Characterization of Nucl.ear RNAi defective (nrde) mutants in *C. elegans*

Proper gene expression is crucial for all cellular processes, and changes in normal gene expression has been implicated in a number of human diseases, including asthma, heart disease, and cancer (Guajardo 2005, Tan 2002, Peng 2005). One form of gene regulation is RNA interference (RNAi). Using forward genetics, we have identified three genes required for nuclear RNAi. These nrde genes regulate gene expression by targeting pre-mRNA in the nucleus. We have found that three of these proteins likely act downstream of previously characterized RNAi proteins known to regulate mRNA levels. Based on sequence identity, at least two of these proteins are conserved. Our results strongly suggest the three proteins act in the same pathway. Greater understanding of the basic mechanisms of RNAi will help maximize its use as a therapeutic to treat disease.

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**COVER SHEET**

TITLE: ___ Characterization of nuclear RNAi defective mutants in *C. elegans*__

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Title

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Abstract

Proper gene expression is crucial for all cellular processes, and changes in normal gene expression has been implicated in a number of human diseases, including asthma, heart disease, and cancer (Guajardo 2005, Tan 2002, Peng 2005). One form of gene regulation is RNA interference (RNAi). Using forward genetics, we have identified three genes required for nuclear RNAi. These genes regulate gene expression by targeting pre-mRNA in the nucleus. We have found that three of these proteins likely act downstream of previously characterized RNAi proteins known to regulate mRNA levels. Based on sequence identity, two of these proteins are conserved. Our results strongly suggest the three proteins act in the same pathway. Greater understanding of the basic mechanisms of RNAi will help maximize its use as a therapeutic to treat disease.

Background

RNA interference (RNAi) is the process by which small RNAs silence target mRNAs based on sequence complementation (Mello & Conte 2004, Sohail *et al.* 2005). Variations of RNAi, called post-transcriptional gene silencing, quelling, or co-suppression, has been found to regulate expression in a wide variety of organisms, including plants, yeast, nematodes, mice, and humans (Tabara *et al* 1999). In *Caenabhorditis elegans*, RNAi is used to target mRNAs for cleavage and degradation. The process begins when dsRNA, exogenously introduced or endogenously produced, is

processed by the protein, Dicer. Dicer binds dsRNA and transforms it into short interfering RNAs (siRNAs) ~19-31 nucleotides (nt) in length (Hammond 2000). The newly formed siRNAs next bind an Argonaute (Ago) protein. Ago, with a complex of additional proteins, binds the siRNA via PAZ and PIWI domains and uses the siRNA as a guide to bind the complementary target mRNA (Kidner 2005, Volpe 2002). The complex of proteins, along with the siRNA and the target mRNA, form the RNA-induced silencing complex (RISC). Once fully formed, RISC can cleave the target mRNA or inhibit its translation, thus extinguishing its expression (Figure 1). RNA-dependent RNA polymerases (RdRP) may amplifly the initial signal caused by the dsRNA by making additional copies of the siRNA by using the target mRNA as a template (Volpe 2002). These additional copies of siRNA or the original dsRNA are able to spread between cells via transmembrane proteins, increasing the number of cells targeted by dsRNA to the entire worm. This process is called systemic RNAi (Fire 1998, Tabara *et al* 1999).

RISC may target complementary nucleic acids arising from many sources during RNAi. RNAi has been demonstrated to target foreign genetic material including viruses and transgenes in plants, and transposons in *C. elegans*. RNAi can be induced in *C. elegans* by feeding the worms bacteria engineered to produced dsRNAs (Mello & Conte 2004). RNAi also works on endogenous sources of nucleic acids as well. DNA and chromatin structure are targeted by RNAi in yeast and plants, and microRNAs formed within cells are essential to properly regulate gene expression (Mello & Conte 2004). Endogenous RNAi has been found to target a number of steps in gene regulation, including cleavage of target mRNA, and binding of target mRNA to prevent translation (Ketting 2001).

Finally, RNAi has also been shown to change DNA sequence. In *Tetrahymena*, RNAi can remove portions of the genome, completely eliminating the DNA sequence (Coyne 1996).

