RNA Helicase 1 interacts with an ABC_{RNAi} Transporter:

Genetic Interactions with *haf-6*

By

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Abstract

The *C. elegans rha-1* gene encodes a conserved helicase with ATP-dependent DEAD/H-box and double-stranded RNA binding domains. *rha-1* is orthologous to the *Drosophila maleless* gene(MLE), an essential component of the dosage compensation machinery that leads to a two-fold increase in transcription of genes located on the single X chromosome of males in comparison to the transcription rate of homologous genes located on an X chromosome in females. The human ortholog, RNA helicase A (RHA), unwinds double-stranded DNA and RNA in a 3' to 5' direction. RHA is a component of several distinct protein complexes, including the RNA-induced silencing complex (RISC), the coding region determinant (CRD)-mediated complex, mRNP granules, and also associates with BRCA1, CREBbp or SMN1 and the RNA polymerase II complex, phosphorylated histones (H2AFX). RHA affects a number of different biological activities, including CRD-mediated mRNA stabilization, RNA splicing, translation, and transcription. It also has been discovered that regulation of RHA is disrupted in many types of cancers. The *C. elegans rha-1* gene is expressed in the gonad where it localizes to the nucleus and the cytoplasm and is required for proper development. Defects in *rha*-1 lead to germline defects, aberrant expression of genes residing in repetitive transgene arrays, and defects in RNAi.

ABC transporter proteins have also been implicated to play a role in RNAi in *C. elegans*. Ten out of the sixty-one ABC transporters are required for efficient RNAi in the germ line; these transporters are called ABC_{RNAi} transporters. All of the ABC_{RNAi} transporter mutants interact genetically with *rde-2* (a novel protein) and *mut-7* (a protein with homology to RnaseD). One of these ABC_{RNAi} transporter genes is *haf-6.* The works presented here show that *rha-1* mutants also interact genetically with *haf-6,* with *rde-2,* and with *mut-7* with respect to RNAi defects. By

contrast, mutations in *rha-1* suppress the transposon mobilization phenotypes observed in *haf-6* mutants. It is also observed that an unknown mutation is genetically interacting with *rha-1* and *haf-6.* We are currently investigating roles for *rha-1* in proper accumulation of *haf-6* and other ABC_{RNAi} transporter mRNAs in an effort to understand the interrelated functions of these proteins in RNAi.

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Chapter 1 RNA Interference

Discovery of RNAi

Anti-sense RNA has been known to inhibit gene expression since before 1990. This ability has identified as co-suppression or post-transcriptional gene silencing in plants [1], and quelling in fungus [2]. Initial work in *C. elegans* was spearheaded by Andrew Fire and Craig Mello who found that double stranded RNA (dsRNA) was better at elucidating interference of *C. elegans* genes than anti-sense or sense strands [3]. Their experiments involved injecting *unc-22* RNA sense, anti-sense, or a mixture of the two into *C. elegans*. Their experiments showed that a mixture of sense and anti-sense RNA gave almost two orders of magnitude larger response than the individual strands alone. They were also able to show that the RNA only interfered with the corresponding sequence of interest and greatly reduced the endogenous mRNA levels. When experiments analyzed localization of RNA, it was found that the RNA was not just localized to the injection site but that the cells from across the worm would also be responsive to the dsRNA. This is referred to as the spreading effect and enables the injected dsRNA to affect many tissues throughout the worm. Further investigation also proved that very little RNA was needed to elicit this RNAi response [3]. This response, suggests that there is an amplification step in the RNAi process that allows only a few dsRNAs to interfere with gene expression. Fire and Mello called this sequence specific response to introduced dsRNA, RNA interference (RNAi) [3].

Conservation of RNAi

The currently accepted hypotheses is that such a strong response has evolved as a defense against viruses before the divergence of plants and animals [4]. This defense system was first discovered in plants[5] and was later found in nematodes [6], fungi [7], insects (such as drosophila [8]), protozoa, and mammals [9]. The RNAi pathway in different organisms seems to

be highly conserved and is made up of similar proteins, comparable mechanisms of action, and an overall conserved response to dsRNA. Although these mechanisms of action are not fully understood, each organism shares a similar response to invading dsRNA.

Functions of RNAi

RNAi is part of a large subset of RNA based responses. Small RNAs and RNAi play a large role in many aspects of cell development and regulation. RNAi can be as simple as an interaction between mRNA and siRNA resulting in direct cleavage of mRNA before it can be translated into protein. It can also regulate gene expression less directly such as through chromatin remodeling and histone modifications [10]. RNAi has also been shown to be involved in the regulation of mitosis and meiosis, as well as play a role in changing developmental stages [11-12].

The canonical RNAi mode of regulation usually occurs at the post-transcriptional level where dsRNA is cleaved into short 21-23nt siRNAs which leads to direct cleavage of the mRNA transcript. *In situ* hybridization was used to exemplify this in *C. elegans* by looking at operons encoding sequence targeted by injected dsRNAs[13]. These experiments also helped prove that the RNAi response was active in both the nucleus and the cytoplasm[13].

Besides direct cleavage of gene targets, RNAi is also involved in nonsense-mediated decay (NMD) of mRNA. Transcripts that have point mutations or premature stops upstream of introns are targeted for degradation via the NMD pathway. A screen for defects in this pathway found seven genes, *smg1-7,* in *C. elegans* that are important for a functional pathway. Further studies showed that these mutants were also defective in RNAi[14].

Another method of RNAi-mediated gene regulation includes transposon silencing and transposon transcription by gene specific methylation of associated nuclesosomes[10]. The RNAi process may lead to recruitment of histone deacetylases that can change the methylation patterns on nucleosomes resulting in suppression of transposon mobilization by condensing the transposon DNA within the genome [15].

RNAi Pathway

RNAi involves many proteins and there are several different pathways that lead to a multitude of outcomes. In most organisms the mechanism is very similar. dsRNA in the cytoplasm is recognized and cleaved by Dicer into siRNAs. These small siRNAs are 20-25 nucleotides in length and have unpaired overhangs on either side. The double stranded siRNAs are then separated into guide strands (anti-sense) and sense strands. The guide strand is then loaded into the RISC (RNA inducing silencing complex) complex. RISC is made up of many different proteins. In each organism and pathway the makeup of RISC is different. Commonly the complexes include a protein with double stranded RNA binding motifs to bring in and position the RNA; it has an Argonaute protein to line up and position the RNA for cleavage. Argonaute proteins bind to siRNAs and guide and help facilitate post-transcriptional gene silencing by cleaving mRNA using their endonuclease activity. Once the guide strand is loaded into the RISC complex, it acts as a template to help RISC find its complimentary target mRNA. Once found, one of the catalytic subunits of RISC, an Argonaut protein, cleaves the complementary strand. The cleaved target mRNA can then be degraded by non-sense mediated mRNA decay, which is able to recognize the mRNA as aberrant. In some organisms, such as

nematodes, the smaller pieces can also be subsequently used in similar pathways leading to further processing of the siRNA, resulting in amplification of the RNAi response.

