A FUNCTION FOR ABC TRANSPORTERS IN RNAi

By

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Abstract

RNA interference (RNAi) is a conserved gene-silencing phenomenon that can be triggered by delivery of double stranded RNA (dsRNA) to cells and is a widely exploited technology in analyses of gene function. While a number of proteins that facilitate RNAi have been identified, current descriptions of RNAi and interrelated mechanisms are far from complete. Here we report that the *Caenorhabditis elegans* gene *haf-6* is required for efficient RNAi. HAF-6 is a member of the ATP binding Cassette (ABC) transporter gene super family. ABC transporters utilize ATP to translocate small molecule substrates across the membranes in which they reside, often against a steep concentration gradient. Collectively, ABC transporters are involved in a variety of activities, including protective or barrier mechanisms that export drugs or toxins from cells (Broeks *et al.*, 1996; Bauer *et al.*, 1999; Begley, 2004; Fromm, 2004), in organellar biogenesis (Aubourg, 1994), and in mechanisms thatprotect against viral infection (Trowsdale *et al.*, 1990; Abele and Tampe, 2004). HAF-6 is expressed predominantly in the intestine and germline and is localized to intracellular reticular organelles. We further demonstrate that nine additional ABC genes from diverse subfamilies are each required for efficient RNAi in *C. elegans*, all expressed in the germ line and may function by aiding in the formation of a functional RDE-2/MUT-7 complex. Thus, the ability to mount a robust RNAi response to dsRNA depends upon the deployment of two ancient systems that respond to environmental assaults: RNAi mechanisms and membrane transport systems that utilize ABC proteins.

Chapter 1

RNA interference mechanism in C. elegans

PTGS and RNAi – History and Discovery

RNA interference, or RNAi, falls under the umbrella of RNA-based gene silencing phenomena that degrade mRNA at the post-transcriptional level (PTGS). Such PTGS mechanisms were well-documented in plants as early as 1990 when Napoli and Jorgensen tried to increase pigmentation in petunia by over-expressing a pigment gene called Chalcone synthase. Interestingly, some plants lost pigmentation and further analyses indicated that the expression levels of both the endogenous and transgenic Chalcone synthase gene were very low (Napoli, Lemieux et al. 1990). This phenomenon was called co-suppression, in which a transgene silences a homologous endogenous gene. Co-suppression has been reported in other organisms such as *Neurospora* (Romano and Macino 1992), *Drosophila* (Pal-Bhadra, Bhadra et al. 1997) and *C. elegans (Dernburg, Zalevsky et al. 2000).*

An interesting phenomenon of gene silencing was observed in *C. elegans* when experimentally introduced antisense RNA was shown to effectively phenocopy *unc-22,* a muscle gene (Fire, Albertson et al. 1991), and *par-1,* required for embryonic polarity (Guo and Kemphues 1995). In fact, a mixture of sense and antisense RNA was shown to silence genes in *C.elegans* more efficiently than either the sense or antisense strand alone (Fire, Xu et al. 1998). This phenomenon of gene silencing mediated by the dsRNA trigger was termed RNA interference or RNAi. RNAi is conserved in eukaryotes (Hannon 2002) and is usually post-transcriptional in nature (Montgomery, Xu et al. 1998). The interference is highly sequence-specific

(Montgomery and Fire 1998) and very few molecules are required to elicit a robust RNAi response (Fire, Xu et al. 1998).

Endogenous functions of RNAi

RNAi and antiviral defense

RNAi may have evolved as a protective mechanism against naturally occuring viral infections. Several lines of evidence support this notion – almost 90% of known plant viruses have an RNA genome (Voinnet 2001); PTGS could be induced in plants by viral infections (Viral Induced Gene Silencing or VIGS) (Ruiz, Voinnet et al. 1998); viruses have evolved mechanisms to suppress PTGS in plants (Anandalakshmi, Pruss et al. 1998). Although there are no known natural viruses infecting *C. elegans*, it has recently been shown that *C. elegans* tissue culture cells lacking RNAi components when infected experimentally with vesicular stomatitis virus showed decreased resistance to infection (Schott, Cureton et al. 2005), whereas mutant strains exhibiting enhanced RNAi showed increased resistance to infection (Wilkins, Dishongh et al. 2005). Another interesting study has revealed that an RNAi component is necessary for antiviral defense against replicating flockhouse viral sequences in *C. elegans* strains (Lu, Maduro et al. 2005). RNAi machinery has also been reported to be essential in *Drosophila* embryos and adults, in order to mount a robust immune response against insect viruses (Fritz, Girardin et al. 2006).

RNAi and transposon silencing

Transposons are active in the *C. elegans* soma but completely silenced in the germ line. In fact, *C. elegans* mutant strains defective in RNAi components like *rde-2, rde-3,* and *mut-7* show an increased rate of transposition in the germ line. (Ketting, Haverkamp et al. 1999; Tabara, Sarkissian et al. 1999; Sijen and Plasterk 2003). Tc1 is the most abundant transposon in the *C. elegans* genome and is characterized by the presence of terminal inverted repeats. Insertion of several copies of the Tc1 transposon into random locations in the genome or read-through transcription of a single copy of a Tc1 transposon with terminal inverted repeats may produce aberrant RNA that is double stranded in nature, which may in turn serve as a trigger for RNAi (Vastenhouw and Plasterk 2004).

RNAi and chromatin

As discussed above, RNAi components are indispensable for maintaining the silenced state of transposons in the germ line. RNAi suppresses transposon hopping by condensing them into heterochromatic domains in the genome (Hannon 2002). This leads us to question whether RNAi machinery facilitates transcriptional gene silencing (TGS) in addition to its long-standing role in PTGS, and if so, are RNAi components associated or indirectly function at the level of chromatin in the nucleus? Several studies support this idea of the role of RNAi in TGS. Fission yeast *S. pombe,* carrying deletions in RNAi gene homologues - argonaute, dicer, and RNA-dependent RNA polymerase (RdRP) - showed accumulation of transcripts whose sequences

were homologous to centromeric repeats (Volpe, Kidner et al. 2002). Volpe, et al. also demonstrated that RNAi function was essential for methylation of lysine 9 of histone H3, a hallmark of heterochromatin, and also demonstrated that RdRP was associated with chromatin by Chromatin Immuno-Precipitation (CHIP) assay in fission yeast. RNAi screens to isolate genes involved in co-suppression in the *C. elegans* germ line identified chromatin associated factors (Robert, Sijen et al. 2005). Another RNAi screen for factors affecting RNAi-TGS also identified chromatin associated factors (Grishok, Sinskey et al. 2005). It is therefore interesting to conclude that RNAi could regulate gene expression by coordinating pathways that operate both at the transcriptional level in the nucleus and post-transcriptional level in the cytoplasm.

RNAi and development

One of the prime biological roles of RNAi discussed so far is to destroy deleterious genomes like foreign viral sequences, aberrant heterochromatic transcripts, and also to silence endogenous transposons, all to the advantage of the survival of the organism. It is important to also realize that organisms use RNAi mechanisms to regulate processes required for their normal development. An interesting example is the RNAi defective *C. elegans dcr*-1 mutant, which exhibits several developmental abnormalities, altered developmental timing, and inability to process *lin-4* and *let-7* microRNA (miRNA) (Ketting, Fischer et al. 2001). *lin-4* and *let-7* miRNA are endogenous non-coding RNA molecules which negatively regulate *lin-14* mRNA

(Lee, Feinbaum et al. 1993; Wightman, Ha et al. 1993) and *lin-41* mRNA (Vella, Choi et al. 2004) respectively by translational repression, which in turn ensures proper developmental timing in *C. elegans.* Innumerable studies have recently shown that miRNAs regulate several developmental processes in plants and animals (Carrington and Ambros 2003) by a mechanism similar to RNAi (Doench, Petersen et al. 2003; Zeng, Yi et al. 2003)

RNAi machinery

Mechanism of RNAi

RNAi is a post-transcriptional mechanism occurring in the cytoplasm. A simplistic illustration of the mechanism is described here and in Figure 1-1. When a cell encounters a dsRNA trigger, it is recognized by an RNase III class enzyme Dicer (Bernstein, Caudy et al. 2001), and cleaved into ~ 21 – 23nt small interfering molecules or siRNA (Hamilton and Baulcombe 1999; Zamore, Tuschl et al. 2000) in an ATP-dependent fashion. Dicer is a highly conserved protein in all eukaryotes. It belongs to the RNase class III endonucleases characterized by an RNase III catalytic domain and dsRNA binding domains. *C. elegans* has one homologue of dicer called DCR-1; *Drosophila* has two dicer homologs, DCR-1 (miRNA processing) (Lee, Nakahara et al. 2004) and DCR-2 (siRNA processing) (Pham, Pellino et al. 2004). siRNAs are double-stranded, have characteristic 2-nt overhangs at their 3' ends and phosphate groups at their 5' ends (Elbashir, Harborth et al. 2001; Elbashir, Martinez et al. 2001). One strand of siRNA is loaded into the RNA-Induced Silencing Complex

(Tavernarakis, Wang et al.) (Hammond, Boettcher et al. 2001). The stability and thermodynamic properties of the two strands of siRNA determine which strand would be incorporated into the RISC (Khvorova, Reynolds et al. 2003; Schwarz, Hutvagner et al. 2003). RDE-4 and RDE-1 are members of the argonaute family of proteins that form an essential component of the RISC complex. RDE-4, a dsRNA binding protein interacts with RDE-1 (Tabara, Sarkissian et al. 1999), and a DExH-BOX helicase to facilitate RNAi (Tabara, Yigit et al. 2002). RDE-4 is also a homolog of R2D2 protein in Drosophila. R2D2 is a dsRNA-binding protein that binds to DCR-2 and aids in the initial cleavage step and loading the siRNA to the RISC complex (Liu, Rand et al. 2003). Once the siRNA is incorporated into the RISC, the latter cleaves a homologous mRNA into 21-23 nucleotide fragments (Zamore, Tuschl et al. 2000). The AGO2 component of RISC has been shown to confer the "slicer" activity or the mRNA cleaving activity of RISC in mammals (Liu, Carmell et al. 2004) and has also been shown to be one of the components of RISC in *Drosophila* (Rand, Ginalski et al. 2004).

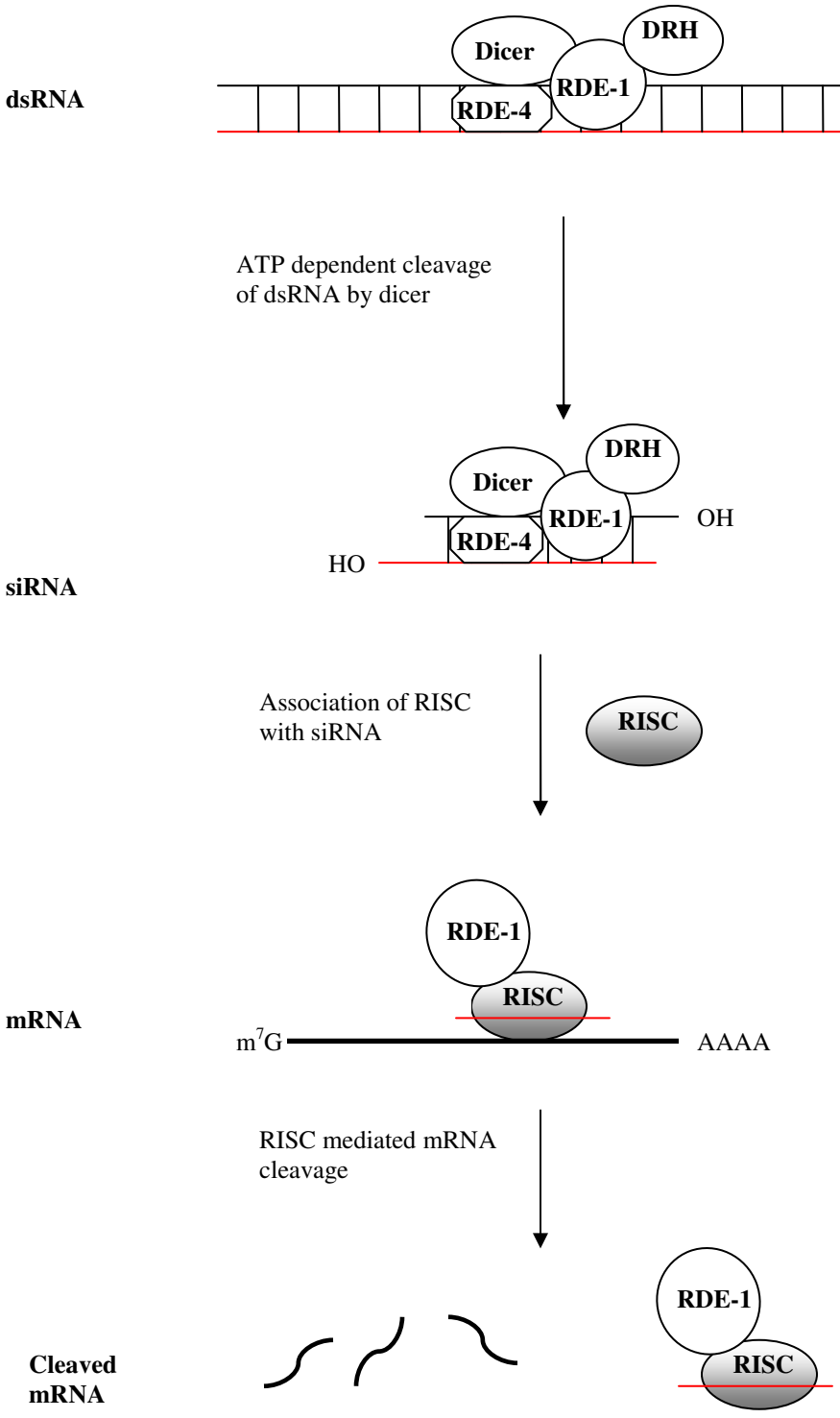


Figure 1-1: Mechanism of RNAi

RNAi and related gene silencing mechanisms

Co-suppression:

Co-suppression is a phenomenon of gene silencing induced by transgenes. This mechanism was first observed when wild-type *C. elegans* strains carrying a HIM-14::GFP transgene showed a *him-14* phenocopy (Dernburg, Zalevsky et al. 2000). Similar co-suppression was observed for other germ-line genes like *spo-11* and *fem-1*. An RNA-based mediator was hypothesized to induce co-suppression in these transgenic lines because the phenocopy was retained to a certain degree even in strains that had lost the transgene array. Also, only those transgenes which were functional transcriptional units were capable of eliciting co-suppression, leading to the idea that an RNA mediator may be involved. This study also demonstrated that genes indispensable for the RNAi mechanism were also involved in co-suppression. *rde-2* and *mut-7* genes were essential for co-suppression, whereas *rde-1* was not. Thus, there seems to be a potential link between the mechanism of RNAi and co-suppression in *C. elegans.*

Nonsense-Mediated Decay:

Nonsense-mediated decay is an mRNA surveillance mechanism that degrades mRNA with premature stop codons. One of many such surveillance mechanisms is maintained by the SMG group of proteins in *C. elegans.* Mutations in *smg* genes seem to affect the RNAi mechanism (Domeier, Morse et al. 2000). When *unc-54* dsRNA sequences were injected into *smg-2* mutant strains, the animals initially became

paralyzed, but recover rapidly from paralysis, unlike wildtype. The *unc-54* RNA levels were also correlated with the recovery rate of *smg-2* mutant strains, whereas the *unc-54* mRNA levels remained low in the wild-type worm. The stability and modification of dsRNA was comparable in both wild-type and mutant strains. Based on these results, it was hypothesized that the *smg-2* mutant strains had the ingredients necessary to initiate the RNAi mechanism, but SMG-2 activity is needed in the later stages to maintain the RNAi activity.

C. elegans as a genetic system to study RNAi

Tremendous progress has been made in dissecting the RNAi pathway, and most of this knowledge originates from studies in the organism *C. elegans.* This study takes advantage of the *C. elegans* genetic system to study the mechanism of RNA interference.

C. elegans is a 1mm-long non-parasitic nematode, whose genetics were first studied by Sydney Brenner (Brenner 1974). *C. elegans* is a self-fertilizing hermaphrodite (XX), and males (XO) are rarely found in the wild-type population. Hermaphrodites give us the ease of propagation, which is usually 3 days at 20°C. While hermaphrodites can be subject to random chemical mutagenesis, males can also be used successfully in genetic crosses to carry mutations between *C. elegans* strains.

