Natural and Unanticipated Modifiers of RNAi Activity in Caenorhabditis elegans

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Abstract

Organisms used as model genomics systems are maintained as isogenic strains, yet evidence of sequence differences between independently maintained wild-type stocks has been substantiated by whole-genome resequencing data and strain-specific phenotypes. Sequence differences may arise from replication errors, transposon mobilization, meiotic gene conversion, or environmental or chemical assault on the genome. Low frequency alleles or mutations with modest effects on phenotypes can contribute to natural variation, and it has proven possible for such sequences to become fixed by adapted evolutionary enrichment and identified by resequencing. Our objective was to identify and analyze single locus genetic defects leading to RNAi resistance in isogenic strains of Caenorhabditis elegans. In so doing, we uncovered a mutation that arose de novo in an existing strain, which initially frustrated our phenotypic analysis. We also report experimental, environmental, and genetic conditions that can complicate phenotypic analysis of RNAi pathway defects. These observations highlight the potential for unanticipated mutations, coupled with genetic and environmental phenomena, to enhance or suppress the effects of known mutations and cause variation between wild-type strains.

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Introduction

RNA silencing is a term commonly used to describe the multiple, overlapping, RNA-directed pathways that are used by cells to regulate gene expression and chromatin functions. RNA silencing mechanisms serve as cellular sentinels, providing fundamental anti-foreign genome defense activities that protect cells against transposon mobilization and organisms against systemic viral infection. RNA silencing pathways include RNA interference (RNAi) mechanisms that are triggered when dsRNA is provided to cells. Hundreds of genes have been implicated in RNA silencing using forward genetic and RNAi-based screens, which is a testament to the scope of these mechanisms in cells. Genetic defects in some members of these pathways can lead to developmental or environmentally-influenced phenotypes in Caenorhabditis elegans. Considering the vital and fundamental nature of RNA silencing functions, it is surprising that relatively few RNA silencing genes are required for viability or fertility, suggesting a functional overlap of RNA silencing activities.

One practical consequence of the interwoven nature of RNA silencing networks is that mutations in these pathways are often tolerated by the organism. Indeed, several unexpected mutations that affect RNA silencing have been identified in laboratory strains as well as in feral isolates [1,2]. The mutations found in laboratory strains are considered background mutations as the ancestry of the mutations can often be traced back to a common source. The lack of a selective disadvantage in the laboratory environment may contribute to the perpetuation of such mutations, especially considering that C. elegans strains are normally cultivated in the laboratory in a manner that does not typically select for animals with “wild-type” levels of RNA silencing activity. Alternatively, a selective advantage under some genetic or environmental conditions might contribute to fixation of RNA silencing mutations.

During the course of our studies on RNA silencing mechanisms, we observed that some of our strains harbored unanticipated mutations that affect RNAi. Unlike previously reported background mutations, these have a more recent genesis. Here we report novel mutations in genes that encode Dicer-interacting proteins, as well as long-lasting maternal effects, that allow for RNAi activity in adults. The recurrent observations of background mutations affecting RNA silencing mechanisms, as well as environmental influences and maternal effects that can influence the robustness of RNAi and thereby obscure underlying RNAi-related mutations, highlights the importance of comparing multiple alleles or differently outcrossed strains, as such unexpected mutations may have pleiotropic effects that could complicate phenotypic analyses.
Materials and Methods

C. elegans strains

Mapping and other strains (from A. Fire): PD2119 [dpy-1(e1) ncl-1(e1865) III], PD2039 [dpy-5(e1)]) unc-51(e1092) I, PD2014 [dpy-10(e244), unc-3(e689) II, PD2027 [unc-17(e243) dpy-4(e1166) IV], PD9064 [dpy-11(e22) unc-60(e723) V], pPD4300 [ccIs4251 (myo-3::GFP) I; syIs2 (egl-15::GFP) IV; ayIs6 (hlh-8::GFP) X]; pPD5863 [ccIs4251 (myo-3::GFP) I; PD1675 [ncl-1(e2199)]]

