Caenorhabditis elegans ABC_RNAi Transporters Interact Genetically With *rde-2* and *mut-7*

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ABSTRACT

RNA interference (RNAi) mechanisms are conserved and consist of an interrelated network of activities that not only respond to exogenous dsRNA, but also perform endogenous functions required in the fine tuning of gene expression and in maintaining genome integrity. Not surprisingly, RNAi functions have widespread influences on cellular function and organismal development. Previously, we observed a reduced capacity to mount an RNAi response in nine *Caenorhabditis elegans* mutants that are defective in ABC transporter genes (ABC_RNAi mutants). Here, we report an exhaustive study of mutants, collectively defective in 49 different ABC transporter genes, that allowed for the categorization of one additional transporter into the ABC_RNAi gene class. Genetic complementation tests reveal functions for ABC_RNAi transporters in the *mut-7/rde-2* branch of the RNAi pathway. These second-site noncomplementation interactions suggest that ABC_RNAi proteins and MUT-7/RDE-2 function together in parallel pathways and/or as multiprotein complexes. Like *mut-7* and *rde-2*, some ABC_RNAi mutants display transposon silencing defects. Finally, our analyses reveal a genetic interaction network of ABC_RNAi gene function with respect to this part of the RNAi pathway. From our results, we speculate that the coordinated activities of ABC_RNAi transporters, through their effects on endogenous RNAi-related mechanisms, ultimately affect chromosome function and integrity.

WHILE the exploitation of cellular RNA interference (RNAi) mechanisms to target specific sequences for silencing has facilitated analysis of gene function, we have only an incomplete description of the endogenous functions that RNAi mechanisms afford to cells. In *Caenorhabditis elegans*, the products of the *mut-7* and *rde-2* genes are required not only for a fully effective RNAi response to dsRNA, but also for endogenous processes that ultimately affect chromosome and microRNA functions. In particular, *mut-7* and *rde-2* mutants display (a) an increased rate of nondisjunction (Him phenotype), (b) aberrant germ-line expression of sequences from a repetitive transgene array in situations where expression is normally silenced, (c) a reduced ability for transgene-induced cosuppression of endogenous genes, (d) a high rate of transposon mobilization from chromosomal sites in the mutant germ line, a tissue that normally represses mobilization of transposons, and (e) defects in the function of endogenous noncoding RNAs (Tabara *et al.* 1999; Dernburg *et al.* 2000;

Tops *et al.* 2005; Lee *et al.* 2006). The *mut-7* gene encodes a protein with an exonuclease domain (Ketting *et al.* 1999). *rde-2* encodes a protein with no discernible motifs and little similarity to proteins outside nematodes (Tops *et al.* 2005). Previous reports indicate both a cytoplasmic and a nuclear localization for MUT-7, whereas RDE-2 is apparently localized exclusively to the cytoplasm where it and MUT-7 reside within a larger protein complex (Tops *et al.* 2005). Precise roles for MUT-7 and RDE-2 in RNAi and endogenous processes have not been established.

We previously reported a role for *C. elegans* ATP-binding cassette (ABC) proteins in RNAi, with emphasis on the analysis of one member of this family, *haf-6*. We also identified an additional eight transporter genes that are similarly required for RNAi: *abt-1*, *pgp-4*, *pgp-11*, *haf-2*, *mrp-1*, *pmp-1*, *wht-1(C05D10.3)*, and *wht-3(C16C10.12)* (Sundaram *et al.* 2006). These initial assays were limited in number and were restricted to observations of RNAi activities in germ line and muscle. However, the genome of *C. elegans* harbors 61 different ABC transporter genes that are collectively expressed in a wide array of tissues (Zhao *et al.* 2004). Thus, we considered that the narrow scope of our initial set of assays might have limited our ability to identify the complete set of ABC_RNAi genes—in particular, those ABC transporter mutants that might have somatic RNAi defects in nonmuscle tissue.

Here we set out to identify additional *C. elegans* ABC transporters that could be classified as ABC_RNAi genes by

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a more thorough assessment of RNAi activities in all available ABC transporter mutant strains. Of the 61 ABC transporter genes, mutations in 49 different genes are represented. We tested each mutant strain for RNAi defects using additional assays, and we performed complementation testing between nonallelic mutants. Our complementation testing utilized ABC transporter mutants and mutants defective for *mut-7* or *rde-2*. Because deletion alleles were used in each of the complementation tests, any noncomplementation interactions that are observed likely represent cases of combined haploinsufficiency. Second-site noncomplementation (SSNC) is often an indicator of physical interactions between proteins (Stearns and Botstein 1988; Campbell *et al.* 1995; Haarer *et al.* 2007); in other cases the interactions reveal functional requirements from multiple pathways that contribute to a given process (Yook *et al.* 2001; Jaspersen *et al.* 2004). In either case, a noncomplementation result implies a common function or influence on a particular process (Hawley and Gilliland 2006). Our results allowed us to classify an additional gene, *haf-9*, as an ABC$_{RNAi}$ gene. We also reveal common and distinct features related to RNAi defects for each of the mutant strains.

ABC transporters are classified into subfamilies on the basis of relative homologies, as well as the number and positioning of conserved transmembrane and ABC domains. The ABC$_{RNAi}$ subset of transporter genes consists of diverse subfamilies (A, B, C, D, and G), and both full transporter and half molecules are represented in this group. A genetic interaction with *mut-7* and *rde-2* is a unifying feature of this diverse group of genes; thus, the ABC$_{RNAi}$ subset can be defined on the basis of function, rather than subfamily classification. The genetic interactions with *mut-7* and *rde-2*, as well as other common phenotypes, point to an ability for ABC$_{RNAi}$ transporters to influence RNAi mechanisms that ultimately affect chromosome function and integrity.

MATERIALS AND METHODS

Strains used in complementation tests with *haf-6(ne335)*: XX47 [*(ne309)*], WM27 [*rde-1(ne219)* V], XX52 [*rsd-2(ne319)* IV], WM30 [*mut-2(ne298)* I], PD8356 [*rde-1(ne300)* V], XX51 [*sid-1 (ne318)*], NL917 [*mut-7(pk204)* III], NL3531 [*rde-2(pk1657)* I], XX50 [*sid-1(ne316)*], NL1820 [*mut-7(pk720)* III], WM29 [*rde-2 (ne221)* I], IG10 [*tol-1(nr2033)* I], PD5085 [*ppw-1(pk2505)*], PD8221 [*rde-4(ne299)* III], CB4043 [*smg-2 (e2008)* I; *him-5 (e1490)* V].

