

# A Member of the Polymerase $\beta$ Nucleotidyltransferase Superfamily Is Required for RNA Interference in *C. elegans*

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## Summary

RNA interference (RNAi) is an ancient, highly conserved mechanism in which small RNA molecules (siRNAs) guide the sequence-specific silencing of gene expression [1]. Several silencing machinery protein components have been identified, including helicases, RNase-related proteins, double- and single-stranded RNA binding proteins, and RNA-dependent RNA polymerase-related proteins [2]. Work on these factors has led to the revelation that RNAi mechanisms intersect with cellular pathways required for development and fertility [3, 4]. Despite rapid progress in understanding key steps in the RNAi pathway, it is clear that many factors required for both RNAi and related developmental mechanisms have not yet been identified. Here, we report the characterization of the *C. elegans* gene *rde-3*. Genetic analysis of presumptive null alleles indicates that *rde-3* is required for siRNA accumulation and for efficient RNAi in all tissues, and it is essential for fertility and viability at high temperatures. RDE-3 contains conserved domains found in the polymerase  $\beta$  nucleotidyltransferase superfamily, which includes conventional poly(A) polymerases, 2'-5' oligoadenylate synthetase (OAS), and yeast Trf4p [5]. These findings implicate a new enzymatic modality in RNAi and suggest possible models for the role of RDE-3 in the RNAi mechanism.

## Results and Discussion

### Molecular Identification of *rde-3*

The *rde-3* locus was originally defined by a single allele [6]. To further characterize this locus, we first conducted additional genetic screens to identify new alleles and then used genetic mapping to define a small interval containing the gene (Figure 1A). We then examined the nucleotide sequence of candidate genes within this region for lesions in *rde-3*. In each allele we found a mutation predicted to disrupt the expression or to change the amino-acid sequence of the gene K04F10.6. In addition, we found a lesion in K04F10.6 in the previously

described mutant *mut-2* (*r459*) [7], suggesting that *mut-2* and *rde-3* are allelic. Finally, we found that a PCR fragment containing only K04F10.6 rescues the somatic RNAi defect of *ne3364*, confirming that K04F10.6 is *rde-3* (Figure 1).

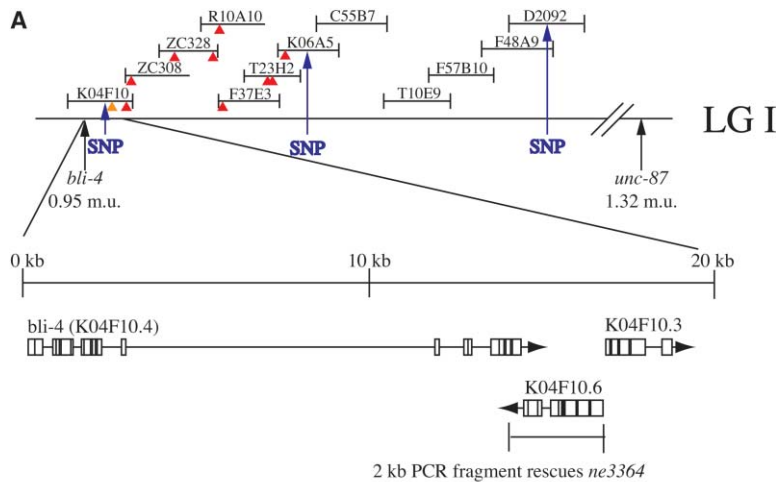
*rde-3* encodes a conserved protein in the polymerase  $\beta$  nucleotidyltransferase superfamily. RDE-3, and other members in this family, including conventional poly(A) polymerases (PAP), 2'-5' oligoadenylate synthetase (OAS), *C. elegans* GLD-2, and budding yeast Trf4p, all contain a nucleotidyltransferase 2 (NTP transferase 2) domain [5]. In addition, RDE-3 and a subset of family members, including GLD-2 and Trf4p, contain an additional domain, of unknown function, named the PAP/25A-associated domain [5]. The *rde-3* alleles *ne298*, *r459*, and *fj14* each contain point mutations predicted to alter conserved amino acids. The *ne3370* allele contains an in-frame deletion of 423 bp (Figure 1B). *ne4243* and *ne3364* each contain nonsense mutations predicted to truncate the protein after 164 and 169 amino acids, respectively, and are thus likely to represent null mutations. Two of the lesions in *rde-3* are predicted to alter conserved residues within the nucleotidyltransferase domain, suggesting that polymerase activity may be important for RDE-3 function (see discussion below).

