TRANSPORT ATPASES: STRUCTURE, MECHANISM AND RELEVANCE TO MULTIPLE DISEASES

ABC transporters and RNAi in *Caenorhabditis elegans*

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Abstract RNAi is an evolutionarily conserved gene-silencing phenomenon that can be triggered by exogenous delivery of double stranded RNA to organisms. In *Caenorhabditis elegans*, the response to dsRNA is remarkably robust, and systemic RNAi responses are often observed. We have taken a genetic approach using this organism to better understand the mechanisms that facilitate RNAi. By analyzing strains of RNAi-defective mutants, we have uncovered an unexpected role for ABC transporters in RNAi and related silencing mechanisms. Ten of the sixty ABC transporter genes encoded in the *C. elegans* genome are required for robust RNAi. We will present data that highlights common features of these genes relative to their roles in RNAi, including genetic interactions with other components of the RNAi machinery. We will also describe unique roles for some transporter genes in endogenous RNAi-related processes.

Keywords RNAi · ABC transporter · *Caenorhabditis elegans* · dsRNA · Mutator

Multiple cellular mechanisms are utilized in RNAi responses to dsRNA

RNA interference (RNAi) is a term coined by Craig Mello to describe a new phenomenon—a potent, sequence-specific gene silencing response that is triggered by extracellular delivery of dsRNA (Fire et al. 1998). The

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1997 discovery of RNAi by Andrew Fire and Craig Mello, and related experiments in the early days of the RNAi field, have been described in many publications (Sen and Blau 2006). The original dsRNA injection experiments were performed in *Caenorhabditis elegans* (Fire et al. 1998), and it soon became clear that RNAi responses are conserved in most eukaryotes (Hannon 2002). Virtually every cell has the capacity to immediately mount an RNAi response against dsRNAs that gain entry into cells. This cellular response has been exploited in the analysis of gene function, allowing for loss-of-function phenotypes to be produced in a variety of different cell types and organisms. RNAi does not result in sequence changes to DNA and the effects are reversible as the pool of dsRNAs becomes depleted. Indeed, the *C. elegans* genome encodes endogenous inhibitors of RNAi (Timmons 2004).

"RNAi" refers to the technique that allows for dsRNA-induced gene silencing and also to the cellular mechanisms that implement gene silencing. The mechanisms are numerous and inter-related and affect gene expression at both transcriptional and post-transcriptional levels. In post-transcriptional RNAi mechanisms, the effector molecule is a small interfering RNA, or siRNA, a double-stranded molecule roughly 22 base pairs in length. siRNAs are cleavage products of the Dicer enzyme, a dsRNA-specific endonuclease found in all eukaryotes. When longer dsRNA molecules are delivered to *C. elegans*, they are cleaved immediately by Dicer into siRNAs. siRNAs can also be chemically synthesized and used in RNAi experiments in *C. elegans*; however, siRNAs do not generally produce as potent a response in comparison to longer dsRNAs due to the increased complexity of siRNA sequences that can be generated from longer dsRNAs. For example, a 1000 bp dsRNA can be "Diced" into at least 40 distinct siRNA sequences, each targeting the same mRNA. siRNAs are

utilized in post-transcriptional silencing mechanisms that involve the activity of RISC (RNAi-induced Silencing Complex), a multiprotein complex in the cytoplasm that facilitates the degradation of mRNAs (Fig. 1a) (Sontheimer 2005). RISC mechanisms are also utilized by cells to attack viral RNAs, perhaps because viral RNAs display dsRNA character or are otherwise recognized as aberrant to the cell. Thus, cytoplasmic RNAi mechanisms have endogenous roles in protecting cells and organisms against viral infections.

In some organisms, transcriptional-level silencing responses are observed upon dsRNA delivery. These responses can lead to modifications in chromatin that include alterations in histone methylation or acetylation patterns as well as methylation of specific DNA sequences (Fig. 1b) (Hannon 2002; Filipowicz 2005; Grewal and Elgin 2007; Paroo et al. 2007). These chromatin-related RNAi activities are utilized by endogenous processes that maintain heterochromatin; cells lacking these RNAi-related functions display defects in centromere function and increased rates of transposon mobilization. These endogenous RNAi functions are essential for normal cell function and development of organisms. Recent studies have greatly increased our knowledge of endogenous RNA molecules and their associated functions in transposon silencing, maintenance of heterochromatin, and influences on developmental decisions (O'Donnell and Boeke 2007). A clearer picture regarding how the various RNAi mechanisms are integrated will undoubtedly contribute to our knowledge of development and enhance our ability to use RNAi to study gene function or to treat disease.