Mutants used in phenotypic analyses: VC1046 [*abce-1 (gk481)*], VC712 [*mrp-4(ok1095)* X], VC561 [*abcf-1(ok830)* V], GH534 [*mrp-4(cd8)* X], VC544 [*abcf-2(ok771)* III], RB1070 [*mrp-6 (ok1027)* X] RB1645 [*abch-1(ok2034)* II], RB1269 [*mrp-8 (ok1360)* III], VC572 [*abcx-1(ok865)* II], NL132 [*pgp-1(pk17)* IV], FX703 [*abt-1(tm703)* IV], VC134 [*pgp-2(gk114)* I], RB842 [*abt-2((ok669)* I], NL131 [*pgp-3(pk18)* X], FX502 [*abt-2(tm502)* I], VC21 [*pgp-4(gk16)* X], FX1050 [*abt-4(tm1050)* V], RB959 [*pgp-5 (ok856)* X], RB817 [*abt-4(ok633)* V], FX663 [*pgp-5(tm663)* X], RB1765 [*abt-5(ok2268)* I], RB1047 [*pgp-6 (ok994)* X], RB1151 [*cft-1*

(ok1180) V], VC286 [*pgp-7(ok528)/szT1;+/szT1* I], MT4983 [*ced-7(n1996)* III], FX830 [*pgp-9(tm830)* V], MT4984 [*ced-7 (n1997* III], FX996 [*pgp-10(tm996)* X], MT8886 [*ced-7(n2024)* III], RB1045 [*pgp-10(ok991)* X], FX843 [*haf-1(tm843)* IV], FX333 [*pgp-11(tm333)* II], RB867 [*haf-1(ok705)* IV], VC26 [*pgp-12 (gk19)* X], VC16 [*haf-2(gk13)* II], RB894 [*pgp-13(ok747)* X], VC1186 [*haf-3(gk549)* V], RB1041 [*pgp-15(ok987)* X], RB1107 [*haf-3(ok1086)* V], RB908 [*pmp-1(ok773)* II], VC449 [*haf-4 (gk240)* I], RB1108[*pmp-3(ok1087)* V], RB1080 [*haf-4(ok1042)* I], FX968 [*pmp-3(tm968)* V], VC252 [*haf-5(gk155)* III], RB675 [*pmp-4(ok396)* IV], VC287 [*haf-5(gk161)* III], VC189 [*pmp-4(ok396)* IV], XX193 [*haf-6(ne335)* I; *him-5(e1490)* V], XX194 [*haf-6(ne335)* I; *ccIs8160(rpL28::GFP); him-5(e1490)* V], XX195 [*haf-6(ne335)* I; *cIs8160(rpL28::GFP)*], XX364 [*haf-6(ne335)* I], FX688 [*wht-1(tm688)* III], VC118 [*haf-7(gk46)* V], RB1007 [*wht-3(ok927)* III], VC15 [*haf-8(gk12)* IV], RB1033[*wht-3(ok962)* III], VC32 [*haf-9(gk23)* I], FX714 [*wht-3(tm714)* III], NL147 [*mrp-1(pk89)* X], RB1058 [*wht-4(ok1007)* II], NL147[*mrp-1(ut153)* X], RB932 [*wht-5(ok806)* IV], RB1713 [*mrp-2(ok2157)* X], VC629 [*wht-6(ok882)* III], RB1028 [*mrp-3(ok955)* X], RB933 [*wht-7(ok812)* III], VC692 [*wht-9(ok1044)* III].

Other strains, outcrossing, and DNA sequence verification: To help prevent or monitor for accidental introduction of RNAi-related mutations that may be present in the background of wild-type N2 populations, we maintain wild-type strains as subtypes. These subtypes have passed our tests for brood size and sensitivity to *pop-1* and *unc-22* foods and lack temperature-sensitive Sterility or Him phenotypes. Many of the phenotypes here were obtained using deletion alleles, and in most cases recapitulating the effects of the mutation using RNAi is not a comparable methodology. For example, in our complementation tests, the animals tested are *trans*-heterozygotes and RNAi would likely induce a null phenotype; therefore, the null mutations and RNAi would produce dissimilar genetic conditions. We repeated experiments using more than one mutant subtype; these were generated by outcrossing the mutant strains to different N2 subtypes. Each N2 subtype, and the corresponding mutant subtype, would be expected to harbor different sets of background mutations.

XX1181 [*mut-7(pk204)* III; *dpy-4(e1166) unc-17(e245)* IV] was generated from crosses of PD2027 [*dpy-4(e1166) unc-17(e245)* IV] and NL917 [*mut-7(pk204)* III]. XX1187 [*rde-2 (ne221)* I; *dpy-4(e1166) unc-17(e245)* IV] was generated from crosses of PD2027 [*dpy-4(e1166) unc-17(e245)* IV] and WM29 [*rde-2(ne221)* I]. XX1569 and XX1570 [*mut-7(pk204)* III] are independent F$_2$ sibling subtypes from a cross of XX1181 and NL917. XX1571-3 [*rde-2(ne221)* I] subtypes were similarly derived from crosses of XX1187 and WM29.

Strains XX1824–1826 harbor the *mut-7(pk201)* mutation. XX1824 [*mut-7(pk201)*; yyEx62.2-*rde-2* cosmid] was derived from a cross between XX1570 mutants and XX506 transgenic animals. XX1825 [*mut-7(pk201)*; yyEx75.1-*haf-6* cDNA (G562R)] was derived from a cross using XX1570 and XX752. XX1826 [*mut-7 (pk201)*; yyEx97.4-*haf-6* cDNA (wild type)] was derived from a cross using XX1570 and XX1318. Strains XX1827–1829 harbor the *rde-2(pk1657)* mutation. XX1827 [*rde-2(pk1657)*; yyEx62.2-*rde-2* cosmid] was derived from a cross between WM29 mutants and XX506 transgenic animals. XX1828 [*rde-2 (pk1657)*; yyEx75.1-*haf-6* cDNA (G562R)] was derived from a cross using WM29 and XX752. XX1829 [*rde-2(pk1657)*; yyEx97.4-*haf-6* cDNA (wild type)] was derived from a cross using WM29 and XX1318.

NL3643 [*unc-22(st136)* IV] was outcrossed to wild-type males (XX171, an N2 strain from the lab of Andrew Fire) five times before it was used in additional crosses. The *unc-22(st136)* allele harbors a transposon insertion, and this outcrossed strain (XX366) has not displayed Mutator activity under any growth condition.

