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A Role for the RNase III Enzyme DCR-1 in RNA Interference and Germ Line Development in *Caenorhabditis elegans*

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Abstract

An early event in RNA interference (RNAi) is the cleavage of the initiating double-stranded RNA (dsRNA) to short pieces, 21 to 23 nucleotides in length. Here we describe a null mutation in *dicer-1* (*dcr-1*), a gene proposed to encode the enzyme that generates these short RNAs. We find that *dcr-1*(−/−) animals have defects in RNAi under some, but not all, conditions. Mutant animals have germ line defects that lead to sterility, suggesting that cleavage of dsRNA to short pieces is a requisite event in normal development.

In many organisms, dsRNA initiates a potent posttranscriptional gene-silencing phenomenon known as RNAi (1,2). First discovered in *Caenorhabditis elegans* in 1998 (3), RNAi is closely related to posttranscriptional gene silencing in plants (4–6), and quelling in fungi (7,8) and likely represents a conserved, ancient pathway. In *C. elegans*, a number of genes are known to be required for efficient RNAi (9–12). Among these, *rde-1* and *rde-4* act early in the pathway and, by an unknown mechanism, facilitate the formation of a heritable, extragenic agent that can transmit RNAi to offspring (9,13). Several genes required for RNAi also have important roles in other cellular processes. For example, *rde-2* and *mut-7*, in addition to acting downstream of *rde-1* and *rde-4*, are necessary for transposon silencing and cosuppression (10,14). A subset of the *smg* genes are important for the persistence of silencing by RNAi and are also involved in nonsense-mediated decay (12). Mutations in *ego-1*, which encodes a protein with homology to an RNA-dependent RNA polymerase, disrupt RNAi for some germ line genes and lead to defects in germ line development (11). Although these genes and others are clearly important for RNAi, as yet, their precise role in the pathway remains unknown.

An important clue in regard to the mechanism of RNAi came with the discovery that RNAi generates 21- to 23-nucleotide (nt) pieces (sense and antisense), called small interfering RNAs (siRNAs) (15–18). siRNAs originate from the initiating dsRNA and are required for targeting the cognate message for degradation. On the basis of the characteristics of these pieces and the requirements of RNAi, it was proposed that a protein with sequence similarity to the dsRNA nuclease, RNase III, as well as an RNA helicase, was involved in the process (2). In support of this idea, siRNAs have been shown to have 3′ overhangs, 2 nt in length, as expected of an RNase III–like activity (18). Further, immunoprecipitation of the *Drosophila* RNase III/helicase enzyme (called Dicer) from extracts yields an immunoprecipitate that can degrade dsRNA into siRNAs, and decreased Dicer levels in vivo correlate with decreased gene silencing (19).

To obtain direct evidence that Dicer is involved in gene silencing, we examined the effectiveness of RNAi in a *C. elegans* strain containing a null mutation in the Dicer homolog

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Note added in proof: While this paper was in press, Dicer was shown to be required for processing of small temporal RNAs (35,36).

(*dcr-1*). DCR-1 is encoded by an 8165– base pair (bp) gene in *C. elegans* and contains an NH_2-terminal DExH/DEAD-box type RNA helicase domain, two RNase III–like domains, and a COOH-terminal dsRNA binding motif (Fig. 1A). Animals with a deletion in *dcr-1* that removes a 2470-bp fragment spanning a region from exon 13 to intron 18 (Fig. 1A) were obtained from the *C. elegans* gene knockout consortium. The deletion removes the NH_2-terminal portion of the first RNase III domain and is also predicted to introduce multiple stop codons into the reading frame. We observed that *dcr-1*(−/−) animals were sterile, suggesting that DCR-1 has an essential role in vivo and also emphasizing that the deletion creates a loss-of-function allele.

In *C. elegans*, RNAi is typically initiated by injecting or feeding dsRNA, and gene silencing is subsequently observed in the F_1 progeny (3,20,21). Because *dcr-1*(−/−) animals were sterile and did not give rise to progeny, we used a transgenic line in which we could monitor RNAi in individual animals, without waiting for subsequent generations. The line was constructed by microinjecting DNA encoding green fluorescent protein (GFP) (*sur5::GFP*), as well as a previously described vector (22) containing an RNA hairpin matching the GFP sequence, under the control of a heat shock promoter [*hsp16–2*$_p$*GFP(IR)*]. In the transgenic line we isolated, heat shock produced an easily discernable RNAi phenotype in heat-shocked animals, so it was not necessary to analyze progeny (Fig. 1B). We mated our transgenic line with *dcr-1*(+/−) animals and examined *dcr-1*(−/−) progeny for RNAi resistance after heat shock. Whereas wild-type animals exhibited robust RNAi interference measured by a loss in GFP fluorescence (98% had substantially reduced GFP fluorescence, $n = 120$), animals homozygous for the *dcr-1* deletion were RNAi defective and continued to exhibit a strong fluorescence (Fig. 1B and Table 1). These results are consistent with the idea that *dcr-1* is required for RNAi.

