* gene, and (H) the predicted *alg-2* gene.

were similar to those induced by *alg-1/alg-2(RNAi), dcr-1(RNAi)* phenotypes were less penetrant. For example, 91% of the *alg-1/alg-2(RNAi)* animals lack the adult-specific alae while only 19% of the *dcr-1(RNAi)* animals completely lack the alae (Figure 2E). This finding could indicate that *dcr-1* has only a relatively minor role in the specification of the alae; alternatively, it might reflect a difficulty in inhibiting *dcr-1* function via RNAi. For example, if *dcr-1* is required for RNAi in *C. elegans* as it appears to be in *Drosophila*, then the use of RNAi to target *dcr-1* may, at best, diminish its activity.

We therefore compared the *dcr-1(RNAi)* phenotype to the phenotype of animals homozygous for mutations in *dcr-1*. We obtained three noncomplementing mutant strains that define the *dcr-1* locus. Two of these, *let-740(s2624)* and *let-740(s2795),* were identified in an extensive genetic screen for mutations balanced by the free duplication *sDp3* (Stewart et al., 1998). The third allele, *dcr-1(ok247),* was made by the *C. elegans* gene knockout consortium. The *let-740(s2624)* and *let-740(s2795)* mutations result in premature stop codons while *dcr-1(ok247)* is an out-of-frame deletion allele re-

moving residues 708 through 1321 (Figure 2G) and terminating after expression of 15 amino acid residues from intronic sequences. All of these lesions are likely to severely disrupt DCR-1 protein expression; the *s2624* allele would encode a protein of only 59 amino acids lacking all of the recognizable functional motifs, while the latter two alleles would encode truncated proteins lacking the PAZ, RNase III, and dsRBP domains. All three mutant *dcr-1* strains exhibit a similar, fully penetrant, sterile phenotype. Homozygous hermaphrodites produce germ cells, including both sperm and oocytes, but for unknown reasons fail to produce embryos. In addition, all three strains exhibit adult cuticle and vulval defects identical to the defects induced by *dcr-1(RNAi)*, including a protruding vulva and occasional vulval bursting as well as faint or missing alae (Figure 2F, and data not shown). Because the *let-740* mutations are allelic to *dcr-1(ok247),* we will henceforth use the more descriptive name, *dcr-1,* to refer to this gene.

The severity of the phenotypes observed in the *dcr-1* homozygous mutants was dependent on the maternal genotype, suggesting that *dcr-1(+)* activity is provided maternally (Figure 2F). If *dcr-1(+)* activity is provided maternally, then RNAi of *dcr-1* into a *dcr-1* heterozygous mother might be expected to enhance the cuticle defects or cause additional phenotypes in the homozygous mutant progeny of the injected animal. Consistent with this possibility, the homozygous mutant class of progeny from *dcr-1* heterozygous mothers injected with *dcr-1* dsRNA arrested as embryos at a developmental stage similar to that observed in the double RNAi targeting *alg-1* and *alg-2* (Figure 2C, see Experimental Procedures). These findings suggest that maternal *dcr-1(+)* activity rescues essential functions of *dcr-1* in the homozygous embryos and larvae and that RNAi of *dcr-1* depletes this maternal activity. Because RNAi of *dcr-1* efficiently inhibits *dcr-1* activities required for larval development without inducing sterility or embryonic lethality, we use *dcr-1(RNAi)* for the subsequent developmental studies described here.

Finally, we asked if homozygous *dcr-1* mutants were sensitive to RNAi. The conceptually straightforward experiment of assaying RNAi in the complete absence of *dcr-1* is, unfortunately, not feasible since *dcr-1* is required for viability of the animal. The best experiments that can be done are to assay for sensitivity to RNAi in animals where *dcr-1* activity has been decreased. We first tested *dcr-1(ok247)* homozygous animals for sensitivity to dsRNA delivered by injection into their mother or directly into the homozyous L4 larvae. In both assays we observed nearly normal levels of RNAi (data not shown). This observation could indicate that maternal *dcr-1(+)* activity can rescue RNAi in *dcr-1* homozygous mutant progeny just as it appears to rescue the developmental and alae defects described above. Consistent with this idea, other RNAi pathway mutants including *rde-1* and *rde-4* homozygotes are strongly rescued by one maternal dose of *rde(+)* activity (Tabara et al., 1999; and data not shown). Because dsRNA targeting *dcr-1* induces strong larval developmental defects, we next asked if *dcr-1(RNAi)* might sufficiently reduce *dcr-1* activity to cause an RNAi-deficient phenotype. For this assay, we injected *dcr-1* dsRNA into adult hermaphrodites and then assayed for sensitivity to RNAi targeting a second gene. In experiments targeting two different genes, we observed a significant reduction of RNAi among the progeny of *dcr-1(RNAi)* animals but not among control animals injected with unrelated dsRNAs (Table 1). These results support the findings from Bernstein et al. (2001) that implicate *Drosophila* Dicer in RNAi and suggest that DCR-1 may have a similar activity in *C. elegans.*