RNA interference was discovered in *C. elegans* (Fire, Xu et al. 1998) and is an ideal organism in which to study RNAi. There is no interferon response to dsRNA in *C. elegans.* The existence of a mechanism that allows systemic signals to enter and exit cells along with the presence of an amplification phenomenon makes RNAi a potent process in *C. elegans.* Delivery of dsRNA can be achieved by several relatively simple methods. dsRNA can be delivered by means of microinjecting (Fire, Xu et al. 1998) into the intestine or germ line, or by soaking the animal in high concentrations of dsRNA (Tabara, Grishok et al. 1998). Tissue-specific RNAi can be achieved by means of endogenously driving the expression of dsRNA through the use of transgenes. A very simple but efficient method to deliver dsRNA is to grow *C. elegans* on a lawn of bacteria that has been engineered to produce dsRNA (Timmons and Fire 1998). This is usually referred to as the feeding based RNAi assay and in this method and soaking, the dsRNA is taken up from the intestine just like other nutrients and spreads through out the organism and to the F1 progeny.

The feeding-based assay was used to design the first screen to isolate RNAi defective mutants (Tabara, Sarkissian et al. 1999). This screen identified mutant strains based on the method-of-delivery of dsRNA targeting the *pos-1* gene required for normal embryonic development. The class of mutants called the RNAi Defective or the *rde* group of mutant strains were defective by both injection an feeding of *pos-1*dsRNA, whereas the second class of mutants were defective only by feeding of *pos-1*dsRNA. One of the mutants identified in this method belonged to the feeding-defective class

and was called *ne335.* To date several genes involved in the RNAi process have been identified, yet our knowledge of the mechanism is far from complete. Thus our efforts to find the identity of *ne335* could be another big step in the field of RNAi and help better understand and dissect the mechanism of RNAi. The results of my graduate work are outlined in the ensuing chapters. Chapter 2 describes the genetic and molecular studies involved in the identification of *ne335* as a member of the ATP Binding Cassette or ABC transporters. It also discusses the interesting discovery of the nine additional ABC transporters involved in RNAi in *C. elegans.* Chapter 3 describes the discovery of two RNAi defective genes *rde-2* and *mut-7* as genetic partners of the 10 ABC transporters involved in RNAi. Our studies suggest that these transporters function upstream of *rde-2/mut-7* complex. Chapter 4 summarizes the findings reported in this dissertation and provides a tentative hypothetical model as to how *ne335* and the other 9 transporters may play a role in RNAi. It is needless to say that our work has made an interesting new discovery for the role of ABC transporters in RNAi and our future studies will be directed to understanding this role better.

Chapter 2

RNAi defects in C. elegans mutant haf-6(ne335)

This chapter describes the cloning and analysis of one of the RNAi defective mutant strains *ne335,* isolated in a feeding based screen for RNAi mutants *(Tabara, Sarkissian et al. 1999).*

Materials and Methods

C. elegans Strains

Worm husbandry and genetic crosses were performed according to standard protocols (Brenner 1974). The following strains, used in mapping, were generous gifts from Dr. Andrew Fire (Stanford University, Stanford, CA): LGI: *dpy-5(e61) unc-54(e1092);* LGIV: *unc-17(e245) dpy-4(e1166);* LGV: *dpy-11(e224) unc-60(e723);* LGII: *dpy-10(e128) unc-52(e669);* and LGIII: *dpy-18(e449) unc-32(e189)*. The *haf-6(ne335)* strain was a generous gift from Craig Mello (University of Massachusetts Medical School, Worcester, MA) (Tabara, Sarkissian et al. 1999). The following mutant strains were used in phenotypic analyses and were obtained from the *C. elegans* Genetics Center (University of Minnesota, Minneapolis, MN) or the National Bioresources Project in Japan: *abt-1(tm703)*; *abt-2(ok669)*; *abt-4(ok633)*; *C05D10.3(tm688)*; *C56E6.1(ok865)*; *cft-1(ok1180)*; *C16C10.12(2 alleles: ok927*, *ok962)*; *ced-7(5 alleles: n2690*, *n1892*, *n2094*, *n1996*, *n1997)*; *F02E11.1(ok1007)*; *F19B6.4(ok806)*; *haf-1(ok705)*; *haf-2(gk13)*; *haf-3(ok1086)*; *haf-4(2 alleles: gk240*, *ok1042)*; *haf-5(2 alleles: gk155*, *gk161)*; *haf-7(gk46)*; *haf-8(gk12)*; *haf-9(gk23)*; *mrp-1(pk89)*; *mrp-3(ok955)*; *mrp-4(ok1095)*; *mrp-6(ok1027)*; *mrp-8(ok1360)*; *NL130: pgp-1(pk17), pgp-*

3(pk18); *NL132: pgp-1(pk17)*; *NL152: pgp-1(pk17)*, *pgp-3(pk18)*, *mrp-1(pk89)*; *pgp-10(ok991)*; *pgp-11(tm333)*; *pgp-12(gk19)*; *pgp-13(ok747)*; *pgp-15(ok987)*; *pgp-2(gk114)*; *NL131: pgp-3(pk18)*; *pgp-4(gk16)*; *pgp-5(ok856)*; *pgp-7(ok528)*; *pmp-1(ok773)*; *pmp-3(ok1087)*; *pmp-4(ok396)*; *RB1047: deletion in pgp-6 and pgp-7(ok994)*; *T26A5.1(ok882)*; *T27E9.7(ok771)*; *F18E2.2(ok830)*; *Y42g9a.6(ok812)*; and *Y49e10.9(ok1044)*.

Feeding Strains and Feeding-based Assays for RNAi Defects

"Feeding plates" harboring bacteria engineered to express dsRNA were prepared using HT115(DE3) host bacteria as described previously (Timmons, Court et al. 2001; Timmons 2006). Plasmid L4440 was the cloning vector for plasmids transformed into this bacterial strain (Timmons and Fire 1998). HT115 (DE3) strains harboring a *pop-1* cDNA insert in L4440 (*pop-1* food) targets the TCF/LEF1 transcription factor and produces sterility in young animals reared on this food. *unc-22* food (Timmons and Fire 1998) targets the muscle-specific *unc-22* transcript and induces a twitching phenotype. Other feeding strains were obtained from MRC/Geneservice RNAi library (Kamath and Ahringer 2003; Kamath, Fraser et al. 2003), or they were generated by inserting PCR-generated fragments of cDNAs into L4440.

RNAi was induced using the feeding protocol by placing animals as L1/L2 larvae onto fresh plates prepared as described previously (Timmons, Court et al. 2001; Hull

and Timmons 2004). In each experiment, control feeding plates with wild-type worms and OP50 plates with mutant worms were used to monitor for effectiveness of the delivery protocol and for potential environmental contributions to the phenocopy, respectively. Assays were performed at 15, 20, and 25°C. *pop-1*(RNAi) was assayed by tabulating the number of viable progeny produced per adult on the feeding plate. This value can be affected by temperature, the time frame of the experiment, and the staging of the animals at the time of placement onto the feeding plate; therefore, the value is comparative, not absolute. In each experiment, RNAi phenocopies in mutant strains were closely compared with similarly treated wild-type worms. Comparative values for *unc-22*(RNAi) activity were obtained by calculating the percentage of F1 animals that twitch.

Positional cloning of haf-6(ne335), Transgene Rescue of RNAi Defects, and Complementation Tests with smg-2

Ingestion of *pos-1* food by wild-type adults can induce lethality in resulting embryos—the presence of viable progeny is evidence of an RNAi defect. *haf-6(ne335)* mutant strains were isolated in a screen for RNAi-defective animals using *pos-1* food (Tabara, Sarkissian et al. 1999). *haf-6(ne335)* is recessive, and *ne335* is the only *haf-6* allele to date. Many RNAi-defective strains have no other phenotypes, and this raises the potential that a second uncharacterized mutation might contribute to RNAi defects in some strains. To assay for, or guard against, this possibility, we

produced different versions of *haf-6(ne335)* strains by outcrossing *ne335* to various wild-type stocks from different origins (e.g., N2 stocks obtained from the *C. elegans* Genetics Center versus N2 stocks maintained for several years in the laboratory of Andrew Fire). We selected for robust RNAi activity, viability, and fertility at 25°C, and for normal brood sizes in wild-type animals before using them in outcrosses. In initial stages of mutant characterization, feeding assays were used to select for *ne335* homozygotes; later, a PCR strategy was used to identify mutant homozygotes from additional outcrosses. Primers 683 GCCATCCTCTCAGCCTAC and 684 CCACCCACGCTCTTACATG were used in genotyping. The differently out crossed *ne335* strains behaved identically in our assays.

A rough genetic map was obtained using two- and three-factor mapping strategies and genetically marked strains. Heterozygous F1 cross-progeny were set aside and allowed to produce F2 progeny. Single F2 progeny were placed at L2/L3 stage onto *pop-1* feeding plates. Homozygous *haf-6* animals gave rise to F3 progeny, and the presence of the marker phenotype was noted for estimations of linkage or recombination frequency. The *ne335* mutation mapped to the left end of chromosome I. A series of overlapping yeast artificial chromosomes (YACs) corresponding to this region were obtained from the *C. elegans* Sequencing Consortium (Sanger Institute, Cambridge, United Kingdom). YAC DNA was obtained by preparing total genomic DNA from yeast. Purified DNA was injected into *ne335* mutant strains and rescue of RNAi defects was assayed using *pop-1* and *elt-2* food.

For complementation testing, crosses were performed using one hermaphrodite per mating on standard OP50 plates. The presence of 50% males in the progeny indicated a successful cross. Complementarity was assessed in reciprocal crosses: 1) *haf-6(ne335)* hermaphrodites were mated with *smg-2(e2008); him-5(e1417)* males, and 2) *smg-2(e2008)* hermaphrodites were mated with *haf-6(ne335); him-5(e1417); ccIs8160* [*rpL28::gfp*] males. The green fluorescent protein (GFP)-expressing transgene (generously provided by Dr. A. Fire) (Timmons, Tabara et al. 2003) facilitated detection of cross-progeny. Cross-progeny were transferred as L1/L2 larvae onto *pop-1* feeding plates; *unc-22* food elicited similar results. Complementation between *smg*-2 and *haf-6* was tested at 20 and 25°C, with similar results.

Plasmids and Transgenic Strains

Transgene lines were established as complex arrays to better ensure expression in the germline (Kelly, Xu et al. 1997). DNA sequences were linearized before injecting. A plasmid harboring a dominant mutation in the rol-6 gene was used as transformation marker; bacteriophage lambda DNA or *C. elegans* genomic DNA was used as carrier.

DNA Segments Used in Plasmid Cloning

1) The *haf-6 cDNA* extends from the initiator ATG, lacks a 5'-untranslated region (UTR), includes its 3'-UTR, and was generated by reverse transcription (RT)-PCR using wild-type RNA as template and primers 472 ATGTCAATTCTATCTAAACTATCCC and 473 CTTTAATATTTCTACTAAAATTTAAA. 2) A *smg*-2 cDNA, lacking in 5'-UTR sequences, harbors an initiator ATG and 3'-UTR and was amplified by RT-PCR using wild-type RNA as template and primers 488 ATGGACGATTCGGACGACGAATATTCGAGAAGCCACGGGG and 489 AATTTTTCTAAAAATTTCGGATTTTCGAGAGAAAATCAAGC. 3) The ends of the *haf-6/smg-2* intergenic region are bounded by the 5'-UTRs corresponding to each gene. This fragment was obtained by PCR amplification using wild-type DNA as template and primers 501 TTTACTAGTGATATCTAAAAACCAGGAAAAATCAATACAAATCAA and 502 TTTTTCTAGAGCTAGCTACTGGAAGAAAAGTGCGTTTTTGAGGGGT. 4) Five hundred and forty-nine base pairs of *let-858* promoter (including its 5'-UTR) were used to drive ubiquitous expression (Kelly, Xu et al. 1997). Primers 343 TTTTTGCATGCGTCGACGCTCTGAAAAACGAAAGTGGA and 344 TTTGGATCCCTTACTATAAAAAAGTTTGAATAC were used to amplify this segment from wild-type DNA. 5) The *smg-2* frameshift (insertion) was generated by first assembling a plasmid with tripartite sequences composed of the *smg-2* and *haf-6* cDNA sequences flanking the *smg-2/haf-6* intergenic region configured in the

chromosomal context with cDNAs in opposite orientation. The tripartite plasmid was then digested with AgeI, which cuts once within the *smg-2* cDNA (1923 base pairs downstream from ATG), and AgeI ends were filled in with T4 DNA polymerase and ligated to regenerate a circular plasmid with a four- base pair insertion in the *smg-2* coding region. 6) The *smg-2* deletion was generated from the tripartite plasmid by digesting the *smg-2* cDNA region with Nde I and religating, resulting in the removal of base pairs 391-2866 (with ATG at position 1). 7) 2385 base pairs of *myo-3* promoter sequence, including its 5'-UTR were used to drive expression in muscle (Fire, Xu et al. 1998). Primers 497 ttagcggatccgctagcAAGCTTGGGCTGCA GGTCGGC and 498 ttagcggatccgcggccgcTCTAGATGGATCTAGTGGTCGTGG were used to amplify the promoter. DNA sequencing confirmed each fragment was error-free. Fragments were cloned into standard vectors and combined to produce the plasmids described using standard cloning techniques. Transgene lines were established as described above.

Transgene-mediated Rescue of RNAi

RNAi activity in transgenic animals was assessed by placing animals as L2/L3 larvae on pop-1 or elt-2 food. Absence of live progeny is indicative of an intact RNAi response.

Transgene-mediated Rescue of Nonsense-mediated Decay (NMD)

Plasmids were injected as described above into *smg-2(r908), unc-54(r293)* hosts, and phenotypes in the resulting transgenic strains were noted. *unc-54(r293)* carries a premature stop codon that produces a paralyzed phenotype in wild type; the phenotype is suppressed in animals defective for *smg-2 (Hodgkin, Papp et al. 1989).* *unc-54* mRNA is not degraded in *smg-2* mutant strains, allowing for expression of a near full-length UNC-54 protein.) Functional SMG-2 activity is evidenced by a paralyzed phenotype.

HAF-6::GFP Reporters

Seven plasmids were designed for assays of protein localization and mutant rescue. GFP sequences were derived from plasmids pPD119.45, pPD118.37, pPD96.02, pPD119.16, and pPD102.33 (obtained from FireLab Vector kit; Andrew Fire). Plasmids were coinjected with carrier DNA and transformation marker sequences (dominant *rol-6*) into wild-type animals and were established as extrachromosomal, complex arrays. GFP fluorescence patterns were monitored in live animals, and the subcellular patterns were similar to those observed using our anti-HAF-6 polyclonal antibody. GFP expression was also monitored using an anti-GFP monoclonal (Sigma-Aldrich, St. Louis, MO) to detect expression in the germline.

Antibodies and Immunofluorescence Microscopy

A peptide corresponding to amino acids 69–82 (IDHLRTTEDQNASMC) was cross-linked to keyhole limpet hemocyanin as hapten [coupled using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride] and injected into rabbits (Harlan Bioproducts for Science, Indianapolis, IN). Immune serum was affinity purified using a column coupled with HAF-6::glutathione *S*-transferase (GST) fusion protein derived from a pGEX-4T-2 vector (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). The HAF-6::GST fusion harbored the first 100 amino acids of HAF-6. The fusion protein was covalently coupled to CNBr-activated Sepharose 4B (Sigma-Aldrich). Remaining antibodies that might cross-react to bacterial proteins were adsorbed onto a second column coupled with bacterial proteins derived from a similar purification protocol as HAF-6::GST. Tissue was prepared for immunofluorescence using a number of different fixation and permeabilization conditions and solutions, including methanol, formaldehyde, or paraformaldehyde fixatives. Animals were permeabilized by freeze-cracking on glass slides using fixative solutions containing either methanol, 4% formaldehyde, or 4% paraformaldehyde in phosphate-buffered saline. Not surprisingly, methanol fixation removed some signal from membranes, confirming the specificity of the antibody preparation; paraformaldehyde fixation resulted in a more pronounced nuclear envelope staining; and formaldehyde fixative or formaldehyde/paraformaldehyde mixes produced consistent staining with patterns similar to reporter GFP patterns in live animals. We also permeabilized animals en masse in Eppendorf tubes (Finney and Ruvkun 1990) with variations on the protocol

that included omission of methanol and/or detergents. Additionally, animals were dissected in fixative using syringe needles to achieve permeabilization. The anti-Complex IV subunit I antibody was purchased from MitoSciences (Eugene, OR); anti-calreticulin antibodies were a gift from Joohong Ahnn (Gwangju Institute of Technology, Gwangju, Korea) (Park, Lee et al. 2001). Fluorescent images were obtained using a Zeiss LSM510 Meta confocal microscope system (Carl Zeiss Microimaging, Thornwood, NY). Goat anti-mouse and goat anti-rabbit secondary antibodies were coupled to Alexa 488 or Alexa 594 (Invitrogen, Carlsbad, CA).