RNAi response in *C. elegans*

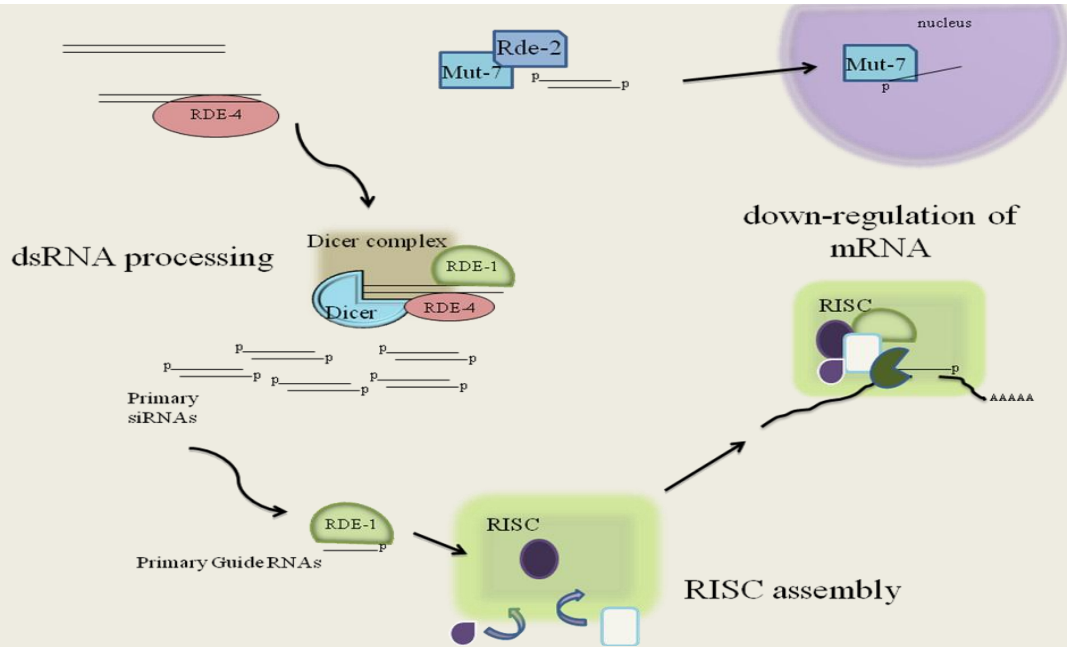

Figure 1. In *C. elegans* when dsRNA is introduced it is processed in the cytoplasm. *Rde-4* traffics the dsRNA to the Dicer Complex where it is cleaved into siRNAs. *Rde-1* takes the guide strand to RISC, and the RISC complex begins to assemble. Once assembled it finds its complementary mRNA. When found an Argonaute protein in the RISC complex cleaves the mRNA. This pathway leads to the down-regulation of mRNA.

RNAi in *C. elegans*

In *C. elegans* the RNAi process is initiated by an RNase III-related enzyme called Dicer. Dicer cleaves long dsRNA that is then used for gene silencing. Dicer is conserved in flies, plants, fungi and mammals[16]. The dsRNA binding protein RDE-4 recognizes foreign dsRNA and shuttles it to Dicer. RDE-4 and Dicer, together with a group of other proteins, then form the Dicer Complex. Once the dsRNA is loaded into the Dicer Complex, Dicer cleaves it into the small 21-23 nucleotide- long siRNAs[17]. It is thought that *RDE-1* then separates the resultant siRNAs into sense and anti-sense strands. *RDE-1* is classified as an Argonaute protein, and

contains a PAZ domain (needed for identification of 3' overhangs seen in siRNAs) and piwi domain (used for associating with piRNAs to aid in the silencing of mobile genetic elements) and is one of the many and important catalytic proteins needed for RNAi [18]. The anti-sense strands, called guide RNAs, are then shuttled by *RDE-1* to the RISC. Within the RISC, the guide strand acts as a template to find the complementary target mRNA. Once the target mRNA is found, one of the RISC-associated proteins cleaves the mRNA[15, 19]. The now aberrant mRNA is then degraded by non-sense mediated mRNA decay, causing the expression of the targeted genes to be knocked down (Figure 1).

It is believed that RNA-dependent RNA polymerases (RdRPs) use the sense strands and the newly cleaved mRNA to produce secondary siRNAs. This would help explain why such small amounts of dsRNA are needed to induce complete silencing of a gene. There are four RdRps in *C. elegans: EGO-1, RRF-1, RRF-2*, and *RRF-3*. Two of these, *RRF-3* and *EGO-1*, are needed for the production of secondary siRNAs. It is known that the process uses a different set of Argonaute proteins in a similar pathway to that of primary siRNAs. Two proteins that are also believed to be important in the secondary RNAi pathway are *MUT-7 and RDE-2.* Mut-7 encodes an exoribonuclease with 3′ to 5′ exonuclease activity. *rde-2* was found when screening for RNAi deficiencies, but its exact function is less understood. Both *MUT-7* and *RDE-2* are involved with X-chromosome nondisjunction and transposon mobilization. *MUT-7* and *RDE-2* are part of a complex found in the cytoplasm. When RNAi processing is underway this complex begins to grow by recruiting more proteins that are needed for the RNAi process. This complex is not necessary for secondary siRNA production but is believed to be required in the pathway between primary mRNA target cleavage and prior to secondary siRNA [20]. The primary siRNAs and secondary siRNAs can be distinguished from each other by differences at the 5' ends. Secondary

siRNAs have di- or triphosphates and primary siRNAs have monophosphates, which can be identified by capping or kinase activity assays[21].

Chapter II ABC Transporters

ABC Transporters

ATP-binding cassette transporters (ABC transporters) are part of a large super family of proteins that are found in all organisms. These transporters have many functions but are all transmembrane proteins that hydrolyze ATP. ABC transporters use the energy from ATP hydrolysis to transport a wide range of substrates across membranes. To be classified as an ABC transporter the protein must have a transmembrane domain and an ATP-binding cassette domain (ABC domain). The transmembrane domain consists of alpha helixes that span the lipid bilayer of membranes. This domain gives the transporter its specificity, and the sequences will vary depending on what type of substrate the transporter is carrying. The ABC domain is highly conserved and resides on the cytoplasm side of the lipid membrane. This is where ATP binding and hydrolysis occur. There are two classes of ABC transporters, full transporters and half transporters. Full transporters are made up of at least two transmembrane domains and two ABC domains. Half transporters have only one transmembrane domain and one ABC domain. To function in the cell these half transporters must find their binding partners to form a complete functioning ABC transporter. Half-transporters will dimerize with a different half transporter or they will form a homodimer so they can become fully functional.

The ABC transporter family is so large and diverse that these transporters are found in almost all cell membranes in all organisms. ABC transporters are vital for various physiological activities. These transporters function to move a variety of substrates in and out of cells such as salts, bile, cholesterol, peptides, lipids, and many other small molecules. They can also act as signals, for example by pumping lipid-modified peptides they can direct cell migration[22]. They have also been implicated in several types of cancers. Overall Forty-eight ABC genes have been found in humans, fourteen of which are thought to be related to cancers such as cystic fibrosis,

adrenoleukodystrophy, Stargardt's disease, drug-resistant tumors, Dubin-Johnson syndrome, Byler's disease, progressive familiar intrahepatic cholestasis, and X-linked sideroblastic anemia[23]. Not only are some of these transporters able to efflux chemotherapeutic drugs but they are also upregulated in some types of multi drug resistant cancers[24].

ABC Transporters in *C. elegans*

C. elegans has sixty-one ABC transporter genes[25-26]. These ABC transporter genes play a vital role in the *C. elegan's* life cycle. Transporters have also been shown to be required for timing in embryonic development[27]. They are known to regulate lysosome biogenesis, part of the fat storage pathway in *C. elegans*(*PGP-2*)[28], and ABC transporters such as *MRP-1* and *PGP-1* are known to efflux chemotherapeutic drugs from cells[29]. Others such as *CED-7* and *CED-1* are involved in removal of unwanted or harmful cells[30-31].

One other important function for ABC transporters is the role they play in RNAi. The Timmons lab identified ten transporters required for efficient RNAi[32-33], and has named these transporters ABC_{RNAi} transporters (Figure 3). Of the sixty-one transporters in *C. elegans* forty-three mutant transporters were tested for RNAi efficiency and nine were found to be deficient. These transporters were then used in tests to see if they would complement mutations in *rde-2* and *mut-7*. All nine transporters were non-complementary meaning they are genetically linked to the *rde-2/mut-7* RNAi pathway. The half transporter *haf-9* was also added to this list of ABC_{RNAi} transporters. On its own it was not defective in the RNAi process, but it did show to be non-complementary with *rde-2* and *mut-7*[32]. These related transporters are all needed for proper RNAi but their expression patterns, protein configurations, and the high homology of all ABC transporters does not lend any specific clues to how these transporters are needed for RNAi.