Table 1. Reduced Sensitivity to RNAi in *dcr-1* (RNAi) Background

dsRNA I	dsRNA II	Resistance to dsRNA II
		non-Unc
none	*unc-22*	0% (n = 434)
mes-2	*unc-22*	0% (n = 824)
dcr-1	*unc-22*	29% (n = 604)
		non-Sqt
none	*sqt-3*	2% (n = 51)
gfp	*sqt-3*	6% (n = 67)
dcr-1	*sqt-3*	89% (n = 309)

dcr-1(RNAi) and *alg-1/alg-2*(RNAi) Cause Retarded Heterochronic Defects

The combination of vulval and adult cuticle maturation defects caused by RNAi of *alg-1/alg-2* and *dcr-1* is reminiscent of phenotypes resulting from mutations in the genes *lin-4* and *let-7* (Lee et al., 1993; Reinhart et al., 2000). The *lin-4* and *let-7* genes promote transitions from earlier to later cell fates and, thus, mutations in these genes cause reiteration of cell divisions typical of earlier larval stages, a hallmark of genes that regulate developmental timing (such genes have been termed "heterochronic genes"). For example, loss-of-function mutations in *let-7* result in a failure of larval seam cells in the hypodermis to progress to the adult-specific program of terminal differentiation indicated by the production of the adult-specific alae and, instead, the cells repeat the late larval type of divisions. These reiterated divisions contribute to an unstable vulval structure and failure to form a cuticle with adult alae.

We determined that the developmental defects in *alg-1/alg-2* and *dcr-1* RNAi animals also result from temporal misspecifications in the seam cell lineages. To aid in the observation of seam cell divisions, we utilized a transgenic strain that drives GFP expression specifically in the seam cell nuclei (see Experimental Procedures). Normally, the ten seam cells present at hatching divide to generate 16 cells during the second larval stage. Although these 16 cells divide at the succeeding third and fourth larval transitions, only one daughter cell maintains the seam cell fate (Sulston and Horvitz, 1977), so that the total number of GFP-expressing seam cells in the adult is 16 (Figure 3A).

RNAi of either *dcr-1* or *alg-1/alg-2* resulted in adults with extra seam cells (Figures 3B and 3C) that arise from reiterated L2 type divisions. This observation is specific for RNAi of *dcr-1* or *alg-1/alg-2* because control RNAi of *mes-2*, a gene not involved in developmental timing, did not affect the seam cell division pattern (data not shown). Most progeny of *dcr-1* and *alg-1/alg-2* dsRNA-injected parents had normal seam cell divisions until the L3 stage, when reiterations of L2 type divisions were common. Many animals showed mixed patterns of stage-specific divisions, a phenotype similar to that ob-

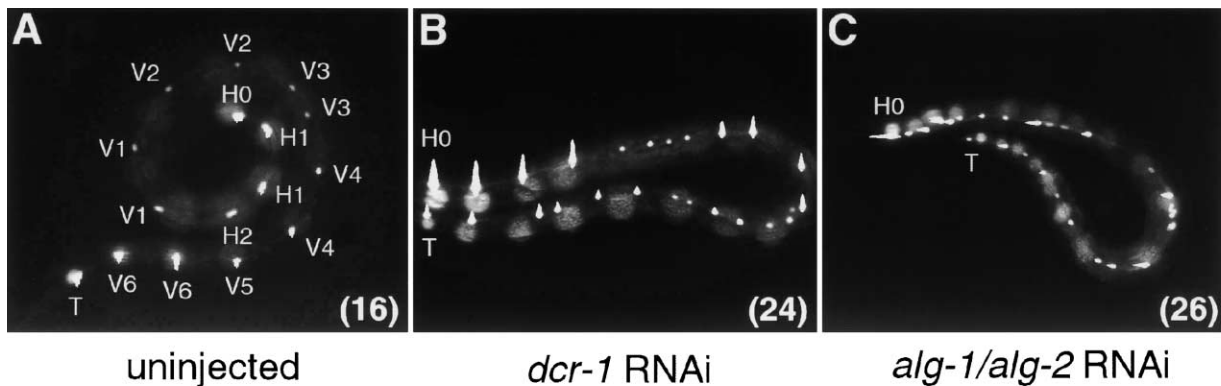


Figure 3. Extra Seam Cells in *dcr-1(RNAi)* and *alg-1/alg-2(RNAi)* Animals

A strain carrying a nuclear localized GFP reporter, expressed specifically in the lateral seam cells, was injected with *dcr-1* or *alg-1* dsRNA. The GFP-positive seam cell nuclei were counted in adult progeny of the injected animals. The 16 normal seam cells are indicated by name for (A) the uninjected control. (B and C) The anterior H0 and posterior T cells are indicated for (B) the *dcr-1(RNAi)* animal and (C) *alg-1/alg-2 (RNAi)* animal. The number of seam cells present is indicated in parentheses.

served previously in heterochronic mutants (*daf-12*, for example; Antebi et al., 1998). The number of seam cells observed in *dcr-1(RNAi)* adults ranged from 16 to 33 with an average of 21, and only 15% showed the normal number of 16 seam cells (n = 52); *alg-1/alg-2(RNAi)* adults exhibited 18–36 seam cells with an average of 25 (n = 81). The *dcr-1* and *alg-1/alg-2 (RNAi)* progeny also repeated L3 or L4 seam cell division programs into adulthood, when normally these cells would stop dividing and become terminally differentiated (data not shown).