In *C. elegans*, RNAi has been extensively studied, and the genes in the process continue to be characterized. In particular, Ago proteins are one of the crucial proteins that have been studied intensively. AGO is a key component of RISC that facilitates the targeting of the mRNA that is to be degraded. By using bioinformatic software programs, Yigit *et al* (2006) identified 27 Ago genes in the *C. elegans* genome. A large number of AGO may indicate functional redundancy and the importance of properly working RNAi or the existence of multiple pathways that utilize a particular AGO (Yigit 2006). It has been shown that a particular AGO, *rde-1*, is required to trigger RNAi in response to exogenous dsRNAs (Yigit 2006). Yigit *et al* (2006) have determined that AGO act sequentially during RNAi, possibly one set specific to the initial siRNA and the second to amplified siRNAs. Additionally, mutants in the AGOs affect diverse functions, including chromosome segregation and fertility (Yigit 2006). The exact roles of the numerous AGO proteins are yet to be determined however.

An interesting feature of the *C. elegans* genome, operons, can be used to study RNAi, including the function of AGOs. Twenty percent of genes in *C. elegans* are found in operons. In the nucleus two or more genes of the operon are expressed as a single pre-mRNA product. The single pre-mRNA is then processed into multiple gene products and transported to the cytoplasm. Previously, Bosher *et al* (1999) found that RNAi can target

pre-mRNAs. Bosher *et al* (1999) observed that dsRNAs targeting the first gene of the operon were found to result in the phenotype of both genes of the operon (Bosher, *et al.*, 1999) (Figure 2). The only time both the first and second gene are in *cis* is in the nucleus. Thus, RNAi can occur in the nucleus. Here we show that one of the 27 AGOs is needed to target pre-mRNA by RNAi. Additionally, we identify several other proteins that are essential to target pre-mRNA by RNAi.

Results

Previously, we screened for the presence of mutants defective in targeting pre-mRNA by RNAi (henceforth termed nuclear RNAi) by taking advantage of the operons of *C. elegans*. *lir-1* lies in an operon with *lin-26*. Null mutants for *lir-1* are viable. However, null mutants for *lin-26* are lethal. Targeting *lir-1* by RNAi in a wild type worm results in death because the pre-mRNA consisting of both *lir-1* and *lin-26* is silenced. *lir-1* lethality by RNAi is due to the loss of *lin-26*. This lethality is a nuclear phenomenon because *lir-1* and *lin-26* are in *cis* only in the nucleus (Bosher *et al* 1999). In nuclear RNAi deficient mutants, worms survive RNAi targeting of *lir-1*. EMS-treated worms were selected for viability when fed dsRNA targeting *lir-1*. We used an RNAi hypersensitive strain, *eri-1* to sensitize the selection. In order to verify that the mutants could still target mRNAs located in the cytoplasm, the mutants were also subject to *pos-1* RNAi. In wild type, RNAi of *pos-1* is lethal. Nuclear RNAi deficient mutants (nrde) were defined as those that survived *lir-1* RNAi, but retained the ability to respond to *pos-1* RNAi. Three complementation groups were defined in the screen, termed *nrde-1*, *nrde-2*, and *nrde-3*.

In order to locate the mutation in the genome, we used single nucleotide polymorphism mapping. Each nrde strain was crossed with a mapping strain. The mapping strain contained single nucleotide polymorphisms (SNPs) that can be used to locate the position of a gene to a particular interval of the genome. The F1 generation was allowed to self and F2 were selected for viability on *lir-1* RNAi. The survivors were genotyped to narrow down the interval in question until the exact location of the gene was found. Once this was determined, the gene in question was sequenced in order to identify the nucleotide base mutation resulting in the nrde phenotype. *nrde-2* was found to be located on chromosome II, position 2.34. A total of six alleles were found. BLASTing nrde-2 showed that this gene normally encodes a conserved protein of no known function. The genes encoding the three alleles of *nrde-1* and 46 alleles of *nrde-3* were identified the same way.