RNAi defects in ABC_{RNAi} transporters detected using *pop-1* assay

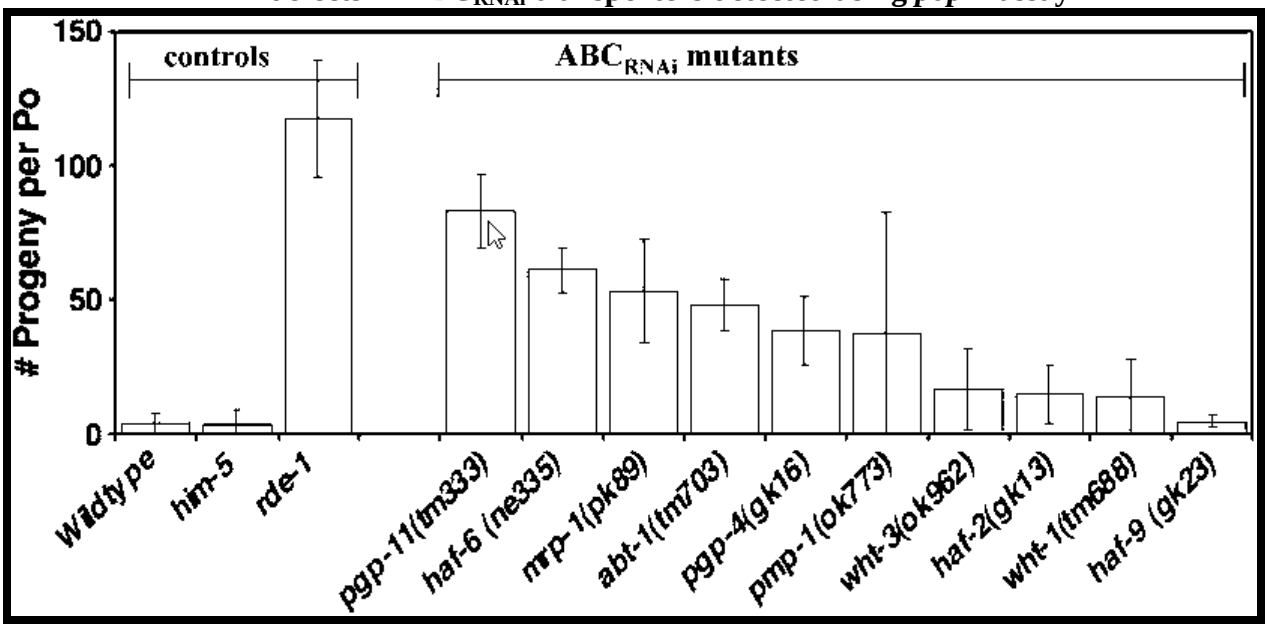

Figure 2. Shows ABC_{RNAi} transporter mutants are defective in RNAi. When these transporter mutants are reared on *pop-1* food they show differing degrees of RNAi defects. This figure was taken from Sundaram, P., et al.[32].

One of these ABC_{RNAi} transporters is *haf-6*. This is a half transporter that is localized to reticular membranes and can be found in the germ line and intestines[33]. *haf-6* has an allele (ne335) which was found to be defective in RNAi processing dependant on how the dsRNA was delivered. The mutant responded to dsRNA when it was injected into the worms but showed to be defective when the dsRNA was introduced through feeding assays[3, 15, 33]. When given low amounts of dsRNA the mutants did not have an RNAi response, however when increased dosages of dsRNA were delivered there was a strong RNAi response. That is, that the mutational effects of defective *haf-6* are dosage sensitive, with respect to dsRNA. These mutants are RNAi defective for genes expressed in the germline.[33].

Chapter III RNA Helicases

RNA Helicases

RNA Helicase proteins are conserved across many organisms such as viruses, bacteria, nematodes, and humans. Usually RNA helicase proteins use ATP hydrolysis to unwind annealed RNA, DNA or RNA to DNA strands. It accomplishes this by moving along the nucleic acid phosphodiester backbone to separate the annealed strands. RNA helicases have many functions within the cell. For example they can displace RNA from protein and can rearrange ribonucleoprotein assemblies [34]. RNA helicases that unwind double stranded RNA are members of the DEAD box family, or the DExH/D family, named after their conserved sequence motif (Asp–Glu–Ala–Asp) [35]. These families of helicases share eight conserved regions that are thought to relate to ATP hydrolysis, helicase activity or RNA binding[36]. Although all proteins having these conserved sequences are called helicases, it is undetermined whether they all have helicase activity.

In humans, RNA helicase A (RHA) unwinds double-stranded DNA and RNA in a 3' to 5' direction. RHA is a component of several distinct protein complexes, including the RNA-induced silencing complex (RISC), the coding region determinant (CRD)-mediated complex, mRNP granules, and also associates with BRCA1, CREBbp or SMN1, the RNA polymerase II complex, and phosphorylated histones (H2AFX) [37-40]. RHA affects a number of different biological activities, including CRD-mediated mRNA stabilization, RNA splicing, translation, and transcription [41]. Regulation of RHA is disrupted in some types of cancers. RHA in humans is a member of the nuclear MEF1 complex that contributes to the up-regulation of *mdr-1* gene expression in multidrug-resistant cancer cells. MDR1 is an ABC transporter and is known to efflux drugs from cancer cells.

RNA Helicase 1 in *C. elegans*

RNA helicase 1 (*Rha-1*) functions as a chromatin regulator, which helps in germline transcriptional silencing in *C. elegans*. Defects in *rha*-1 lead to germline defects, aberrant expression of genes residing in repetitive transgene arrays in situations where the transgene is normally silenced, and defects in RNAi. Orthologs of *Rha-1* are found in mice, cows, and show a 60% homology with *Drosophila maleless* gene MLE and human RHA. *Drosophila* MLE is an essential component of the dosage compensation machinery that leads to a two-fold increase in transcription of genes located on the single X chromosome of males in comparison to the transcription rate of homologous genes located on an X chromosome in females. All of these orthologs share a conserved ATP domain and an RNA binding DEAD box domain [42].

To study this RNA helicase, the Walstrom lab used the mutation strain *rha-1* (tm329). This mutation is a 1,059 bp deletion beginning in exon four. Studies show that this mutant strain produces a genetic null[43]. These studies showed 1) that the absence of rha-1 at restrictive temperatures caused disruption of normal chromatin organization, 2) that rha-1 is recessive, 3) that the defects effect both hermaphrodites and males (males showed stronger defects in gamete production), 4) and defects in mitotic proliferation (where mitosis begins correctly but cannot be maintained) due to histone modifications[43].

Chapter IV Materials and Methods

I) *Testing for* RNAi *deficiency by pop-1 feeding assays*

Bacteria that lack RNaseIII can be engineered to express double stranded RNA. These bacteria can then be fed to *C. elegans* allowing the dsRNA entry into the worm. The worm's natural responses will produce an RNAi response to the dsRNA and target the mRNA of interest for down regulation. To prepare the bacteria, HT115 (DE3) cells were transformed with a plasmid that contained segments of dsRNA corresponding to the *pop-1* transcript. This dsRNA will target TCF and LEF1 transcription factors that are needed for proper embryogenisis. The transformed bacteria was induced and plated onto small agarose plates that contained tetracycline, ampicillin and Isopropyl β-D-1-thiogalactopyranoside (IPTG) [43-45]. One to four larval stage 1 (L1) to larval stage 2(L2) worms were placed on these newly transformed bacteria plates. As the worms matured they ingested the bacteria and if they had a functional RNAi pathway they produced no progeny. If the worms RNAi pathway was disrupted ingestion of the dsRNA would not trigger an RNAi response and they would have progeny.