Deletion strains used to map rde-4(ne309) (from the Caenorhabditis Genetics Center): BC4637 [dF130(2427) unc-3(e189) III; dpy-5(e1)] TY1353 [nD10 unc-3(e189)/unc-39(e189) III; dpy-17(e164) III], BC4638 [dF127(c2428) dpy-4(e164) unc-3(e189) III; dpy-5(e1)], BC697 [dF121(2008) unc-32(e189) III; dpy-19(e1147) III], MT1298 [dF16/unc-36(251) dpy-19(e1259) III; dpy-5(e1)], BC4697 [nD40 dpy-18(e364) II; tC1 (III-V)], D6801 [unc-32(e189) I; dF121/unc-36(251) II; C7018 unc-3(e189) III; dpy-6(e1256) I], C4118 [nD16/unc-32(e189) I; dpy-18(e364) III], GE2158 [nD2/qC1 dpy-19(e1259) gfp-1(q339) III], GE2189 [unc-32(e189) I; dF17/dC1 dpy-19(e1259) gfp-1(q339) III; him-3(e1147) IV], BW1355 [dpy-18(e364) III; dpy-4(e1147) e2501 gfp-1(q339) III], BW1369 [unc-32(e189) dpy-18(e364) I; dF17/dC1 dpy-19(e1259) gfp-1(q339) III], NG2681 [nD10 unc-32(e189) II; dpy-19(e1259) gfp-1(q339) III; CX2914 [nD16/dpy-17(e164) unc-32(e189)]]

Other strains (from the Caenorhabditis Genetics Center): CB879 [him-1(e1879)], AZ244 [unc-119(e323) III; rtu-57], BB1 [dcr-1(ek247)/unc-32(e189) III], NL1820 [mut-7(k726) I]

New strains: XX636 [rde-4(ne209)]; him-1(e1879) I, XX637 [dpy-1(e1)] unc-19(e32) III], XX47 [rde-4(ne309)], XX1849 [him-1(e1879) I; rde-4(ne309) III], XX1076 [cPD4251/myo-3::GFP I; yyEx1.1 (myo-3::gfp hairpin, rol-6(e1006))]; XX528 [hasf-6(ne335) cPD4251/myo-3::GFP I; yyEx1.1 (myo-3::gfp hairpin, rol-6(e1006))], XX1151 [cL4440/myo-3::GFP I; him-3(ne444) IV; rde-1(yj1)] V, XX1278 [cL4440/myo-3::GFP I; him-3(ne444) IV; rde-1(yj1) V; XX1835 [rde-4(ne299)]; cL4421/myo-3::GFP; yyEx1.1 (myo-3::promoter::gfp hairpin; rol-6(e1006)]], XX1823 [him-3(ne440) I; cL4421/myo-3::promoter::GFP I]; Strains XX103, XX172, XX183, XX193, XX194, XX195, XX364, and XX528 are RNAi-defective strains that are homozygous for the haf-6(ne353) mutation.

Transgene strains: The cL4425 and yyEx1.1 transgenes used here have been described [3,4]. The two transgenes were introduced together in wild-type and mutant backgrounds using standard genetics. Both transgenes utilize a myo-3 promoter, which is transcriptionally active in muscle from late embryogenesis through adulthood. cL4425 expresses a GFP reporter, and was obtained by inducing an extrachromosomal array to integrate into the middle of chromosome I near the rde-2 gene [5]. The yyEx1.1 transgene contained a myo-3 promoter that drives gfp dsRNA expression in muscle from repetitive extrachromosomal DNA sequences that were assembled by the worm from injected plasmids. The cL4425 transgene allows for muscle-specific accumulation of gfp mRNA and of GFP reporter protein and is the target of RNAi in this double transgene RNAi system.