The ABC$_{RNAi}$ mutants identified previously [*abt-1(tm703)*, *haf-2(gk13)*, *haf-6(ne335)*, *mrp-1(pk89)*, *pgp-4(gk16)*, *pgp-11(tm333)*, *pmp-1(ok773)*, *wht-1/C05D10.3(tm688)*, *wht-3/C16C10.12(ok962)*, and the newly identified *haf-9(gk23)*] were tested for RNAi activity as unoutcrossed lines and again after three rounds of outcrossing to wild-type males (XX171). Outcrossed lines were established from cloned individuals, and extensive PCR analyses were performed on the cloned adult and on the ensuing population to ensure homozygosity for each deletion. Most strains that displayed noncomplementing interactions were subjected to additional rounds of outcrossing using wild-type XX937 males (a selected subtype from an N2 stock obtained from the Caenorhabditis Genetics Center, DR subclone of CB origin) or XX366, a transposon-insertion line that does not display Mutator activity.

We sequenced DNA prepared from each of the *haf-6*, *rde-2*, and *mut-7* noncomplementing strains, and in each mutant we verified that the other two genes were wild type in sequence. DNA sequences were obtained from genomic DNA as well as from RT–PCR products. cDNAs were easily generated, indicating gene expression, and the splicing patterns were as expected. (Both *rde-2* mRNA isoforms were observed in all mutant strains.) We sequenced an *rde-2* cDNA 1737 bp in length from the following strains: XX194 [*haf-6(ne335) I; him-5(e1490)* V], NL917 [*(mut-7(pk201)* III] (260 bp from the middle of this cDNA were not read), and NL1820 [*mut-7(pk720)* III]. We sequenced a *haf-6* cDNA 2000 bp in length from XX1569 [*mut-7(pk204)* III], XX1571 [*rde-2(ne221)* I], NL1820 [*mut-7(pk720)* III], and NL3531 [*rde-2(pk1657)* I]. A total of 2943 bp of *mut-7* cDNA were sequenced in XX194 [*haf-6(ne335) I; him-5(e1490)* V], XX1571 [*rde-2(ne221)* I], and NL3531 [*rde-2(pk1657)* I]. Sequence information from genomic DNA isolated from each mutant strain validated our results derived from cDNAs and provided information in gaps.

Feeding strains and feeding-based assays for RNAi defects: Feeding plates harboring bacteria engineered to express dsRNA were prepared using the HT115(DE3) host bacteria as described (TIMMONS *et al.* 2001; HULL and TIMMONS 2004; SUNDARAM *et al.* 2006). Plasmids transformed into this bacterial strain were derived from L4440 vector (TIMMONS and FIRE 1998). *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 a gene expressed in muscle and induces a Twitching phenotype. Other feeding strains were obtained from the MRC Geneservice RNAi library (KAMATH *et al.* 2003). Male stocks were established for each mutant strain by rearing animals at 20° on feeding strains with plasmids targeting *him* genes. *mut-7* and *rde-2* animals are Him, and we observed that mutant males sire progeny only when the males arose by means unrelated to the *mut-7/rde-2* defect. When *rde-2* or *mut-7* males are produced via RNAi phenocopy of Him pathway genes, matings can be successful. RNAi assays were performed as described (TIMMONS *et al.* 2001; HULL and TIMMONS 2004; SUNDARAM *et al.* 2006) at 15°, 20°, and 25°.

Generation of transgene lines and transgene-based rescue of RNAi: Transgene lines were established as complex arrays to better ensure expression in the germ line (KELLY *et al.* 1997). Plasmid DNA sequences were linearized before injecting. *unc-25::gfp* sequences were used as a dominant transformation marker; bacteriophage lambda DNA was used as carrier DNA. Cosmid F21C3 harbors wild-type *rde-2* genomic sequence and provides for rescue of the RNAi defects in *rde-2* mutants. Plasmid pLT417 harbors *let-858 promoter::let-858 5'-UTR::haf-6 cDNA::let-858 3'-UTR* sequences. The *haf-6* coding region in pLT417 harbors a G562R substitution that affects a conserved amino acid in the ATP-binding cassette domain, and this plasmid provides partial rescue of RNAi defects in *haf-6*

mutants. Plasmid pLT372 is identical to pLT417 except that the *haf-6* cDNA sequence is wild type; this plasmid rescues the RNAi defects in *haf-6* mutants (SUNDARAM *et al.* 2006). Transgene arrays were generated in wild type and crossed into mutant backgrounds. Strains were verified for homozygosity by RNAi behavior (of nontransgenic siblings), temperature-sensitive sterility phenotypes, PCR, and/or DNA sequencing, as appropriate to the strain. RNAi activity was assessed by placing animals as L2/L3 larvae on *pop-1* food. Absence of live progeny is indicative of an intact RNAi response.

Complementation testing: Conclusions from our complementation tests were based on two or more independent crosses that were tested using different preparations of *pop-1* feeding plates. Crosses were performed using one hermaphrodite per mating on standard OP50-seeded plates with no obvious contamination. The presence of 50% males in the progeny indicated a successful cross. RNAi activity was assessed by noting the number of F_2 progeny produced per F_1 animal placed on *pop-1* food. The assays were performed under identical conditions in an incubator set at 20°. We did not assess RNAi activity in *trans*-heterozygous males. In Figure 2A, *mut-7* and *rde-2* mutants were used as the hermaphrodite parent with *haf-6* males to generate *trans*-heterozygotes; reciprocal crosses with *haf-6* as parental hermaphrodite produced progeny with normal RNAi activity. Complementation testing was performed using all combinations of *rde-2(ne221)*, *mut-7(pk204)* (both harbor point mutations that result in a premature stop codon and a S812L substitution, respectively), *rde-2(pk1657)*, and *mut-7(pk720)* (both harbor large deletions). In Tables 2 and 3, conclusions were drawn using data from at least two sets of independent genetic crosses and the progeny from each cross was tested on different preparations of *pop-1* food. The F_1 cross-progeny were placed as L2/L3 larvae on plates and the number of F_2 animals per F_1 adult was tabulated. We used the following classification scheme in our assessments: −, 0–5 F_2's per F_1 *trans*-heterozygote (indicative of wild-type RNAi activity); *w*, 6–20 F_2's per F_1; +, 21–50 F_2's per F_1; and ++, >50 F_2's per F_1 (indicative of strong RNAi defects). *Trans*-heterozygous combinations that displayed − and *w* results were considered complementary (C); combinations that produced + and ++ results were considered noncomplementary (NC); allele sets that produced + results were considered weak (W); and sets that displayed wide experiment-to-experiment variations (− and ++) were labeled inconsistent (I). Strains that displayed noncomplementation were subjected to three rounds of additional outcrossing and were retested for RNAi defects. A weak result could reflect a maternally supplied protein with a short half-life in the germ line. Alternatively, a weak RNAi defect might indicate that the paternal gene is transcriptionally activated relatively late in the critical period. Such conditions may also contribute to inconsistent results as well.