Based on BLAST searches, *nrde-3* is an AGO. In order to further define *nrde-3*'s role as an AGO, several mutations were made to study *nrde-3*. First, the protein was fused to GFP, forming NRDE-3::GFP. NRDE-3::GFP was found to be located primarily in the nucleus (Figure 3A). To verify that the presence of GFP was not affecting results, the RNAi phenotype *of nrde-3::GFP* was compared to the wild type strain and *nrde-3*. The phenotypic results were the same in both the GFP and the wild type strains, indicating that GFP did not affect RNAi. Next, in a background lacking endogenous siRNAs (*eri-1*), NRDE-3::GFP was found in the cytoplasm. However, upon feeding of dsRNA, NRDE-3::GFP translocated to the nucleus in the *eri-1* background (Figure 3B).

Interestingly, *nrde-3* encodes a nuclear localization signal (NLS). The NLS signals to the cell to transport the protein to the nucleus. We perturbed the NLS from the *nrde-3::GFP* strain by substituting three amino acids of the NLS to create *nrde-3 *NLS::GFP*. NRDE-3 *NLS::GFP was located in the cytoplasm upon exposure to dsRNAs and with no dsRNA exposure (Figure 3C). This demonstrates that the NLS is necessary to translocate NRDE-3 to the nucleus.

Many proteins are known to play a role in the cytoplasmic pathway of RNAi, including *sid-1*, a transmembrane protein necessary for systemic RNAi (Winston *et al* 2002). To study how NRDE-3 may interact with these proteins, we crossed the mutants strains with NRDE-3::GFP and visualized NRDE-3::GFP. In *sid-1; nrde-3::GFP*, NRDE-3::GFP is found to be localized to the nucleus. Thus the lack of SID-1 does not affect NRDE-3::GFP localization to the nucleus. Another protein found to be required for cytoplasmic RNAi targeting mRNAs is *mut-2*. *mut-2* was previously found to be required to silence via exogenously introduced dsRNAs, acting at some point before the formation of siRNAs (Tabara *et al* 1999). MUT-2 was found to be required for NRDE-3::GFP localization to the nucleus (Figure 4). Therefore, the nuclear pathway appears to share at least some components of the cytoplasmic pathway.

We have shown that three different proteins are required for nuclear RNAi. In order to further understand how these proteins are related, we created double mutants in an *eri-1* background of each type: *nrde-1; nrde-2*, *nrde-1;nrde-3*, and *nrde-2; nrde-3*. These mutants were not as viable as wild type, had smaller brood sizes, and greater lethality

than wild type. The ability of the mutants to perform RNAi was also compromised. Normally in an *eri-1* background, worms are very sensitive to *unc-15* siRNAs. UNC-15 encodes for a protein that regulates neurotransmitter release (Kohn *et al* 2000). *eri-1* worms become paralyzed but are able to produce offspring. *rde-1* worms appear wild type when exposed to *unc-15* dsRNA indicating that *unc-15* protein expression is unaffected. *rde-1* encodes for an AGO known to be essential to both cytoplasmic and nuclear RNAi. Compared to the controls, *nrde* single and double mutants lie between the two extremes. The degree of paralysis varies per mutant. Double mutants in the NRDE proteins in comparison to single mutants did not demonstrate synergistic defects in responding to *unc-15* RNAi. No additive effect in the double mutants implies that the proteins act in the same pathway rather than multiple, parallel pathways (Figure 5).

Although the genes found appear to play a crucial role in nuclear RNAi, the genes identified were found in an *eri-1* background. In order to determine whether additional products could be identified in a wild type background, an additional screen was completed. First the worms were mutagenized using EMS and then subjected to *lir-1* RNAi and then *pos-1* RNAi. From this screen, a number of mutants were identified, including several genes known to be involved in the cytoplasmic RNAi. In addition, four novel nrde mutants were found, dubbed nrde-4, 5, 6, and 7. Complementation testing was completed to demonstrate that each mutation defines a different gene. Mapping is underway.