C. elegans is a particularly attractive system for studying RNAi mechanisms. RNAi-defective mutants are readily isolated, and most of the mutants that have been isolated are viable and fertile. *C. elegans* also lacks an interferon response. Mammalian interferon responses to dsRNA are not sequence-specific in nature and can result in global translational arrest of all mRNAs. Thus, the lack of interferon responses in *C. elegans* allows for cleaner studies of RNAi mechanisms in comparison to studies in mammalian systems. The cells of *C. elegans* are translucent, and GFP resporters work quite well, allowing for observations of RNAi in individual cells. Additionally, a number of methods are available for dsRNA delivery in *C. elegans*. For example, dsRNA can be injected directly into the animals. Alternatively, because bacteria are the laboratory food source for *C. elegans*, animals can be co-cultured with bacteria engineered to express dsRNA. This bacterial "feeding method" was used to screen for mutants that are defective in RNAi (Tabara et al. 1999).

HAF-6 is required for efficient RNAi in *C. elegans*

One of our RNAi-defective mutants harbored a 35 base pair deletion in exon 3 of the *haf-6* gene (Sundaram et al. 2006), one of sixty ABC transporter genes in the *C. elegans* genome. The HAF-6 protein is a half-transporter member of the B subfamily, and harbors extensive regions of identity with human transporters (26–47% identity with ABCB3, B2, B9, B10 and B8). In order to better understand how
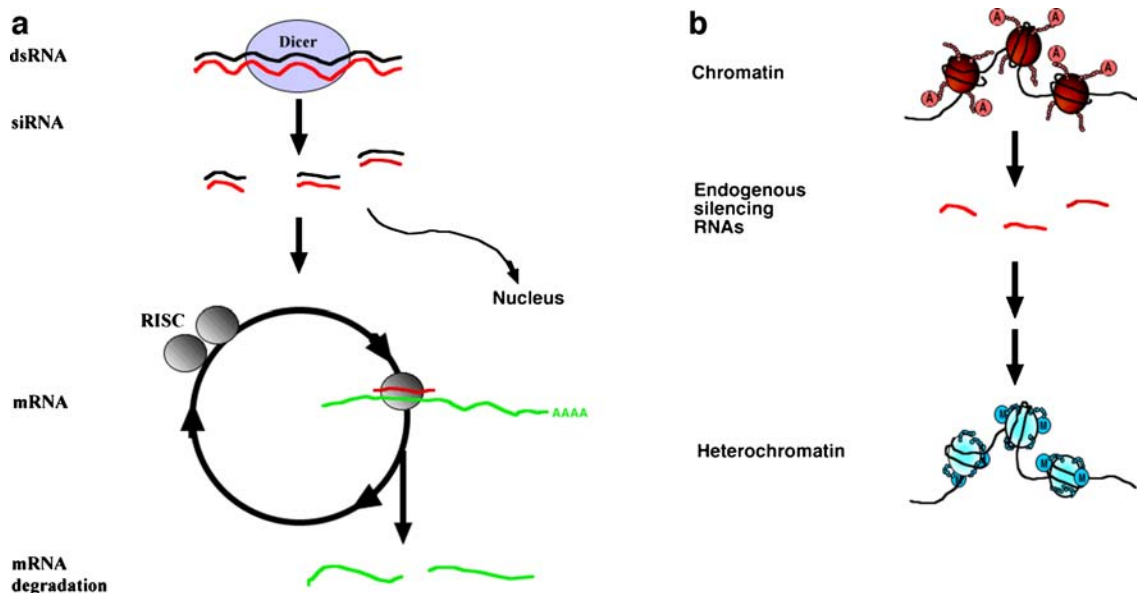