RESULTS

Forty of 49 *C.elegans* ABC transporter mutants tested are not RNAi defective: The *C. elegans* genome harbors 61 ABC transporter genes. Previously, we identified nine ABC transporter mutants that are defective in RNAi (SUNDARAM *et al.* 2006). In these earlier experiments, bacterial strains were used as a dsRNA delivery vehicle to deliver *pop-1* dsRNA or *unc-22* dsRNA to animals. When ingested by *C. elegans*, these bacterial strains can induce RNAi responses. We refer to this methodology as a "feeding" protocol and to the specific reagents as "*pop-1* food" or "*unc-22* food." The *pop-1* gene encodes a Wnt-

responsive Tcf/Lef-related protein with an HMG box and is required for germ-line development; the *unc-22* gene encodes a large extracellular matrix protein required for muscle function. Ingestion of *pop-1* food elicits sterility in wild-type animals, and *unc-22* food elicits a twitching phenocopy. In contrast, RNAi-defective mutants that ingest either of these foods produce progeny and have normal movements.

In earlier experiments, most of the ABC transporter mutants we tested displayed normal RNAi activities, except for the nine mutants we now refer to as ABC$_{RNAi}$ mutants. The fact that only about one-sixth of the ABC transporter genes, and not all ABC transporter genes, are required for efficient RNAi leads one to speculate that there is some degree of specificity with regard to how ABC transporters might affect RNAi function. The goal of the experiments reported here is to address the question of specificity by determining if additional ABC transporters are required for efficient RNAi, functions that may have been missed in previous examinations. Because the RNAi defects in ABC transporter mutants were revealed using feeding protocols, and because RNAi feeding protocols have some degree of experiment-to-experiment variability, we initially retested all available ABC transporter mutants. We first simply repeated our previous observations using *pop-1* and *unc-22* foods. All tests were again conducted at three different temperatures (15°, 20°, and 25°) to uncover potential heat or cold sensitivities, and the experiments were performed on the original strains as well as strains that had been subjected to additional outcrossings in our lab (see materials and methods). The results obtained did not differ from our previous reports (Sundaram *et al.* 2006 and data not shown).

We next utilized a more comprehensive set of feeding strains, targeting a wider array of genes expressed mostly in the soma (Table 1). Again, this more comprehensive set of assays did not reveal additional ABC$_{RNAi}$ mutants; RNAi activity was observed in all of the strains except those already designated as ABC$_{RNAi}$ (data not shown). Because all the ABC$_{RNAi}$ transporter mutants display RNAi defects when bacterial feeding strains are used to target genes expressed in the germ line, this broader set of data allows us to speculate that ABC$_{RNAi}$ transporter functions are particularly required in the germ line.

We directly compared the relative expressivity of the RNAi defects in the ABC$_{RNAi}$ strains by assessing each of their RNAi activities simultaneously, using an identical preparation of *pop-1* food. Some ABC$_{RNAi}$ strains display strong RNAi defects, while in other strains the defects are rather weak (Figure 1).

haf-6, mut-7, and rde-2 alleles are noncomplementing: Previous reports have documented SSNC interactions between alleles of *rde-2* and *mut-7(ne311)*, a *mut-7* allele with a missense mutation in the presumptive nuclease domain (Tops *et al.* 2005). During the course of our genetic analyses of *haf-6(ne335)* mutants, we observed

SSNC between alleles of *rde-2* and *haf-6*: doubly heterozygous *haf-6(ne335)* +/+ *rde-2(ne221)* animals were RNAi defective (Figure 2A). Using feeding-based assays to score for RNAi defects, we also observed SSNC using *rde-2(ne221)* and a different allele of *mut-7—mut-7(pk204)* (Figure 2A). This allele harbors a nonsense codon and disrupts the ability of MUT-7 to bind RDE-2 (Tops *et al.* 2005). We then extended the syllogism by testing whether the RNAi defects in *mut-7* could be complemented by mutations in *haf-6*, and, indeed, we observed noncomplementation between *mut-7(pk204)* and *haf-6(ne335)* (Figure 2A). Similar noncomplementation results were obtained using mutants harboring deletions in *rde-2* and *mut-7*, *rde-2(pk1657)*, and *mut-7(pk720)*. Thus *rde-2*, *mut-7*, and *haf-6* constitute a noncomplementing group of alleles with respect to RNAi activity.

RNAi defects in *haf-6* mutants are dosage dependent with respect to the amount of dsRNA introduced (Sundaram *et al.* 2006), and we considered that the SSNC results may indicate dosage sensitivity with respect to HAF-6, MUT-7, and RDE-2 proteins as well. This hypothesis is supported by the observation that the RNAi defects in *mut-7* and *rde-2* mutants can be partially rescued by extra copies of *haf-6(+)* DNA (Figure 2B). We also observed an ability of *rde-2* sequences to partially rescue the RNAi defects in *haf-6* mutants. Wild-type *rde-2* sequences do not provide rescue of RNAi defects in *mut-7(pk204)* (Figure 2B); however, these mutants harbor a version of MUT-7 that cannot bind RDE-2. Thus, rescue of RNAi defects in *mut-7(pk204)* animals by overexpression of HAF-6 may occur independently of an RDE-2/MUT-7 protein complex.

All ABC$_{RNAi}$ mutants display genetic interactions with *mut-7* and *rde-2* mutants: We reasoned that we might use complementation testing with *mut-7* or *rde-2* alleles as an assay to help reveal additional ABC transporter genes that are required for RNAi. We therefore extended our complementation testing to all available ABC transporter mutant strains. The vast majority of the ABC transporter alleles in these experiments harbor deletions; whereas both deletions and point mutations in *mut-7* and *rde-2* alleles are available and were used in our assays. The *mut-7* point mutations displayed similar genetic behavior to the deletion strains, and this was the case for *rde-2* as well. Because the results we obtained using *mut-7* strains were similar to those obtained using *rde-2* mutants, we refer to the use of either of these two mutant strains, their activities, and their genetic behaviors as *mut-7/rde-2*.