## Discussion

RNAi regulating levels of endogenous dsRNAs is known to be crucial to proper development in *C. elegans.* However, the role RNAi plays in gene regulation in *C. elegans* is not entirely known (Olsen & Ambros 1999). RNAi has been found to additionally target exogenously introduced "selfish genes" such as transgenes, transposons, and viruses in other organisms. Many genes found to be necessary for RNAi in *C. elegans* inhibit transposons. It has been proposed that RNAi acts as an innate immune system against foreign RNA in *C. elegans*, although, to date, *C. elegans* has no known pathogenic viruses (Shaham 2006). Combating these selfish genes potentially require a nuclear set of proteins to prevent their integration into the genome. Here, we have identified a number of proteins which may be involved in the nucleus to perform RNAi. We have shown that NRDE-3 localization to the nucleus is an example of a component of the RNAi pathway that may act specifically to target genes at the level of pre-mRNA. Although NRDE-3 is an Ago family protein, it is highly unlikely that is cleaves the target mRNA. The possibly lack of endonuclease activity in NRDE-3 demonstrates that other proteins yet to be determined may act to actually down-regulate the pre-mRNA. The localization of NRDE-1 and NRDE-2 is currently under study and whether these proteins are localized to the nucleus will contribute to our understanding of nuclear RNAi.

Although we have shown how NRDE-3::GFP translocates to the nucleus in the cytoplasmic RNAi mutant, *sid-1*, we do not understand how or whether the nuclear and cytoplasmic pathways of RNAi diverge. To further understand the target mRNAs of

NRDE-3, we have identified one of the NRDE-3 binding RNAs to be one of the most targeted endogenous genes of the cytoplasmic pathway, E01G4.5 (pers. comm. Pavelec 2008). We will fuse E01G4.5 to GFP, or introduce the sequence to an intron of GFP, and use the engineered protein as a tool to determine the ability of nuclear RNAi mutants to target endogenous genes. We will RNAi known RNAi genes and look for misregulation of E01G4.5 as evidence that the RNAi gene down-regulated is necessary for the nuclear RNAi pathway.

The nuclear pathway studied was screened for mutants in targeting exogenously introduced siRNAs, the nuclear location of NRDE-3 hints at a role in genome control. In plants and yeast, RNAi is known to affect gene expression by influencing heterochromatin formation though DNA methylation. DNA does not appear to be methylated in *C. elegans* nor is a conventional DNA-methyltransferase encoded (Bird 2002). However, methylation of histones may be possible (Simpson 1986). Further studies using ChIP to study the effects of nuclear RNAi mutants on histone modification would demonstrate whether nrde mutants affect gene expression at the level of transcription.

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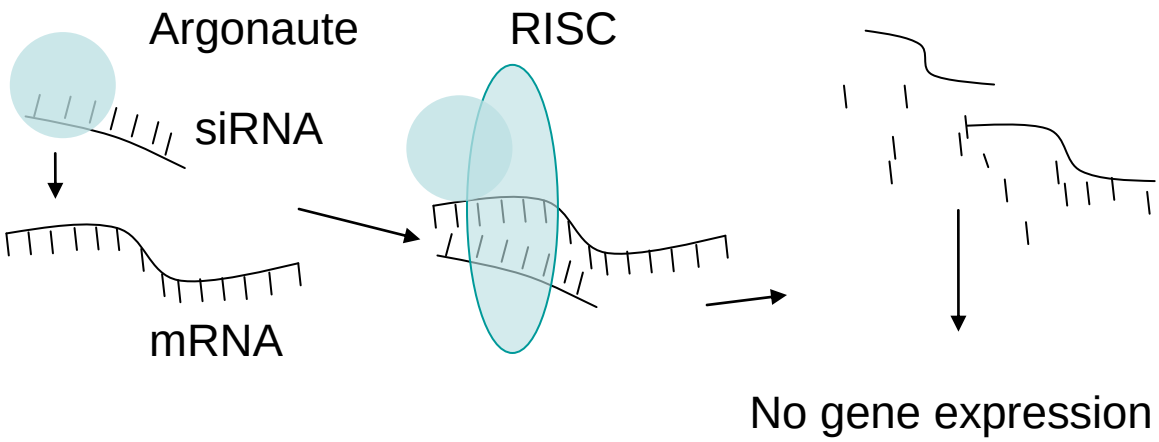