We consistently observe inappropriate seam cell division patterns in L3 through later stages in *dcr-1(RNAi)* and *alg-1/alg-2(RNAi)* animals. However, because of the likely incomplete RNAi of *dcr-1* and the redundancy of *alg-1* and *alg-2,* it is not possible to establish the precise point in larval development where these genes are first required. Additional support that these genes may act earlier in larval development comes from the seam cell division pattern displayed by the more strongly affected animals obtained by coinjecting dsRNAs targeting portions of both *alg-1* and *alg-2* . In these experiments, reiterations of L1-type divisions were observed, in addition to repetition of later stage patterns (data not shown).

dcr-1 and *alg-1/alg-2* Regulate Stage-Specific Gene Expression

The similarity of phenotypes described above to those of the heterochronic genes *lin-4* and *let-7* raised the possibility that *alg-1*, *alg-2*, and *dcr-1* might act upstream of the *lin-4* or *let-7* stRNAs or might be necessary for their regulatory activities. The targets of *lin-4* and *let-7* include the *lin-14* and *lin-41* mRNAs. Genetic studies suggest that *lin-4* and *let-7* stRNAs directly regulate *lin-14* and *lin-41* through complementary sequences in their 3′UTRs (Lee et al., 1993; Wightman et al., 1993; Slack et al., 2000; Reinhart et al., 2000). Because the retarded phenotypes of *lin-4* and *let-7* are caused in part by failure to downregulate their target genes, mutations in *lin-14* and *lin-41* partially suppress the *lin-4* and *let-7* mutant phenotypes (Ambros, 1989; Reinhart et al., 2000; Slack et al., 2000). To determine if *alg-1/alg-2* and *dcr-1* RNAi animals exhibit a similar genetic relationship with *lin-14* and *lin-41* mutants, we performed dsRNA injections in the *lin-14* and *lin-41* mutant backgrounds. We found significant suppression of the RNAi-induced *alg-1/alg-2* and *dcr-1* heterochronic phenotypes, including alae and vulval defects, by the *lin-14(n179)* and *lin-41(ma104)* nonnull mutations (Figure 4). In addition, the penetrant germline phenotype associated with *alg-1/alg-2(RNAi)* was partially suppressed by the *lin-41* and *lin-14* mutations (Figure 4, see also Figure 2D), but the synthetic lethal phenotype associated with double *alg-1/alg-2(RNAi)* was not suppressed (data not shown). In control RNAi experiments, the *lin-14* and *lin-41* mutant strains were fully sensitive to RNAi. These findings are consistent with the idea that the retarded heterochronic phenotypes induced by *alg-1/alg-2* and *dcr-1 (RNAi)* are caused, at least in part, by misregulation of *lin-14* and *lin-41*.

Elements in the 3′UTRs of *lin-14* and *lin-41* mRNAs are responsible for negative regulation mediated by the *lin-4* and *let-7* stRNAs. If *alg-1*, *alg-2*, and *dcr-1* are necessary for *lin-4* and *let-7* function, then we would expect misregulation of reporter genes that carry the *lin-14* and *lin-41* 3′UTR elements. To test for misregulation of the *lin-14* 3′UTR, we used a transgene containing a dominant mutation in the cuticle collagen gene *rol-6(su1006)* fused to the *lin-14* 3′UTR. In wild-type animals the *lin-14* 3′UTR downregulates the expression of the dominant *rol-6* reporter gene in a *lin-4*-dependent fashion, leading to a non-Rolling phenotype in 100% of animals bearing the transgene (n = 825). In contrast, 54% of *lin-4(e912)* animals bearing the same transgene exhibit a Rolling phenotype (n = 253). While injection of this strain with control *mes-2* dsRNA produced virtually no rolling progeny (n = 256), injection of *dcr-1* dsRNA caused rolling in half of the progeny (n = 296), indicating a marked interference with downregulation of the *rol-6/lin-14* 3′UTR reporter gene.

We tested *alg-1/alg-2(RNAi)* animals for misregulation of the *lin-41* 3′UTR by using a transgene bearing a LacZ::*lin-41*/3′UTR gene fusion, which is expressed early in larval development but then undergoes *let-7*-dependent downregulation prior to adulthood (Reinhart