Primers

rde-1 primers: 164: ggaagaatgccaagaaagtc; 165: tgggtgatatctttag;
170: tatcataaacaggaaagacta; 171: tcatatctctctccatcgtt; 207: aaccctgccgcacatgaatg; 717: atatatagtcgtaatttccccga; 718: ataattgggaagagactcagg;
1142: gatctcttgagggcccaacacaagtc; 1143: gttctgtaaaccaatgagttag;
1144: gtagtatttgaataatggtaggagaa; 1150: cacccgttaattgtggctcgagaag;
1249: gcgtatcaccagtaggtgtagatgaa; 1250: tccccggaagaaagaaagagaagc

Tcl primers: 1333: tctgataacttgaatttcggtttag; 1334: atcattcaagactgaatttcggtttag;
1335: gaaacattaactaggacgcaataactt; 1336: tctgatttgaagaactcataagttc;
1337: gaaacattaactaggacgcaataactt; 1338: gaagaatgccaagaaagtc;

PCR- based detection of rde-1(yy-11) allele in mixed populations

Animals were washed with water and suspended in NET buffer (100 mM NaCl, 20 mM EDTA, 50 mM Tris-HCl, pH 8.0) and frozen. 1:200 volume of 10% SDS and 1 mg proteinase K were added to thawed pellets, which were incubated at 65°C for 2–4 hours, then phenol extracted, and ethanol precipitated. Primer set 1249/207 anneals to the rde-1 gene, producing an amplified product of 640 bp in wild-type animals. (This primer set preferentially amplifies the wild-type allele in mixed populations of rde-1(yy11) and wild-type animals.) Primer set 1249/1333 specifically amplifies the rde-1(yj11) insertion, yielding an amplification product of 279 bp.

dsRNA delivery by “feeding”

Standard C. elegans plates supplemented with cholesterol, ampicillin, tetracycline, and IPTG were seeded with the Escherichia coli strain HT115(DE3) harboring feeding plasmids as described [5,6,7,8]. Transformed bacteria harboring pop-1 cDNA in L4440 vector transcribe dsRNA that targets the TCF/LEF1 transcription factor, inducing sterility in young animals reared on this food, while the dpy-11 food targets a protein that affects body shape, eliciting a shorter and fatter appearance in comparison to wild type. Control experiments utilized wild-type worms and empty L440 vectors. Experiments were performed at 15°C, 20°C, and 25°C.

Genetic analysis of RDE-4 maternal effects

To assess the extent of RDE-4 maternal effects, we first generated strains such as XX1835, which harbors the rde-4(299) mutation, the yyEx1.1 gfp dsRNA-expressing extra-chromosomal array, and the integrated cL4425 GFP reporter. Homozygous rde-4 animals (XX1835, as well as a similar strain harboring the rde-4(ne309) mutation) were mated with XX1823 males, which harbor the GFP reporter. F1 progeny harboring the yyEx1.1 gfp dsRNA-expressing transgene array marked with a dominant “roller” transformation marker, as well as the GFP reporter, were individually placed on standard C. elegans culture plates. F2 animals harboring the “roller” phenotype were scored for RNAi activity as young adults and subsequently genotyped by DNA sequencing of the rde-4 gene. 25% of such F2 progeny are predicted to be homozygous for the rde-4 mutation. Before sequence analysis, some F2 adults were again placed as individuals on standard
culture plates and allowed to produce F3 animals. The transgenic F3 animals were scored for RNAi activity and some were also sacrificed for DNA sequencing. 25% of the plates with such F3 progeny are predicted to be homozygous for the rde-1 mutation. Animals were tracked, and the genotype was correlated with phenotype at the end of each experiment. We scored animals as RNAi+ if they displayed GFP fluorescence in fewer than 30% of muscle cells and RNAi− when GFP fluorescence in was observed in 70–90% of muscle cells. (All RNAi− animals in these assays displayed bright GFP fluorescence in >90% of muscle cells.) Experiments were conducted at 25°C, 22°C and 15°C.

Results and Discussion

A novel allele of rde-1 arose spontaneously in a transgenic stock

As an extension of experiments designed to uncover roles for the Caenorhabditis elegans ABC transporter gene haf-6 in RNA interference [9,10], we built strains to determine if haf-6 is required for RNAi when dsRNA is transcribed from transgenes. The strains harbored two distinct configurations of transgenic DNA such that gfp sequences served as both trigger and target of RNAi, allowing for direct visual inspection of RNAi activity. In a wild-type background, this dual transgene system allows for easy visualization of an RNAi response against gfp. Our experiments using these transgenic strains led to observations of RNAi defects (Figure 1). The RNAi defects were not rescued by the introduction of wild-type haf-6 sequences, and we ascertained that the transgene-mediated RNAi defects were due to an unknown mutation, which we termed yy11, present in the background of the transgenic haf-6(ne335) strain.