### *rde-3* Is Required for Fertility and Viability as well as siRNA Accumulation

The previous characterization of *ne298* showed that *rde-3* had additional phenotypes, including a Him (High incidence of males) phenotype that reflects an increase in the nondisjunction of the X chromosome and a Mut (Mutator) phenotype resulting from the activation of endogenous transposon families [6]. The new *rde-3* alleles, including the presumptive null alleles, exhibit phenotypes similar to those of *rde-3* (*ne298*), suggesting that all of these mutants represent strong loss-of-function alleles. *rde-3* mutants do not exhibit the specific developmental defects expected from a loss in the function of the miRNAs *lin-4* or *let-7* (data not shown), suggesting that, as with other mutator-class *rde* mutants, its developmental functions in fertility and viability may reflect a role in other as-yet-unknown mechanisms.

We next examined the sensitivity of these alleles to RNAi targeting genes expressed in different tissues: *pos-1* (germline) and *unc-22* (muscle). The three alleles tested (*ne298*, *ne3364*, and *ne3370*) showed similar levels of resistance to RNAi (Figures 2A and 2B). Note that the dead embryos observed in Figure 2A include a background of approximately 20% inviable embryos that arise spontaneously because of the incompletely penetrant lethal phenotype associated with *rde-3* alleles. We found that the presumptive null allele, *ne3364*, like *ne298* [6], is sensitive to RNAi induced by a transgene that simultaneously drives the expression of sense and antisense *unc-22* RNA. Two independent transgenic lines were analyzed in these experiments; the penetrance of the *unc-22* twitching phenotype was indistinguishable from that induced by the same transgenes

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**B** *rde-3*/K04F10.6

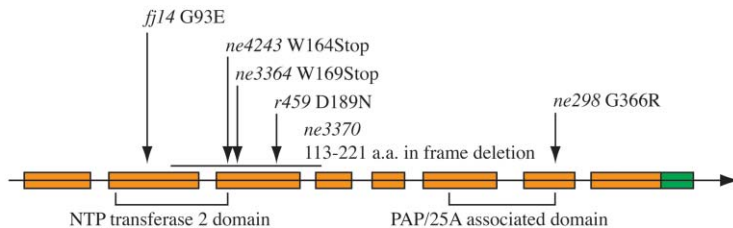


Figure 1. Molecular Identification of *rde-3*

(A) Schematic diagram of the *rde-3* genetic interval. *rde-3* was mapped genetically to an interval of approximately 0.37 map units, very close and to the right of *bli-4*. Single nucleotide polymorphisms (blue arrows) were used to narrow the interval to 7 cosmids. Candidate genes shown as red triangles were sequenced. Mutations were found in K04F10.6 (orange triangle). A single-gene K04F10.6 PCR fragment was used for rescue experiments (forward primer: 5'-GAT TGT ATT GTT TCT TTT GTC TTA TAC CGG G-3', reverse primer: 5'-CGT TGG AGA AAC GAA GAA TGT GCA TAG-3').

(B) Line and block diagram of the intron/exon structure of *rde-3*. Reverse-transcriptase PCR was used to confirm the exon structure predicted by Wormbase. RDE-3 contains two conserved domains, including an NTP transferase 2 domain at its N terminus and PAP/25A (poly(A) polymerase/2'-5' oligoadenylate)-associated domain at its C terminus. The molecular lesions in six *rde-3* alleles are indicated above the diagram.

when expressed in wild-type strains (data not shown). These findings indicate that RDE-3 is essential for RNAi induced by feeding, but is not required for RNAi induced by transgene-driven expression of dsRNA. RDE-3 has one close homolog and at least ten more distantly related family members in *C. elegans*. Therefore, it is possible that the ability of *rde-3* mutants to respond to transgene-driven RNAi reflects compensation in this pathway from one or more of these homologs. Alternatively, it is possible that RDE-3 function is required for RNAi amplification and that RDE-3 is thus less critical when very large amounts of dsRNA are delivered by transgene-driven expression directly in target tissues (for more on these possibilities, see the discussion below and the model in Figure 4B).