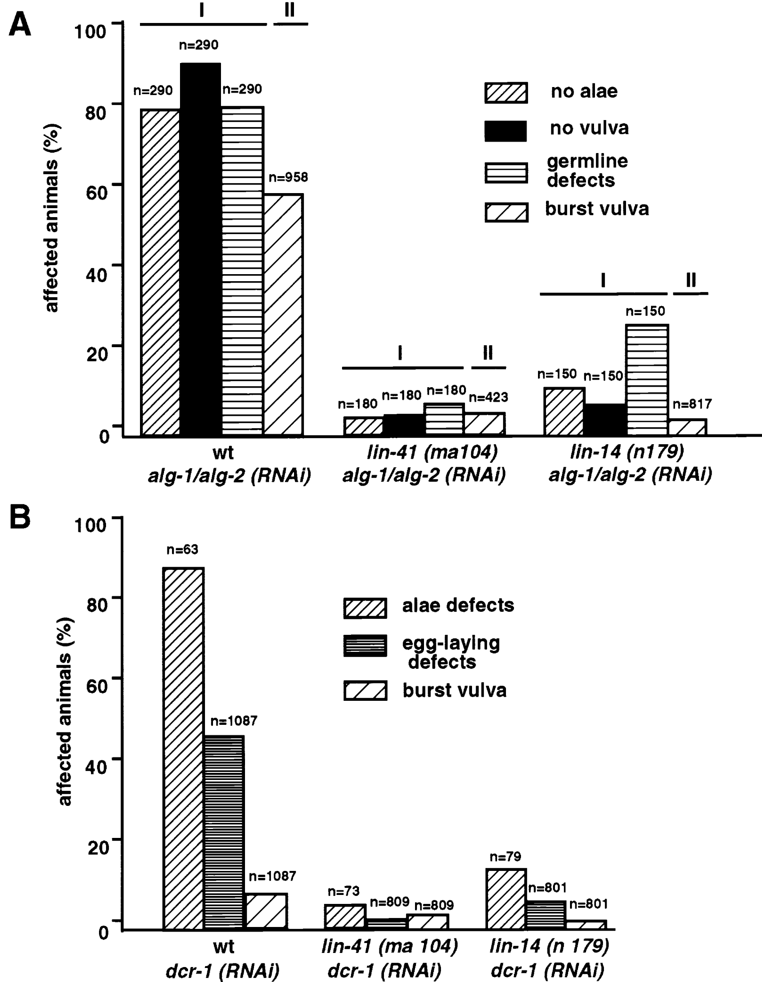


Figure 4. Genetic Suppression of *dcr-1* and *alg-1/alg-2(RNAi)* by *lin-41* and *lin-14* Mutants

(A and B) Adult progeny of injected wild-type, *lin-41(ma104)*, and *lin-14(n179)* animals were assayed for RNAi induced phenotypes as indicated. (A) *alg-1/alg-2(RNAi)* and (B) *dcr-1(RNAi)*. The number of animals scored (n) for each phenotype category is indicated. The alae, vulva, and germline were observed using the compound microscope, while egg-laying and vulval bursting phenotypes were scored in the dissecting microscope. Two different dsRNAs were used for *alg-1/alg-2(RNAi)*, indicated by the Roman numerals above the bars in (A): (I) Full-length *alg-1* dsRNA, used in the alae, vulva, and germline assays, induces a high percentage of vulvaless animals that preclude scoring the vulval bursting phenotype. Therefore, dsRNA prepared from (II) the *alg-1* partial cDNA clone yk403g7 was used to induce the weaker vulval bursting phenotype.

et al., 2000; Slack et al., 2000). Only 12% (n = 25) of control adult worms expressed LacZ from the *lin-41* 3′UTR fusion gene, while 48% (n = 23) of *alg-1/alg-2(RNAi)* adult animals expressed the fusion gene, consistent with derepression of the LacZ::*lin-41*/3′UTR gene fusion. This 4-fold increase in the number of adults expressing the LacZ::*lin-41*/3′UTR gene fusion after *alg-1/alg-2(RNAi)* is similar to the effect of a *let-7(−)* mutation (Reinhart et al., 2000). The findings that reporter genes bearing the *lin-14* and *lin-41* 3′UTRs are upregulated by *dcr-1* and *alg-1/alg-2* inhibition, together with the observation that *lin-14* and *lin-41* mutations suppress the retarded heterochronic phenotypes caused by *dcr-1* and *alg-1/alg-2* RNAi, are consistent with the model that *dcr-1*, *alg-1*, and *alg-2* function in the *lin-4* and *let-7* pathway to regulate larval development.

dcr-1 and *alg-1/alg-2(RNAi)* Animals Exhibit Defects in stRNA Processing

lin-4 and *let-7* are expressed as longer, approximately 70 nt RNAs that are predicted to fold into structures containing regions of double-stranded RNA. Because *Drosophila* Dicer cleaves introduced dsRNAs into fragments of approximately 22 nt (Bernstein et al., 2001), we hypothesized that the heterochronic phenotypes caused by *dcr-1 (RNAi)* may be due to a defect in the processing of the larger, potentially dsRNA, forms of *lin-4* and *let-7* into the 22 nt stRNAs. To test this idea we collected progeny from mothers subjected to *dcr-1(RNAi)* and performed Northern blot analyses to monitor the size and abundance of the *lin-4* and *let-7* RNAs. Because *alg-1/alg-2 (RNAi)* causes a similar heterochronic phenotype but acts at an unknown step in the pathway, we also monitored *lin-4* and *let-7* processing in *alg-1/alg-2 (RNAi)* animals.