Fig. 1 A broad outline of RISC-based silencing mechanisms that act in the cytoplasm (**a**) and endogenous RNA-directed mechanisms that act in the nucleus (**b**). RISC-based mechanisms lead to mRNA cleavage using siRNA sequences as guide. Transcriptional silencing mechanisms can affect histone configurations in chromatin, leading to alterations in gene expression

haf-6 might influence RNAi mechanisms, we performed experiments designed to uncover the subcellular localization of the HAF-6 protein. We analyzed expression in live animals using GFP-tagged versions of HAF-6, and we also performed immunofluorescence experiments on fixed tissues using anti-HAF-6 antibodies. The GFP reporter was attached to a full-length HAF-6 protein, and because this fusion protein was able to rescue the RNAi defects in mutants, the GFP pattern that we observed likely has physiological relevance. We observed HAF-6 in intracellular membranes that correspond to the endoplasmic reticulum.

We were surprised to learn that ABC transporters have a role in RNAi as over one hundred different proteins are required for RNAi and relatively few are membrane proteins. In *C. elegans*, the SID proteins localize to the plasma membrane and they apparently facilitate cellular uptake of dsRNA molecules (Jose and Hunter 2007). We considered that the HAF-6 transmembrane protein might also have a similar function, however, this possibility is unlikely due to its intracellular location. We hypothesize that HAF-6 might be involved in intracellular trafficking downstream of the SID proteins; alternatively, HAF-6 may function in the trafficking of substrates to particular subcellular regions where they are used for RNAi biochemistry.

Some of our experiments suggest that ATPase activity is required in the RNAi-related functions of HAF-6. For example, a *haf-6* cDNA with a wild-type sequence can fully rescue the RNAi defects in *haf-6* mutants, whereas a cDNA harboring a point mutation that causes a substitution near the conserved ABC transporter signature motif, LSGGQ, in the ATP binding cassette did not provide for complete rescue of the RNAi defects. One interpretation of this result is that efficient transport of substrate by HAF-6 is required for full RNAi activity. Additional biochemical experiments are required in order to validate our assumptions.

Why might a multicellular organism require *haf*-6 in order to perform efficient RNAi? We reasoned that *haf*-6 might be required for RNAi in particular tissues; alternatively, *haf*-6 might be part of a mechanism that facilitates systemic RNAi responses. One important clue regarding requirements for *haf*-6 in RNAi is the observation that *haf*-6 mutants are not completely RNAi defective. The RNAi defects are readily observed when the bacterial feeding protocol is used to deliver dsRNA. Bacterial feeding delivers a low, yet continuous, dosage of dsRNA to the animals. By contrast, when large concentrations of dsRNA are injected into the mutants, robust RNAi activity is observed. Thus *haf*-6 mutants are dosage-sensitive with respect to dsRNA. In experiments that utilize the bacterial feeding protocol, animals first ingest the bacterial cells, and dsRNA is released into the gut by grinding and digestion of the bacteria in the intestine. The dsRNA must then be taken up by intestinal cells and disseminated to other tissues in order for RNAi to be observed in somatic cells other than intestine. Since we observe RNAi activity in the muscle cells of mutant animals when they are reared on dsRNA-expressing bacteria, we presume that systemic trafficking mechanisms for dsRNA are intact in the mutants. Additionally, even though we observe RNAi defects in intestinal cells of *haf-6* mutants, dsRNA is still able to traffick from intestine to muscle in mutant animals. Taken together, these data suggest that, unlike the SID transmembrane proteins, HAF-6 does not participate in cell-to-cell trafficking of dsRNAs. However, we cannot rule out additional mechanisms that might contribute to systemic silencing that have not yet been uncovered.