Gene silencing by RNAi is known to involve the degradation of the targeted mRNA (16,23). To obtain molecular evidence that *dcr-1* is required for RNAi, as well as to monitor the effects of the *dcr-1* deletion on RNAi of other genes, we used semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) to measure mRNA levels after RNAi. When dsRNA corresponding to the *mpk-1* gene was injected into L4 worms, wild-type animals exhibited reduced *mpk-1* mRNA levels, whereas *mpk-1* mRNA remained abundant in *dcr-1*(−/−) animals (Fig. 2A). These results, and similar results with dsRNA to *gld-1* (24), again suggest that DCR-1 is required for RNAi. However, when animals were injected with dsRNA to *unc-54* (Fig. 2B), decreased levels of *unc-54* mRNA were observed in both wild-type and *dcr-1*(−/−) animals, consistent with a normal RNAi response.

In the above experiments, *dcr-1*(−/−) animals were resistant to RNAi of genes expressed in the germ line (*mpk-1* and *gld-1*) but showed a normal RNAi response for *unc-54*, a somatic gene. Thus, *dcr-1* may be similar to the genes *mut-7* and *rde-2* (9), or *ego-1* (11), which are required for RNAi of germ line expressed genes, but not for somatic genes. Consistent with this idea, we found that *dcr-1* mutants showed a normal RNAi response when dsRNA corresponding to another somatic gene, *unc-22*, was injected (78% of animals twitched in levamisole, $n = 23$). *dcr-1*(−/−) animals were also sensitive to RNAi when fed bacteria expressing *unc -22* dsRNA (100% of animals twitched in levamisole, $n = 22$).

We also noticed that the requirement of RNAi for the *dcr-1* gene depended on the method used to deliver the dsRNA. For example, *dcr-1* was required for RNAi of the GFP transgene by the heat shock–inducible RNA hairpin (Fig. 1B). However, when we injected *gfp* dsRNA into the *dcr-1*(−/−) animals carrying the GFP transgene, 100% of *dcr-1*(−/−) animals had reduced fluorescence ($n = 14$).

Our data indicate that *dcr-1*(−/−) animals are defective for RNAi in some but not all cases. Possibly, gene silencing by dsRNA can occur by multiple pathways, some that require DCR-1

and some that do not. Alternatively, the RNAi defects of *dcr-1*(−/−) animals may be partially rescued by maternal dicer (mRNA or protein) that persists in the F_1 progeny. However, if this is the case, the maternal DCR-1 must not be available, or sufficient, to rescue all RNAi.

To begin to understand the role of *dcr-1* in germ line development, we compared germ line morphology in wild-type and *dcr-1*(−/−) adult hermaphrodites using differential interference contrast microscopy and 4′,6′-diamidino-2-phenylindole (DAPI) epifluorescence staining (Fig. 3). Normally, in adult wild-type animals, the germ line develops in a defined and largely invariant manner. Moving from the distal region proximally, germ cells proliferate, enter meiosis, and differentiate into oocytes in the loop region and proximal gonad (25). Oocytes are fertilized as they are pulled through the spermatheca into the uterus (25). In *dcr-1*(−/−) animals, we do not observe any gross defects in chromosome morphology in the distal region of the gonad; however, several defects are seen in the proximal region. Elongated oocytes are found even before the loop of the gonad (Fig. 3B). Furthermore, as they migrate proximally, oocytes appear misshapen, lack clear delineation, and remain unfertilized. Nuclei in proximal oocytes also appear abnormal, often appearing nonspherical. DAPI staining reveals areas of intense staining in enlarged proximal nuclei suggestive of DNA replication without cell division (endomitosis; Fig. 3, C to E). We also observed vulval bursting in many, but not all, *dcr-1*(−/−) animals (Fig. 3B). The burst vulva phenotype is also observed in animals containing a mutation in *let-7*, which encodes a 21- to 22-nt RNA important for developmental timing (26,27). Because the mature *let-7* RNA is similar to the size of putative Dicer products and is thought to be processed from a base-paired hairpin, the burst vulva phenotype may indicate that *let-7* processing is defective in the *dcr-1* mutants. Taken together, the phenotypes of *dcr-1* (−/−) animals indicate that DCR-1 has multiple and important roles in vivo.