In order to identify the nature of the gene with the yy11 mutation, we employed standard genetic methodology to separate the recessive haf-6(ne335) and yy11 mutations (Figure 1A,B). The resulting single mutant yy11 homozygotes displayed RNAi defects in response to transgene-delivered dsRNAs, as well as defects in response to ingestion of dsRNAs targeting genes expressed in the germ line or soma. By contrast, the haf-6(ne335) single mutants were RNAi-defective in response to ingested dsRNA, as expected, but did not display RNAi defects in response to transgenes. Thus, the transgene-mediated RNAi defect was solely due to the unknown yy11 mutation.

We mapped the yy11 mutation to chromosome V. Considering that the yy11 mutation elicits a strong RNAi defect, we reasoned that the corresponding gene might already have been uncovered in screens for RNAi-defective mutants. We therefore performed screens for RNAi-defective mutants. We therefore performed

Figure 1. RNAi activity in rde-1(yy11) and haf-6(ne335) mutants. The yy11 allele, present as a background mutation in a haf-6(ne335) transgenic stock, was genetically dissected from the haf-6(ne335) mutation and analyzed separately for RNAi activity. (A) yy11 provides resistance to transgene-delivered dsRNAs, as evidenced by lack of gene silencing of the GFP reporter in homozygous animals. By contrast, haf-6(ne335) single mutants display wild-type RNAi activity in this transgene assay. (B) Animals homozygous for the yy11 allele are RNAi resistant when dsRNAs are delivered by ingestion of bacteria that express dsRNAs. Thus, the rde-1(yy11) mutants displayed strong RNAi phenotypes irrespective of delivery method (A, B), while, as expected, the haf-6(ne335) single mutant was RNAi defective by ingestion only (A, B). Bacteria expressing dsRNA targeting the pop-1 and dpy-11 genes were used in feeding experiments. (C) Genetic and molecular analysis revealed the nature of the yy11 mutation—a transposon insertion in exon 9 of rde-1. RNA sequences flanking the insertion site are indicated. doi:10.1371/journal.pone.0050191.g001

difference between the two transposons) may reflect the recent nature of the transposition event. A potentially active transposon in rde-1, coupled with the ability to select for rde-1 activity using RNAi methodology, raises the possibility of using the rde-1(yy11) allele as a screening or assessment tool for transposon silencing or RNAi activities.

The rde-1(yy11) allele was attained serendipitously—it was first observed in a haf-6(ne335); rde-1(yy11) double mutant (Figure 1). Two of the ancestors of this stock had been exposed to mutagens, which may have resulted in the transposition event that gave rise to the rde-1(yy11) allele [12]. One of these ancestral strains was the original haf-6(ne335) isolate; a second ancestral strain was exposed to mutagens in order to induce integration of the oxl-4251 GFP reporter sequence [3]. We therefore hypothesized that the transposition event leading to the rde-1(yy11) mutation might have originated in such ancestral strains and thereafter lingered as a low frequency mutation. We designed a PCR-based assay to test for the presence of the Tc1 insertion using PCR and amplification conditions that would allow for the detection of the rde-1(yy11) allele in a mixed population of worms (Figure 2A). We applied this assay to progenitor strains, and strains later derived from them, for the presence of the rde-1(yy11) allele. We detected the allele in a frozen archived stock from the original, un-outcrossed haf-6(ne335) isolate (Figure 2B). haf-6 mutants display mutator activity [10]; therefore, the rde-1(yy11) insertion may be a reflection of the transposon silencing defect in haf-6 mutants.
alleles (640 bp product, control PCR reaction using primers designed to amplify wild-type strain) to rule out maternal contamination. Each lane represents a PCR reaction from the population. It is likely that the strategy we used to build the transgenic strain in Figure 1A had been out-crossed to the wild-type animals, the stock nor from genomic DNA isolated from all worms in similar strain. Additionally, the transposon insertion helps to confirm the null phenotype, allowing for a more complete phenotypic analysis. Existing rde-1 alleles with experimentally established RNAi defects harbor small alterations to the DNA and/or protein sequence. These alleles are amino acid substitutions or disruptions that are downstream of the yy11 insertion: for example, a E141K substitution in the rde-1(ne219) allele; G485E in rde-1(ne4086); I562L in rde-1(ne4085); G1016E in rde-1(ne297); and a Q825STOP mutation in rde-1(ne300). The Tc1 insertion in yy11 occurs after amino acid G683, and is a more severe disruption than existing point mutations, meaning that it is also likely to be a null mutation.