The RNAi defects in *mut-7/rde-2* mutants were complemented by all the ABC transporter mutants that display wild-type RNAi activity, with the exception of *haf-9* (Tables 2 and 3 and supplemental Tables 1 and 2 at http://www.genetics.org/supplemental/). Therefore, we now include *haf-9* in our list of ABC$_{RNAi}$ genes, despite the fact that homozygous mutants with a single-gene defect in *haf-9* are wild type for RNAi activity. Our

TABLE 1

ABC transporter mutants defective in 40 different genes are not defective in RNAi

Gene targeted	Expected expression pattern	Observed RNAi phenocopy
acn-1 (BROOKS *et al.* 2003; FRAND *et al.* 2005)	Hypodermis, seam cells, excretory gland	Sterile and Unc, the few surviving progeny die as embryos or larvae
bar-1 (EISENMANN *et al.* 1998; NATARAJAN *et al.* 2004)	Vulval precursor cells, seam cells, somatic gonad, P12	Ruptured vulva, egg-laying defective, Protruding vulva
bli-3 (FRASER *et al.* 2000; FRAND *et al.* 2005)	Peripheral to muscle bundles, hypodermal cells	Sterile, treated animals and surviving progeny display blistered cuticles
ceh-13 (WITTMANN *et al.* 1997; BRUNSCHWIG *et al.* 1999; STREIT *et al.* 2002)	A, D, E, MS lineages	Small, Unc, larval arrest
cks-1(dom-6) (POLINKO and STROME 2000)	Germ line, embryo	Embryonic lethality
pop-1 (LIN *et al.* 1995, 1998; SIEGFRIED *et al.* 2004; DESHPANDE *et al.* 2005)	Germ line, embryo, vulval precursor cells, Q neuroblasts, hypodermis	Sterility
elt-2 (FUKUSHIGE *et al.* 1998, 1999)	Intestine	Early larval arrest
F38E11.5 (KAMATH *et al.* 2003; SIMMER *et al.* 2003; RUAL *et al.* 2004)	Somatic gonad, embryo	Sick, slow growing, and sterile
peb-1 (THATCHER *et al.* 2001)	Pharynx, vulva, hindgut	Unc, sterile
sma-1 (MCKEOWN *et al.* 1998; PRAITIS *et al.* 2005)	Hypodermal cells, pharynx, excretory cell, intestine, some neurons	Slow growth, small, Dumpy
unc-15 (RUAL *et al.* 2004; DESHPANDE *et al.* 2005)	Various muscles	Paralysis
unc-22 (MOERMAN *et al.* 1988)	Muscle	Twitching
unc-112 (ROGALSKI *et al.* 2000; EDENS *et al.* 2001)	Various muscles	Paralysis
wnk-1 (KAMATH *et al.* 2003; SIMMER *et al.* 2003)	Excretory canal, hypodermis	Egg-laying abnormal

The reagents utilized for RNAi assays and how RNAi defects were monitored are described. The RNAi target is listed in the first column. Bacteria were used as the dsRNA delivery vehicle. The collected information regarding expression patterns is listed in the second column. The RNAi phenocopies we observed in wild-type animals are listed in the third column. Experiments were performed on strains defective for 49 different ABC transporter genes. Tests for RNAi activity were performed at 15°, 20°, and 25°.

collective results imply that the SSNC assays are a more sensitive indicator for RNAi function than feeding-based RNAi assays. This additional set of data points to specificity with regard to the ability of ABC transporters to influence RNAi mechanisms, as >75% of the 49 ABC transporter mutants tested complement the RNAi defects in *mut-7/rde-2*. In addition, we hypothesize that the RNAi defects in ABC transporter mutants are specific to the *mut-7/rde-2* branch of the RNAi pathway, as a number of strains with defects in other RNAi pathways, such as the RNA-induced silencing complex (RISC) pathway or pathways that facilitate dsRNA trafficking, complemented the RNAi defects in *mut-7* and *rde-2* (Table 2 and supplemental Table 1). Thus not only are the SSNC tests sensitive assays for the detection of RNAi-related functions for ABC transporters, but also the assays help narrow the ABC$_{RNAi}$ functions to a particular branch of the RNAi network (LEE *et al.* 2006; YIGIT *et al.* 2006).

Long-lasting parent-of-origin effects influence whether RNAi phenotypes are observed in *trans*-heterozygous progeny: In the sections above, we demonstrated SSNC interactions between ABC$_{RNAi}$ and *mut-7/rde-2* mutants: *trans*-heterozygous progeny displayed RNAi defects. We also observed a parent-of-origin effect in those SSNC interactions. In complementation tests using *mut-7/rde-2* as the parental hermaphrodite and ABC$_{RNAi}$ mutants as parental males, RNAi defects were observed in *trans*-heterozygous progeny (Table 3 and supplemental Table 2). However, in most reciprocal crosses utilizing *mut-7* or *rde-2* as the parental male, the resulting *trans*-heterozygous progeny displayed a wild-type level of RNAi activity (Table 3 and supplemental Table 2). In control experiments, a parent-of-origin effect is not revealed in simple crosses utilizing wild-type males and hermaphrodites that are homozygous for *mut-7* (or *rde-2*): wild-type levels of RNAi activity are
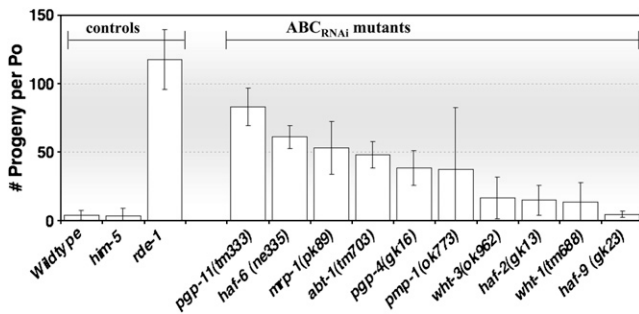
FIGURE 1.—The RNAi defects are unequal in ABC$_{RNAi}$ mutants. ABC$_{RNAi}$ mutants were reared on *pop-1* food and the number of progeny produced by each strain was tabulated. Bars represent the average of values obtained from three plates, each with five P$_o$ animals. The experiment utilized identical conditions for each plate. Error bars denote SD. (Some *pmp-1* parental animals were lost from one plate, hence the larger error.)

can be provided from paternal sources in animals that are singly heterozygous for *mut-7* or *rde-2*. However, in *trans*-heterozygous *(mut-7/rde-2)/+ ; +/ABC$_{RNAi}$* progeny that originate from oocytes lacking maternal MUT-7 or RDE-2, paternal *mut-7/rde-2* does not provide rescue of RNAi defects. In these *trans*-heterozygous progeny, the maternal load of any ABC$_{RNAi}$ transporters should be inherited at wild-type levels. How the genetic condition of heterozygosity for an ABC$_{RNAi}$ gene prevents paternal rescue of *mut-7/rde-2* is a question that remains to be answered.