**Figure 1. The current model for the RNAi Mechanism**
The protein Dicer transforms dsRNA into siRNAs, which form a complex called RISC. RISC cleaves mRNA at the sites homologous to the siRNAs of which it was made to prevent transcription

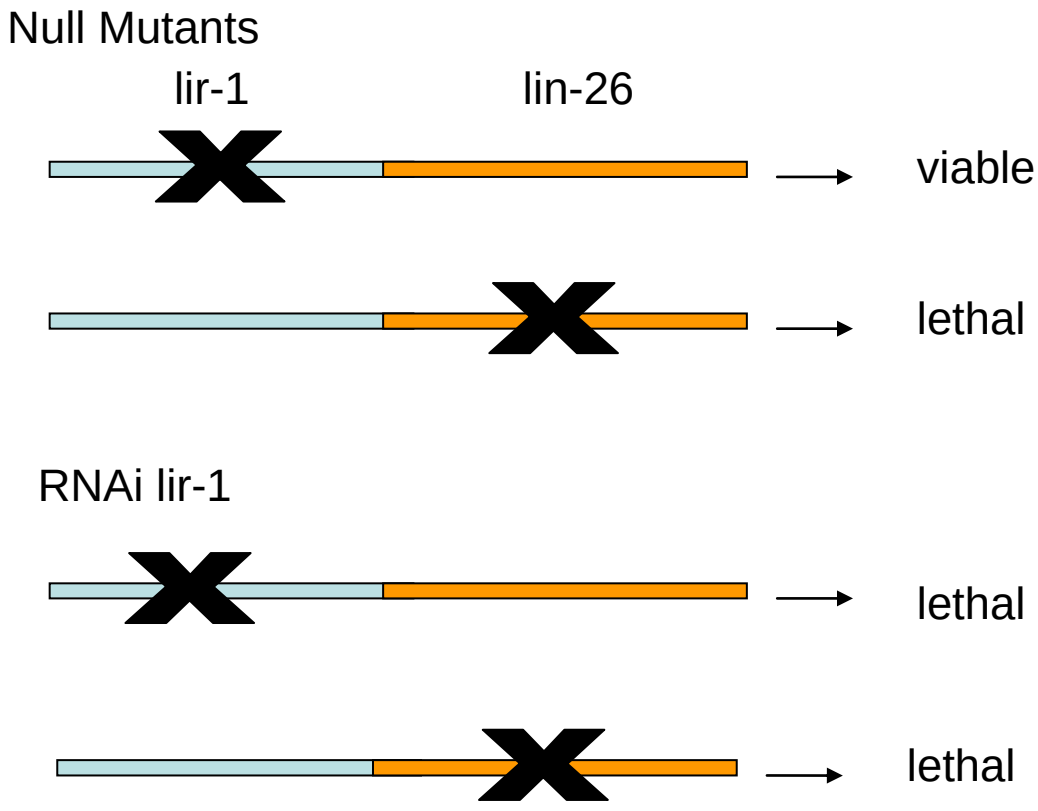
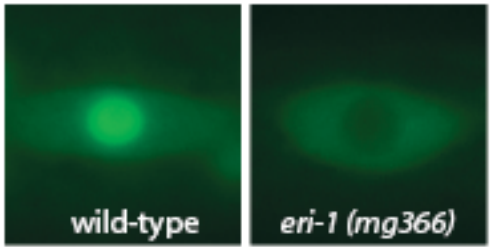



**Figure 2. Nuclear RNAi compared to mutant phenotypes.**
Targeting *lir-1* with dsRNAs leads to a phenotype reminiscent of the *lin-26* null mutant.