Maternal effects can mask the presence of mutations in genes with RNAi function

We previously described a strain with an RNAi-defective allele, ne309, that does not mount an RNAi response to ingested dsRNAs [4]. We mapped the ne309 mutation to chromosome III, and we performed complementation tests using large deletons to localize the ne309 mutation and to help confirm that the strain harbored a single RNAi-defective allele (Figure 3A). Deletions lacking DNA sequences near the center of chromosome III failed to complement ne309, which led us to perform additional complementation tests between ne309 and RNAi-defective strains with mutations in specific genes that map to this region. Among these genes was rde-4, which failed to complement ne309. Upon sequencing of the rde-4 gene in ne309 mutants, we found a mutation consisting of a single T insertion in exon 1. The T insertion in ne309 also has a non-identical T insertion within the same codon for A la123 [13] (Figure 3C).

Our preliminary analyses of rde-4(ne309) homozygotes revealed that the mutants were RNAi deficient in their response to ingested dsRNA, yet they displayed a systemic RNAi response to transgene-delivered dsRNA [4]. These results are inconsistent with reports of fairly strong RNAi defects in rde-4 mutants, irrespective of dsRNA delivery method [13]. We therefore re-investigated the RNAi response to transgene-delivered dsRNA in rde-4(ne309) mutants. We first ensured that the strains in this study did not harbor previously described background mutations that affect RNAi activity; for example, we used strains that had been extensively out-crossed in order to remove any incidental mutations that might affect RNAi. We again used the two transgene system to target silencing of a gfp reporter to assess RNAi activity (as in Figure 1). In these experiments, we observed strong RNAi defects in rde-4(ne309) and rde-4(ne299) mutants, which is consistent with previous reports of strong RNAi defects in rde-4. By contrast, we also observed RNAi competency in rde-4 homozygotes due to maternal effects (Figure 4A and Tables 1 and 2).

An RNAi response was observed in the adult somatic tissues of rde-4 homozygotes born from a heterozygous parent, which is surprisingly long-lasting for a maternal effect (Figure 4A). Such long-lasting maternal effects have also been observed for dcr-1 mutants [14,15]; however, unlike rde-4 mutants, dcr-1 animals are sterile. There are several factors which may contribute to the perdurance of RNAi activity in rde-4 mutants displaying such maternal effects. C. elegans harbors dsRNA amplification mecha-
nisms that, in part, involve the activity of RNA-dependent RNA Polymerases and the production of secondary siRNAs [16]. Thus, any maternally deposited silencing RNAs, along with maternally deposited RDE-4 could contribute to secondary siRNA production early in development, allowing for later RNAi activity, long after the maternally deposited RDE-4 and dsRNA are eliminated. Another factor that could contribute to RNAi activity in rde-4 adults might involve early modifications to chromatin, triggered by maternally deposited molecules, and persistence of the modifications through larval development [17], perhaps maintained by later exposure to dsRNA. Maternal effects have previously been observed for rde-4, yet such experiments made use of dsRNAs that were injected into heterozygous animals, derived from heterozygous rde-4 parents, display RNAi activity due to maternal effect (third row). rde-4 F3 animals, derived from homozygous rde-4 parents, display RNAi defects, as evidenced by the lack of GFP reporter silencing (fourth row). Each generation of animals was derived from a series of genetic crosses, with genotypes depicted on the right. Similar results were observed for both rde-4(ne299) and rde-4(ne309) alleles. The animals depicted in (A) are adults. (B) Early stage wild-type L1 larvae harboring both gfp dsRNA trigger and gfp mRNA target sequences do not display RNAi. In later stages, RNAi activity in these wild-type animals becomes apparent, as depicted in (A), top left. 40 x magnification.