In *C. elegans*, antisense siRNAs accumulate in animals undergoing RNAi. Some mutants defective in RNAi, such as *rde-1* and *mut-7*, fail to accumulate siRNAs, whereas other mutants, such as *mut-14*, show no defect in siRNA accumulation [8]. To further understand why RNAi is defective in *rde-3*, we examined the level of siRNA in *rde-3* mutants. As shown in Figure 2C, wild-type animals accumulate antisense *pos-1* siRNAs when they are exposed to RNAi targeting *pos-1*. However, siRNAs are not detected in either *ne298* or *ne3364* worms. This result suggests that RDE-3 activity is required upstream of the accumulation of siRNA during RNAi.

***rde-3* Is a Member of a Functionally Diverse Multigene Family**

The *rde-3* family includes several *C. elegans* members, as well as numerous homologs in other organisms.

These homologs have been implicated in a variety of cellular mechanisms, including polyadenylation of transcripts in the nucleus (PAP) and cytoplasm (GLD-2) [9]. The OAS members of this family are upregulated by the interferon antiviral response in mammalian cells [10, 11]. Viral dsRNA is thought to bind to OAS and to activate the synthesis of 2'-5' adenylyl oligomers, which in turn activate RNase L [12]. RNase L then degrades cytoplasmic RNAs of both the host and virus in a non-sequence-specific manner, preventing further viral amplification. The budding yeast protein Trf4p has been implicated in the polyadenylation and recruitment of improperly processed tRNAs to the exosome [13], and it has also been implicated in DNA synthesis and chromosome cohesion [14].

Like GLD-2, OAS, and Trf4p, RDE-3 lacks any recognizable RNA binding motif. GLD-2 is thought to interact with target mRNAs through its association with the KH-domain RNA binding protein GLD-3 [9]. Mammalian OAS family members bind to dsRNA via an undefined RNA binding motif that appears to involve several positively charged residues localized on the surface of the protein [15]. It is not presently known how Trf4p recognizes its targets. Further studies will be required to determine whether and how RDE-3 binds to RNA.

Proteins in this superfamily have two conserved features at the active site of the enzyme: a helical turn that includes a highly conserved glycine-serine (GS) motif and a conserved aspartic-acid triad [5]. Interestingly, *rde-3* (*fj14*) contains a glycine-to-glutamate mutation in the GS motif, and *rde-3* (*r459*) contains an aspartic-acid-to-asparagine mutation in the third aspartic acid of the