Freshly transformed bacteria (less than five days from transformation) were used for each experiment. Wild type and RNAi deficient strains were plated at the same time as the experimental plates to ensure that the transformation and expression of the double stranded DNA was working. The plates were assayed five days after placing the L1/L2 worms. They were scored by counting the number of F1 progeny per adult. Many things can affect the effectiveness of food, including the age of the transformation, the temperature, and the stage at which the worms were placed on the food. As a result only experiments with wild type worms having zero progeny were scored.

II) *Isolating Mutations*

1215(tm329) was suspected of having additional mutations besides the known *rha-1* mutation. Primers 683 GCCATCCTCTCAGCCTAC and 684 CCACCCACGCTCTTACATG were used to determine that the xx1215 stock had the 35 base pair deletion called *haf-6* (ne335). To determine whether the xx1215 stock had the *yy6* mutation crosses were preformed against wild-type worms (xx538) to isolate each known mutation individually. xx1215 was crossed to wild-type; if the cross was successful ,indicated by the presence of males in the F1 progeny, the progeny were moved to a new plate. Then the F2 generation was cloned out by moving one F2 worm to an individual plate. Once the F2 generation gave rise to F3's the F2's were used for PCR analysis of the *haf-6* gene. Next the F3 generation was tested for the *rha-1* mutation (tm329) using the primers 942 AAAACGGCGCCATCCACTCC and 943 CCATCAAAAAGAGTTACGCG. This led to the production of strains with *rha-1* mutations with no *haf-6* deletion and *haf-6* mutants with no *rha-1* mutation. These new strains, along with controls, were placed on *pop-1* feeding plates as described in the methods section I.

III) *Complementation Tests*

To determine if *rha-1 haf-6 yy6 mut-7* and *rde-2* are in the same pathway complementation tests were performed. Male stocks were made by using a feeding assay where bacteria express dsRNA that corresponds to a kinesin gene, when disrupted this leads to increased incidences of nondisjunction that leads to the production of males. *rde-2* and *mut-7* stocks did not give rise to a sustainable male stock so few tests were done with these strains. The males were then crossed to homozygous mutation strains. From these crosses all of the F1 progeny were moved to *pop-1* food. If the cross was successful the heterozygous F1's were then

moved, in groups of four, to *pop-1* plates. The plates were scored five days from the time they were originally placed on *pop-1* food. The mutations being tested were considered complementary if there was 0 to 2.5 F1's per adult; they were considered noncomplementary mutations if they had 2.5 or more F1's per adult. Controls were set up by crossing mutations to wild-type strains and assaying in the same manner. All controls led to complementation.

IV) Transposon Mobilization Rates

Transposon mobilization is a measure of chromosome maintenance. Mobilization means that the transposon has been removed from where it was and has moved into another region of that chromosome. To measure transposon mobilization, mutants were crossed into an *unc-22* strain that harbors a transposon inserted into this gene. This transposon insertion leads to the disruption of the *unc-22* muscle gene. This gene is needed for regular sigmoid movement, when the gene is disrupted the worm twitches as it moves. *rha-1* males were crossed to *unc-22* hermaphrodites and the resulting progeny were screened using PCR to check for the *haf-6* deletion and the *rha-1* mutation. These new strains were then sent to Dr. Katharine Wolstrom's lab for analysis. They analyzed the strains by counting the number of total worms and the number of non-twitching worms. If you divide the non-twitching worms by the total number of worms counted and multiply by 100% this number is called percent revertance. Percent revertance is the percentage of worms that have mobilized the transposon out of the *unc-22* gene.

V) *Preparing C. elegans genomic DNA*

Large-scale preps were used to make genomic DNA. Plates were made using the standard Nematode Growth Medium (NGM) protocol but substituting agarose for the agar [46]. The

plates were then seeded with a sequenced *E. coli* strain, w3110. An equal amount of worms were placed on each plate. The worms were then grown at 22°C until the *E. coli* was almost gone and a sufficient number of adult-stage worms were present. M9 Minimal Media (42mM Na2HPO4, 24mM KH2PO4, 9mM NaCl, 19mM NH4Cl, 1mM MgSO4 , 0.1mM CaCl2 , 2.0% glucose) was then used to rinse the worms into a vial where they were allowed to settle. The M9 was then removed and replaced with fresh M9. This was done two times to thoroughly wash the worms. After the last wash, lysozyme was added at a concentration of 10 mg per milliliter of settled worms. The vial was then incubated at room temperature for two hours with shaking. Once the incubation cycle was completed, the worms were washed three times with M9 to remove any lysed bacteria. Two milliliters of NTE (100 mM NaCl 50 mM Tris-HCl 20 mM EDTA pH7.5), 100 ul of 10% SDS and 1µl (10mg/ml) proteinase K was added to the settled worm pellet and this was incubated for two additional hours at 65°C. 600 ul of phenol: chloroform (1:1) was added per milliliter of worm pellet. This solution was then vortexed and centrifuged in a Fisher Scientific Marathon 16 KM centrifuge at 15000xG for five minutes. The aqueous phase was then moved to a new tube and ethanol extracted. The DNA/RNA pellet was re-suspended in TE and RNase A was added to each tube and incubated at 37°C for two hours. The DNA was then added to the DNeasy Blood and Tissue Kit (Qiagen). The kit protocol was followed for cleaning the DNA and then eluted with water that was buffered with salts to pH 8.0. Concentrations were estimated by electrophoresis on a 0.7% agarose gel. The samples were then dried and sent for sequencing.

VI) qRT-PCR

To isolate mRNA from *C. elegans,* trizol extraction and a Pure Link RNA Mini Kit (Ambion) were used. Sixty worms were placed in trizol and vortexed at 4°C for 20 minutes. Chloroform was then added and the sample was vortexed again. After sitting for 3 minutes at room temperature the sample was centrifuged in a Fisher Scientific Marathon 16 KM centrifuge at 15000xG for 15 minutes at 4°C. The aqueous layer was removed and chloroform was again added, vortexed, and centrifuged. The aqueous layer was removed and 70% ethanol was added to it. This solution was then placed onto the Pure Link column and the manufacture's procedure for RNA purification was followed. The RNA/DNA was eluted from the column with water buffered with salts to pH8.0. To remove the DNA from the sample, the DNA-free Kit (Applied Biosystems) was used. A nanodrop spectrophotometer was used to estimate the concentration of RNA.