Results

ne335 Is an Allele of haf-6

The *ne335* allele was isolated based on its "method-of-delivery-dependent" RNAi defects (Tabara, Sarkissian et al. 1999). *ne335* mutant strains elicit RNAi when dsRNA is introduced by injection (Fire, Xu et al. 1998), but they are RNAi defective when worms are reared on "dsRNA food" (bacteria that express dsRNA corresponding to a particular worm gene) (Timmons, Court et al. 2001). We mapped the *ne335* mutation to the left end of chromosome I using standard methodology and *pop-1* or *unc-22* food to identify homozygotes and recombinants (Figure 2-1). The single gene Y48G8AL.11 rescued the RNAi defects in *ne335* (Figure 2-4, A, B). Y48G8AL.11 is named *haf-6* based on its homology to half-molecule ABC

transporters. Although full ABC transporter proteins have two sets of transmembrane-spanning domains and two domains with homology to ATP-binding cassettes, half-transporters have only one of each domain (Figure 2-3). The molecular lesion in the *ne335* allele is a 35-base pair deletion in exon 3 of the *haf-6* gene (Figure 2-2), and the mutated gene is predicted to produce a partial protein with only the first two transmembrane-spanning helices but no ATPase domain.

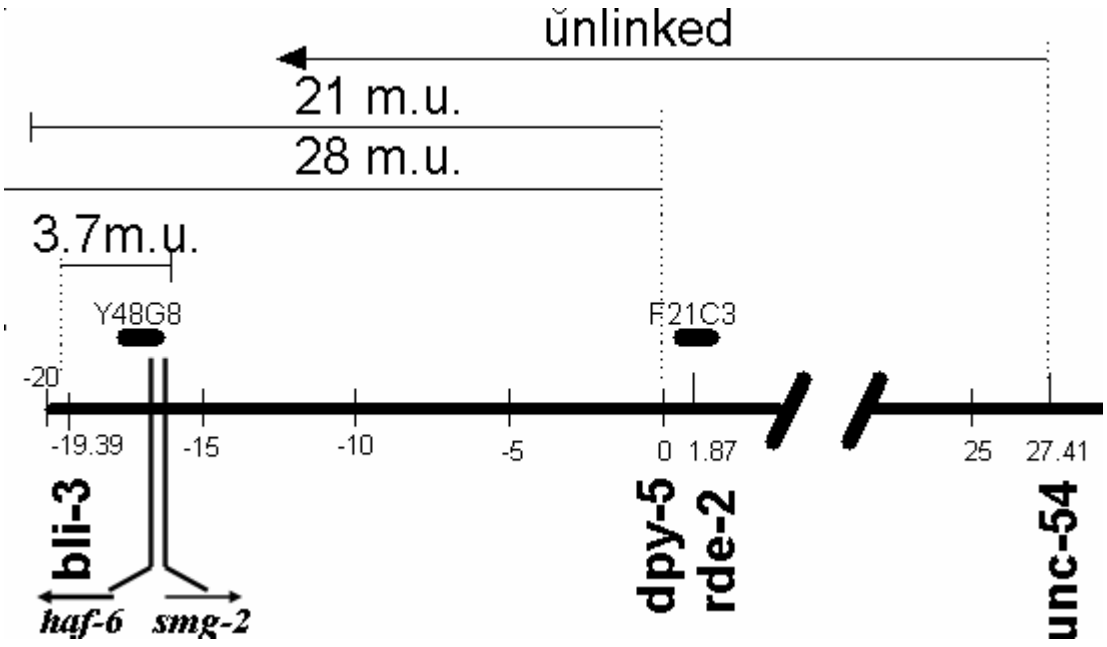


Figure 2-1: *haf-6* maps to the left arm of chromosome I. We scored 300–500 F2 progeny animals in each mapping experiment. YAC Y48G8, which rescues the RNAi defect in *haf-6(ne335)* mutant strains, is indicated. The *haf*-6 gene is approximately 700 bp upstream of *smg*-2 and is

oppositely transcribed, an indication that the genes may have regulatory elements that are shared or that compete. Both genes reside in Y48G8.

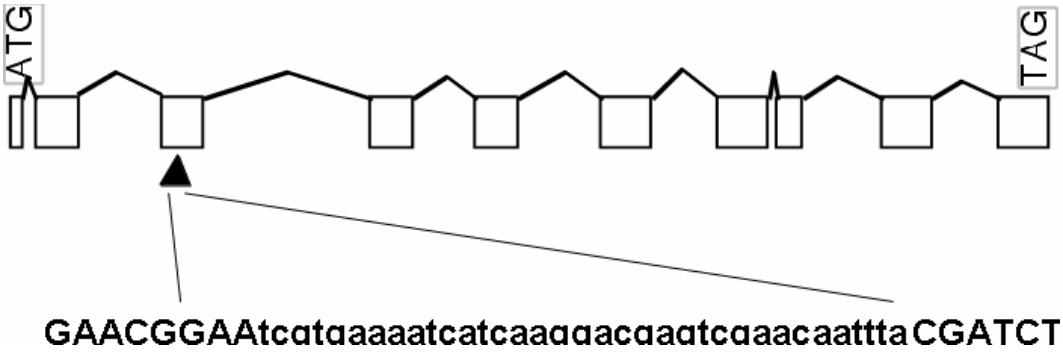


Figure 2-2: intron/exon structure of *haf-6*. We found an additional 303 base pairs of coding region not predicted in Wormbase (release WS146). The 35-base pair deletion in *haf-6(ne335)* is indicated by a filled triangle. The deleted region is lowercase. The five horizontal lines at the bottom of diagram highlight the relative positions of the five predicted transmembrane helices. The thick lines represent exons; thin lines indicate introns.

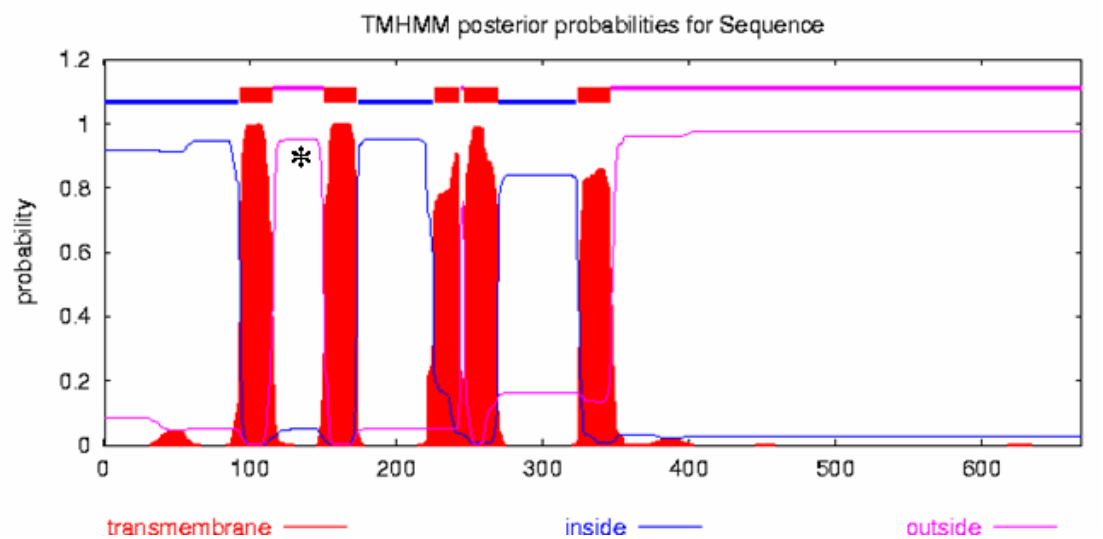


Figure 2-3: Predicted transmembrane topology of HAF-6 (Krogh *et al.*, 2001); the ABC is C-terminal. The set of five transmembrane helices constitutes a transmembrane domain (TM).

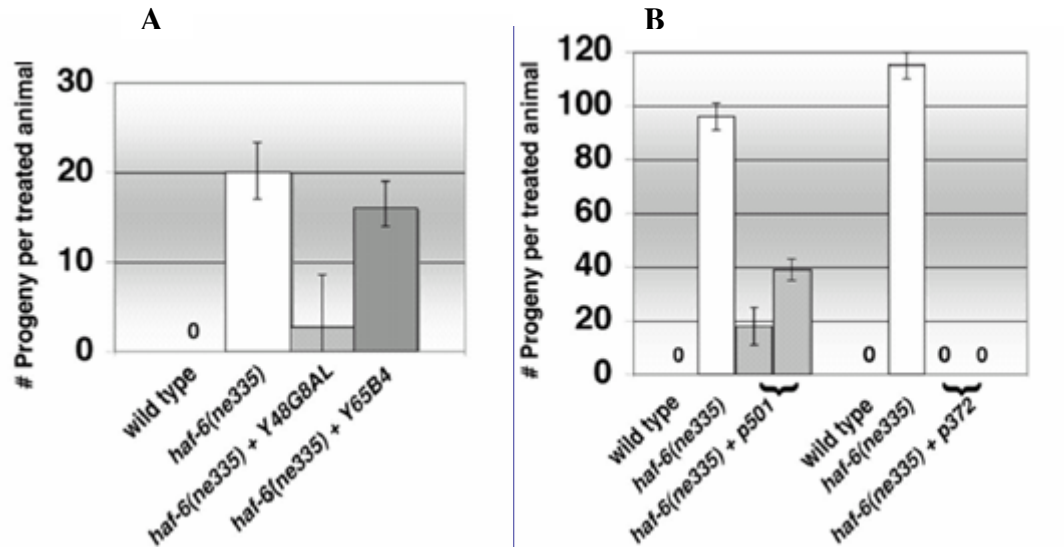


Figure 2- 4: (A) Rescue of RNAi defects in haf-6(ne335) mutant strains by Y48G8 but not from an unrelated YAC. RNAi defects were assessed on pop-1 feeding plates at 20°C. Bars represent the average of five experiments; error bars represent SD. In total, seven overlapping YACs were tested; data from Y65B4 are included in the figure as a representative negative control. Only

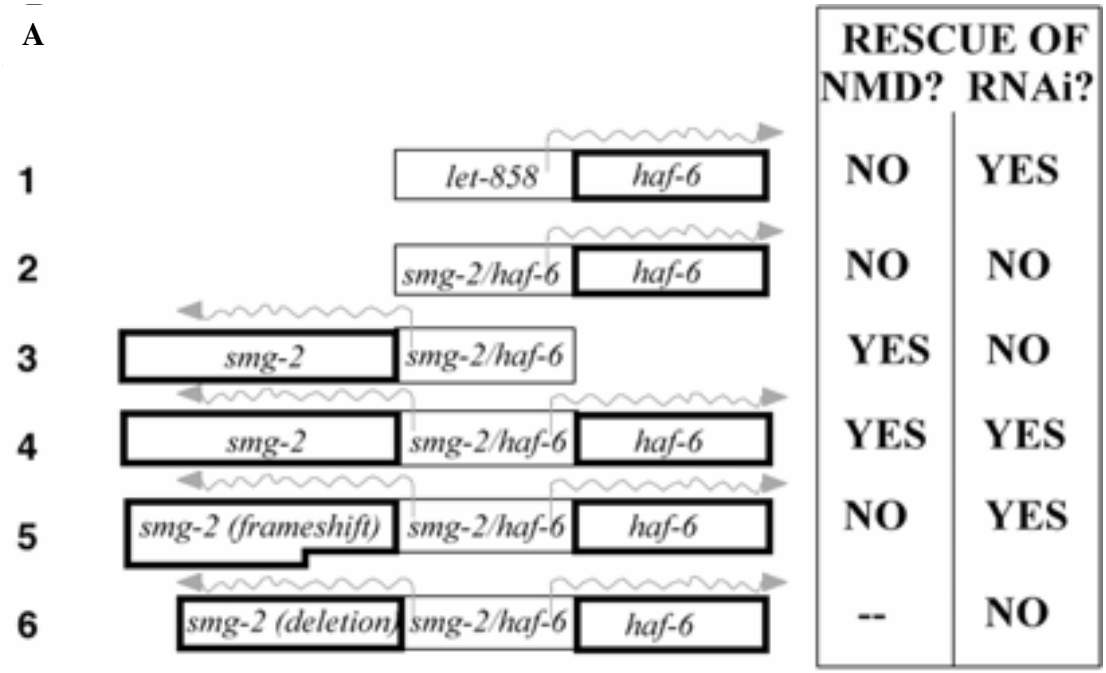
Y48G8 provided rescue, demonstrating that specific sequences, as opposed to the presence of a transgene, provide rescue. (B) Rescue of RNAi defects in *haf-6* mutant strains. The pLT501 and pLT372 plasmids harbor a full-length *haf-6* cDNA driven from *haf-6* upstream/promoter sequences. The HAF-6 sequence in pLT501 harbors a G562R amino acid substitution; plasmid pLT372 is wild type for HAF-6. Bars represent an average of two experiments; error bars denote the range. Gray bars represent different transgenic lines derived independently from the same DNA injection mix.

The Neighboring Gene, smg-2, Does Not Contribute to the RNAi Defect in haf-6(ne335)

Although robust RNAi phenocopies are observed in response to delivery of dsRNA, the effect is not permanent. Faster recovery of RNAi has been observed in *smg-2* mutant strains injected with dsRNA in comparison with similarly injected wild-type animals (Domeier, Morse et al. 2000). The *smg-2* gene is located ~700 base pairs upstream of *haf*-6, and the genes are oppositely transcribed (Figure 2-1). Because the two genes are juxtaposed and may have overlapping regulatory elements, we considered that *smg-2* activity might be impaired in *ne335* mutant strains, contributing to the RNAi defects we observe. SMG-2 activity is required for some aspects of mRNA surveillance, a mechanism that degrades defective mRNAs, in particular those mRNAs harboring a premature stop codon. *smg-2* mutant strains act as allele-specific suppressors of NMD(Hodgkin, Papp et al. 1989; Page, Carr et al.

1999; Anders, Grimson et al. 2003; Grimson, O'Connor et al. 2004), and we used this property in functional assays for SMG-2 activity.

We took a transgenic approach to determine whether *smg-2* activity might be affected by the mutation in *haf-6* (Figure 2-5A). cDNA sequences, and not genomic regions, for *haf-6* and *smg-2* were used due to the presence of unclonable inverted repeats in several introns of both genes; furthermore, no cosmids were subcloned from this region by the *C. elegans* DNA Sequencing Consortium. The *haf*-6 cDNA alone rescued the RNAi defects in *haf-6(ne335)* when driven ubiquitously from a *let-858* promoter (Figure 2-5A, construct 1). The *smg-2* cDNA alone did not rescue the RNAi defects in *haf-6(ne335)* mutant strains, but it did rescue NMD defects in *smg-2* mutant strains (Figure 2-5A, construct 3). A single construct harboring both cDNAs flanking the intergenic region was able to rescue both the NMD defect in *smg-2* and the RNAi defect in *haf-6(ne335)* mutant strains, as expected (Figure 2-5A, construct 4). Plasmids with this tripartite configuration of DNA segments that harbored a *smg*-2 cDNA with a frameshift mutation (Figure 2-5A, construct 5) or with a deletion (Figure 2-5A, construct 5) were generated. These plasmids did not allow rescue of NMD, as expected; furthermore, construct 5 did rescue RNAi defects in *haf-6(ne335)*. Extra copies of *smg-2* in the *haf-6(ne335)* mutant background did not improve the RNAi defects in our transgene-based assays; thus, the *ne335* mutation has no apparent effect on *smg-2*. By contrast, we observed that part of the *smg-2* coding region is required for full *haf-6* expression (Figure 2-5A, constructs 2 and 6 compared with constructs 4 and 5), possibly acting as a transcriptional enhancer.