Endogenous RNAi processes are affected in *haf-6* mutants

haf-6 mutants are RNAi-defective in a dosage-sensitive manner; increasing the dosage of dsRNA improves the RNAi response. *mut-7* and *rde-2* mutants display similar dosage-sensitive RNAi defects, and *haf-6*, *rde-2*, and *mut-7* mutants also display similar defects in endogenous processes that affect normal development. *rde-2* encodes a protein with little similarity outside nematodes (Tops et al. 2005), and *mut-7* encodes a protein with a 5′-3′ exonuclease domain (Ketting et al. 1999). Results from yeast two-hybrid studies demonstrated that RDE-2 and MUT-7 proteins physically associate in the cytoplasm, and MUT-7 protein is also found in the nucleus (Tops et al. 2005).

mut-7 and *rde-2* mutants were previously demonstrated to display an increased rate of transposon mobilization in the germ line (Tabara et al. 1999), a tissue that normally silences the mobilization of these parasitic genomic elements. Because *mut-7*, *rde-2*, and *haf-6* mutants display RNAi defects that are similar in nature, we measured transposon mobilization rates in *haf-6* mutants and found that *haf-6* mutants are also defective in silencing transposons in the germ line. However unlike *mut-7* and *rde-2*, which display transposon mobilization activity at all temperatures, *haf-6* mutants display transposon mobilization activity only when reared at elevated temperatures. Thus, *haf-6* function is required for transposon silencing in unfavorable environments that include elevated temperatures.

haf-6, mut-7 and *rde-2* interact genetically

Second-site noncomplementation interactions (SSNC) have been previously reported for *mut-7* and *rde-2* (Tops et al. 2005) (Fig. 2a). In SSNC tests, doubly heterozygous animals are produced in a genetic cross using parental animals with mutations in two different genes. The doubly heterozygous progeny are then monitored for phenotypes,
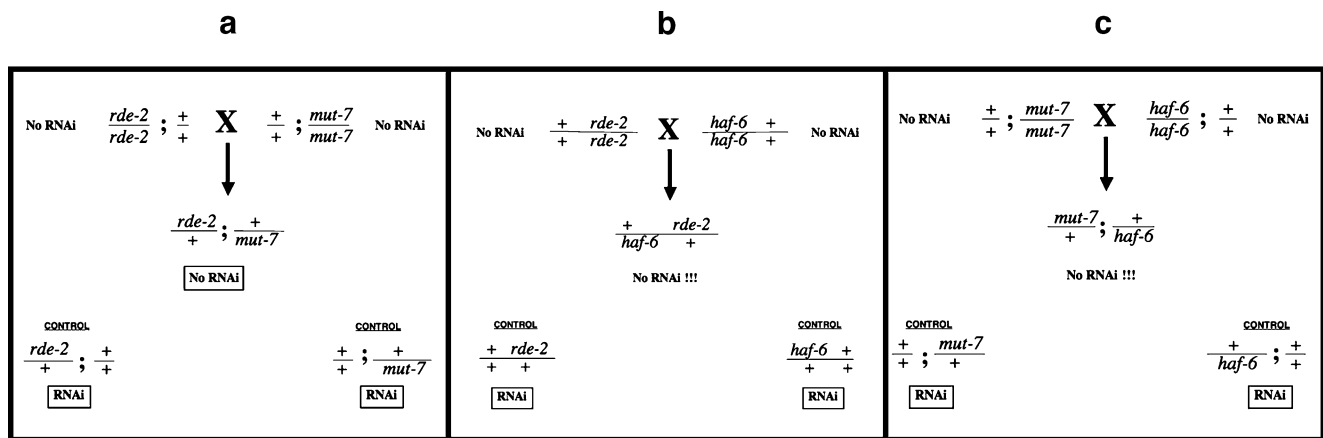
a   **b**   **c**

Fig. 2 Second-site noncomplementation interactions between alleles of *mut-7, rde-2* and *haf-6*. The top line in each of the three diagrams represents a genetic cross between two different strains that are homozygous for different mutations. The resulting doubly heterozygous progeny are assayed for RNAi activity. Results from tests using control, single heterozygotes is listed in the bottom corners of each diagram. **a** *rde-2* and *mut-7* display SSNC interactions (Tops et al. 2005); **b** *rde-2* and *haf-6* display SSNC interactions; and **c** *mut-7* and *haf-6* display SSNC interactions

such as reductions in RNAi activity. In general, most pairs of unrelated mutations do not display SSNC interactions—doubly heterozygous animals display wild-type phenotypes. The rather unusual SSNC interaction can indicate that a physical association of the corresponding proteins is required in order to elicit a common function—in this case, RNAi. Alternatively, SSNC interactions may indicate an involvement of the two gene products in parallel pathways whose combined activities are required for a common function (Hawley and Gilliland 2006). The SSNC interaction between *mut-7* and *rde-2* mutants likely reflects the fact that the corresponding proteins can physically associate, as was previously demonstrated (Tops et al. 2005).