Biochemical evidence (19), combined with the presence of domains in *dcr-1* with clear homology to dsRNA nucleases, suggests that *dcr-1* itself encodes an enzyme responsible for cleaving dsRNA into siRNAs. Our studies show that DCR-1 is essential for RNAi under some conditions and plays an important role in germ line development. Another gene in *C. elegans*, *ego-1* (11), is also important for RNAi and normal germ line development. Certainly this is a clue in regard to the natural function of these genes, but further studies will be required to understand these functions. In regard to DCR-1, our favorite hypothesis is that this protein plays a role in degrading dsRNA and that the latter has important functions in the germ line. Sense and antisense transcripts have long been known to exist in germ line cells (28), and it is intriguing to speculate that these RNAs are part of a natural gene-silencing pathway, potent enough to require regulation, possibly by DCR-1.

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29. Wild-type or *dcr-1*(+/−) L2 progeny were cloned to individual plates, heat shocked on 2 consecutive days (33°C, 1 hour), and scored for GFP fluorescence and sterility on the following day. Before and between heat shock, all animals were maintained at 20°C.
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33. dsRNA was prepared by annealing equal amounts of sense and antisense RNAs in 50 mM Pipes (pH 6.7). RNAs were transcribed from PCR templates amplified from parent sequences with primers containing T7 or T3 promoter sequences. dsRNA (0.5 mg/ml) was injected into L4 hermaphrodites.
34. Total RNA was isolated as described (*12*) 48 hours after injection; cDNA was synthesized with random hexamers and Superscript II (Life Technologies). Semiquantitative PCR was performed with ^{32}P-radio-labeled primers; PCR products were fractionated by polyacrylamide gel electrophoresis and quantified by PhosphorImage analysis. Primers spanned at least one exon boundary in regions separate from those targeted by the injected dsRNA. Reactions were normalized to *gld-1* RNA levels.
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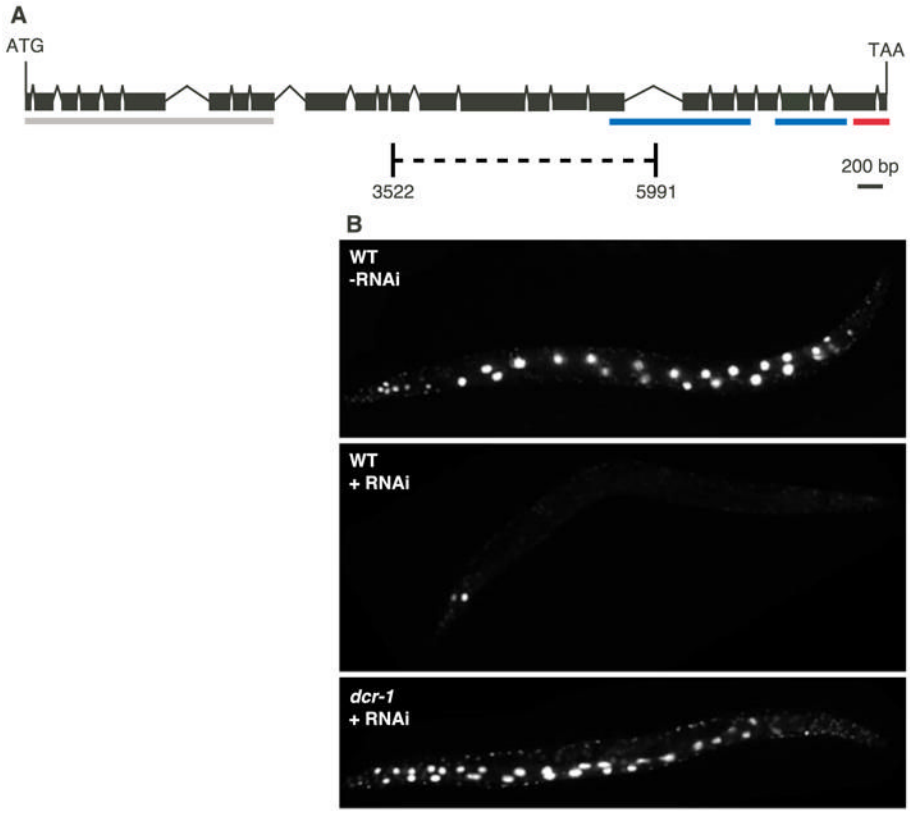