B

Genotype	RNAi?
+ / +	+
haf-6(ne335) / haf-6(ne335)	--
smg-2(r908) / smg-2(r908)	+
smg-2(e2008) / smg-2(e2008)	+
haf-6(ne335) + / + smg-2(e2008)	+
haf-6(ne335) + / + smg-2(r908)	+

Figure 2-5:

***smg-2* does not contribute to the RNAi defects in *haf-6(ne335)*. (A) DNA constructs injected into mutant strains were configured as indicated. Transgenic lines for each plasmid were established in both *haf-6(ne335)* and *smg-2(r908), unc-54(r293)* mutant backgrounds. Each *haf-6(ne335)* transgenic strain was tested for RNAi activity; each *smg-2(r908), unc-54(r293)* transgenic strain**

was tested for NMD as described in *Materials and Methods*. *haf*-6 mutant animals without transgenes did not display RNAi activity in these assays; likewise, the nontransgenic *smg-2, unc-54* mutant strain did not display NMD. Rescue of RNAi defects in *haf*-6 mutant strains was dependent upon proper expression of *haf-6* sequences; rescue of NMD in *smg-2* mutant strains was dependent on intact SMG-2 activity. The design of constructs 3–6 reflects the chromosomal, oppositely oriented, arrangement of the genes. A *smg-2* coding segment is required for full *haf-6* expression (compare 2 and 4 with 6 and 5). (B) No RNAi defects are observed in *smg-2* mutant strains using feeding assays, and the *haf-6* and *smg-2* alleles do not complement. (Both *pop-1* and *unc-22* foods were used in these experiments.)

Additional data support the conclusion that *smg-2* activity is not affected by the 35-base pair deletion in *haf-6(ne335)* and that the RNAi defects we observe are solely due to defects in *haf-6*: 1) The *smg-2* sequence is wild type in *haf-6* mutant strains, ruling out the possibility that mutations in two genes coexist in this strain. 2) We easily obtained *smg-2* cDNA by RT-PCR using RNA from *haf-6* mutant strains as template, suggesting the *smg-2* gene is expressed (our unpublished data). 3) The RNAi defects in *haf-6* are complemented by *smg-2* mutant strains (Figure 2-5B). 4) When our feeding assays (*pop-1* and *unc-22* foods) are used to test the RNAi capability of *smg-2(r908)* and *smg-2(e2008)* mutant strains, we observed no RNAi defects at any growth temperature (15, 20, or 25°C; Figure 2-5B). The proximity of the genes, the presence of *haf-6* regulatory elements in the *smg-2* coding region, and the potential for other forms of shared regulation raise the possibility that some *smg-2*

mutant strains, especially those with large deletions, might also have disruptions in *haf-6*, complicating analyses of RNAi defects in these strains.

An Intact ABC Domain Is Required for HAF-6 Activity in RNAi

We verified our subcloned fragments by DNA sequencing and found that the *haf-6* cDNA in plasmid pLT501 (Figure 2-4B) had acquired a point mutation during PCR amplification. [This mutation is not present in the genome of wild type or *haf-6(ne335)*.] The mutation is predicted to result in an amino acid substitution of Arg for Gly at amino acid position 562. Gly562 is strictly conserved and resides only three amino acids upstream from the signature conserved motif (LSGGQ) specific to ABC transporters, and G562 likely plays an important role in nucleotide binding. The G562R mutation rescued RNAi defects in *haf-6(ne335)*; however, the relative ability of the R562G form to rescue RNAi was notably reduced in comparison with the ability of intact *haf-6* sequences to rescue (Figure 2-4B, plasmid 501 has the G562R mutation and plasmid 372 is wild type). Our interpretation of the results is that the G562R substitution produces a HAF-6 protein with a weakened ability to bind and/or hydrolyze nucleotides with the result that the RNAi activity is also weakened. Thus the RNAi role for HAF-6 likely requires substrate transport.

Phenotypic Analysis of haf-6(ne335)

haf-6 mutant animals are viable and fertile, yet display method-of-delivery–dependent RNAi defects. We used the full spectrum of dsRNA delivery methods available in *C. elegans* to better understand the nature of the RNAi defects in *haf-6(ne335)*. We report two main observations.

First, the RNAi defect in *haf-6(ne335)* mutant strains is dosage sensitive with respect to the amount of dsRNA. An RNAi response is observed when relatively high concentrations of dsRNA are injected; lower concentrations are less effective in eliciting an RNAi response in mutant strains (Figure 2-6A). Experiments using soaking methods also reveal a concentration-dependent RNAi response (Figure 2-6B)—treatment of mutant strains with a relatively high concentration of dsRNA was required for a robust RNAi phenocopy in the population. With soaking, wide variations in penetrance of RNAi phenocopies can be attributed to individual variations in the amount ingested. Although soaking and injection can elicit RNAi in mutant strains, feeding is most effective in revealing their RNAi defects (Figure 2-6C).

Second, we observe RNAi defects in *haf-6(ne335)* for genes that are expressed in germline and intestine but not other tissues (Figure 2-7). We initially considered that *haf-6*, a method-of-delivery–dependent mutant, might be defective in the dissemination of feeding-derived dsRNAs from the intestine to other tissues. However, some systemic RNAi responses are intact in *haf-6* mutant strains, as

evidenced by the appearance of RNAi phenocopies 1) in somatic tissue when dsRNA is introduced by feeding and 2) in treated animals and their progeny from dsRNA introduced by soaking and injection. Thus, an efficient RNAi response to dsRNA, in particular to dsRNA delivered by bacterial feeding, is sensitive to the relative levels of HAF-6 activity as well as to the dosage of dsRNA.

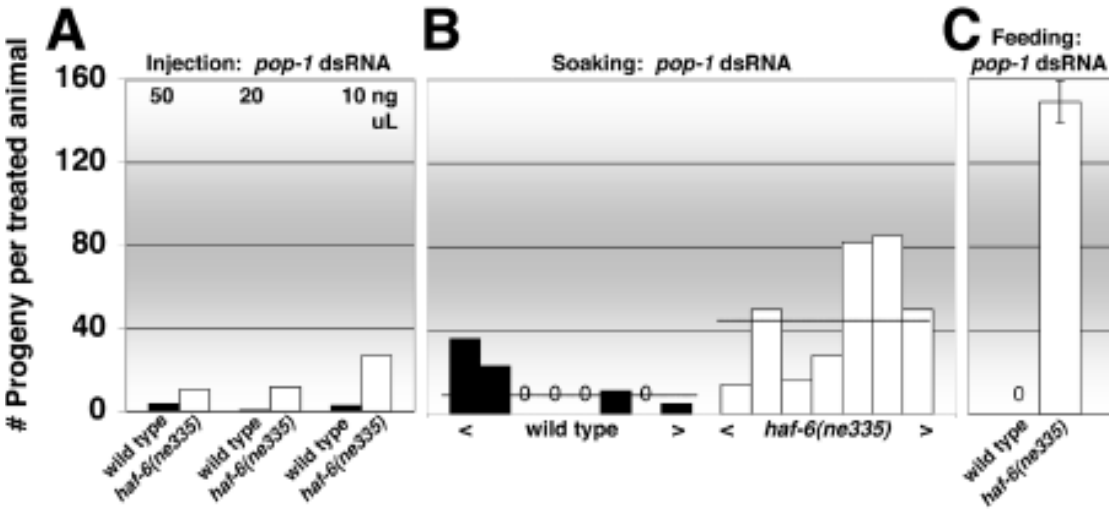


Figure 2- 6

***haf-6* mutant strains display dose-dependent and tissue-specific RNAi defects. Injection (A), soaking (B), and feeding (C) methods were used to deliver dsRNA. (A–C) *pop-1*(RNAi) phenocopies (sterility) were assessed by scoring the number of F1 progeny per treated animal for a defined time frame. (A) Various dilutions (from 10 to 50 ng/µl) of *pop-1* dsRNA were injected into the body cavity of L3/L4 animals that were subsequently cultured on standard plates at 20°C and also at 25°C (our unpublished data). No temperature-dependent differences in RNAi activity were observed. Ten to 18 animals (average 12.2) were injected in each set. (B) Animals were soaked in 50 ng/µl *pop-1* dsRNA as L1/L2 at 20°C, and each animal was recovered onto individual culture plates. Bars represent the number of progeny produced over a defined time**

period from treated individuals. The horizontal lines above each set of bars represent the average number of progeny produced for the particular strain: 3.3 progeny/adult in wild type; 44.6 for haf-6(ne335). Individual soaked mutant strains displayed a more variable phenocopy penetrance than did wild type; however, average overall RNAi response in mutant strains was lower than wild type. The variation in response is likely correlated to the amount of material ingested by individuals and hence to the dosage of dsRNA. (C) Animals were placed onto pop-1 food as L1/L2 and monitored for sterility at 20°C over a defined time, identical conditions were used in each set of experiments.

	RNAi phenocopies from dsRNAs delivered by bacterial feeding						
dsRNA food : Expression:	*unc-15* (somatic)	*elt-2* (somatic)	*unc-112* (somatic)	*F38E11.5* (somatic) and (germline)	*dhc-1* (germline)	*dom-6* germline)	*pas-6* (germline)
Wildtype	Paralyzed	L1 arrest	Paralyzed	Sick/sterile	Embryonic lethal	Embryonic lethal	Embryonic lethal
ne335	Paralyzed	Live F1s	Paralyzed	Sick/sterile	Live F1s	Live F1s	Live F1s
rde-2(ne221)	Paralyzed	Live F1s	Paralyzed	Sick /sterile	Live F1s	Live F1s	Live F1s

Figure 2-7: *haf*-6 mutant strains are RNAi defective in germline and intestine. The feeding protocol was used to deliver dsRNAs corresponding to the genes indicated. Animals were placed at L1/L2 stage onto feeding plates. All feeding strains elicited an RNAi phenocopy in wild-type. Experiments were performed at 15, 20, and 25°C, with similar results.

Subcellular Localization of HAF-6

Most organisms harbor multiple ABC protein genes (Schmitt, Benabdelhak et al. 2003)—60 ABC transporter genes have been identified in *C. elegans* (Sheps, Ralph et al. 2004). Collectively, the different ABC transporters localize to most membranes in the cell, including the plasma membrane, endoplasmic reticulum, and mitochondria. Determining the subcellular localization of HAF-6 is key to understanding the role of HAF-6 in RNAi and to the identification of transportable substrates that are relevant to its RNAi function. HAF-6 is a half-transporter member of the B subfamily, and an additional eight half-transporter genes in subfamily B are encoded by the *C. elegans* genome (Sheps, Ralph et al. 2004). The precise functions, substrates, and subcellular localizations of half-transporters in *C. elegans* have not been fully investigated. Mammalian half-transporters from subgroup B function as heterodimers, and possibly homodimers in the endoplasmic reticulum; others localize to mitochondria or lysosomes (Monaco *et al*., 1990; Spies *et al*., 1990; Allikmets *et al*., 1996; Csere *et al*., 1998; Hogue *et al*., 1999; Yamaguchi *et al*., 1999; Mitsuhashi *et al*., 2000; Zhang *et al*., 2000a,b). Because not all members of the same subfamily are similarly localized, subfamily classification does not allow predictions of subcellular localization. Additionally, localization domains have been identified for a relatively

few transporter proteins, and the degree of evolutionary conservation of such motifs has yet to be determined.

We first built plasmids to express HAF-6::GFP fusion proteins in *C. elegans* (Figure 2-8). The different constructs produced three variations of GFP fusions: an N-terminal GFP::HAF-6 protein, a C-terminal HAF-6:: GFP protein, and an internal GFP sequence in-frame within the entire HAF-6 protein. Expression of GFP fusion proteins was driven from *haf-6/smg-2* promoter segments that provided rescue in previous experiments (Figure 2-5, construct 5), from a *myo-3* promoter segment that drives expression in muscle, or from a *let-858* promoter that drives expression in all cells. Each construct was injected into wild-type and *haf-6* mutant animals as described in *Materials and Methods*, and transgenic lines were established. GFP accumulation was observed in only two plasmid configurations (Figure 2-8): 1) Muscle cells in transgenic lines harboring plasmid pLT428 expressed GFP; however, the expression was weak and was observed only in a few anterior muscle cells in each animal. Additionally, not all transgenic animals (with the Roller transformation marker phenotype) displayed GFP fluorescence, and GFP expression was not meiotically stable—no GFP was observed in these lines past the F3 generation. However, in GFP-expressing muscle cells, we observed intracellular fluorescence that was clearly reticular and not mitochondrial (our unpublished data). 2) The second construct, pLT531, displayed a more robust GFP fluorescence (Figure 2-9A). HAF-6::GFP expression in this plasmid was regulated by a *let-858* promoter that drove ubiquitous expression. Fluorescence from the same HAF-6::GFP construct did not accumulate to

visible levels when driven from an endogenous *haf-6/smg-2* promoter (pLT438 and pLT510). Because the *let-858* promoter drives ubiquitous expression, we anticipated that GFP would be observed in all cells of transgenic lines harboring pLT531. However, we observed GFP expression mostly in the germline and intestine, those tissues that display RNAi defects (Figure 2-9). (Germline expression of GFP was observed using anti-GFP antibodies and immunofluorescent microscopy.) The more limited tissue expression pattern may reflect the absence of a heterodimeric binding partner in the nonfluorescent tissues.