The same amounts of wild-type DNA and mutant DNA were converted into CDNA using SuperScript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen). The purified cDNA was then used for qRT-PCR using Absolute QPCR SYBR Green Mix (Thermo Scientific). The qRT-PCR was set up using approximately 2.1 ng of DNA and 5mmol each of forward and reverse primer. The samples were then placed in the MJ Research Bio-Rad Real Time PCR Opticon Engine for analysis. The PCR procedure had an annealing temperature of 54°C and an extension at 72°C. The fluorescence was measured after each of the 39 extensions. At the end of all cycles a melting curve from 40-95°C was prepared for each well to ensure specific product formation.

qRT-PCR primers were designed to flank introns to ensure only mRNA was being amplified. They were designed to produce short 85-208 base pair products. Two genes were

chosen to act as controls: *cdc-42* and *tba-1*. These genes are known to be evenly expressed throughout the lifetime of the worm. Primers were designed to walk along the transcript of the *haf-6* and *smg-2* genes while *mrp-1* primers were designed to check for several different isoforms of Mrp-1. The primers were as follows:

Primer Number	Sequence	Region
1088	AAAGCGCCGTGCAAAGCAGCAAA	haf-6 (exon 1)
1089	GCTCCAAACCCACAAATTCGCCAT	haf-6 (exon 2)
1090	ATGCGGTCGGACTTGTTTCAGAAG	haf-6 (exon 3)
1091	CTGGCTAACGCAGAGCTTGAATGA	haf-6 (exon 4)
1092	TGCAACGTCAGTCGAGGAGAACAT	haf-6 (exon 7)
1093	ATCCACTCGGAAACCTGGACACAA	haf-6 (exon 8)
1094	TATTGGGAACACGCAGGACTCTCA	smg-2 (exon 1)
1095	CCACGTCGTGAAATGGCAAATCGT	smg-2 (exon 2)
1096	AAACACGTTGCTCGGGAACTCTGA	smg-2 (exon 5)
1097	TGTGCTTCTCGGTATTCGACCGTT	smg-2 (exon 4)
1098	AGAATGTGCCCATCCCAGCACATA	smg-2 (exon 8)
1099	ATCCATTGCTGCTTCAGCTTGTGG	smg-2 (exon 9)
1100	TCCCGTTAGTTCGTGAACTGGTGT	mrp-1 (exon 1) mrp-1 a,b,c,d3,e
1101	GCTGGCCACAATTTGTTACTTGCG	mrp-1 (exon 2)
1102	AGATGGTCAGACGATGCCAAGGAA	mrp-1 (exon 11) mrp-1 a,b,c,d3,e
1103	TGATGATTGACGCAGCACAAACGG	mrp-1 (exon 12)
1104	ATATGGACGTGGTGGATGAGCGTT	mrp-1 (exon 13) mrp-1 e
1105	AACGCTTCAATTGCCGAGAAGTGC	mrp-1 (exon 14)
1106	TGTTTATCCGGACTGCCGTATCGT	mrp-1 (exon 13) mrp-1a,d3
1107	TTGTTGGAGCCATCGTTCAAGCTG	mrp-1 (exon 13) mrp-1b,d2
1108	TGATTATTTGGGCAACTCCGTGGG	mrp-1 (exon 13) mrp-1c
1109	TGCATGCCATGGGTGCTTACAA	mrp-1 (exon 13) mrp-1d.1
1110	CGATGCTGAGACTCTCTGATGAA	mrp-1 (exon 13) mrp-1d.1
1111	CTTGTTTGCTTCTCCGTGGTTGCT	cdc-42
1112	TCTCCAACATCCGTTGACACTGGT	cdc-42
1113	AGCTTGCTCGATCTTCTCATCGGA	eIF-3.c
1114	AGCCAACTCTCCGTCCAATCTTTG	eIF-3.c
1115	ATTGGCTGCGTACACTCCACTGAT	tba-1
1116	ATCGCACTTCACCATTTGGTTGGC	tba-1

Chapter V Results and Discussion

haf-6 deletion as a background mutation

Studies done by the Timmons lab have shown that Haf-6 is an ABC_{RNAi} transporter and plays an important role in the RNAi pathway. During regular maintenance of stocks, a strain that had a mutation other than *haf-6*(ne335) was used as a control for a *haf-6* PCR reaction. The results of this PCR showed that the strain had the *haf-6* 35bp deletion. Further studies of different mutation strains from different labs showed that the *haf-6*(ne335) deletion could be found in the background of many previously unidentified strains. Table 1 shows some of the strains that were tested and contained the *haf-6*(ne335) deletion. Some of the strains were then crossed to wild-type worms so that they contained only single mutations. Interestingly, several of these mutant strains were shown in the literature to have no defects in the RNAi pathway. This is contradictory to the hypothesis that *haf-6* is vital for a functional RNAi pathway.

To further investigate the role of *haf-6, pop-1* feeding assays were used to identify defects in the RNAi pathway. *pop-1* feeding assays are based on plasmid-expressing bacteria that are used as a food source for *C. elegans*. This plasmid expresses a dsRNA of the *C. elegans pop-1* gene which is needed for embryonic development. If the RNAi pathway is intact then the dsRNA is processed and down regulates *pop*-1. If the worms have an intact RNAi pathway they will have no viable progeny, if they are defective they will have progeny. The results showed that many of the mutant strains unsuspectingly carrying the *haf-6* deletion had a functional RNAi pathway (Table 1). Contradictory to this, the *haf-6*(ne335) mutation has been thoroughly studied and it has been shown that it is defective in RNAi. These results lead us to the conclusion that some strains contain a third mutation that is suppressing the *haf-6* phenotype. This unknown mutation has been labeled *yy6*. The only way to discern if *yy6* is present in a strain is to check for the *haf-6* deletion and to test for RNAi activity. If the strain has both we assume that the *yy6* mutation is present. Studies have shown that this new mutation, *yy6*, is an intergenic mutation.

Table 1. *haf-6* deletion is found in several stocks

STRAIN	KNOWN MUTATION	*haf-6* LOCUS	OTHER MUTATION	RNAi ACTIVITY
XX937	wild type	+	--	+
XX195		*haf-6(ne335)*	--	--
RB886	*adr-2(ok735)*	*haf-6(ne335)*	*yy6?*	+/--
RB1641	*dcap-2(ok2023)*	*haf-6(ne335)*	*yy6*	+
KS99	*egl-27(ok11)*	*haf-6(ne335)*	*yy6*	+
VC883	*had-2(ok1479*	*haf-6(ne335)*	*yy6*	+
RB1067	*his-24(ok1024)*	*haf-6(ne335)*	*yy6*	+/--
XX951	*his-24(ok1024)*	+	--	+
RB1089	*hpl-1(ok1060)*	*haf-6(ne335)*	*yy6?*	+/--
RB995	*hpl-2(ok916)*	*haf-6(ne335)*	*yy6*	+
RB996	*hpl-2(ok917)*	*haf-6(ne335)*	*yy6*	+
RB1090	*hpl-2(ok1061)*	*haf-6(ne335)*	*yy6*	+
VC201	*itsn-1(ok268)*	*haf-6(ne335)*	*yy6*	+/--
XX952	*itsn-1(ok268)*	+	--	+
SS222	*mes-3(bn21)*	*haf-6(ne335)*	*yy6*	+
SS580	*pgl-1(bn102)*	*haf-6(ne335)*	*yy6?*	+/--
KMW1	*rha-1(tm329)*	*haf-6(ne335)*	*yy6*	--
XX953	*rha-1(tm329)*	+	--	+/--
RB1025	*set-2(ok952)*	*haf-6(ne335)*	*yy6*	+
HC75	*sid-1(qt2); ccls4251*	*haf-6(ne335)*	*yy6*	--
VC199	*sir-2.1(ok434)*	*haf-6(ne335)*	*yy6*	+
RB774	*zfp-1(ok554)*	*haf-6(ne335)*	*yy6*	+
CB1893	*unc-17(e113) dpy(e184)*	*haf-6(ne335)*	*yy6?*	+/--
XX1332	*unc-17(e113) dpy(e184)*	+	+	+/--
DR103	*dpy-10(e128 unc-4(e120*	*haf-6(ne335)*	*yy6*	+
XX1012	*dpy-10(e128 unc-4(e120*	+	--	+
CB2065	*dpy-11(e224) unc-76(e911)*	*haf-6(ne335)*	*yy6*	+
XX1330	*dpy-11(e224) unc-76(e911)*	+	--	+
PD8160	*dpy-20 ; ccIn8160[rpl28::gfp]*	*haf-6(ne335)*	*yy6*	+
XX101	*dpy-20 ; ccIn8160[rpl28::gfp] ; him-5*	*haf-6(ne335)*	*yy6*	+
PD4251	*dpy-20(e1282 : ccIs4251[myo-3::gfp]*	*haf-6(ne335)*	*yy6*	+

Table 1: The *haf-6* deletion can be found in the background of many different mutation strains from many different labs. *yy6* is also found in most instances that the *haf-6* deletion is seen. (+) stands for wild type, (--) means defective and (+/--) means slightly defective with respect to RNAi activity.