Fig. 1.

dcr-1 mutants are deficient for RNAi. (**A**) The gene structure of *dcr-1* (K12H4.8, chromosome III) is shown with a dotted line indicating the deletion found in *dcr-1(ok247)*. The deletion extends from nucleotides 3522 to 5991, relative to the ATG codon. Regions encoding the RNA helicase domain (gray), the RNase III domains (blue), and the dsRNA binding motif (red) are underlined. To ensure that the observed phenotypes in *dcr-1(ok247)* animals were due to a mutation in *dcr-1*, we outcrossed animals heterozygous at the locus against wild-type animals (N2) eight times. (**B**) Wild-type (WT) or *dcr-1(ok247)* animals expressing a GFP transgene were compared by fluorescence imaging before (−RNAi) or after (+RNAi) heat shock to induce the GFP RNA hairpin (29). The *sur-5::GFP* transgene is expressed in many cells, with brightest expression in the intestine (30). GFP fluorescence in wild-type animals is reduced after the heat shock treatment, whereas *dcr-1(ok247)* mutants continue to exhibit high levels of GFP fluorescence comparable to non–heat-shocked animals. Even in wild-type animals, GFP fluorescence remains after heat shock in neuronal tissues, which are often refractory to RNAi. Heterozygous individuals behaved like wild-type animals, exhibiting a substantial loss in GFP fluorescence (24). Transgenic lines were constructed with standard protocols (31), and *C. elegans* strains were maintained as described (32).

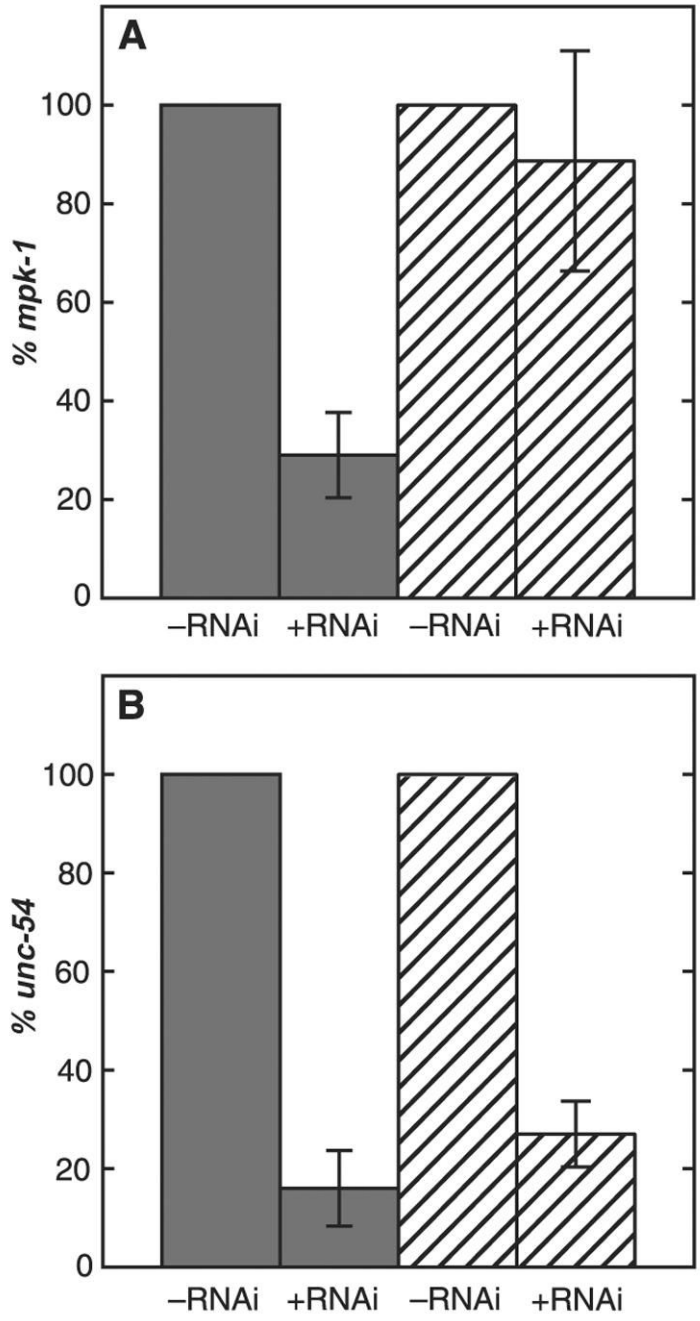

Fig. 2.