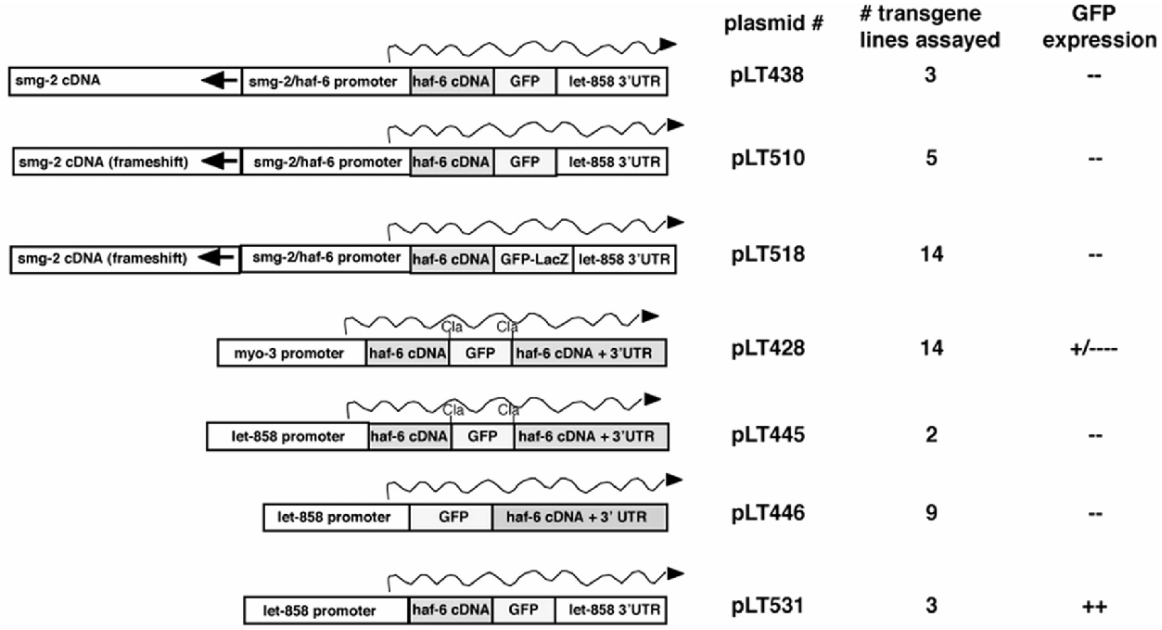
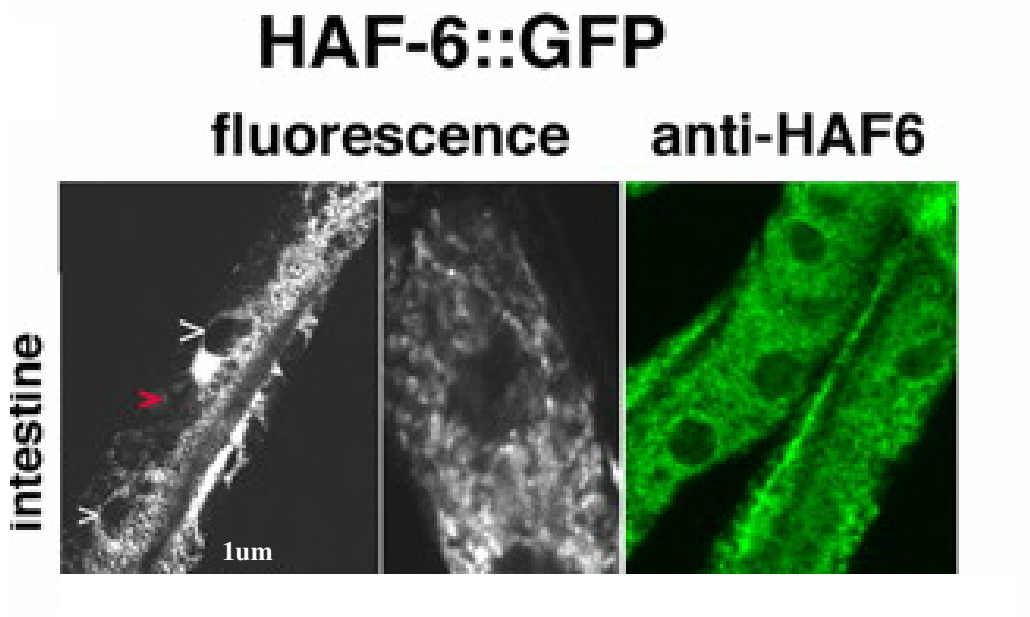
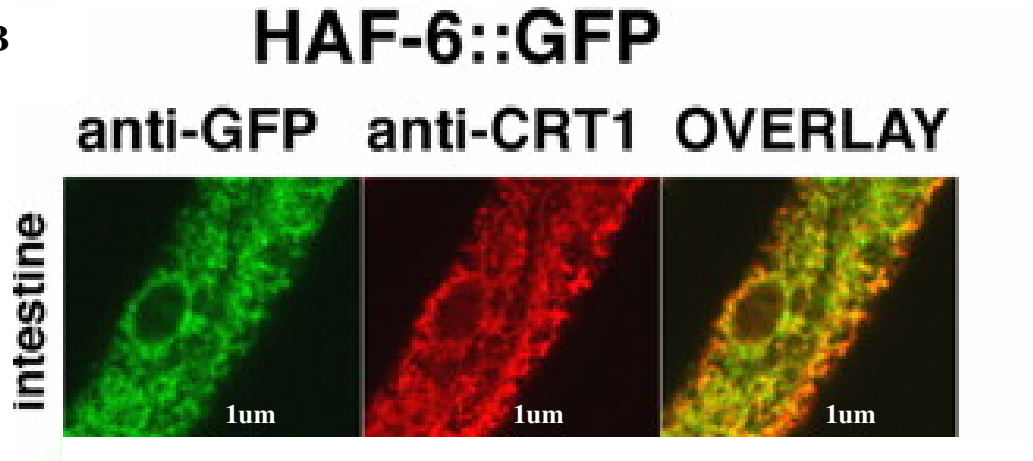


Figure 2- 8: GFP reporter plasmid configurations used to build transgenes. Plasmids were configured as indicated and injected into wild type and *haf-6* mutant strains. The number of independently established lines is indicated. GFP fusion proteins expressed in muscle from plasmid pLT428 displayed weak fluorescence, and the ability of the transgenic animals to express GFP was lost over generational time. We observed a clear ER localization pattern for GFP in the few muscle cells with fluorescence. Only plasmid pLT531 elicited stable GFP fluorescence. The pLT531 plasmid was injected into *haf-6(ne335)* mutant strains to generate strain XX915. XX915 animals displayed GFP fluorescence in a pattern similar to that observed in wild type, and the RNAi phenotypes were rescued.

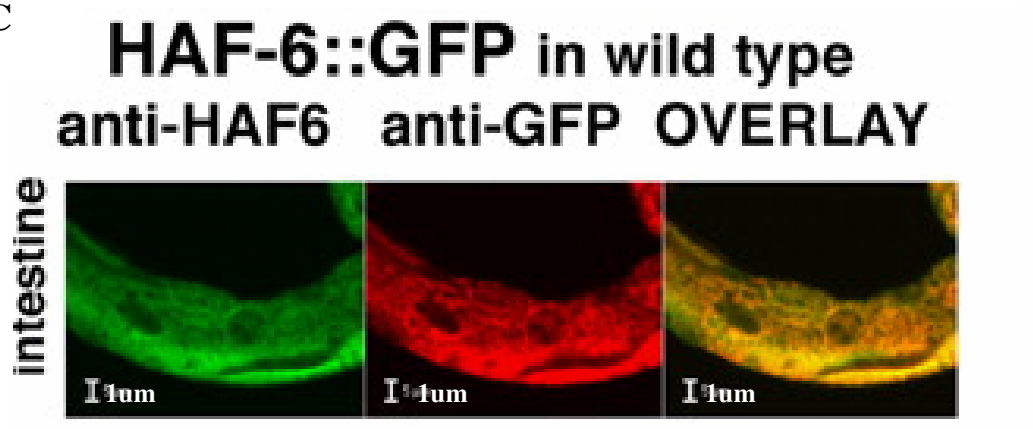
A


HAF-6::GFP
fluorescence
anti-HAF6
intestine
1um

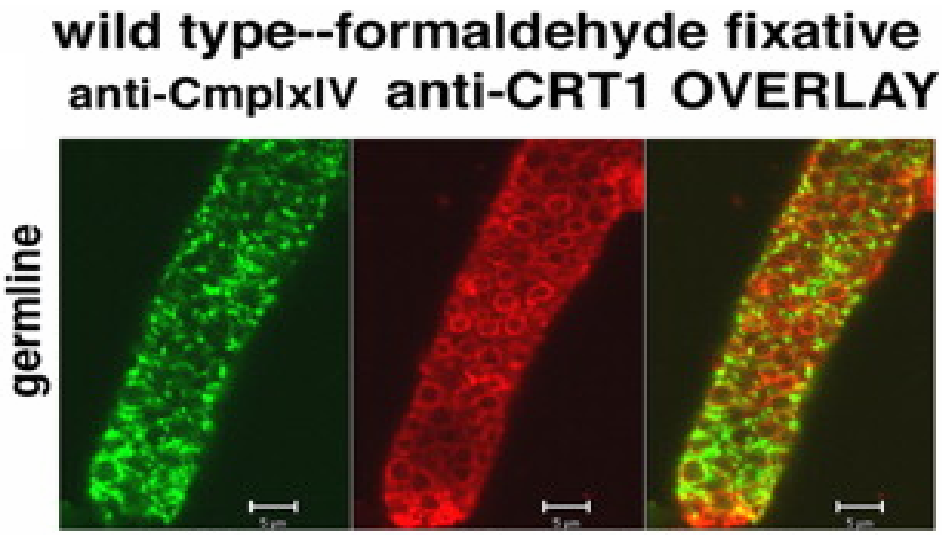
B


HAF-6::GFP
anti-GFP
anti-CRT1
OVERLAY
intestine
1um
1um
1um

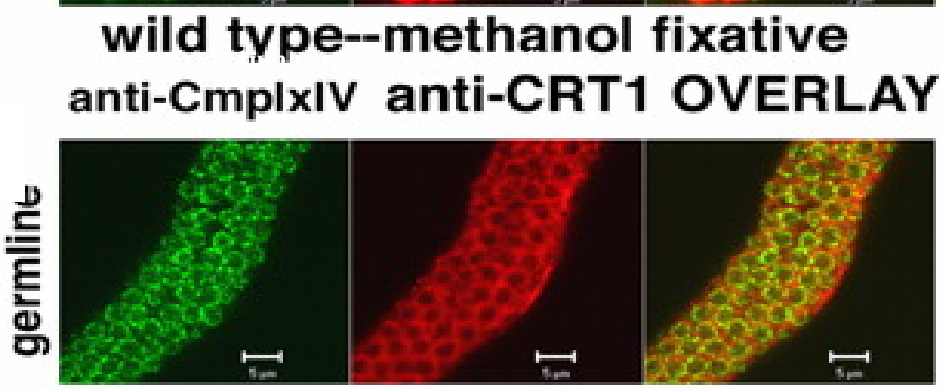
C


HAF-6::GFP in wild type
anti-HAF6
anti-GFP
OVERLAY
intestine
1um
1um
1um

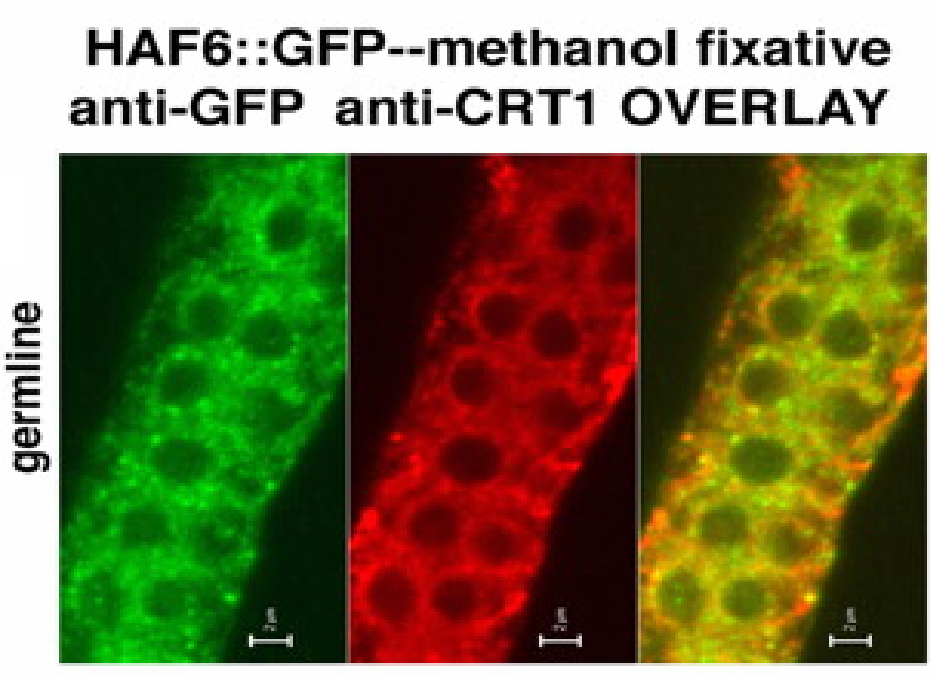
D


wild type--formaldehyde fixative
anti-CmplxIV anti-CRT1 OVERLAY
germline

E


wild type--methanol fixative
anti-CmplxIV anti-CRT1 OVERLAY
germline

F


HAF6::GFP--methanol fixative
anti-GFP anti-CRT1 OVERLAY
germline

G

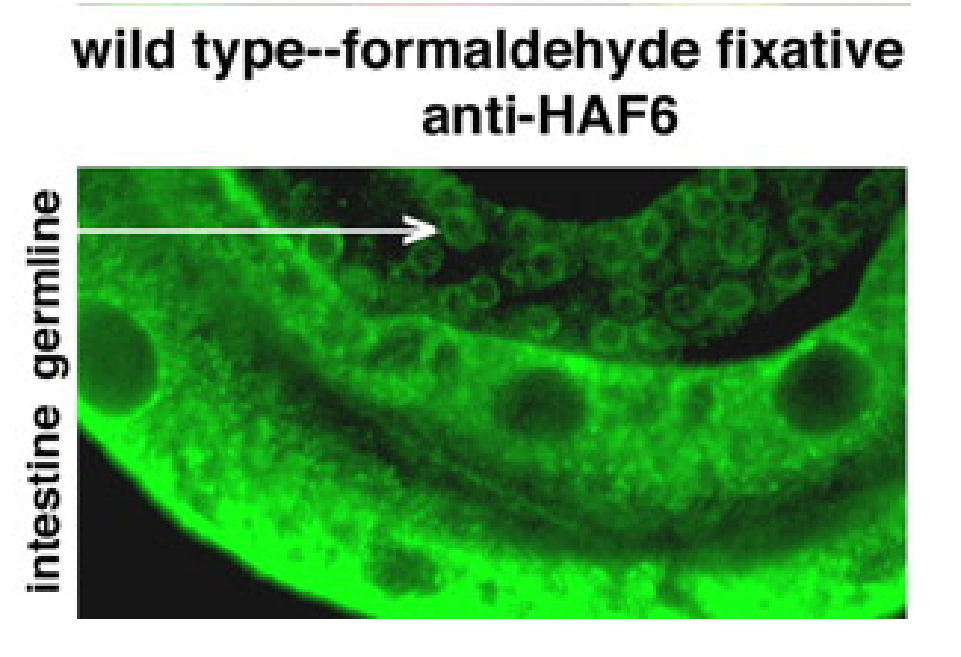


Figure 2- 9 HAF-6 is expressed in intestine and germline and localizes to intracellular, reticular membranes. Endogenous GFP fluorescence from a rescuing HAF-6::GFP fusion protein (Figure 4, plasmid pLT531) and immunolocalizations using anti-HAF-6 and control antibodies were used to investigate subcellular distribution patterns. (A) HAF-6::GFP fluorescence patterns in live animals (left and center) are similar to the patterns observed in fixed animals decorated with an anti-HAF-6 antibody (right). White arrows, intestinal nuclei; red arrows, autofluorescent granules. (B) The HAF-6::GFP protein (left) is localized to the same compartment as calreticulin (CRT-1), a primarily ER resident with a C-terminal KDEL sequence. Similar results were obtained in cohybridization experiments using the anti-HAF-6 antibody with anti-CRT-1. HAF-6 is a transmembrane protein, and 100% spectral overlap with CRT-1 is not anticipated; nonetheless, co-compartmentalizaion is observed. (C) Cohybridization experiments were performed in transgenic animals expressing both endogenous HAF-6 and a HAF-6::GFP fusion protein. The signals from anti-GFP and anti-HAF-6 overlapped, indicating that the fusion protein is expressed in all the compartments where the endogenous protein is located. (D) A control experiment demonstrating that subcellular compartments can be distinguished in

germline tissue fixed with formaldehyde. Mouse anti-Complex IV subunit I antibodies recognize mitochondrial proteins. Rabbit anti-CRT-1 antibodies recognize proteins localized mostly to the endoplasmic reticulum. (E) A control experiment demonstrating that distinct compartmentalization patterns are also observed using methanol as fixative. (F) HAF-6 localization patterns do not resemble mitochondrial patterns in germline. HAF-6 patterns are reticular in germline. (G) HAF-6 protein is perinuclear in germline tissue; perinuclear staining is less prevalent in intestinal cells. Both tissues shown are from the same animal. Confocal images were captured using a Zeiss LSM 510 META. Bars, 5 µm on all panels except A (10 µm) and F and G (2 µm).

The RNAi defect in mutant animals was rescued by the HAF-6::GFP fusion protein; however, this does not preclude the possibility that some GFP fluorescence might originate from misfolded or nonfunctional proteins trapped in the endoplasmic reticulum. We therefore validated our GFP fluorescence observations using immunolocalization techniques and an antibody specific to HAF-6 protein.

ABC transporter proteins have a high degree of amino acid similarity. The N-terminal region of HAF-6 has little similarity to other ABC proteins in *C. elegans*, so we generated an anti-peptide polyclonal antibody targeting this region. The deletion in the *haf-6(n335)* is not predicted to affect the region that encodes the epitope. Indeed, we do observe antibody staining in the mutant, indicating that HAF-6 protein, probably a truncated version, is expressed. [We were not able to isolate a full-length cDNA from *haf-6(ne335)* RNA by RT-PCR and anticipated that the RNA might be

more susceptible to degradation.] The specificity of the antibody is demonstrated by the observation of similar staining patterns in experiments using anti-HAF-6 antibody versus experiments using GFP reporters from a construct that provides rescue of the RNAi defects. Furthermore, we have developed antibodies against other HAF proteins, and we observe patterns that are distinct from HAF-6 (our unpublished data).