Some combinations of ABC$_{RNAi}$ mutations also fail to complement: We extended our analyses to see if pairs of different ABC$_{RNAi}$ mutants would complement one another (Table 3). We anticipated that we might gain information regarding redundancy with respect to ABC transporter functions in RNAi or results that might indicate heterodimerization partners for the half transporters. We observed a few pairs of noncomplementing ABC$_{RNAi}$ transporter alleles and several instances where the SSNC results were parent-of-origin dependent (Table 3). In general, most combinations of ABC transporter mutants were complementary, highlighting the specificity of these interactions and ruling out the possibility of a common background mutation in each of the strains.

Most of our noncomplementation experiments utilized *pop-1* food. We considered that the RNAi defects we observed in *trans*-heterozygous animals might reflect an
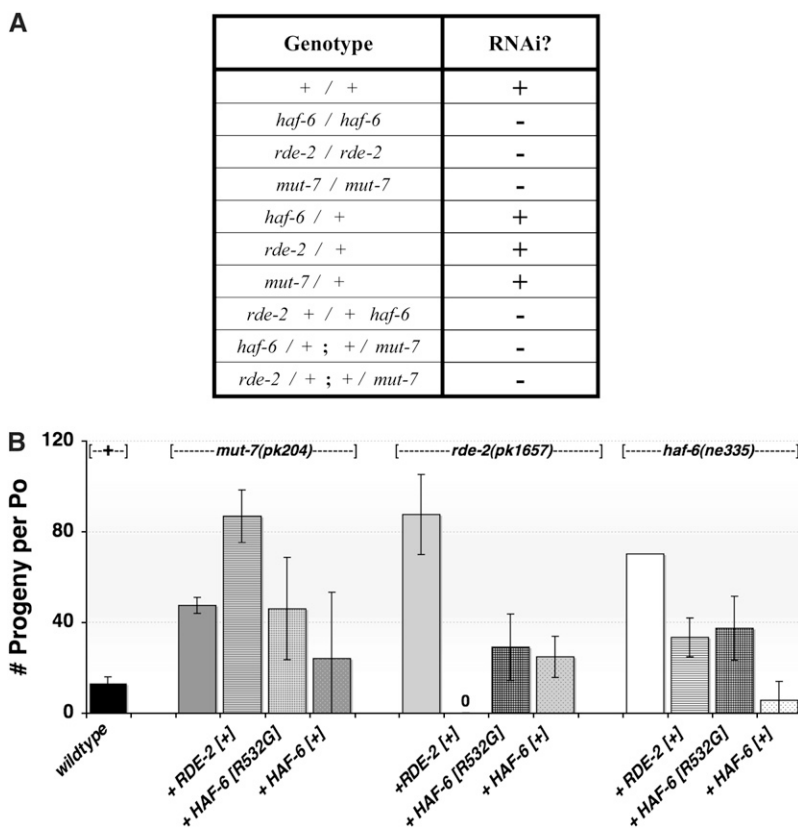
displayed in both sets of genetically identical progeny produced from reciprocal crosses. Thus, a dependency on maternal MUT-7/RDE-2 is revealed only when the animal is also heterozygous for an ABC$_{RNAi}$ transporter gene.

The RNAi defects in *trans*-heterozygotes are surprisingly long lasting. Animals were placed on feeding plates as young larvae, and RNAi activities were assessed later in development, in the germ line of the ensuing adult. *mut-7* and *rde-2* mutations are recessive, and RNAi activity

Genotype	RNAi?
+ / +	+
haf-6 / haf-6	−
rde-2 / rde-2	−
mut-7 / mut-7	−
haf-6 / +	+
rde-2 / +	+
mut-7 / +	+
rde-2 + / + haf-6	−
haf-6 / + ; + / mut-7	−
rde-2 / + ; + / mut-7	−

FIGURE 2.—*haf-6, mut-7,* and *rde-2* strains constitute a noncomplementing set of mutants with RNAi defects. (A) Animals with the genotype indicated in the left column were placed on *pop-1* food as described (MATERIALS AND METHODS). The number of progeny produced from each animal was tabulated. + denotes RNAi activity (the average number of progeny produced ranged from 0 to 7 per adult animal in this experiment); − denotes an RNAi defect or lack of response to *pop-1* food (an average of 83–154 progeny per animal was observed). *mut-7* and *rde-2* mutants were the hermaphrodite parent in each of these experiments. Similar noncomplementation results were observed using strains harboring deletions in *mut-7* and *rde-2* in comparison to strains harboring point mutations. (B) RNAi defects are cross-complemented using transgenes. RNAi activity was assessed using *pop-1* food. The animals were homozygous for the mutation at the top of the graph; the *x*-axis label indicates the additional sequences that were provided by transgenes. Wild-type RDE-2^{+} protein was provided by cosmid F21C3. HAF-6[R532G] protein was provided by plasmid pLT417; this plasmid provides incomplete rescue of the RNAi defects in *haf-6* mutants. Wild-type HAF-6 protein is expressed from pLT372, which harbors a *haf-6* cDNA (SUNDARAM *et al.* 2006). Bars represent an average of three experiments; error bars indicate SD.

TABLE 2

A genetic interaction with *mut-7* or *rde-2* is not a general feature of ABC transporter mutants

Subfamily	Mutation	Complementation with	
		mut-7	rde-2
E	*abce-1(gk481)*	No males	
F	*abcf-1(ok830)*	No males	
F	*abcf-2 (ok771)*	C	C
H	*abch-1(ok2034)*	C	C
H	*abcx-1(ok865)*	C	C
A	*abt-2(ok669)*	C	C
	abt-2(tm502)	C	C
A	*abt-4(ok633)*	C	C
	abt-4(tm1050)	C	C
A	*abt-5(ok2268)*	C	C
A	*ced-7(n1996)*	C	C
	ced-7(n1997)	C	C
	ced-7(n2094)	C	C
C	*cft-1(ok1180)*	I	I
B	*haf-1(ok705)*	C	C
	haf-1(tm843)	C	C
B	*haf-3 (ok1086)*	C	C
	haf-3 (gk549)	C	C
B	*haf-4(gk240)*	I	I
	haf-4(ok1042)	C	C
B	*haf-5(gk161)*	C	C
	haf-5(gk155)	C	C
B	*haf-7(gk46)*	C	C
B	*haf-8(gk12)*	C	C
C	*mrp-2(ok2157)*	C	C
C	*mrp-3(ok995)*	C	C
C	*mrp-4 (ok1095)*	C	C
C	*mrp-4 (cd8)*	C	C
C	*mrp-6(ok1027)*	C	C
C	*mrp-8(ok1360)*	C	C
B	*pgp-1(pk17)*	C	C
B	*pgp-2(gk114)*	C	C
B	*pgp-3(pk18)*	C	C
B	*pgp-5(ok856)*	C	C
	pgp-5(tm663)	C	C
B	*pgp-6(ok994)*	C	C
B	*pgp-7(ok528)*	C	C
B	*pgp-9(tm830)*	C	C
B	*pgp-10(ok991)*	C	C
	pgp-10(tm996)	C	C
B	*pgp-12(gk19)*	C	C
B	*pgp-13(ok747)*	C	C
B	*pgp-15(ok987)*	C	C
D	*pmp-3(ok1087)*	C	C
	pmp-3(tm968)	C	C
D	*pmp-4(ok396)*	C	C
G	*wht-3(ok927)*	C	C
	wht-3(tm714)	C	C
G	*wht-4(ok1007)*	C	C
G	*wht-5(ok806)*	C	C
G	*wht-6(ok882)*	C	C
G	*wht-7(ok812)*	C	C
G	*wht-9(ok1044)*	C	C
Other RNAi-related mutants			
K08H10.7	*rde-1(ne219)*	C	C