Mapping *yy6*

Mapping of *yy6* showed that it was on chromosome 1 along with the *haf-6* gene. This is consistent with the observation that the *yy6* mutation is always inherited with the *haf-6* mutation. Mapping *yy6* to a smaller region proved to be unsuccessful, so it was decided to send this newly discovered triple mutant strain, 1215(tm329), for sequencing. Along with this strain several others were sent for sequencing, a xx538 wild-type, xx1683*haf-6 yy6 him-5* and xx195 *haf-6*. The libraries have been made and data has been obtained but it is still undergoing analysis. Knowing the nature of the yy6 mutation could greatly help in discovering how it is interacting with an ABC_{RNAi} transporter and an RNA helicase in the RNAi pathway.

1215(tm329) is a triple mutant

Mutation strain KMW1 (1215(tm329)) was thoroughly studied by the Walstrom lab. This strain was screened for the *haf-6* (ne335) deletion by the Timmons lab and it harbored the deletion. As well as being RNAi defective, *rha-1* mutations have defects in gamete production and chromosomal regulation. To see if this strain also contained the third mutation (*yy6*), the strain was crossed with a wild-type worm and the mutants were isolated. Previous studies have shown that *yy6* is close to *haf-6* so they are inherited together. Once the mutants were isolated and confirmed by PCR, *pop-1* feeding assays were used to identify RNAi defects (figure 3). These tests showed that the *haf-6* strain isolated from 1215(tm329) was not RNAi defective. This leads us to conclude that this strain must contain the mutation *yy6*. The *rha-1* mutation that was isolated from 1215(tm329) was found to be only slightly RNAi defective. This result can be compared to the xx953 (tm329) strain that was made by out-crossing 1215(tm329) to wild-type worms. Interestingly the triple mutant was strongly defective in RNAi processing but when the

rha-1 mutation is isolated it is only weakly defective and the *haf-6, yy6* strain is not defective at all. Further studies need to be done to see if all of the phenotypes previously seen in 1215(tm329) are still present in the absence of the *haf-6* and *yy6* mutations and to find out how this third *rha-1* mutation is interacting with the *haf-6 yy6* mutations and suppressing their phenotype.

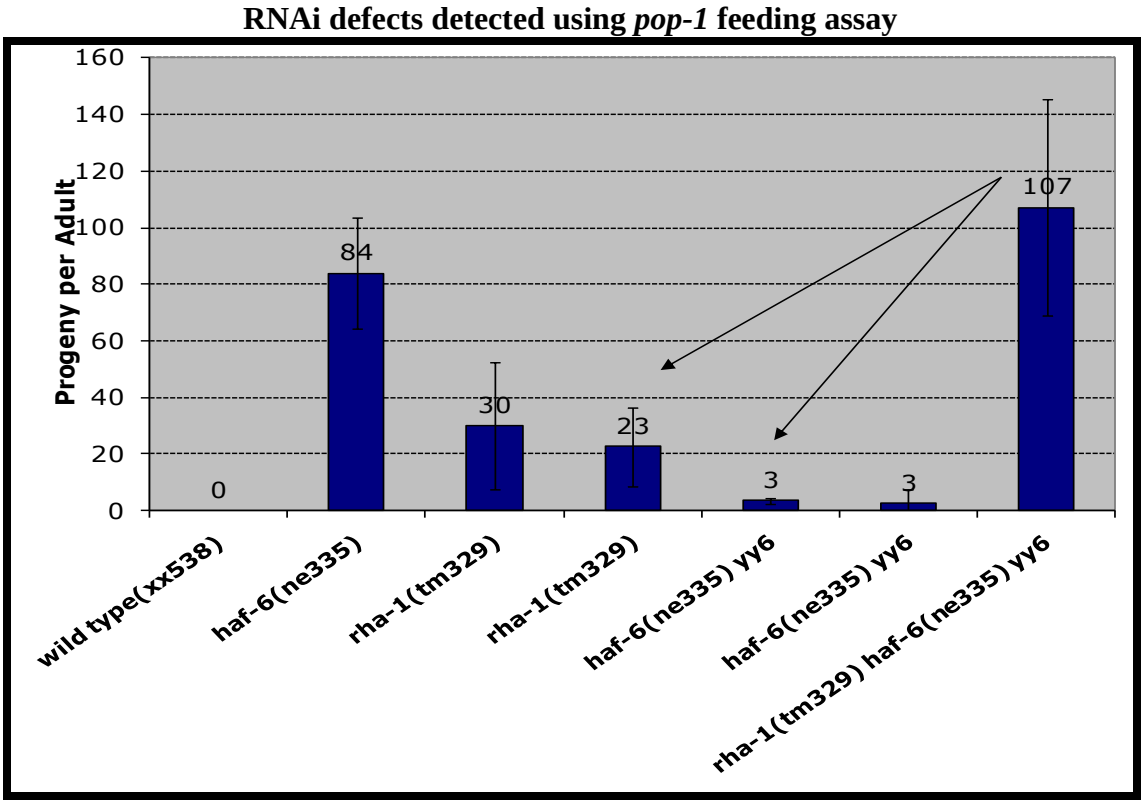

Figure 3. *pop-1* feeding assay to look for RNAi defects in different mutant backgrounds. Wild-type worms show no RNAi defects so they produce no progeny. *haf-6* mutants show strong defects in RNAi. *rha-1* (tm329) is a strain that has been out-crossed and contains only the *rha-1* mutation. The next two strains were isolated from 1215(tm329). The *rha-1* mutation that was isolated from the triple mutant strain shows the same deficiencies in RNAi. The *haf-6* mutation that was isolated from the triple mutants shows almost no defects in the RNAi pathway, which leads us to believe that it contains the yy6 mutation. Next is a *haf-6* strain that has the yy6 mutation and serves as a control. The triple mutant shows the strongest RNAi defects.

Genetic interactions

The next experiment was designed to see genetic interactions between *rha-1 haf-6* and *yy6*. ABC_{RNAi} transporters are known to genetically interact in the Mut-7 / Rde-2 pathway. *rha-1* is interacting with the ABC_{RNAi} transporter *haf-6* and has been shown to interact with *mut-7* and *rde-2* but that was before the *haf-6* was discovered to be in the background of this strain. To ensure that this interaction was due to the *rha*-1 mutation and not because of the *haf-6* mutation we set up complementation tests to look for genetic interactions. The resulting F1 progeny were then analyzed for RNAi defects. The results of these experiments can be seen in Table 2. The data reveal a genetic interaction between all of the mutants, because each mutant shows non-complementation with the other mutants. This table represents cross progeny, which means each box represents an F1 progeny that has inherited half of its genes from each parent. Table 2 shows that all of the mutants are non-complementary to *mut-7* and *rde-2* meaning that they genetically interact in this pathway. The data also show that; 1) *haf-6* genetically interacts with *rha-1,* 2) *yy6* changes the interaction with *haf-6* and *rha-1* so that it is now complementary and the RNAi pathway is working correctly, 3) there is a paternal effect seen between *haf-6* and *rha-1* (when the *rha-1* mutation comes from the hermaphrodite there is a stronger deficiency in the RNAi process), and 4) *yy6* acts recessively, but *rha-1* must be homozygous to change the interaction between *haf-6* and *yy6*. The data suggest that *haf-6 rha-1* and *yy6* interact with *mut-7/rde-2* and all are genetically interacting with each other.