Immunofluorescence staining revealed that HAF-6 was predominantly expressed in intestinal and germ line tissue (Figure 2- 9), a pattern that mirrors the RNAi defects observed in mutant strains (Figure 2-7). Thus *haf-6* RNAi defects are apparently limited to the tissues where HAF-6 is expressed. In double-labeling experiments, the anti-HAF-6 antibody did not decorate the same cellular compartment as a mitochondrial antibody (anti-Complex IV). We did observe co-compartmentalization using anti-HAF-6 and anti-Calreticulin antibodies, indicating that HAF-6 likely resides in the endoplasmic reticulum and not in mitochondria, as was expected from comparisons of sequence similarity to human half-transporters. Interestingly, HAF-6 localization extended to perinuclear regions in germline tissue, which is consistent with an endoplasmic reticulum (ER) localization (Figure 2-9G). However, localization of HAF-6 to the nuclear periphery was not prominent in intestinal tissue. Furthermore, the subcellular patterns observed in experiments using anti-HAF-6 antibody were similar to the patterns of GFP expression observed in transgenic animals expressing the HAF-6::GFP fusion protein (Figure 2-9A).

Additional ABC Transporters Facilitate RNAi

The *C. elegans* genome encodes 61 genes with sequence homology to ABC transporters (Sheps, Ralph et al. 2004). Strains that harbor defects in 43 of these genes were tested for RNAi activity using *pop-1* and *unc-22* food (see *Materials and Methods*). We observed strong defects in eight additional strains (Table 1)—this set includes full-transporter as well as half-transporter molecules from a wide distribution of subfamilies. Some transporter genes have overlapping patterns of expression in somatic tissue, whereas others have nonoverlapping and more restricted patterns of expression (Zhao, Sheps et al. 2004). Given the high degree of sequence homology between ABC transporters, the diversity of expression patterns, and the variety of configurations of transmembrane and ABC motifs in this set of genes, we cannot yet ascribe an RNAi function to a common sequence-related feature. Because all the strains in this set are RNAi-defective for germline targets, we predict a germline function for these transporters. Most surprising is the observation that nine ABC proteins are nonredundantly required for efficient RNAi; however, because the RNAi defect in any single mutant strains is rather weak, their effects may be additive.

Table 1: Additional ABC transporters are required for efficient RNAi in *C. elegans*

ABC Sub-Family	Strain	RNAi activity on: *pop-1* food?	RNAi activity on: *unc-22* food?	Predicted topology
A	*abt-1(tm0703)*	defective	wild type	(6TM-ABC)2
B	*pgp-4(gk16)*	defective	wild type	(6TM-ABC)2
B	*pgp-11(tm33)*	defective	wild type	(6TM-ABC)2
B	*haf-2(gk13)*	defective	defective	8TM-ABC
B	*haf-6(ne335)*	defective	defective-heat sensitive	5TM-NFB
C	*mrp-1(pk89)*	defective—cold sensitive	wild type	(12/6TM-ABC)2
D	*pmp-1(ok773)*	defective	wild type	4TM-ABC
G	*C16C10.12(ok962)*	defective	wild type	ABC-4TM
G	*C05D10.3(tm688)*	defective	wild type	ABC-4TM

Deletion strains of ABC transporters were reared on *pop-1* and *unc-22* feeding plates at 15°C, 20°C, 25°C, and 26°C. *pop-1* RNAi phenocopies (Sterility) and *unc-22* phenocopies (Twitching) were assessed and compared to those of wild type. Notations on the predicted topology and ABC classification systems were derived from (Sheps *et al.*, 2004). Strains testing negative in these assays harbored mutations in the following genes (see Materials): *abt-2 ; abt-4 ; C56E6.1; ced-7 ; cft-1 ; F02E11.1 ; F19B6.4 ; haf-1 ; haf-3 ; haf-4 ; haf-5 ; haf-7 ; haf-8 ; haf-9 ; mrp-3 ; mrp-4 ; mrp-6 ; mrp-8 ; pgp-1 ; pgp-2 ; pgp-3 ; pgp-5 ; pgp-6 ; pgp-7 ; pgp-10 ; pgp-12 ; pgp-13 ; pmp-3 ; pmp-4 ; pmp-15 ; T26A5.1 ; T27E9.7 ; TAG-167 ; Y42g9a.6 ; Y49e10.9.*

Discussion

The *haf-6* gene encodes a half-molecule transporter with homology to members of the B subclass of ABC transporters. Most organisms harbor many different ABC transporter genes, and the *C. elegans* genome has at least 60 (Sheps, Ralph et al. 2004). Transporters are classified into subgroups based on the overall level of homology and the numbers and arrangement of the two conserved domains: the transmembrane domain, made up of multiple transmembrane-spanning helices, and the ABCs. ABC transporters can be found in virtually all membranes of the cell, including those of subcellular organelles, although some ABC proteins lack transmembrane-spanning domains. The small molecule substrates transported by the collective ABC transporters include lipids, peptides, ions, nucleotides, carbohydrates, and drugs. Substrates can be transported into or out of cells or between intracellular compartments, and substrate transport is involved in various essential and nonessential cellular activities.

Generally, the amino acid sequence of an ABC transporter allows few conclusions to be drawn regarding the substrates that might be transported. We first anticipated that the homology between HAF-6 and the better-studied human half-transporter molecules would provide clues to function, or perhaps sub cellular localization. HAF-6 has the most overall sequence similarity to human transporters that localize to mitochondria. However, HAF-6 seems to localize to reticular membranes rather than mitochondrial membranes in our observations of live animals expressing GFP and of

antibody-stained fixed tissue. Thus, the overall amount of homology between transporters from different species does not allow predictions regarding subcellular localization.

We observed RNAi defects in eight additional ABC transporter-defective strains. Transporters are often expressed in a tissue-specific manner in multicellular organisms, and expression patterns for most of the nine transporters in *C. elegans* have been reported (Zhao, Sheps et al. 2004). *pgp-4* and *pgp-11* expression was observed in the excretory cell; *pgp-11* is also expressed in intestinal tissue, as is *mrp-1* and *pmp-1*; additional *mrp-1* expression was observed in the pharynx and vulva, and C05D10.3 gene expression was observed in embryos and the gut of males (Zhao, Sheps et al. 2004). The expression patterns were determined using transgenes configured to drive expression of GFP in live animals from promoter (upstream) sequences of an ABC transporter gene. However, it is generally the case that transgenes are silenced in the germline and in young embryos , (Kelly, Xu et al. 1997) so this method is not a reliable indicator of gene expression in these tissues. The RNAi defects we observed using *pop-1* food (Table 1) indicate a role in the germline for these genes.

Of the nine transporters that facilitate RNAi, *haf-2* is the only gene expressed in muscle (Zhao, Sheps et al. 2004), and *haf-2(gk13)* is the only mutant we tested with a strong muscle-specific RNAi defect. Because the remaining eight mutant strains did not display *unc-22*(RNAi) phenocopies in response to ingestion of *unc-22* food, the

RNAi defects are likely only observed in tissues where the ABC transporter in question is normally expressed. None of the mutant strains is likely to be strongly defective in the dissemination of silencing signals derived from ingestion of bacterial food, because RNAi is active in some somatic tissues (muscle). However, a fully systemic RNAi phenocopy in response to ingested dsRNAs may depend upon the proper function and expression of multiple ABC transporters in multiple tissues. We anticipate that tissue-specific RNAi defects may be observed in additional ABC transporter mutant strains. We are currently testing the remaining mutant strains using bacterial feeding strains to target genes with expression patterns that overlap with that of the ABC transporter in question.

Some ABC transporters export heavy metals, drugs, and other toxins from cells, thereby providing protection from harsh environments. A developmental program that results in arrested dauer larvae, which are better adapted for survival in harsh conditions, is initiated when *C. elegans* encounters environmental stress. The ABC transporter *mrp-1* allows animals to survive in environments laden with heavy metals (Broeks, Gerrard et al. 1996) and has been demonstrated to function in preventing dauer formation (Yabe, Suzuki et al. 2005). Here, we report that *mrp-1* mutant strains harbor RNAi defects (Table 1). Thus, an unappreciated role for ABC proteins during stress may be to influence RNAi mechanisms and thereby affect developmental reprogramming.

ABC transporters are required for various physiological activities, including protective or barrier mechanisms that export drugs or toxins from cells (Broeks, Gerrard et al. 1996; Bauer, Wolfger et al. 1999; Begley 2004; Schulz and Frommer 2004) and mechanisms that protect against viral infection (Trowsdale, Hanson et al. 1990; Chen, Abele et al. 2004). A variety of human pleiotropic disorders are linked to mutations in ABC transporters (Dean 2005) , and transporters are up-regulated in some drug-resistant cancers (Gottesman and Ambudkar 2001; Gottesman, Ludwig et al. 2006). Because alterations in ABC transporter function contribute to a number of pathologies, understanding how ABC transporters influence endogenous RNAi mechanisms will prove relevant to disease etiology as well as environmental adaptation. Furthermore, an ability to influence RNAi may help explain the patterns of evolutionary conservation of this diverse group of genes. We propose several models to reason how ABC protein function might influence RNAi: 1) ABC transporters may provide an appropriate intracellular environment for proper RNAi biochemistry, 2) ABC proteins may actively signal RNAi mechanisms through the transport of specific substrates; or 3) ABC protein activity may redirect the activity of associated proteins toward an RNAi function.

Chapter 3

RNAi functions of C. elegans ABC protein function may converge downstream in the RDE-2/MUT-7 complex

C. elegans mutant strains *rde-2* and *mut-7* were isolated in several genetic screens designed to isolated RNAi defective mutant strains (Tabara, Sarkissian et al. 1999) as well as strains with increased transposon activity in the germ line (Ketting, Haverkamp et al. 1999). MUT-7 encodes a 3' to 5'exonuclease (Ketting, Haverkamp et al. 1999). Fractionation studies have shown that MUT-7 proteins exist as complexes of ~250 KDa in both cytosol and nuclear fractions and this complex shifts in size to ~350KDa in the presence of dsRNA only in the nuclear fraction, suggesting that other proteins may associate with MUT-7 to execute the RNAi mechanism (Tops, Tabara et al. 2005). Studies indicate that *mut-7* functions downstream of *rde-4* - after the siRNA production step (Grishok, Tabara et al. 2000) and is needed for *in vivo* detection of siRNAs in *C. elegans* (Tijsterman, Ketting et al. 2002). RDE-2 encodes a *C. elegans* specific protein (Tops, Tabara et al. 2005) with no discernible motifs. Most of what is known about RDE-2 comes from its association with the MUT-7 protein. *rde-2* and *mut-7* show genetic interaction evident by non-complementation of RNAi defects in a doubly heterozygous mutant strains (Tops, Tabara et al. 2005). The RDE-2 protein is present in a complex with MUT-7 in the cytosol and was also shown to interact with MUT-7 using a yeast two-hybrid assay (Tops, Tabara et al. 2005). Interaction between *mut-7* and *rde-2* is further supported by the similar phenotypes exhibited by these mutant strains. Both mut-7 and rde-2 are required for RNAi in the germ line and not soma (Tabara, Sarkissian et al. 1999). They also show an increased rate of transposition in the germ line, elevated levels of males in the population and defects in co-suppression (Ketting, Haverkamp et al.

1999; Tabara, Sarkissian et al. 1999; Dernburg, Zalevsky et al. 2000; Grishok, Tabara et al. 2000).

This chapter describes genetic interactions of the RNAi defective *C. elegans* mutant strains with *rde-2* and *mut-7* as well as a collective description of *rde-2* and *mut-7* phenotypes exhibited by them.

Materials and Methods

C. elegans strains

The following *C. elegans* strains carrying deletions in *C. elegans* ABC genes were obtained from CGC (University of Minnesota, Minneapolis, MN) or the National Bioresources Project in Japan: and used in phenotypic analysis. *abt-1(tm0703), abt-2(tm502) I, abt-4(ok633) V, abt-4(tm1050) V, ced-7(n1892) III, ced-7(n1996) III, ced-7(n1997) III, ced-7(n2094) III, ced-7(n2690) III, cft-1 (ok1180)V, C05D10.3(tm688), haf-1 + C30H6.9 (ok705)IV, haf-1 (tm843), (ok1086) V, haf-2(gk13), haf-9(gk23), haf-4 (gk240) I, haf-4 (ok1042) I, haf-5 (gk161) III, haf-5 (gk155) III, haf-7 (gk46) V, haf-8 (gk12) IV, mrp-1(pk89), mrp-3(ok995) X, mrp-4 (ok1095) X, mrp-6 (ok1027) X, mrp-8(ok1360) III, pgp-1(pk17), pgp-1(pk17);pgp-3(pk18) IV, [pgp-1(pk17) pgp-3(pk18) mrp-1(pk89)], (gk114) I, pgp-3 (pk18) X, pgp-4(gk16) , pgp-5 (ok856) X, pgp-5(tm663), pgp-6 and pgp-7(ok994) X, pgp-7(ok528), pgp-9 (tm830) V, C54D1.1(ok991) X, pgp-10(tm996) X, (gk19) X, pgp-13(ok747) X, pgp-15 (ok987)X, pmp-1(ok773), pmp-3 (ok1087) V, pmp-4 (ok396), rde-2(ne221)I,*

rsd-2(ne319), rsd-6(pk3000) I, sid-1(qt2) V, C56E6.1 (ok865), C16C10.12 (ok927) III, C16C10.12 (ok962) III, C16C10.12 (tm714), F02E11.1 (ok1007) II, F18E2.2 (ok830) V, F19B6.4 (ok806) IV, T26A5.1 (ok882) III, T27E9.7 (ok771) III, Y39E4B.1(gk481), (ok812) Y42G9A.6 III, 48E10.9 (ok1044) III. At least 4 attempts were made to outcross background mutations in all the *C. elegans* ABC deletion strains, before the strains were employed for further phenotypic analysis.

RNAi defects in the germ line using pop-1 dsRNA

C. elegans strains were assayed for their RNAi defects on *pop-1* dsRNA at 20C. We picked 5 P0 animals per plate as L1/L2s and the number of progeny was counted and reported as F1 / P0.

Complementation assay

For complementation testing, crosses were performed using one hermaphrodite per mating on standard OP50 plates. The presence of 50% males in the progeny indicated a successful cross. The F1 cross-progeny were transferred as L1/L2 onto different batches of *pop-1* feeding plates. The extent of complementation was determined based on the number of F2s /P0 on *pop-1* dsRNA. 0-5 F2s /P0 were classified as complementation or indicated by the negative "-" sign, 5-20 F2s /P0 were classified as weak complementation or "w," 20-50 F2s /P0 as "+" complementors and, 50 and above F2s /P0 as strong or "++" group. Several rounds of complementation tests were

done and the results were compared. Complementarity was also assessed in reciprocal crosses.

Temperature sensitive RNAi phenotypes of C. elegans ABC strains similar to rde-2 and mut-7

Brood size and unc-22 assay

C. elegans strains were assayed for their reproductive rate on OP50 by placing 5PO animals per plate as L2/L3s at 25° C, 20°C, and 15°C. The number of progeny was counted and reported as F1/P0. Temperature sensitive RNAi defects were assayed by placing strains on *unc-22* dsRNA. Twitching phenotype was reported as number of strong twitchers vs. non-twitchers at both 25° C and 20°C

Mutator assay

C. elegans strains carrying deletions in the ABC genes were crossed into strains carrying a transposon insertion in the *unc-22* gene and hence exhibiting a twitching phenotype. 5 twitching PO animals were picked on to each OP50 plate at 26°C, 15°C, and 20°C. Plates were monitored for several days while avoiding starving conditions, and the numbers of non-twitching F1/P0 were scored. These revertants were a measure of transposon mobilization in the germ line of the respective strains.

Results

Following the discovery of RNAi defects in *C. elegans* strains defective for ABC transporter HAF-6 (Sundaram, Echalier et al. 2006), we analyzed other *C. elegans* ABC transporters for their requirement in the mechanism of RNA interference. *C. elegans* has 60 ABC transporters (Sheps, Ralph et al. 2004), for which deletions are available for 50 ABC transporters through CGC. RNAi defects were found for additional ABC mutant strains (Sundaram, Echalier et al. 2006) and their phenotypes were analyzed further.

RNAi defects on pop-1 dsRNA

C. elegans ABC mutant strains were tested for RNAi defects in the germ line through the use of *pop-1* dsRNA at 20°C. As reported before (Sundaram, Echalier et al. 2006), in addition to *haf-6(ne335)* mutant strains, 9 other ABC strains were observed to be RNAi defective by this assay. No temperature-dependence phenotype was observed in this assay. The results at 20°C are comparable to 25°C, except for the strains of *haf-9, haf-6, rde-2* and *mut-7*, all of which have a sterile germ line at higher temperatures (Figure 3- 1).