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The relatively late-acting nature of the promoter in this system provides additional insurance that dsRNA is not present in early developmental stages, and potentially allows time for any maternally deposited RDE-4 or dsRNA molecules to be cleared before new gfp dsRNA is transcribed. Indeed, 100% of the newly hatched L1 larvae from this dual transgene system do not display an RNAi phenocopy even in wild-type animals (as depicted in Figure 4B), an indication that any dsRNA molecules or chromatin modifications remaining at this stage have been eliminated or are functionally ineffective. Thus, the observation of RNAi activity in rde-4 homozygotes at adult stage (Figure 4A) provides an indication that maternally-deposited RDE-4 persists until later stages, when it then acts upon dsRNA that is newly transcribed.

In addition to maternal effects, there are other conditions that can influence the level of RNAi activity observed in rde-4 mutants. For example, RNAi activity has been observed in homozygous rde-4 mutants when the dosage of dsRNA is high. Indeed, we previously observed an RNAi response in rde-4(ne309) mutants to injected dsRNA [4]. Earlier reports also demonstrated that rde-4 mutants can display an RNAi response when large amounts of dsRNA are delivered by transgenes [22]. We can then infer several factors that may influence the level of dsRNA accumulation from
transgenes: the copy number of dsRNA-expressing cassettes in transgene arrays, the promoter strength, and the expression status of the transgene–transgenes configured as extrachromosomal arrays are targets of epigenetic mechanisms that can lead to increased levels of transcriptional repression in a generational fashion, especially in the germ line [21]. Our earlier observations that rde-4(ne309) mutants were capable of mounting a systemic RNAi response to dsRNA expressed in muscle were likely influenced by the rde-4 maternal effects, the young age of the dsRNA-expressing array newly formed arrays are metastable with respect to transcriptional status and may be capable of higher levels of transcription than an array maintained for many generations [21], and by the relatively low abundance of the gfp mRNA target used at that time [4]. Finally, environmental influences, such as temperature, have also been observed to affect the RNAi activity in wild-type as well as rde-4 and other RNAi-related mutants [22,23]. Taken together, these considerations highlight the possibility that dsRNA concentrations and environmental conditions might mask the presence of an underlying and unanticipated RNAi defect caused by a background mutation in a non-essential RNAi pathway component.

Conclusions

The Dicer double-stranded RNA-specific endonuclease plays a central role in many RNA silencing pathways and is an essential gene in *Caenorhabditis elegans* and other organisms. The DCR-1 protein, in part, catalyzes the conversion of dsRNAs into small siRNAs, facilitates the formation of the RISC complex, and processes miRNA genes in *C. elegans*. DCR-1 is directed towards particular functions in the context of the associated proteins. For example, the ERI/DCR complex contains DCR-1 as well as ERI-1, RRF-3, ERI-3, and ERI-5 [24]. The ERI/DCR complex is important for the proper function of endogenous siRNAs, a pathway that is relevant to proper sperm development [25]. In another distinct DCR-1 complex can be found the RDE-1 and RDE-4 proteins. Among other activities, this complex is required for an RNAi response to experimentally-delivered dsRNA. RDE-4 is a dsRNA binding protein required for production of siRNAs from long dsRNAs [26], while the RDE-1 Argonaute protein facilitates removal of the passenger strand from the siRNA, which is necessary in order for proper function of the guide strand [27]. Mutations in genes encoding the *eri* class of DCR-1 interacting proteins enhance RNAi activity, while mutations in rde-1 and rde-4 strongly abrogate RNAi. Despite the important endogenous roles played by Dicer and associated proteins, most of the proteins associated with Dicer are not essential. Thus, when such genes are