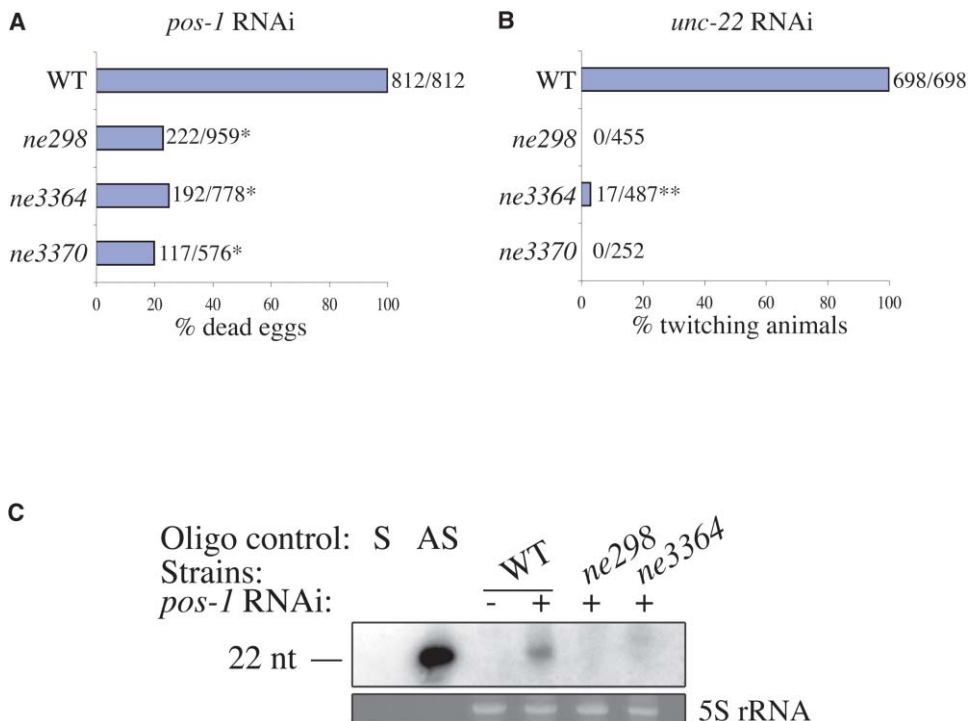


Figure 2. *rde-3* Is Defective in RNAi

(A and B) Graphical representations of the sensitivity of three *rde-3* mutant strains to RNAi induced by microinjection of: (A) double-stranded *pos-1* RNA (1 mg/ml) or (B) double-stranded *unc-22* RNA (1 mg/ml). Young adult animals were injected with dsRNA and were transferred individually to fresh plates every 12 hr. Phenotypes were scored for the progeny produced 24–48 hr after injection. The number of sensitive animals over the total number of animals scored is presented next to each bar. \*: The data for the three *rde-3* strains includes a background of approximately 20% inviable embryos that arise spontaneously because of the incompletely penetrant embryonic lethal phenotype of *rde-3*. \*\*: These progeny were very weakly twitching in comparison to the wild-type.

(C) *rde-3* is defective in siRNA accumulation. Northern blot analysis of siRNAs in wild-type and *rde-3* mutant animals is shown. Total RNAs were extracted with TRI Reagent (Molecular Research Center) from embryos. Small RNAs were further purified with MirVana (Ambion). Ten micrograms of RNAs were separated in 15% UREA-PAGE and transferred to nitrocellulose membrane. Northern analysis was performed with <sup>32</sup>P-labeled sense riboprobe covering the whole *pos-1* coding region [18]. Ethidium bromide staining of 5S rRNAs was used for loading control. Sense (S) and antisense (AS) *pos-1* RNA oligos of 22 nt were included as hybridization controls.

catalytic triad (Figure 3). These findings suggest that the polymerase activity of RDE-3 is likely to be important for its function. These residues are also conserved in a fission-yeast homolog, Cid12 (Figure 3). Interestingly, Cid12 has recently been shown to function in an RNAi-like mechanism required for chromatin silencing at centromeric repeats [16]. Cid12 associates with the RNA-dependent RNA polymerase Rdp1 and is required for the accumulation of siRNAs involved in the silencing of centromeric chromatin in *S. pombe*. Despite their apparent functional similarity as factors required for siRNA accumulation, RDE-3 and Cid12 do not appear to represent orthologous proteins. The *C. elegans* gene ZK858.1 has a much greater degree of sequence identity with Cid12 (Figure 3). It is conceivable that RDE-3 and Cid12/ZK858.1 provide similar functions in distinct branches of the RNAi pathway—for example, in post-transcriptional versus transcriptional silencing.

There are many possible models for how RDE-3 might function in RNAi. In one model, RDE-3 may respond to dsRNA in a manner analogous to OAS, producing

oligoadenylate. However, rather than inducing a general nonspecific mRNA destruction, it may stimulate nucleases required for siRNA-directed mRNA turnover. Alternatively, RDE-3 could function indirectly to facilitate RNAi by insuring a proper balance of RNA metabolism in the cell. For example, RDE-3 could function as its homolog GLD-2 does in the polyadenylation of a subset of mRNAs in the cytoplasm. In the absence of RDE-3, defects in the polyadenylation of these mRNAs could lead to an accumulation of aberrant transcripts that enter the RNAi pathway and titrate limiting factors, reducing the ability of cells to initiate RNAi in response to foreign dsRNA (Figure 4A). Similarly, RDE-3 could function as Trf4p does in the turnover of improperly processed tRNAs or of other RNAs normally destined for the exosome. Defects in such a mechanism could, once again (as shown in Figure 4A), lead to inappropriate recognition of these aberrant RNAs and competition for limiting components of the RNAi machinery.

Another model worth considering (Figure 4B) is that RDE-3 is required for the amplification of RNAi in re-