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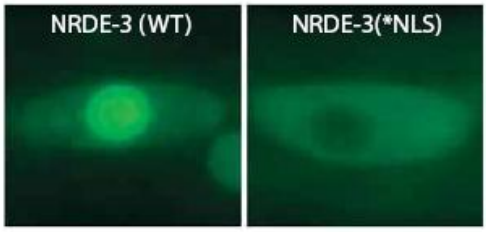


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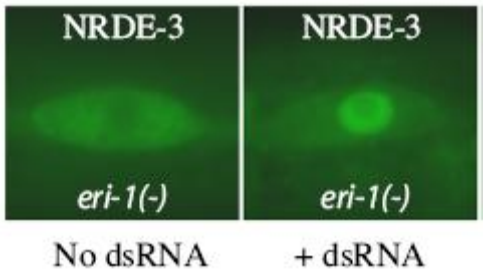


C

**Figure 3. NRDE-3::GFP translocation.**
A, NRDE-3::GFP is localized to the nucleus without feeding with dsRNAs. In the *eri-1* background, NRDE-3::GFP is compared to *nrde-3::GFP*. B, The strain *nrde-3 *NLS::GFP* localization is compared to *nrde-3::GFP*. C, NRDE-3::GFP localization in the *eri-1* background is compared with and without dsRNA feeding.

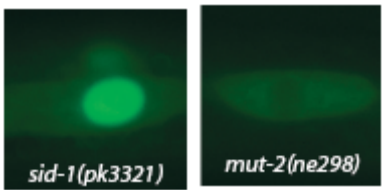


**Figure 4. NRDE-3::GFP Localization in cytoplasmic RNAi mutants.**
NRDE-3::GFP localization is visualized in *sid-1* and *mut-2* mutant backgrounds.

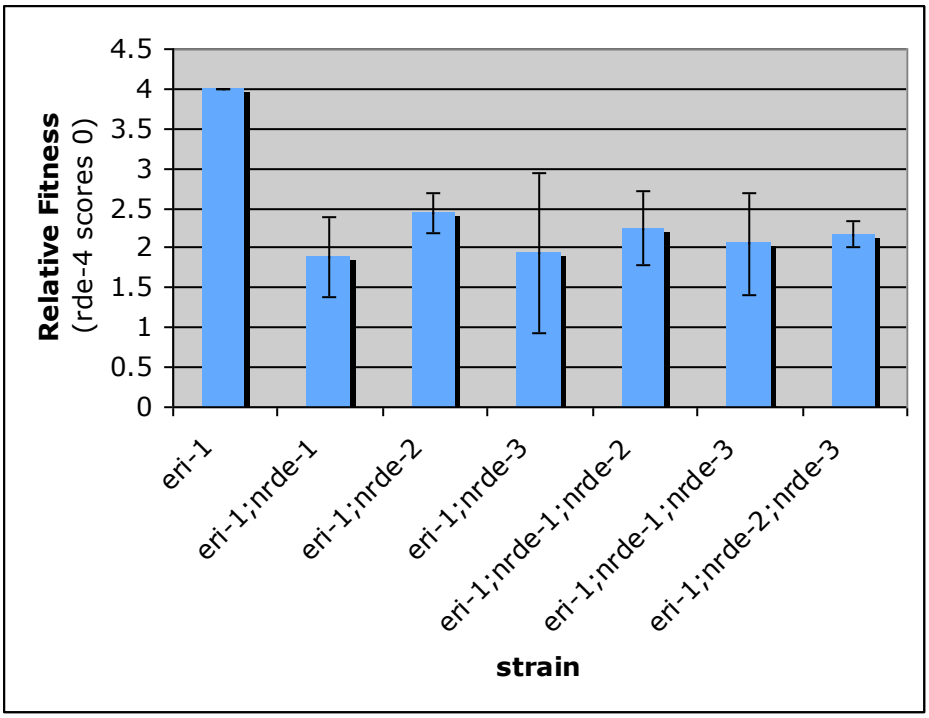


**Figure 5. RNAi phenotyping for synergy between nrde mutants.**
Single and double nrde mutants were scored for *unc-15* RNAi phenotype in the *eri-1* background. A score of 0 was the *rde-1* (necessary for cytoplasmic RNAi) *unc-15* RNAi phenotype. The score of 4 was the *eri-1* (enhanced exogenous RNAi) *unc-15* RNAi phenotype.