(*continued*)

Subfamily	Mutation	Complementation with	
		mut-7	rde-2
T20G5.11	*rde-4(ne299)*	C	C
C04F5.1	*sid-1(ne318)*	C	C
F52G2.2	*rsd-2(ne319)*	C	C
F16D3.2	*rsd-6(pk2011)*	C	C

Complementation tests were performed using *mut-7(pk204)* or *rde-2(ne221)* strains as the hermaphrodite parent; however, the reciprocal cross was performed for *abce-1* and *abcf-1* as we were unable to generate male stocks from these mutants. *trans*-heterozygous progeny were tested for RNAi activity using *pop-1* food. False positives were observed in *cft-1* mutants due to contamination on the plates. C, complementing alleles; NC, noncomplementing alleles; W, weakly complementing alleles; I, alleles that inconsistently complement (see MATERIALS AND METHODS for a description of the scoring system and supplemental Table 3 at http://www.genetics.org/supplemental/ for data).

ability of this genetic condition to influence the *pop-1* pathway. To address this question, we retested noncomplementing pairs of alleles using *cks-1* food and again observed noncomplementation. *cks-1* is a conserved cell-cycle regulator required for cyclin-dependent kinase activity and proper cell division (POLINKO and STROME 2000; SHIRAYAMA *et al.* 2006), confirming that the defect in *trans*-heterozygotes resides in the RNAi pathway and not in the *pop-1* pathway (supplemental Table 2).

Conditional phenotypes are observed in ABC$_{RNAi}$ mutants: The SSNC interactions we uncovered led us to investigate whether the ABC$_{RNAi}$ genes, like *mut-7* or *rde-2*, are required for endogenous RNAi-associated processes. We first looked to see if ABC$_{RNAi}$ mutants shared common phenotypes with *mut-7* and *rde-2*. Unlike *mut-7* and *rde-2*, the ABC$_{RNAi}$ mutants do not have a high rate of chromosome nondisjunction, as they do not display a high incidence of males (Him) phenotype under any of the environmental conditions we tested, including elevated temperature, the presence of exogenous dsRNAs or heavy metal cations, or starvation. However, we did observe additional phenotypes in some ABC$_{RNAi}$ transporter mutants that are indicative of defects in RNAi mechanisms. We report three main observations.

First, the brood sizes are reduced in some mutants reared under conditions of elevated temperature (Figure 3A). While most mutants have normal fertility, *haf-6* and *haf-9* mutants produce fewer progeny at elevated temperatures. *mut-7* and *rde-2* mutants also have reductions in brood sizes, and it is widely appreciated that both *rde-2* and *mut-7* stocks will eventually stop producing progeny when reared at 25° for several generations.

Second, the RNAi defects in *haf-6(ne335)*, *mut-7(pk204)*, and *rde-2(ne221)* mutants are temperature sensitive for

TABLE 3

RNAi defects in cross-progeny

	♂	♀ abt-1	haf-2	haf-6	haf-9	mrp-1	pgp-4	pgp-11	pmp-1	wht-1	wht-3	rde-2	mut-7
A	abt-1	NC	I	C	C	C	C	C	C	C	NC	NC	NC
B	haf-2	C	NC	C	C	C	C	C	C	C	C	NC	NC
B	haf-6	C	C	NC	C	C	I	C	C	C	C	NC	NC
B	haf-9	C	C	C	NA	I[a]	C	C	C	C	C	NC	NC
C	mrp-1	C	I	C	C	NC	C	I[a]	C	C	I[a]	NC	NC
B	pgp-4	C	I	I	C	C	NC	I	I	I	I[a]	NC[a]	NC[a]
B	pgp-11	C	I	NC	C	NC	NC	NC	C	C	NC	NC	NC
D	pmp-1	C	C	C	C	C	I	C	NC	I	I	NC	NC
G	wht-1	C	C	C	C	C	C	C	C	NC	C	NC	NC
G	wht-3	I[a]	NC	C	C	I	C	C	C	C	NC	NC	NC
–	rde-2	I	C	C	C	C	W	C	C	C	I	NC	NC
–	mut-7	I	C	C	C	C	C	C	C	C	W	NC	NC

All ABC transporter genes in the ABC$_{RNAi}$ category display genetic interactions with *mut-7* and *rde-2*. Reciprocal crosses were performed for each set of mutants; cross-progeny were tested for RNAi activity using *pop-1* food. Column headings show the homozygous mutation from the hermaphrodite parent; row headings indicate the parental male genotype; entries in table body show complementation results observed in *trans*-heterozygous progeny. C, complementing alleles; NC, noncomplementing alleles; W, weakly complementing alleles; I, alleles that inconsistently complement. Animals homozygous for *haf-9* are not RNAi defective.