Analyses of mRNA levels show that *dcr-1*(−/−) mutants are defective for RNAi of some but not all genes. dsRNA corresponding to *mpk-1* (**A**) or *unc-54* (**B**) was injected into L4 animals (33), and endogenous mRNA levels were quantified for single worms by semiquantitative RT-PCR (34). Bars show average mRNA levels ($n = 3$) for wild-type (solid) and *dcr-1*(−/−) animals (hatched) with (+) or without (−) dsRNA injection. Error bars show SEM.

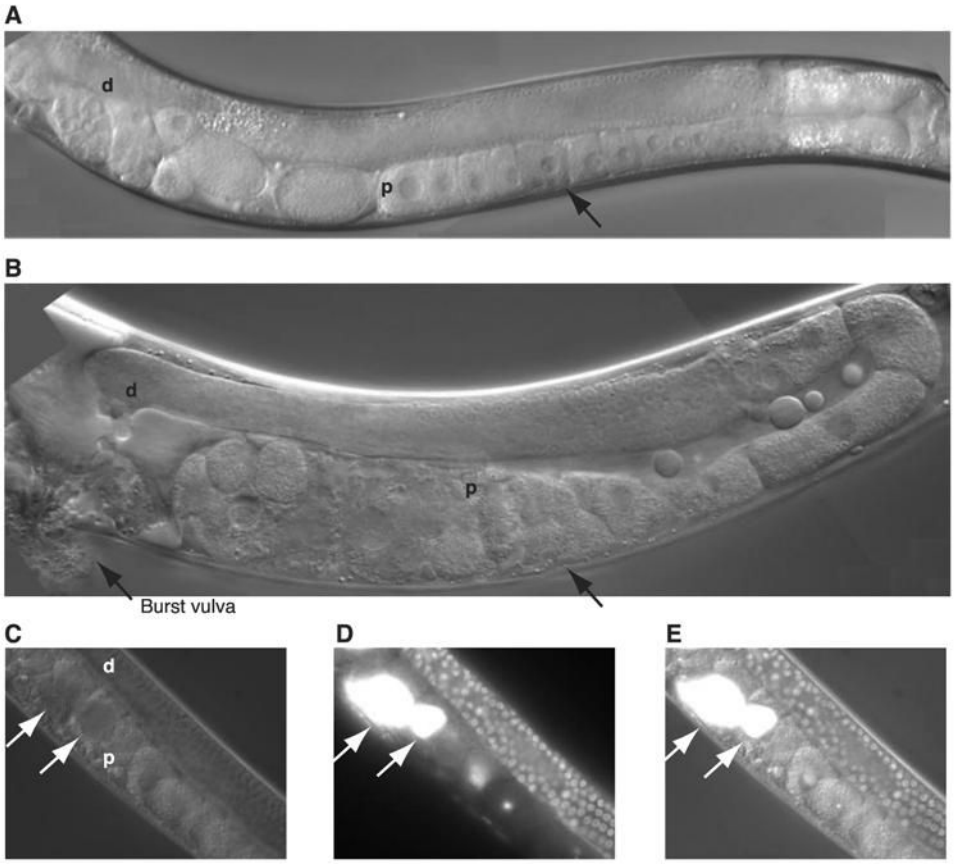

Fig. 3.

Germ line development is abnormal in *dcr-1*(−/−) animals. (**A** through **C**) DIC micrographs. Distal (d) and proximal (p) orientation is indicated. (A) Wild-type adult hermaphrodite. The arrow points to a normal oocyte in the proximal gonad. (B) *dcr-1* adult hermaphrodite with a burst vulva. An abnormal misshapen oocyte is indicated by an arrow in the proximal gonad (arrow). (C) *dcr-1* adult hermaphrodite with enlarged oocyte nuclei (arrows). (**D**) Fluorescent micrograph of the DAPI-stained animal of (C). Areas of intense staining colocalize to nuclei (arrows). (**E**) Merged micrograph of images in (C) and (D).

Table 1

dcr-1 mutants are RNAi defective.

Trial	Percentage of *dcr-1(ok247)* animals exhibiting fluorescence
1	94* (*n* = 33)
2	95(*n* = 19)
3	94* (*n* = 16)

* The remaining 6% displayed an intermediate phenotype.