### Table 1. Analysis of RDE-4 maternal effects.

| Crosses at 22°C | # F1 animals | # F2 animals | # F3 animals |
|-----------------|--------------|--------------|--------------|
| Phenotype       | RNAI+ (Dim)  | RNAI− (Bright) | RNAI+ (Dim)  | RNAI− (Bright) | RNAI+ (Dim)  | RNAI− (Bright) |
| cross A         | 47           | 0            | 14           | 0             | 4            | 2             |
| cross B         | 68           | 0            | 15           | 0             | 6            | 2             |
| cross C         | 73           | 0            | 31           | 0             | 17           | 6             |
| Total:          | 27           | 10           |              |               |              |               |

RDE-4 maternal effects are fully penetrant and expressive. Progeny animals from crosses depicted in Figure 4A were scored in each generation for RNAi activity (see Materials and Methods). Results from three different crosses are depicted. Three experiments were conducted at 22°C. The maternal effects were not altered by growth temperature. *rde-4/+* F1 heterozygotes displayed wild-type RNAi activity, as expected. All of the F2 animals, including *rde-4/rde-4* homozygotes, displayed wild-type RNAi activity due to RDE-4 maternal effects. Some of the F2 progeny were chosen at random, allowed to produce F3 progeny, and subsequently genotyped by DNA sequencing. Those F2 progeny that were homozygous for *rde-4* produced F3 progeny that were RNAI− at 100% penetrance and expressivity; those F2 progeny that were heterozygous or wild type produced F3 progeny that were RNAI+ at 100% penetrance and expressivity, which is in keeping with a strong RDE-4 maternal effect. The ratio of wild-type to homozygous *rde-4* F2 animals was approximately 3:1, as predicted, and this is reflected in the RNAI activity of the F3s as well. Similar results were observed for both *rde-4(ne299)* and *rde-4(ne309)* alleles.

![Figure 4A](image-url)

### Table 2. Analysis of RDE-4 maternal effects.

| Crosses at 15°C | # F1 animals | # F2 animals | # F3 animals |
|-----------------|--------------|--------------|--------------|
| Phenotype       | RNAI+ (Dim)  | RNAI− (Bright) | RNAI+ (Dim)  | RNAI− (Bright) | RNAI+ (Dim)  | RNAI− (Bright) |
| cross A         | all          | 0            | all          | 0             | 14           | 4             |
| cross B         | all          | 0            | all          | 0             | 6            | 3             |
| cross C         | all          | 0            | all          | 0             | 3            | 1             |
| cross D         | all          | 0            | all          | 0             | 7            | 1             |
| Total:          | 30           | 9            |              |               |              |               |

RDE-4 maternal effects are not affected by temperature. Four separate experiments, performed as described for Table 1, were conducted at 15°C using *rde-4(ne309)*. Similar results were obtained using *rde-4(ne299)* alleles.
unknowingly mutated, there is potential for genetic complication and misinterpretation of data.

Here we report observations of rde-4 and rde-1 mutations that highlight the potential for maternal effects, environmental conditions, dsRNA dosage and recently derived mutations to complicate phenotypic analyses. The rde-1(yj11) mutation, a recently derived transposon insertion, spontaneously appeared in a strain that harbored a known RNAi-defective mutation. As a result, the effects of the background rde-1 mutation were at first mistakenly attributed to the known mutation. Fortunately, the rde-1(yj11) allele was localized to only one set of experiments, and the mutation was identified before further complications arose. The phenotypic complications related to rde-1(yj11) were opposite to those observed for rde-4(ne309). In this case, the systemic RNAi defects in a known, but not yet identified, RNAi-defective mutant were masked by maternal effects and a high ratio of dsRNA trigger molecules to mRNA target. There are many non-essential RNAi pathway mutants with RNAi defects that are more subtle than those observed in rde-4, and many of these mutants are dosage-sensitive with respect to the amount of dsRNA as well. Therefore, unanticipated background mutations in such genes have the potential to complicate phenotypic analysis in a similar manner.

It has become increasingly apparent in the C. elegans RNAi field that the possibility of background mutations must be considered.

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