Both *dcr-1* and *alg-1/alg-2(RNAi)* animals exhibited a marked accumulation of the *lin-4* long form at both L3-L4 and adult stages (Figure 5A, upper panel). The same RNA preparations from the *dcr-1* or *alg-1/alg-2 (RNAi)* animals were probed for the expression of *let-7*. We found that, as with *lin-4*, *let-7* processing depends on *dcr-1* activity (Figure 5A, bottom panel) but, in contrast, did not appear to depend on *alg-1/alg-2* activity. We next monitored *lin-4* and *let-7* stRNA processing in *dcr-1(ok247)* homozygotes and in animals specifically depleted for either *alg-1* or *alg-2*. In this experiment RNAs prepared from each population were simultaneously probed for expression of *lin-4* and *let-7* RNA (Figure 5B). As with *dcr-1(RNAi)*, the *ok247* homozygotes exhibited a significant accumulation of both *lin-4* and *let-7* long forms (Figure 5B, lanes 3 and 4). A gene-specific dsRNA

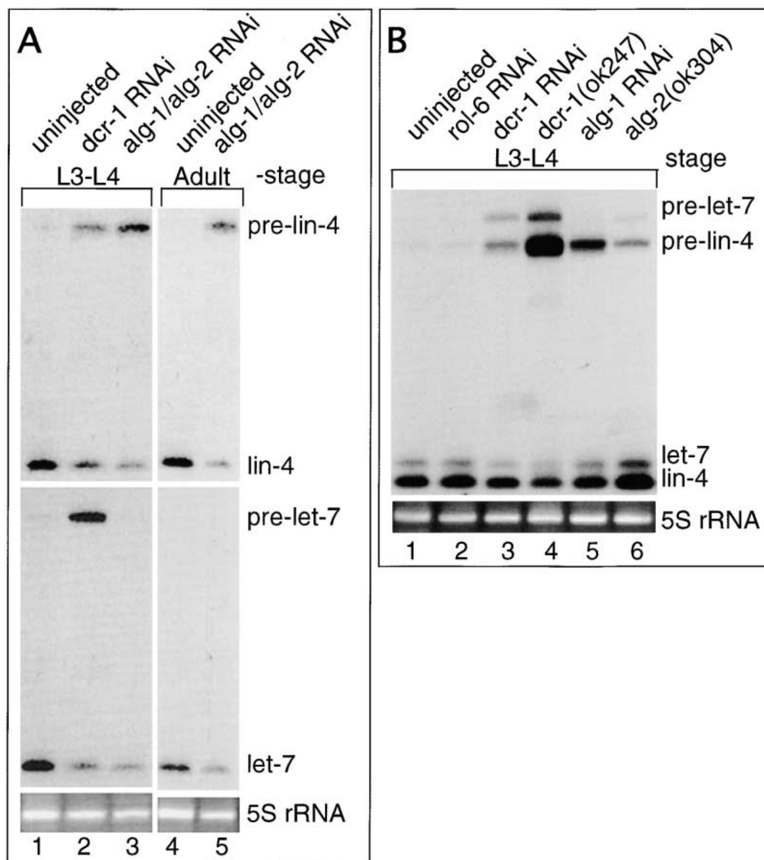


Figure 5. *dcr-1* and *alg-1/alg-2* Activities Are Required for Efficient Expression of *let-7* and *lin-4* stRNAs

(A and B) Northern blot of total RNA isolated from staged populations of worms as indicated. (A) The top panel shows a blot probed for *lin-4* RNA while the bottom panel shows the same blot after stripping and reprobing for *let-7* RNA. (B) A second experiment in which the blot was probed simultaneously to detect both *lin-4* and *let-7* RNAs. The precursor and mature forms of each stRNA are indicated. 5S rRNA serves as a loading control.

targeting *alg-1* induced accumulation of the pre-*lin-4* RNA but not pre-*let-7* (Figure 5B lane 4), and similarly, *alg-2(ok304)* animals exhibited a slight accumulation of pre-*lin-4* and little or no accumulation of pre-*let-7* (Figure 5B, lane 5).

The quantity of the short forms of the *lin-4* and *let-7* stRNAs consistently appeared to be reduced in RNA populations prepared from *alg-1/alg-2(RNAi)*, *dcr-1(RNAi)*, and *dcr-1(ok247)* animals (Figures 5A and 5B), while control RNA populations prepared from animals undergoing RNAi of the cuticle collagen gene *rol-6* exhibited normal levels of *lin-4* and *let-7* stRNAs (Figure 5B, compare lanes 1 and 2). This apparent reduction in *let-7* stRNA level was observed even in *alg-1/alg-2(RNAi)* populations where no significant accumulation of pre-*let-7* was observed. These findings suggest that *alg-1/alg-2* activities may be more important for the stability or function of *let-7* stRNA than for its processing from the larger form. Alternatively, *alg-1/alg-2* might also be involved in *let-7* processing but the *let-7* long form may be less stable, so that unprocessed *let-7* does not accumulate in the absence of *alg-1/alg-2* activity.

Discussion

RNAi and Natural Tiny RNAs

RNAi shares several features with developmental gene regulation mediated by the stRNA encoding genes *lin-4* and *let-7*. In RNAi, experimentally introduced double-stranded RNA is processed into small RNAs of approximately 22 nt. These small RNAs have been termed small interfering "siRNAs" because they appear to guide a nuclease in the destruction of complementary target mRNAs (Elbashir et al., 2001a). The developmental regulators, *lin-4* and *let-7*, are expressed as RNAs of approximately 70 nt that are predicted to fold into stable stem-loop structures that may be the precursors of the small temporal "stRNAs." Thus, the folded 70 nt *lin-4* and *let-7* RNAs may be analogous to the dsRNAs that trigger RNAi while the stRNA products may be analogous to the siRNAs that direct mRNA destruction. Genetic and molecular evidence presented here extend this analogy, linking stRNA production and function to a processing machinery and to regulatory proteins related to those that mediate RNAi. Specifically, we have shown that the efficient processing of the *lin-4* and *let-7* stRNAs from larger precursors depends on the activity of DCR-1, a *C. elegans* homolog of the *Drosophila* multifunctional RNase III related protein, Dicer, that has been shown in *Drosophila* cell extracts to process dsRNA into siRNAs that can mediate RNAi (Bernstein et al., 2001). Further, we have shown that *alg-1* and *alg-2*, two homologs of the RNAi pathway gene *rde-1*, are required for efficient stRNA expression, and along with *dcr-1* function to promote *lin-4* and *let-7* activities in temporal development. Thus, the expression of the tiny RNAs that mediate RNAi and developmental gene regulation appear to share a requirement for DCR-1 activity, while RDE-1 and its homologs provide parallel functions in these pathways (Figure 6). Our findings are consistent with a model in