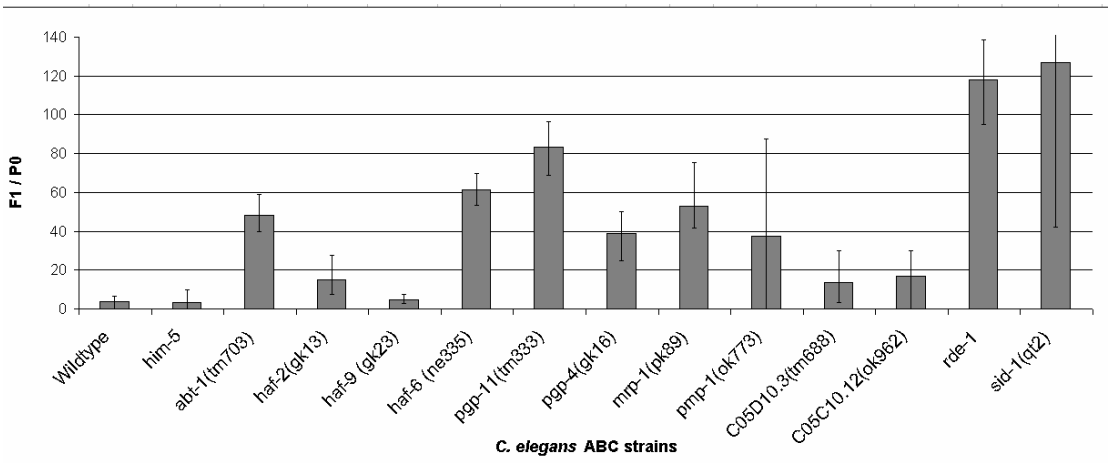


Figure 3- 1 RNAi defects of ABC deletion strains on *pop-1* dsRNA. The strains assayed in this experiment are listed on the x-axis. 5 P0 animals were picked onto *pop-1* dsRNA plates at 20Cs. The exact number of F1s that hatched out on each plate was counted and the F1 / P0 ratio was calculated as indicated on the y-axis.

Temperature-dependent defects in the soma (muscle)

The *haf-6(ne335)* (Sundaram, Echalier et al. 2006) ABC mutant is defective for all germ line targets tested, but not for the somatic targets that we have tested except *elt-2* and *unc-22. elt-2* is expressed exclusively in the intestine and it has been shown that *haf-6(ne335)* is expressed in the intestinal tissue of *C. elegans* (Sundaram, Echalier et al. 2006). It can therefore be reasoned that *haf-6(ne335)* is required for RNAi in the tissues where it is expressed.

UNC-22 is an abundant muscle protein, the loss of function of which leads to the twitching phenotype in worms. Strains that are RNAi defective do not twitch and are

referred to as non-twitchers. *haf-6(ne335)* has not been detected to be expressed in the muscle. However, *haf-6(ne335)* is defective in this tissue in a temperature dependent manner. In other words, *haf-6(ne335)* exhibits RNAi defects for the *unc-22* gene (non-twitchers) at 25°C (Figure 3- 2) while they seem not to be resistant to RNAi of the *unc-22* gene (twitchers) at lower temperatures (Figure 3- 3). This intriguing defect specific to the *unc-22* gene is exhibited only by the *haf-6(ne335) C. elegans* mutant and not by the 9 additional RNAi defective ABC mutant strains.

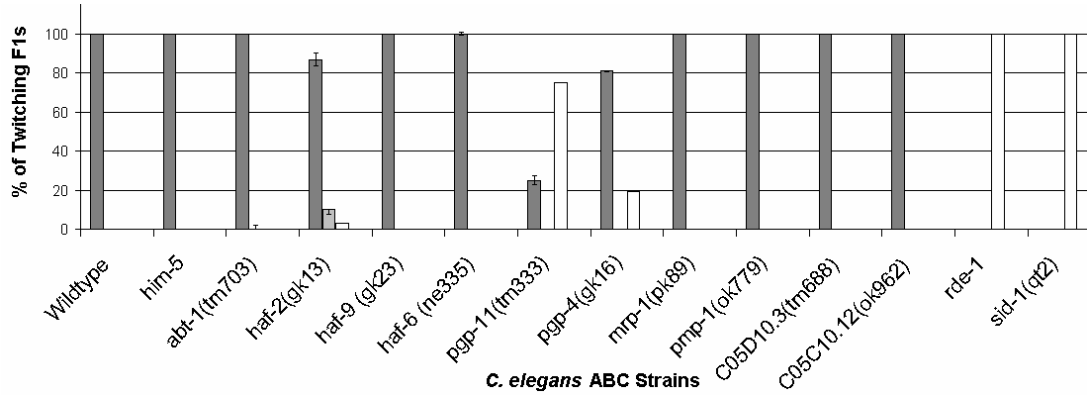


Figure 3- 2: *haf-6(ne335)* displays RNAi phenocopy for *unc-22* at 20C. Worms were scored as strong or weak twitchers vs. non-twitchers. As noted *haf-6(ne335)* shows NO RNAi defects like most of the other ABC mutant strains.

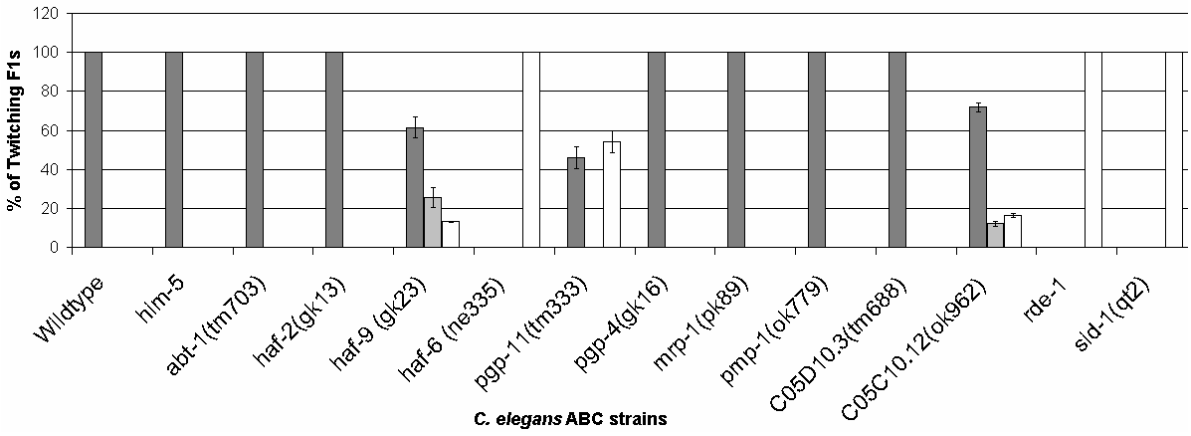


Figure 3- 3: *haf-6(ne335)* exhibits RNAi defects for *unc-22* at 25C.
5 P0 worms were picked on *unc-22* dsRNA. The laid eggs were allowed to hatch and the twitching phenotype was assessed in the F1s beginning L3 until adult stage. The # of twitcher F1s were counted and expressed as a percentage. Worms were scored as strong or weak twitchers vs. non-twitchers. As noted *haf-6(ne335)* shows strong RNAi defects compared to the other ABC mutant strains.

Strong twitchers (RNAi) **Weak twitchers** **Non-twitchers (RNAi defective)**

Genetic Interaction with the RNAi defective mutant strains rde-2 and mut-7

C. elegans ABC mutant strains show genetic interaction and share common phenotypes with two other RNAi defective mutant strains *rde-2 and mut-7.* This genetic interaction was first encountered when *haf-6(ne335)* mutant failed to complement *rde-2(ne221).* Wild-type *rde-2* sequences could rescue RNAi defects in *haf-6(ne335)* mutant strains and *vice versa* (data not shown) reinforces interactions between these proteins. The *rde-2* gene was sequenced in a *haf-6(ne335)* mutant and *haf-6* gene in a *rde-2(ne221)* mutant and the sequences were wild-type. Thus the genetic interaction between *rde-2(ne221)* and *haf-6(ne335)* is a case on non-allelic non-complementation.

It has been shown previously that *rde-2(ne221)* and *mut-7* interact with each other both genetically and physically (Tops, Tabara et al. 2005). Thus complementation tests were also performed between *haf-6(ne335)* and *mut-7,* and we found similar non-complementation. The *rde-2* gene in *mut-7* mutant is also wild-type.

To better understand the role of ABC transporters in RNAi and if this non-complementation with *rde-2(ne221)* and *mut-7* was specific to the *haf-6(ne335)* mutant, we wanted to extend this genetic analysis to the other ABC transporter strains defective in RNAi in *C. elegans.* The results were remarkable – all the ABC transporter strains with defects in RNAi showed non-complementation with *rde-2(ne221)* and *mut-7.* This suggests that the functions of the ABC proteins involved in RNAi may all converge downstream at the RDE-2/MUT-7 complex. This non-complementation was observed only when the hermaphrodite used in the complementation test was either *rde-2(ne221)* or *mut-7.* In the reciprocal cross however, we typically observed no genetic interactions, suggesting that a maternal requirement of *rde-2* and *mut-7* gene products is required for efficient RNAi (Figure 3- 4).

♂ \ ♀		*abt-1*	*pgp-4*	*pgp-11*	*haf-2*	*haf-6*	*haf-9*	*mrp-1*	*pmp-1*	*C05D10.3*	*C16C10.12*	*rde-2*	*mut-7*
A	*abt-1*	NC	C	C	I	C	C	C	C	C	NC	NC	NC
B	*pgp-4*	C	NC	C	C	W	C	C	C	C	I	NC	NC
B	*pgp-11*	C	NC	NC	I	NC	C	NC	C	C	NC		NC
B	*haf-2*	C	C	C	NC	C	C	C	C	C	C	NC	NC
B	*haf-6*	C	I	C	C	NC	C	C	C	C	C	NC	NC
B	*haf-9*	C	C	C	C	C	NC	I	C	C	C	NC	NC
C	*mrp-1*	C	C	I	I	C	C	NC	C	C	I	NC	NC
D	*pmp-1*	C	I	C	C	C	C	C	NC	I	I	NC	NC
G	*C05D10.3*	C	C	C	C	C	C	C	C	NC	C	I	NC
G	*C16C10.12*	I	C	C	NC	C	C	I	C	C	NC	NC	NC
--	*rde-2*	I	W	C	C	C	C	C	C	C	I	NC	NC
--	*mut-7*	NC	NC	C	C	C	C	C	C	C	W	NC	NC

Figure 3-4: Complementation tests between ABC transporters with RNAi defects and *rde-2/mut-7*. The chart summarizes the RNAi defects in the cross-progeny or F1s. Males from the respective ABC mutant strains were picked onto OP50 plates and mated with a single hermaphrodite of either the *rde-2(ne221)* or the *mut-7* strain and also the other hermaphrodite ABC strains. All crosses were set at 20C. Successful crosses were identified by the presence of ~50% males in the F1 progeny. 4-5 F1 hermaphrodites were picked onto fresh *pop-1* dsRNA and allowed to lay eggs for ~6 days at 20C. The average number of live F2s /P0 on *pop-1* dsRNA was calculated. Based on the F2s/P0 the heterozygous allele combinations were classified as complementation or non-complementation groups. A scale based on F2 / P0 was developed. 0 -5 F2 / P0 was assigned a '-' sign or complementing pair of alleles (C) , 5 - 20 F2 / P0 was assigned a 'W' sign or weakly complementing pair of alleles, 20 - 50 F2 / P0 was assigned a '+' sign or non-complementing pair of alleles (NC) and 50 - 100 F2 / P0 was assigned a '++' sign or strongly complementing pair of alleles (NC), Multiples crosses were set and several sets of F1 cross progeny were tested on different batches of *pop-1* dsRNA, before the final results were decided. Almost all ABC/*rde-2 or mut-7* heterozygotes fell into the '++' non-complementation group. Some results were indecisive or inconsistent and is presented by 'I.' Reciprocal crosses were also set and F1s analyzed in a similar way.

From the interesting results outlined above, we find that almost all the RNAi defective *C. elegans* ABC mutant strains interacts genetically with *rde-2(ne221)* and *mut-7*. In order to make the strong argument that these genetic interactions are indeed specific to the deletions or mutations in the ABC genes and not a random event, we

performed a control assay, where we crossed all the available *C. elegans* ABC strains with *rde-2(ne221)* and *mut-7* hermaphrodites. ABC genes not involved in RNAi did complement *rde-2(ne221)* and *mut-7*, indicating the specificity of our non-complementation results (Figure 3- 5).

Gene (allele)	*rde-2*	*mut-7*	Gene (allele)	*rde-2*	*mut-7*
abt-2(ok669) I.	C	C	*pgp-6 & pgp-7(ok994) X*	C	C
abt-2(tm502) I	C	C	*pgp-7(ok528)*	C	C
abt-4(ok633) V	C	C	*pgp-9 (tm830) V*	C	C
abt-4(tm1050) V	C	C	*pgp-10 (ok991) X*	C	C
ced-7(n1996) III	C	C	*pgp-10(tm996) X*	C	C
ced-7(n1997) III	C	C	*pgp-12(gk19) X*	C	C
ced-7(n2094) III	C	C	*pgp-13(ok747) X*	C	C
cft-1 (ok1180)V	I	I	*pgp-15 (ok987)X*	C	C
haf-1 + C30H6.9 (ok705)IV	C	C	*pmp-3 (ok1087) V*	C	C
haf-1 (tm843)	C	C	*pmp-3(tm968)*		
haf-3(ok1086) V	C	C	*pmp-4 (ok396) VC189*	C	C
haf-4 (gk240) I	I	I	C08H9.2	C	C
haf-4 (ok1042) I	C	C	*C56E6.1 (ok865)*		
haf-5 (gk161) III	C	C	*C16C10.12 (ok927) III*	C	C
haf-5 (gk155) III	C	C	*C16C10.12 (tm714)*	C	C
haf-7 (gk46) V	C	C	*F02E11.1 (ok1007) II*	C	C
haf-8 (gk12) IV	C	C	*F18E2.2 (ok830) V*		
mrp-3(ok995) X	C	C	*F19B6.4 (ok806) IV*	C	C
mrp-4 (ok1095) X	C	C	*T26A5.1 (ok882) III*	C	C
mrp-6 (ok1027) X	C	C	*T27E9.7 (ok771) III*	C	C
mrp-8(ok1360) III	C	C	*Y39E4B.1(gk481)*		
pgp-1(pk17)]	C	C	*Y42G9A.6 (ok812) III*	C	C
[pgp-1(pk17);pgp-3(pk18) IV	C	C	*Y48E10.9 (ok1044) III*	C	C
pgp-1(pk17) pgp-3(pk18) mrp-1(pk89)]	C	W	*rde-1*	C	C
pgp-2(gk114) I	C	C	*rde-4*	C	C
pgp-3 (pk18) X	C	C	*sid-1*	C	C
pgp-5 (ok856) X	C	C	*rsd-2*	C	C
pgp-5(tm663)	C	C	*rsd-6*	C	C

Figure 3-5: RNAi defects in cross progeny. Assay was performed similar to that described in Figure 3. 'C' refers to complementation.

Following the discovery of strong genetic interactions between the RNAi defective ABC mutant strains and *rde-2/mut-7*, we set out to analyze if the RNAi defective ABC mutant strains also exhibited phenotypes similar to *rde-2/mut-7* strains.

rde-2 and *mut-7* mutants are RNAi defective, germ line sterile at higher temperatures, mutators with activation of transposons in the germ line at elevated temperatures, Him (High incidence of males), defects in co-suppression and miRNA function. A Him phenotype was not observed in the RNAi defective ABC mutant strains. Co-suppression has not been studied yet. Interestingly, RNAi defective ABC mutant strains exhibited temperature dependent phenotypes like transposon activity and germ line sterility like *rde-2* and *mut-7.* These phenotypes are reported and discussed below.