Table 2. RNAi defects seen in complementation progeny

♂ \ ♀	*mut-7*	*rde-2*	*haf-6*	*rha-1*	*haf-6 yy6*	*haf-6 yy6 rha-1*	*haf-6 rha-1*
mut-7		+					
rde-2	+						
haf-6	+	+		++	--		
rha-1	+	+	+		--	++	+
haf-6 yy6	+	+	--	--		--	--
haf-6 yy6 rha-1	++	++		++	--		
haf-6 rha-1	++	+		++			

-- = 0 - 2.5 F1:Po (complementary mutations)
+ =2.5 - 18 F1:Po
++ =18 - >50 F1:Po (non-complementary mutations)

Complementation tests were set up using different combinations of mutations and then the heterozygous F1 progeny were moved to *pop-1* food to look for RNAi defects. All mutations are non-complementary with *mut-7* and *rde-2* suggesting that all of these mutations are in the same pathway. This chart shows that rha-1 may have stronger maternal effects than *haf-6*. When *rha-1* is homozygous you can see its interaction on the RNAi pathway but when heterozygous RNAi activity is not affected by *rha-1*. Crosses also reveal that even when *yy6* is heterozygous it still is affecting the RNAi pathway.

Transposon mobilization

haf-6 has been shown to be a mutator gene which can lead to an increased incidence of transposon mobilization, as well as having a role in chromosome regulation. To examine transposon mobilization rates, mutant strains were crossed with *unc-22* twitchers. *unc-22* worms twitch due to a transposon inserted into the *unc-22* muscle gene. Revertance can be measured by counting the number of non-twitching worms, where the transposon has hopped out of the muscle gene. This gives a measure of chromatin regulation. The results shown in Table 3 indicate that there is an increased amount of transposon mobilization in *haf-6* mutants at restrictive temperatures. The *pop-1* feeding assay showed that *yy6* and *rha-1* rescue the defects caused by the *haf-6* deletion. This rescue can also be seen in the revertant percentages, which are back to 0% in the *unc-22 haf-6 yy6* mutants meaning that transposon mobilization is not occurring. When the *rha-1* mutation is added to this background we do not see a change in phenotype. In the *pop-1* feeding assays we saw that *rha-1* was effecting the *yy6 haf-6* interaction. In this mutator assay we see that the *rha-1* mutation is having no effect on transposon mobilization phenotypes of *haf-6 yy6* genes. This different outcome leads us to believe that this could be a very germline specific response. *pop-1* feeding assays are based on a continuous feeding of dsRNA against a germline protein that is required for proper development of gametes. Both Haf-6 and Rha-1 have been shown to be expressed in the germline and help with regulation of germline proteins. *Unc-22* is a muscle protein and might be under different chromosomal regulation than germline genes.

Table 3. Transposon Mobilization

		% revertants	
Strain Genotype	**16 °C**	**20 °C**	**25 °C**
unc-22 (st136)	**0 N=15,416**	**0 N=43,076**	**0.015 N=20,128)**
unc-22(st136); haf-6(ne335)	**0 N=18,864**	**0 N=21,548**	**0.085 N=5,877**
unc-22(st136); haf-6(ne335) yy6	**0 N=22,592**	**0 N=29,721**	**0 N=20,050**
unc-22(st136); haf-6(ne335) yy6; rha-1(tm329);	**0 N=23,438**	**0 N=25,004**	**0 N=12,494**
unc-22(st136); haf-6(ne335) + ; rha-1(tm329);	**0 N=18,744**	**0 N=42,974**	**0 N=14,008**

***unc-22* was crossed into *haf-6 rha-1* and *yy6* mutants. Revertants was then measured by counting non-twitching worms out of total worms. The *yy6* mutation rescues the transposon mobilization phenotype seen in *haf-6* mutants but *rha-1* has no effect on the *yy6 haf-6* interactions in this assay.**

Protein expression levels

haf-6 and *smg-2* are neighboring genes on chromosome one. These two genes are transcribed in opposite directions from a shared promoter region (Figure 4). *smg-2* phenotypes are sometimes seen in *haf-6* mutants; these include phenotypes such as the males going sterile and hermaphrodites having protruding vulvas. Previous work has been done that showed *haf-6* mutants are rescued by *haf-6* cDNA but not by *smg-2* cDNA. The reverse has shown that *smg-2* mutants are only rescued by *smg-2* cDNA. It is known that some transcription elements for *haf-6* are in the *smg-2* gene. This lead us to the question that if *smg-2* contains *haf-6* regulatory elements does *haf-6* contain *smg-2* regulatory elements that could be affected by the *haf-6* 35bp deletion.

To test this we used quantitative reverse transcriptase PCR (qRT-PCR) to look at expression levels of *smg-2* mRNA in wild-type worms and in *haf-6* mutant worms. Figure 5

shows the results of two independent experiments using two different sets of *haf-6* and wild-type cDNA. Two genes, *cdc-42* and *tba-1*, were used as loading controls to ensure equal amounts of DNA were loaded into each reaction. Both experiments show that when you compare *haf-6* mutant cDNA to wild-type CDNA and adjust for concentration by *cdc-42* or *tba-1* that the expression levels of *smg-2* does not significantly differ between the two strains. *haf-6* primers were used to show that *haf-6* mRNA expression levels are significantly decreased when *haf-6* mutant cDNA is compared to wild-type cDNA. This is as expected because the 35bp deletion shifts transcription out of frame and leads to a nonfunctional protein.

Gene map of *smg-2* and *haf-6*

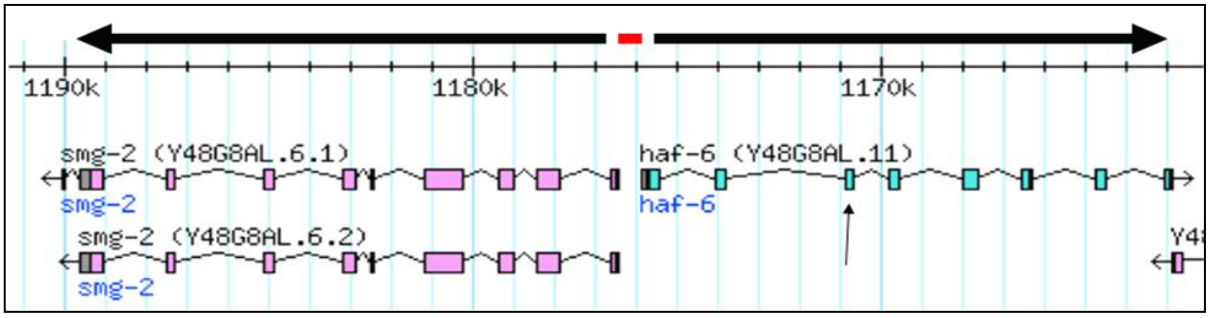

Figure 4. Gene map of *smg-2* and *haf-6*. The red square represents the shared 800bp promoter region. The dark arrows indicate the direction of transcription. The small arrow is pointing to exon three where there is a 35bp deletion in *haf-6* mutants.