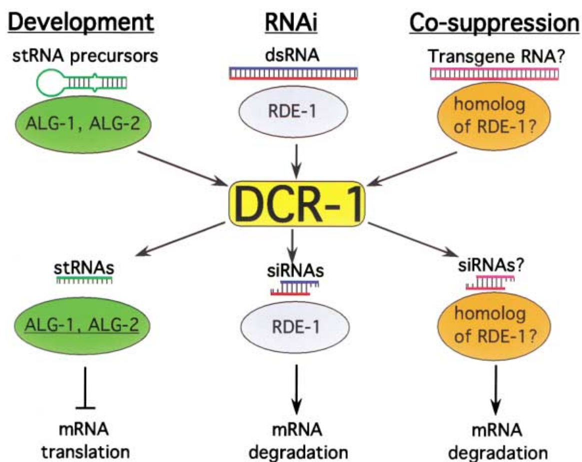


Figure 6. Model

which members of the RDE-1 and DCR-1 families act not only in gene silencing but also with naturally expressed dsRNAs to execute cellular and developmental gene regulatory events.

Differences between RNAi and stRNA Mechanisms

Although there are compelling similarities between RNAi and developmental regulation by *lin-4* and *let-7* there are also several important differences. In RNAi, the dsRNAs utilized, typically contain long stretches of perfect base pairing (Parrish et al., 2000). The stRNA precursors, however, are predicted to contain at most 6, for *lin-4*, and 13, for *let-7*, uninterrupted Watson-Crick base pairs. Whereas cleavage of the perfectly base-paired RNAs that initiate RNAi yields both sense and antisense, or potentially double-stranded siRNAs (Hamilton and Baulcombe, 1999; Hammond et al., 2000; Zamore et al., 2000; Elbashir et al., 2001a), only one strand of the *lin-4* and *let-7* stRNAs is detected (A.E.P. and G.R., unpublished observations). Thus, after generation of the mature stRNA, the remaining sequences must undergo rapid degradation.

The RNAi and stRNA pathways also appear to induce distinct outcomes: RNA destruction versus translation inhibition. In RNAi the target mRNA is rapidly degraded (Montgomery et al., 1998; Tuschl et al., 1999). Although the RNase responsible for target RNA destruction is not yet known, it is thought that the antisense strand of the siRNA acts as a guide in mRNA destruction, by base-pairing with the target mRNA. The stRNAs also specifically downregulate the expression of their target genes. Although details of the mechanism by which stRNAs cause decreased expression are unknown, the regulation of *lin-14* by *lin-4* occurs at the translational level. Upon expression of *lin-4* RNA, the levels of LIN-14 protein rapidly decline, but *lin-14* mRNA levels remain constant and appear to remain associated with polyribosomes (Wightman et al., 1993; Olsen and Ambros 1999). Because *let-7*-mediated regulation of LIN-41 protein expression may only occur in a subset of cells (Slack et al., 2000), it is, as yet, unclear if the mRNA levels or polyribosome loading of this target is affected by the expression of *let-7* RNA.

The distinction between mRNA destruction by RNAi and inhibition of translation by the *lin-4* regulatory RNA could reside in the target mRNA sequence or in the particular region of the mRNA targeted. Whereas siRNAs can target sequences anywhere in the mature mRNA, stRNAs pair with specific sites in the 3′UTRs of their target genes. And just as the precursors of the stRNAs have imperfect internal complementarity, the stRNAs contain imperfect complementarity to their target sequences. Imperfect pairing could permit access to RNA nucleotides by sequence-specific RNA binding proteins or conversely might reduce the affinity with which a nuclease could cleave the mRNA/stRNA hybrid. Alternatively, both siRNAs and stRNAs may induce similar modifications of their target mRNAs while flanking sequences provide for context dependent interactions that cause inhibition of translation in the case of *lin-14* but promote destruction of other mRNAs.

RDE-1 Family Members and Small RNA Cofactors in Development and PTGS

There are 24 members of the RDE-1/AGO1/PIWI family in *C. elegans* (Figure 1). The degree of conservation between certain members of this family is striking. For example, ALG-1 and ALG-2 exhibit 41% identity with AGO1 from *Arabidopsis* and 67%–69% identity with AGO1 relatives in animals. The common ancestor of worms and humans appears to have had both an AGO1 ortholog and a second, already-divergent family member that has given rise to the PIWI family of genes (Figure 1). The fact that divergent members of this family, including *rde-1*, *qde-2,* and *ago-1*, all function in gene silencing suggests that PTGS mechanisms represent an important ancestral function of genes within this family.