Temperature-dependent brood size

C. elegans ABC mutant strains were assayed for their reproductive rates at different temperatures 26°C, 25°C, 20°C, and 15°C. The brood sizes of *haf-9, haf-6, mut-7,* and *rde-2* (discussed later in this chapter) showed very low reproductive rates compared to other ABC mutant strains. At 26C and 25C *haf-9, haf-6, rde-2* and *mut-7* showed a similar temperature dependent sterile phenotype. This similarity reiterates the genetic interaction displayed between *haf-6* with *rde-2,* and *mut-7* strains. When the assays were conducted at 20°C, the strains showed healthier brood size. No significant observations were made in the reproductive rate at 15°C (Figure 3- 6).

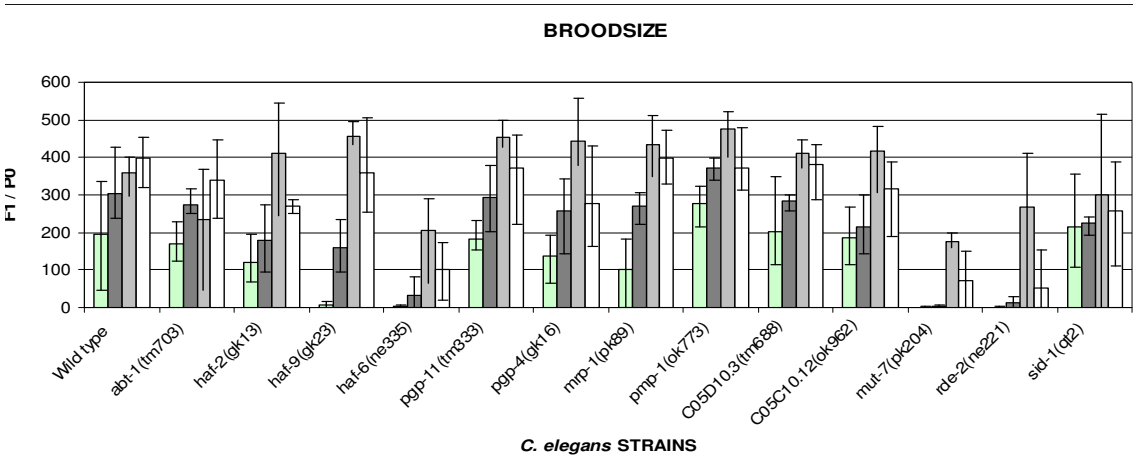


Figure 3- 6: Brood size of ABC deletion strains as well as *rde-2* and *mut-7*. The strains assayed in this experiment are listed on the x-axis. 5 P0 animals were picked onto OP50 plates at different temperatures: 26°C, 2°5C, 20°C, and 15°C. P0 animals were allowed to lay eggs for 3 – 10 days. The exact number of F1s that hatched out on each plate was counted and the F1 / P0 ratio was calculated as indicated on the y-axis. Care was taken to avoid environmental stress conditions like contamination, starvation etc. It is important to note that every mutant has different reproductive rates which were also influenced by temperature. The experiment at 26°C and 25°C took ~4 days whereas the assay took ~6 days at 20°C and ~10 days at 15°C

26°C 25°C 20°C 15°C

Temperature dependent transposition in the germ line (Mutator activity)

The wild-type *C. elegans* germ line shows no transposon activity. However, RNAi defective mutant strains like *rde-2* and *mut-7* show an increased rate of transposition in the germ line. Although one of the endogenous roles of RNAi is to silence transposition in the germ line, not all RNAi defective mutant strains show mutator activity.

Mutator activity can be assayed by using a twitching *C. elegans* strain, in which the *unc-22* gene is interrupted by a Tc1 transposon. When this mutant *unc-22* gene with the transposon is crossed into the RNAi defective mutant backgrounds like *rde-2* and *mut-7,* transposition occurs in the germ line of the parent animal resulting in non-twitching F1s. The number of non-twitching F1s is therefore a measure of mutator activity.

Mutator assay was performed in the RNAi defective ABC mutant backgrounds and we found *haf-6(ne335)* in particular to share the mutator phenotype with *rde-2* and *mut-7* at 25C (Figure 3- 7). A few other ABC mutant strains that showed mutator activity are *haf-9 (gk23), C05D10.3 (tm688),* and *mrp-1(pk89).* It is conceivable that these ABC proteins, along with HAF-6, may play an important role in RNAi in conjunction with the RDE-2/MUT-7 protein complex.

STRAIN	% REVERTANTS		
	15˚C	20˚C	25˚C
unc-22(st136)		0% (0 / 16,500)	0% (0 / 25,000)
rde-2(ne221) ; unc-22(st136)		**0.06% (5 / 8,650)**	**0.40% (90 / 22,200)**
mut-7(ne221) ; unc-22(st136)			
abt-1(tm0703) ; unc-22(st136)	0% (0 / 16,000)	0% (0 / 16,000)	0% (0 / 38,000)
C05D10.3(tm688) ; unc-22(st136)	0% (0 / 16,000)	0% (0 / 16,000)	**0.06% (26 / 42,000)**
C16C10.12(ok927) ; unc-22(st136)			
C16C10.12(ok962) ; unc-22(st136)	0% (0 / 16,000)	0% (0 / 16,000)	0% (0 / 38,000)
haf-2(gk13) ; unc-22(st136)	0% (0 / 16,000)	0% (0 / 16,000)	0% (0 / 38,000)
haf-6(ne335) ; unc-22(st136)	0% (0 / 21,150)	0% (0 / 21,000)	**0.09% (17 / 18,000)**
haf-9 (gk23) ; unc-22(st136)	0% (0 / 16,000)	0% (0 / 16,000)	**0.04% (15 / 41,200)**
mrp-1(pk89) ; unc-22(st136)	0% (0 / 16,000)	0% (0 / 16,000)	**0.02% (8 / 41,000)**
pgp-4(gk16) ; unc-22(st136)	0% (0 / 16,000)	0% (0 / 16,000)	0% (0 / 38,000)
pgp-11(tm33) ; unc-22(st136)	0% (0 / 16,000)	0% (0 / 16,000)	0% (0 / 38,000)
pmp-1(ok773) ; unc-22(st136)	0% (0 / 16,000)	0% (0 / 16,000)	0% (0 / 38,000)

Figure 3- 7: Mutator activity in the RNAi defective *C. elegans* ABC strains. 4-5 twitcher PO animals were picked onto OP50 plates at 25C, 20C and 15C. The number of non-twitcher F1s were counted and reported as % of non-twitchers. Starvation and contamination was avoided. ~25,000 F1s or more were assayed for the non-twitching phenotype. These strains were built and tested for mutator activity by Wang Han.

Discussion

rde-2 and *mut-7* are two RNAi defective mutant strains implicated in the RNAi mechanism as early as 1999, when the first screen for RNAi defective *C. elegans* mutant strains were performed (Ketting, Haverkamp et al. 1999; Tabara, Sarkissian et al. 1999). RDE-2 is a *C. elegans* specific protein that interacts with MUT-7 both *in vivo* and *in vitro (Tops, Tabara et al. 2005). mut-7* encodes a 3' to 5'exonuclease (Ketting, Haverkamp et al. 1999), and the MUT-7 protein exists as complexes with RDE-2 in the cytosol. In the presence of dsRNA, MUT-7 is found exclusively in the nuclear fraction, not associated with RDE-2 (Tops, Tabara et al. 2005).

rde-2 and *mut-7* mutant strains exhibit phenotypes like increased chromosomal non-disjunction leading to the increased production of males (XO), expression of transgenes usually silenced in the germ line of *C. elegans,* high transposition rate in the germ line of *C. elegans*, and defects in co-suppression. One commonality in all the *rde-2* and *mut-7* mutant phenotypes is that they occur in the germ line and are at least genetically required to maintain genome integrity and function. In other words, RDE-2 and MUT-7 proteins may function directly or indirectly in association with other proteins at the level of DNA in the germ line.

C. elegans has 10 ABC mutant strains defective in the RNAi mechanism. It has been demonstrated by our studies that these proteins are all required in the germ line for

efficient RNAi (Sundaram, Echalier et al. 2006). They show genetic interaction with the *rde-2* and *mut-7* mutant strains and also share some of their common phenotypes.

RDE-2, MUT-7, and ABC transporter proteins involved in RNAi function in the germ line and all of their functions probably culminate in the formation of a functional RDE-2 / MUT-7 complex in the cytosol. The domains of RDE-2 and MUT-7 that physically interact with each other have been mapped (Tops, Tabara et al. 2005). It has been shown that the N-terminus domain of RDE-2 is inhibitory to the MUT-7 interaction and deleting this domain increases this interaction several fold. Although ABC transporters are transmembrane proteins and may or may not physically interact with RDE-2, one idea is that they may be transporting substrates that probably sequester the N-terminus of RDE-2, thereby promoting its interaction with MUT-7. Thus the ABC transporters involved in RNAi may be fully or partially responsible for strengthening the RDE-2 / MUT-7 interaction, which makes RNAi an efficient mechanism.

Although our non-allelic non-complementation results may support the view discussed above, it is noteworthy that most of the RNAi defective transporters do not exhibit the mutator phenotype like *rde-2* and *mut-7,* and none of the transporters are Him. One of the possibilities is that the function of ABC transporters and their relation to *rde-2* and *mut-7* may be tissue-specific (discussed in chapter 4).

It is important to mention that the other *rde-2 / mut-7* phenotypes like co-suppression and germ line desilencing of transgenes have not yet been analyzed.

Thus ABC transporters may be necessary for an efficient RNAi process by promoting the formation of a functional RDE-2 / MUT-7 complex. They may have other functions that could simply be developmental or may be needed to maintain normal homeostasis in the cytoplasm, while not having any role in the mechanism of RNAi. However, we cannot rule out the fact that development and RNAi are two inter-linked processes.

Chapter 4

Hypothetical model for the role of ABC transporters in RNAi in C. elegans

RNA interference is usually a post-transcriptional mechanism, although our studies have provided clues that it may be a coordinated response that initially originates either in the cytoplasm in response to exogenous dsRNA or in the nucleus in response to accidentally transcribed aberrant endogenous transcripts. Once initiated, irrespective of the site of origin, it culminates in the nucleus by protecting the genome from damage. Emerging new studies are also finding newer links that suggest that RNAi is more of a nuclear phenomenon.

ABC transporters are transmembrane proteins, and several substrates traverse through them utilizing the energy derived from ATP. They have been conserved in the genomes of yeast, fruit flies, humans, and worms (Bauer, Wolfger et al. 1999; Gottesman and Ambudkar 2001; Schulz and Frommer 2004; Sundaram, Echalier et al. 2006). Human ABC transporters have been studied in great depth with respect to multi-drug resistance in cancer, sterol/cholesterol transport, chloride ion channels in cystic fibrosis, etc. (Dean and Allikmets 2001; Dean, Hamon et al. 2001; Dean, Rzhetsky et al. 2001; Dean 2005), but their involvement in RNAi has only been recently reported from our studies (Sundaram, Echalier et al. 2006).

The data discussed in this dissertation provides us with two major new findings – the genetic interactors of ABC transporters (RDE-2 and MUT-7) and their germ line function. As discussed in the previous chapters, these ABC transporters may transport substances that either directly or indirectly mediates the formation of RDE-2 and MUT-7 complexes necessary for an efficient RNAi mechanism. In this regard, one of the direct questions to be addressed is the substrates of these transporters. Future studies that may help identify the precise localization of these ABC proteins in the germ line may give us clues about the substrate specificity. Given the fact that these transporters are redundant in nature, they may transport at least a few substrates in common. Or they may transport completely different substrates based on their organelle of residence.

Although several more discoveries have to be made in order to decipher the mechanism by which ABC transporters may mediate RNAi, our research work has given us some solid insights in to the mechanism. Here we describe a hypothetical model that shows how ABC transporters in conjunction with RDE-2/MUT-7 complexes may mediate RNAi. HAF- 6 transporter is expressed in the ER where as other ABC transporters may be expressed in different organelles of the germ line. Based on our non-complementation assay we know that all the ABC transporters involved in RNAi interact genetically with RDE-2/MUT-7.

Hence, we hypothesize that the 10 ABC transporters involved in RNAi aid in the formation of a functional RDE-2/MUT-7 in the cytoplasm by an unknown mechanism. The identity of the substrate these ABC transporters transport and how these substrates help in the formation of a functional RDE-2/MUT-7 complex are the two immediate questions that need to be answered in order to understand the role of the ABC transporters in RNAi. One idea is that the substrate probably sequesters the inhibitory N-terminus of the RDE-2 protein thereby promoting the formation of a stable RDE-2/MUT-7 complex. Another idea is that the substrate may simply help maintain a homeostatic environment for the RNAi mechanism to take place. Once the RDE-2/MUT-7 complex is formed in the cytoplasm and senses the dsRNA, the MUT-7 protein may associate with other proteins in the nucleus where it protects the genome by silencing transposons and other repetitive sequences.

Our study puts forth several interesting questions that need to be addressed in the near future. One is the identity of the substrate. Studies in humans have shown that Transporters Associated with Antigens or TAP molecules reside in the ER similar to the HAF-6 molecule. TAP processes and transports peptides that in turn bind to Class I Major Histocompatability Complex or MHC. Based on the similarity in the organelle residence, HAF-6 may transport peptides similar to the TAP.

It would be interesting to look at the expression patterns of all the 9 additional ABC

proteins. One of the interesting aspects that could be studied in these RNAi defective ABC mutant strains is their ability to process miRNAs. miRNAs are tied to several developmental processes and RNAi has a long standing role in the normal development of the organism. Thus there are several areas that one could explore the role of ABC transporters, that will certainly help put together the scattered pieces of the RNAi puzzle.

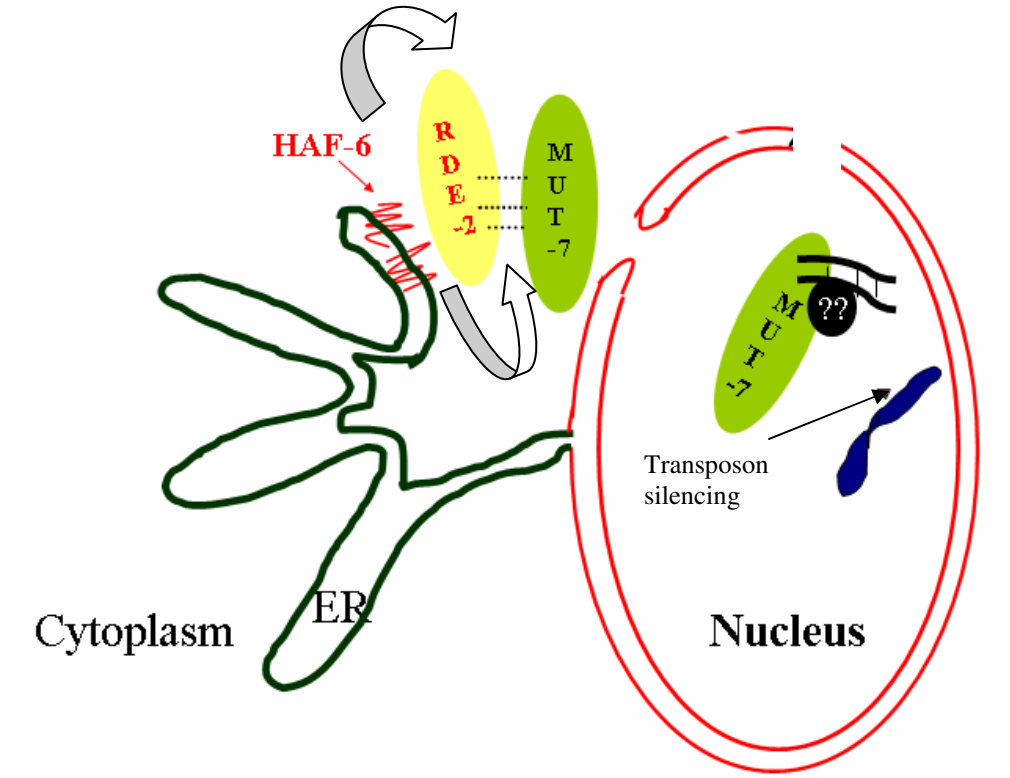


Figure 4-1: A hypothetical model for how ABC transporters like HAF-6 and their genetic counterparts may be involved in the mechanism of RNAi in *C. elegans*. The red compartment represents the nucleus and the area outside the cytoplasm. HAF-6 is expressed in the ER RDE-2/MUT-7 complexes are present in the cytoplasm and in the presence of dsRNA, MUT-7 translocates in to the nucleus. The blue structure represents a chromosome and the filled triangle a transposon.

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