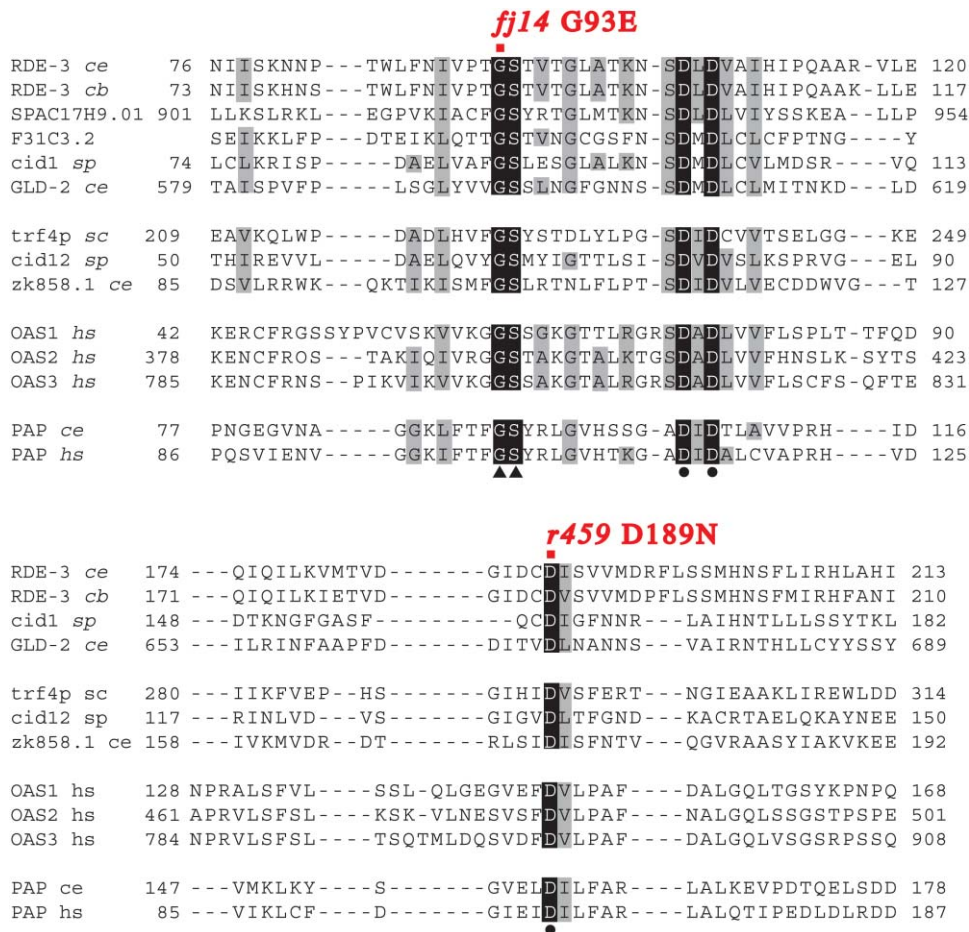


Figure 3. *rde-3* Mutants Alter Conserved Residues in the Nucleotidyltransferase Domain

Sequence alignment of RDE-3 with members of the polymerase  $\beta$  nucleotidyltransferase superfamily. Proteins are divided into subgroups on the basis of sequence identity. Residues identical in all proteins are highlighted in black, and conservative amino-acid substitutions are highlighted in gray. The conserved GS residues are marked by black triangles. The aspartic-acid residues of the predicted catalytic triad are indicated with black circles. The lesions in *fj14* and *r459* are indicated. The following abbreviations were used: *ce*, *Caenorhabditis elegans*; *cb*, *Caenorhabditis briggsae*; *hs*, *Homo sapiens*; *sc*, *Saccharomyces cerevisiae*; and *sp*, *Schizosaccharomyces pombe*. The sequences were aligned with Clustal W [19]. Some divergent sequences among subgroups were aligned manually.

response to low levels of the inducing trigger dsRNA. This model is consistent with our own observation that RDE-3 is not required for RNAi initiated by transgene-driven dsRNA (see above) and with the finding from *S. pombe* that the RDE-3 homolog Cid12 interacts with RdRP. In *C. elegans*, the detectable accumulation of siRNAs during RNAi requires RdRP activity (D. Conte and C.C.M., unpublished data; [17]) and is thought to involve an RdRP-dependent synthesis of new dsRNA after an initial round of target mRNA recognition. After ingestion, small quantities of dsRNA entering target tissues may be processed by Dicer to generate low abundance siRNAs. These primary siRNA could then direct a first round of target-mRNA cleavage. RDE-3 might then polyadenylate the nascent 3' end of this cleavage product, stabilizing it and permitting its recognition by RdRP (Figure 4B). In the absence of RDE-3, the initial cleavage product may be too unstable to be recognized efficiently by

RdRP, preventing amplification and the consequent accumulation of siRNA. Although their precise functions remain to be discovered, the findings that (1) RDE-3 and Cid12 are required for RNAi-related mechanisms, and (2) mutations in these factors result in similar defects in siRNA accumulation in these divergent species, strongly suggest that members of this protein family are likely to represent integral components of small-RNA-mediated silencing pathways.