During the course of our analysis of *haf-6*, we noticed SSNC interactions between *haf-6* and *rde-2* (Fig. 2b). We extended these observations to see if *mut-7* and *haf-6* mutants also displayed SSNC interactions, and we observed interactions between these two genes also (Fig. 2c). Thus *mut-7, rde-2,* and *haf-6* constitute a set of interacting genes that are required for RNAi. Taking into consideration these genetic interactions as well as the subcellular localizations of the corresponding proteins, we consider that HAF-6 is required to traffic specific substrates toward an RNAi pathway that includes MUT-7/RDE-2 proteins; although we cannot rule out the possibility that all three proteins physically interact (Fig. 3).

Additional ABC transporters are required for efficient RNAi

The genome of *C. elegans* harbors approximately sixty ABC transporter genes (Sheps et al. 2004), and mutations in 45 different transporter genes have been isolated in various labs. We hypothesized that additional ABC

transporters might also influence RNAi mechanisms, and we therefore obtained all available strains with defects in ABC transporter genes and tested them for RNAi activity. We observed RNAi defects in an additional eight ABC transporter mutants (Sundaram et al. 2006). These mutants are defective in both full transporter genes as well as half transporters, and reside in multiple subfamilies. Thus, an ability to influence RNAi function is observed in only 30%
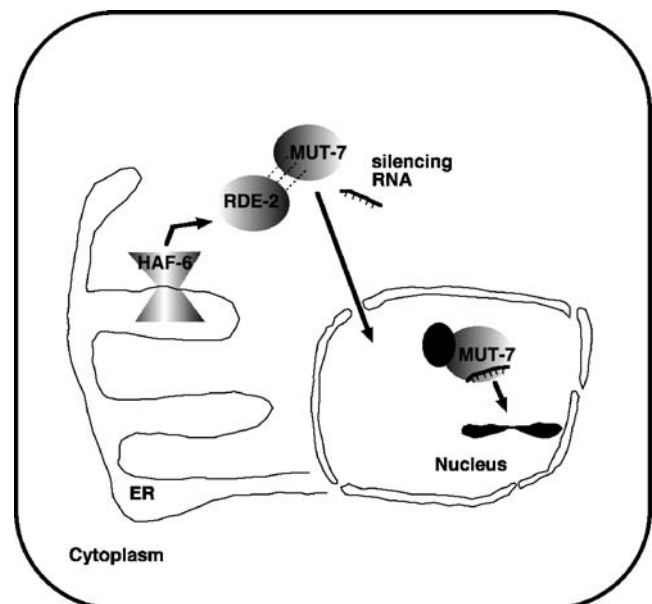

Fig. 3 Our current model for ABC_{RNAi} activity. ABC_{RNAi}, *mut-7,* and *rde-2* mutants display genetic interactions and have similar phenotypes, yet the proteins reside in distinct subcellular compartments. The MUT-7 protein harbors a 5′-3′ exonuclease domain, while RDE-2 is a novel protein, with no conserved motifs. We hypothesize that transport of specific substrates by ABC_{RNAi} transporters can influence RNAi-related mechanisms that include MUT-7 and RDE-2 as components. These RNAi-related mechanisms ultimately affect chromosome functions in the nucleus

of the ABC transporter mutants tested, and we refer to this class of transporters as ABC$_{RNAi}$.