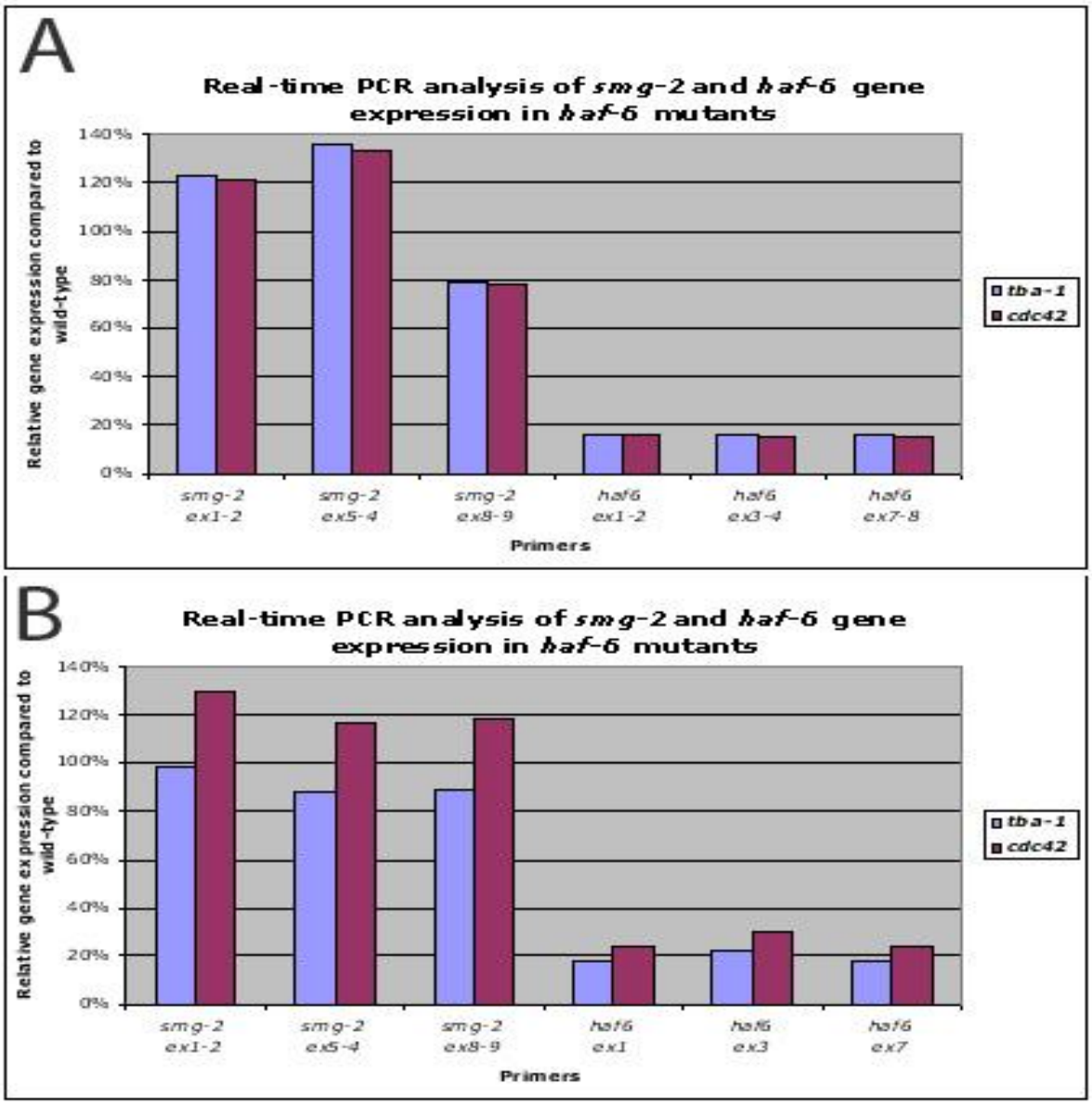

Figure 5. Graph showing that *smg-2* expression levels are not affected in a *haf-6* mutant background. A and B are duplicate experiments using different isolated sets of *haf*-6 and *wild-type* cDNA.

Chapter VI Summary of Significance

The work in the Timmons lab on ABC transporters and their function in the RNAi pathway was the starting point for these projects. The discovery that the *haf-6(ne335)* mutation could be unsuspectingly found in many strains that have properly functioning RNAi led us to discover these interacting genes. Screens for strains carrying the *haf-6(ne335)* directed us to the very interesting mutant *rha-1* strain. Dr, Katherine Wolstrom had characterized this mutant in detail and found that these mutants have reduced germ cell meiosis, defective mitosis, temperature-sensitive sterility, and that it genetically interacts in the *mut-7/rde-2* pathway. When these studies were done it was not know that *haf-6(ne335)* was in the background. This list of phenotypes can also describe *haf-6* mutants. Our studies were focused on these mutations and how they are interacting in the RNAi pathway.

pop-1 feeding assays are an effective and reliable way to see if RNAi processing is occurring properly. These assays revealed that a second mutation must be accompanying the (*haf-6 ne335*) deletion when found in strains that have a functioning RNAi pathway. The discovery of *yy6* and its ability to rescue the defects in RNAi and transposon mobilization in *haf-6* strains leaves many questions. The identification of another gene that can directly interact with these two mutations in an RNAi dependent maner but not in transposnon mobilization shows how many different and parallel pathways need the same proteins. Mutant *rha-1* strains are weakly RNAi defective but with the *haf-6* and *yy6* mutations added the defects in RNAi are significantly stronger. Many studies need to be done to see how an RNA helicase protein, an ABC transporter and this newly discovered mutation are acting in conjunction in the RNA pathway. Identification of the substrate that haf-6 is transporting in the ER could lead to a greater understanding of its role in RNAi. In humans RhaA has been to associate in the cytoplasm in the RISC complex, but this has not been discovered yet in *C. elegans*. RhaA has many functions

within the cell more studies need to be done to see how *rha-1* shares these very diverse functions.

Transposons are mobile genetic elements that play a major but silent role in most eukaryotic organisms. These elements make up a significant fraction of the genome. A piece of DNA that has the ability to move from position to position along a chromosome can have devastating effects on its host cell. Transposons provide a way for genetic diversity but insertion of a transposable element into a gene can also cause mutations. This mutagenic activity can lead to disease. ABC transporters and RNA helicases in cancer show an increased rate of transposon mobilization. RNAi is one mechanism that is believed to regulate these transposable elements. Transposon mobilization assays allow us to see if these RNAi deficient mutants are also involved in this chromosomal specific pathway. *haf-6* and *yy6* seem to function in the regulation of chromosomes as seen by the *haf-6* mutants inability to keep its transposon silenced and *yy6*s ability to rescue this defect. The fact that *rha-1* does not show a defect in properly maintaining its transposable elements leads us to believe that its function in the RNAi pathway might reside outside of the nucleuses.

The appearance of *smg-2* phenotypes in *haf-6* strains led us to question whether the *smg-2* genes were being affected by the deletion in *haf-6* mutants. *smg-2* phenotypes include protruding vulvas and male sterility, both which are seen in *haf-6* strains under working lab condition. These two genes share a common 800 bp promoter region and it is known that the *smg-2* gene contains regulatory elements that effect *haf-6* transcription. The appearance of these phenotypes and the fact that they share a promoter region lead us to see if expression levels of *smg-2* were affected in a *haf-6* background. qRT-PCR studies showed that *smg-2* expression

levels are not changed in *haf-6* mutants. These experiments were done under normal working conditions. Further studies need to be completed to ensure that environmental conditions will not change these expression rates.

Human RhaA (ortholog of *C. elegans rha-1*) is present in the MEF transcription factor complex. In cancer cells RhaA up-regulates the expression of MDR1 (multidrug resistannce gene 1) which is an ABC transporter. MDR1s function in the cell is to remove a wide variety of drugs and xenobiotics. The need for this natural defence againt possibly toxic agents is important but in cancer cells the up-regulation of this gene leads to efflux of drugs that are required to treat cancer cells. *C. elegans* ortholog to MDR1 is *mrp-1-mrp-4* (multidrug resistance protein 1-4). In *C. elegans mrp-1* is needed for proper RNAi and functions to help remove heavy metals from cells. Our preliminary results (data not shown) indicate that *mrp-1* mRNA levels are not affected in a *rha-1* mutant background. This data does not prove that *rha-1* is not affecting *mrp-1* levels, but leads us to believe that other mechanisms might help in the regulation of *mrp-1*. More studies need to be done to see if by stressing the cells in such a way to mimic cancer in *C. elegans* might alter the expression levels of not only *mrp-1* but the other three *mrp* genes.

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