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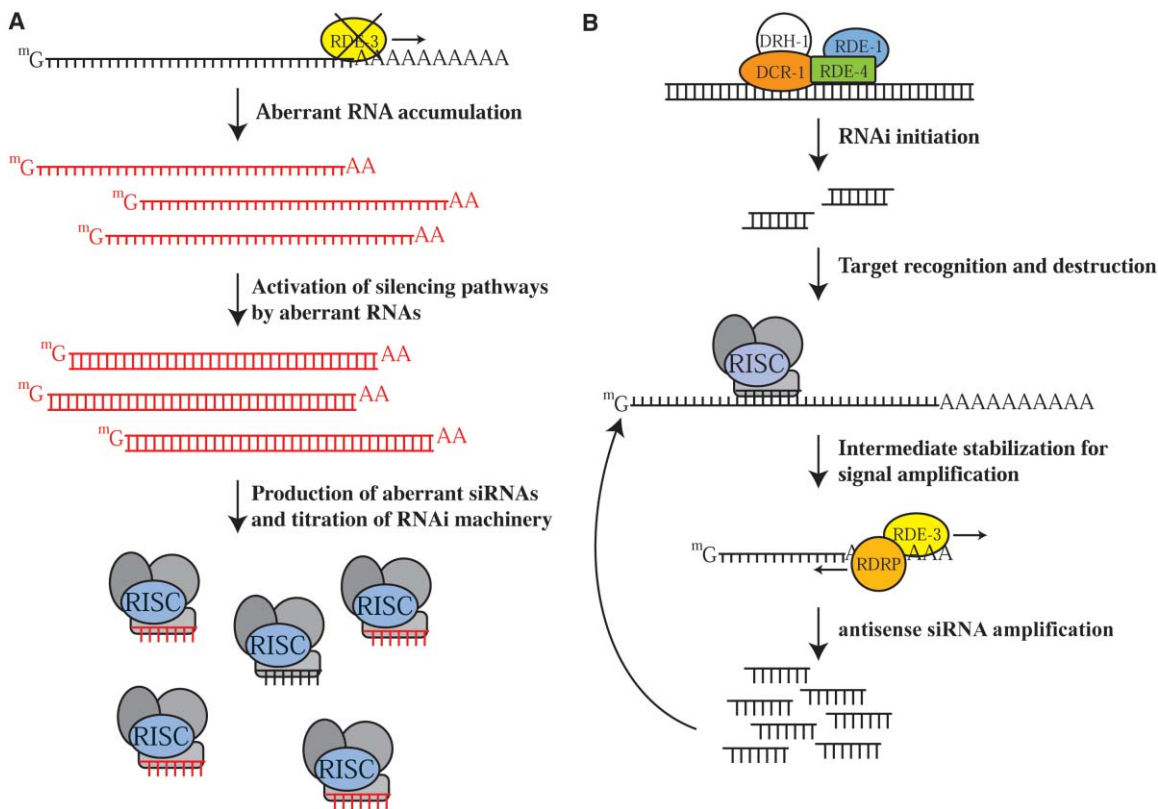


Figure 4. Models

Schematic diagrams illustrating possible roles for RDE-3: (A) as a factor that maintains the RNA metabolism balance required for efficient RNAi or (B) as a direct component of the RNAi pathway. (A) depicts RDE-3 as a poly(A) polymerase required for processing a subset of mRNAs in the cell. RDE-3 loss (indicated by the “X”) could lead to accumulation of “aberrant” transcripts (red) with short poly(A) tails that can enter the RNAi pathway, titrating the RNA-induced silencing complex (RISC) or other limiting factors necessary for efficient RNAi. In (B), RNAi-initiating proteins, including Dicer (DCR-1), which processes long dsRNA into siRNAs, a Dicer-related helicase (DRH-1), an argonaute protein (RDE-1), and the dsRNA binding protein (RDE-4) [20], are shown processing foreign dsRNA into primary siRNA. These extremely-low-abundance primary siRNAs then target mRNA in an initial round of cleavage mediated by components of the RISC. RDE-3 is then proposed to polyadenylate the 5' cleavage product, stabilizing this intermediate and allowing RdRP to amplify the response, generating abundant secondary siRNAs.

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