All ABC$_{RNAi}$ transporter genes interact genetically with *mut-7* and *rde-2*

We reasoned that we might be able to use SSNC assays as a means to identify additional members of the ABC$_{RNAi}$ class. We therefore performed SSNC tests between all available ABC transporter mutants and *mut-7* mutants, as well as between ABC transporter and *rde-2* mutant strains. All the ABC transporters previously categorized as ABC$_{RNAi}$ members failed to complement both *mut-7* and *rde-2*. In addition, *haf-9* mutants interacted with both *mut-7* and *rde-2*; therefore, we now consider *haf-9* as a member of the ABC$_{RNAi}$ subclass of transporters, even though animals that are homozygous for a *haf-9* deletion do not display RNAi defects. Since HAF-9, HAF-2, and HAF-6 may form different combinations of homodimers and/or heterodimers, we consider that RNAi activity in *haf-9* homozygotes may be provided by dimerization configurations from the other two proteins. None of the remaining thirty-five ABC transporters displayed genetic interactions with either *mut-7* or *rde-2* mutants. Our collective results suggest that the ability to influence RNAi pathways is specific to the ABC$_{RNAi}$ subclass of transporters. Additionally, the RNAi-related functions of ABC$_{RNAi}$ transporters is apparently specific to the branch of the RNAi network that includes *mut-7* and *rde-2*, as genetic interactions between ABC$_{RNAi}$ genes and other branches of the RNAi pathway were not observed.

Some ABC$_{RNAi}$ transporters are required for transposon silencing

The genomes of most organisms harbor numerous transposons, many of which are capable of mobilizing and causing insertional mutations. However transposon mobilization is held in check by various cellular mechanisms, including RNAi. Previous reports have noted a requirement for the *mut-7* and *rde-2* genes in the prevention of transposon mobilization (Tabara et al. 1999). We also observed an enhanced rate of transposon mobilization in *haf-6* mutants, when animals are reared at higher temperatures. We extended our characterization of ABC$_{RNAi}$ genes by testing all members of the ABC$_{RNAi}$ class for transposon mobilization activity, and we observed a temperature-sensitive increase in transposon activity in three other members of this class, including *haf-9*. Thus ABC$_{RNAi}$ transporters, *mut-7,* and *rde-2* share several common features with respect to RNAi activity: (a) all these genes are required in the germ line; (b) all ABC$_{RNAi}$ genes interact genetically with *mut-7* and *rde-2*; and (c) *mut-7, rde-2,* and some ABC$_{RNAi}$ mutants display increased transposon activity.

Summary

From our exhaustive studies of mutants, collectively defective in forty-five different ABC transporter genes, we were able to classify an additional gene, *haf-9,* as an ABC$_{RNAi}$ gene. Our studies rigorously distinguish ABC$_{RNAi}$ transporters from the remaining ABC transporter genes with respect to RNAi function, and our studies point to a role for ABC$_{RNAi}$ transporters in endogenous silencing processes that affect chromosome function. We speculate that ABC$_{RNAi}$ functions may allow for multiple means of influencing *mut-7/rde-2*-related RNAi mechanisms, thereby affecting chromosome function or even specific gene expression. In this regard, ABC$_{RNAi}$ transporters may provide an important link between environmental conditions and RNAi mechanisms that can influence chromosome function.

References

Filipowicz W (2005) Cell 122:17–20
Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC (1998) Nature 391:806–811
Grewal SI, Elgin SC (2007) Nature 447:399–406
Hannon GJ (2002) Nature 418:244–251
Hawley RS, Gilliland WD (2006) Genetics 174:5–15
Jose AM, Hunter CP (2007) Annu Rev Genet 41
Ketting RF, Haverkamp TH, van Luenen HG, Plasterk RH (1999) Cell 99:133–141
O'Donnell KA, Boeke JD (2007) Cell 129:37–44
Paroo Z, Liu Q, Wang X (2007) Cell Res 17:187–194
Sen GL, Blau HM (2006) Faseb J 20:1293–1299
Sheps JA, Ralph S, Zhao Z, Baillie DL, Ling V (2004) Genome Biol 5:R15
Sontheimer EJ (2005) Nat Rev Mol Cell Biol 6(2):127–138
Sundaram P, Echalier B, Han W, Hull D, Timmons L (2006) Mol Biol Cell 17:3678–3688
Tabara H, Sarkissian M, Kelly WG, Fleenor J, Grishok A, Timmons L, Fire A, Mello CC (1999) Cell 99:123–132
Timmons L (2004) Bioessays 26:715–718
Tops BB, Tabara H, Sijen T, Simmer F, Mello CC, Plasterk RH, Ketting RF (2005) Nucleic Acids Res 33:347–355