Developmental functions have also been reported for members of the *piwi* and *ago1* families in both animals and plants (Bohmert et al., 1998; Moussian et al., 1998; Cox et al., 1998; Cikaluk et al., 1999; Kataoka et al., 2001). One feature that emerges from studies of these developmental phenotypes is that many of these genes appear to regulate germ cell and stem cell functions. Perhaps germ cells and stem cells have developed PTGS mechanisms for suppressing viral and transposon pathogens that might otherwise degrade the genome and, thus, the totipotency of these cells. The developmental phenotypes associated with mutations in members of the *rde-1* gene family could thus reflect a general loss of gene silencing important for stem cell maintenance or differentiation. However, the findings reported here suggest an alternative possibility. We have shown that *rde-1*-related genes, *alg-1* and *alg-2*, function with natural small RNA cofactors in specific developmental gene regulation events. Thus, we speculate that the *Drosophila* genes *piwi*, *aubergine*, and *ago1*, the *Arabidopsis* gene *ago1*, and perhaps many other members of this family in *C. elegans* and other organisms may similarly have small endogenous RNA cofactors with which they function to regulate specific target mRNAs.

While there are 24 members of the *rde-1/Argonaute* gene family in *C. elegans*, there are fewer in *Arabidopsis*, humans, and *Drosophila*. Only the *Piwi* and *Argonaute* subtypes are conserved in many species, while RDE-1 as well as most of the other *C. elegans* family members

are more divergent. Perhaps the family of tiny RNAs that may act with these proteins has also undergone expansion in *C. elegans*. Whether the ancestral function of RDE-1-related genes was in developmental control or sequence-directed immunity, it is clear that a great potential exists for exploiting these proteins, along with small RNAs as guides, to direct the regulation of specific gene targets in the cell.

Previous work has indicated that RDE-1 plays an upstream role in the initiation of interference in response to dsRNA in *C. elegans* (Grishok et al., 2000). Findings described here suggest that ALG-1 and ALG-2 may play a similar upstream role in the *lin-4* and *let-7* stRNA pathways. Thus, distinct members of the extended family of RDE-1 homologs in *C. elegans* may play specific roles in RNAi and stRNA pathways. We speculate that one or more of the other *C. elegans* RDE-1 family members may provide a similar function in cosuppression in *C. elegans* (Figure 6). One attractive possibility is that these diversified factors provide specificity to their respective pathways. This might involve a role in the recognition of the distinct trigger sequences or in insuring that the processed small RNAs are assembled into distinct downstream complexes. Perhaps members of the RDE-1 family remain associated with the RNA sequences throughout processing and provide specificity needed to ensure that the small RNAs produced are targeted to the appropriate downstream complex, for example, to mediate mRNA destruction versus translation inhibition (Figure 6).

A role for RDE-1 family members in both small RNA production and targeting could explain why the inhibition of *alg-1/alg-2* induces such a dramatic effect on *lin-4* and *let-7* function while at best reducing but not eliminating the processed stRNA. Similarly, recent studies of small RNA accumulation during RNAi suggest that *rde-1* is not essential for small RNA production after exposure to dsRNA (S.P. and A.F.; A.G. and C.C.M., unpublished observations) and yet *rde-1(+)* activity is absolutely required for interference. Conceivably, dsRNA processing might still occur in the absence of RDE-1 or its homologs but the resulting siRNAs or stRNAs may not be assembled into the appropriate downstream complexes and therefore fail to function. Nevertheless, the finding that *alg-1/alg-2(RNAi)* dramatically affects the accumulation of the *lin-4* precursor supports a role for these factors either upstream of, or at the same step as DCR-1 (Figure 6).

The Role of DCR-1 in RNAi and stRNA Processing

We have shown that *dcr-1* is an essential gene and is also required for RNAi in *C. elegans*. In the model proposed above, *dcr-1*, which appears to be a single copy gene in *C. elegans,* could play a role in dsRNA processing important in many gene silencing and developmental pathways. DCR-1 has several motifs that might be expected in a dsRNA processing enzyme, including a helicase, a dsRNA binding domain, and two RNase III type dsRNA exonuclease domains. Thus, we propose that DCR-1 functions in multiple pathways important for developmental and PTGS mechanisms, and may be guided in its processing of distinct substrates by members of the RDE-1 family (Figure 6). Consistent with a relatively specific role for *dcr-1*, we found that mature ribosomal RNAs, which are also produced by RNase III type processing, accumulate to normal levels in animals with reduced *dcr-1* activity (data not shown).

The combination of a maternally provided *dcr-1* activity and zygotic sterility make it difficult to unambiguously answer the question of whether this protein is absolutely essential for RNAi and stRNA pathways. Nevertheless, the reiteration of L2 fates revealed by the seam cell lineage analysis of *dcr-1(RNAi)* animals, and the suppression of those phenotypes by mutations in *lin-14* or *lin-41* are unique phenotypic and genetic signatures that strongly support the model where *lin-4* and *let-7* processing is dependent on *dcr-1*(+) activity. Perhaps the embryonic and larval lethal phenotypes associated with *dcr-1* inhibition and the developmental phenotypes associated with the *Arabidopsis* homolog, *caf 1*, reflect a role for members of this gene family in the processing of other as yet unidentified small regulatory RNAs. Thus, tiny RNAs may function in a broader range of gene regulatory and developmental events than the temporal transitions mediated by the founding members of the class, the *lin-4* and *let-7* stRNAs.

A recent study (P. Zamore, personal communication) has shown that a human homolog of *dcr-1* is important for processing of the *let-7* stRNA precursor in cultured human cells, suggesting that the regulatory interactions observed in *C. elegans* are conserved. A prediction from the current study is that human *let-7* processing or function will also require a human member of the RDE-1/AGO1/PIWI family of genes. Indeed, it is likely that the ramified family of RDE-1/AGO1/PIWI related proteins has coevolved with numerous small RNA encoding genes analogous to *lin-4* and *let-7*, and that many such genes await discovery in plant and animal genomes.

Experimental Procedures

RNA Interference Assays

RNAi methods were as described in Grishok et al., 2000. cDNA clones and corresponding targeted genes were as follows: yk403g7, F48F7.1 (*alg-1*); yk199g3, yk433a5, T07D3.7 (*alg-2*); yk397e11, D2030.6 (*prg-1*); yk87d2, ZK757.3b; yk359b5, T22B3.2; yk36g4, R06C7.1; yk102d8, yk358e1, R09A1.1; yk20f1, T23D8.7; yk21a5, C16C10.3; yk448d6, R04A9.2; yk548f2, F58G1.1; yk249a12, C06A1.4; yk550c3, C18E3.7; yk307e2, K12B6.1; yk240e7, C04F12.1.

PCR was used to generate templates for dsRNA synthesis using primers with T7 promoter sequences and gene-specific sequences as follows: 5′-GGC GAT TCG CTG ACA TCG-3′ and 5′-GGC AAA ATA CAT GAC GTT GTT C-3′ for full-length *alg-2*, 5′-GGC GAT TCG CTG ACA TCG-3′ and 5′-GCA AAA TGA TTG GCT CGC A-3′ for unique 200 bp fragment of *alg-2*, 5′-GGC GG CCG CAA TAT TTG-3′ and 5′-GGT TCT CCA ATT GAG ACA CT-3′ for unique 300 bp fragment of *alg-1*. 5′-GGC TTT GCT TTC TTT GCT GCT-3′ and 5′-GGT AAT GAT GAT ATC TCT CCA CTT-3′ for *dcr-1*. 5′-G CCC AGC ACA TCA ACT CCC TCA GG-3′ and 5′-C ACC CCA ATT CGG TGC TCT CCG GCG-3′ for *mes-2*. The *alg-1* full-length dsRNA was made from the plasmid pCCM508.

Combinations of full-length *alg-1* dsRNA with *prg-1* dsRNA (yk397e11) or with dsRNAs corresponding to predicted genes T23D8.7, T22D3.2, and ZK757.3 did not result in phenotypes more severe than single *alg-1* (RNAi).

dcr-1(RNAi) of *dcr-1* Heterozygous Animals

Injections of *dcr-1* dsRNA were performed into animals heterozygous for *dcr-1(ok247)* and a GFP marked *hT2* balancer chromosome. This strain had 21% (n = 235) *dcr-1* GFP(−) adult homozygous animals. After injection of 13 *dcr-1(ok247)/hT2::GFP* mothers, zero GFP(−) animals were observed (n = 144). Embryonic lethalilty after

dcr-1(RNAi) was measured using the *dcr-1(ok247) /qC1* strain, which uninjected segregates 3% (n = 521) arrested embryos. After injection of *dcr-1* dsRNA, 12 *dcr-1*/qC1 mothers, produced 36% (n = 481) F1 embryos arrested at the 2-fold to 3-fold stage of morphogenesis.

Determination of Seam Cell Numbers

The SCM::GFP strain provided by Joel Rothman, which contains a nuclear localized GFP marker expressed specifically in seam cells, was injected with dsRNAs for *mes-2, dcr-1*, *alg-1*, and *alg-1*+*alg-2*. Populations of F1 progeny from uninjected controls and dsRNA injected animals were staged and representative animals were examined for seam cell number and division patterns by observing the GFP marker under UV light at the L1, L2, L3, L4, and adult stages.

Northern Analyses of *let-7* and *lin-4* RNAs

Homozygous mutant worms or F1 progeny of uninjected and dsRNA injected worms were collected at the L3-L4 and adult stages. Total RNA isolation and Northern analyses were performed as previously described in Lee et al., 1993 except hybridization and wash steps were performed at 50°C. Oligonucleotides used as Northern probes were *let-7*: 5′-AACTATACAACCTACTACCTCACCGGATCC-3′ and *lin-4*: 5′-ATAGTACACTCACACTTGAGGTCTCAGGG. 5S rRNA was detected by ethidium bromide staining of polyacrylamide gels prior to transfer.

Analysis of the *let-7* Regulated lacZ::*lin-41* 3′ UTR Reporter Gene

Worms containing the mgEx540 reporter array were injected with dsRNA against *alg-1* and transgenic progeny as well as uninjected controls were collected for fixing and staining with Xgal to detect expression of lacZ::*lin-41*.

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