[a] Noncomplementation was more obvious after further outcrosses of stocks (see Table 2 legend and MATERIALS AND METHODS for description of scoring and supplemental Table 2 for data).

one target, *unc-22*, when the feeding method is used to deliver dsRNA (Figure 3B). No temperature sensitivity is observed when *unc-22* dsRNA is introduced by soaking, injection, or transgene expression of *unc-22* sequences in muscle (data not shown). To date, *unc-22* is the only sequence for which we have observed a method- and temperature-sensitive RNAi defect. This phenotype is not a general consequence of defects in RNAi and is not
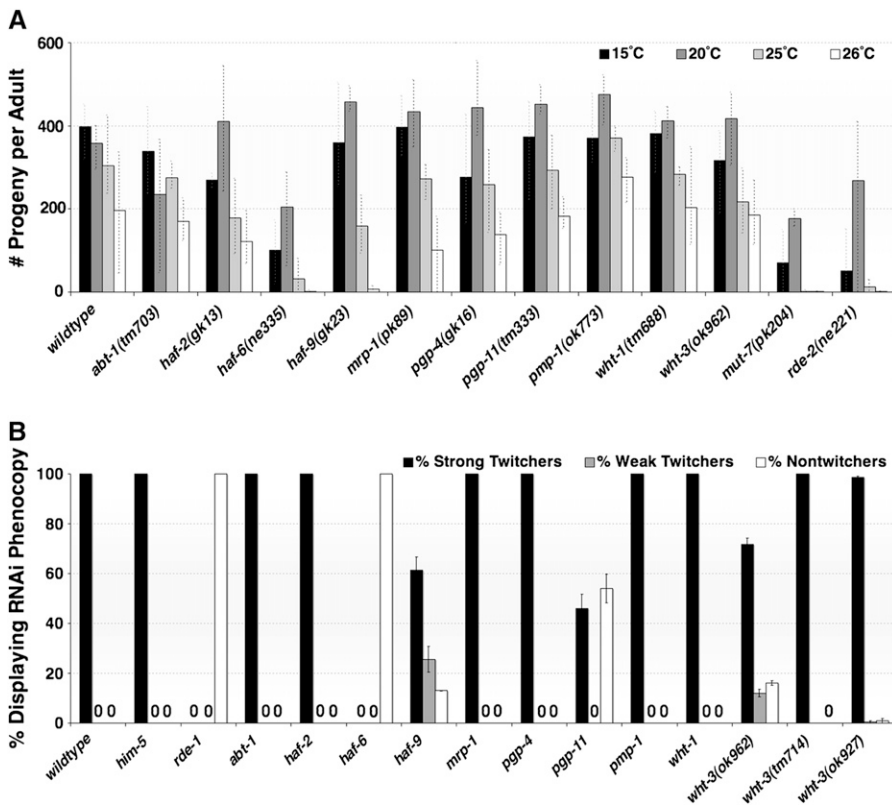
FIGURE 3.—ABC$_{RNAi}$ mutants display additional temperature-sensitive phenotypes. (A) The brood sizes from each strain were assessed at different temperatures under otherwise identical conditions. Progeny were counted and removed from plates daily for 5 days (15° and 20° experiments) or 3 days (25°). Bars represent results from duplicate experiments. Error bars denote SD. (B) Animals with the indicated genotypes were placed on *unc-22* food and Twitching phenocopies were assessed at 15°, 20°, and 25°. No twitching was observed in *rde-1* or in *sid-1* mutants at any temperature, as expected. Twitching was observed in all other strains reared at 15° and 20°; the results from the 25° experiment are displayed here. Animals from each plate were classified as nontwitchers, weak twitchers (good movement with twitching), or strong twitchers (paralysis and twitching). Bars represent the average of three experiments; error bars denote SD.

TABLE 4

Mutants have increased rates of transposon mobilization

Strain	% revertants		
	15°	20°	25°
unc-22(st136)	0 (0/33,000)	0 (0/32,000)	0 (0/41,000)
rde-2(ne221); unc-22(st136)		0.08 (10/12,750)	0.40 (90/22,200)
mut-7(ne221); unc-22(st136)			3.74 (127/3,400)
abt-1(tm0703); unc-22(st136)	0 (0/26,000)	0 (0/24,500)	0 (0/38,000)
haf-2(gk13); unc-22(st136)	0 (0/25,500)	0 (0/27,220)	0 (0/38,000)
haf-6(ne335); unc-22(st136)	0 (0/21,150)	0 (0/21,150)	0.09 (17/18,000)
haf-9 (gk23); unc-22(st136)	0 (0/27,300)	0 (0/25,500)	0.04 (15/41,200)
mrp-1(pk89); unc-22(st136)	0 (0/26,000)	0 (0/26,050)	0.02 (8/41,000)
pgp-4(gk16); unc-22(st136)	0 (0/24,850)	0 (0/25,100)	0 (0/38,000)
pgp-11(tm33); unc-22(st136)	0 (0/28,500)	0 (0/28,000)	0 (0/38,000)
pmp-1(ok773); unc-22(st136)	0 (0/26,900)	0 (0/28,700)	0 (0/38,000)
wht-1(tm688); unc-22(st136)	0 (0/28,300)	0 (0/22,000)	0.06 (26/42,000)
wht-3(ok962); unc-22(st136)	0 (0/26,800)	0 (0/25,000)	0 (0/38,000)
wht-3(ok927); unc-22(st136)		0 (0/13,500)	0 (0/11,800)

Strains are marked with an *unc-22(st136)* allele, which harbors a Tc1 transposable element. Germ-line excisions were assessed by noting the number of nontwitching revertants that appeared in the progeny of homozygous mutants. Revertants were set aside and a 3:1 phenotypic ratio was observed in the progeny from each, as expected; PCR was performed on some revertants and the amplified *unc-22* sequence was the size expected for wild type.

observed in RISC-defective animals or in animals defective for systemic RNAi due to loss of *sid-1* (data not shown). However, we have observed similar temperature-sensitive RNAi defects from *unc-22* food in *rsd-2* and *rsd-6* mutants (W. HAN, unpublished results). We hypothesize that this temperature- and method-of-delivery-dependent RNAi phenotype is a hallmark for a subset of RNAi-defective mutants that includes *rde-2, mut-7, haf-6, rsd-2,* and *rsd-6*.

Finally, *mut-7* and *rde-2* mutants display an increased rate of transposon mobilization (Mutator activity), and some ABC_RNAi mutants display temperature-sensitive Mutator activity as well (Table 4). Strains with Mutator activity include *haf-6(ne335), haf-9(gk23), wht-1(tm688),* and *mrp-1(pk89)*. While *mut-7* and *rde-2* animals display Mutator activity at all temperatures, all the ABC_RNAi mutants are temperature sensitive for this phenotype. We hypothesize that this collective set of temperature-sensitive phenotypes in *haf-6* and other ABC_RNAi mutants reflects endogenous roles for ABC_RNAi transporters in cellular processes that also require the function of

MUT-7/RDE-2, roles that ultimately affect chromosome integrity.

DISCUSSION

A subclass of *C. elegans* ABC_RNAi transporters can be defined on the basis of function: This study rigorously distinguishes ABC_RNAi transporters from the remaining ABC transporter genes and points to roles for ABC_RNAi transporters in silencing events that affect chromosome function. The *C. elegans* genome encodes ~61 ABC transporter proteins that collectively reside on most intracellular membranes. Because ABC transporter-dependent movement of substrates is essential for subcellular organelle biogenesis and function, and because substrates can be transported between organelles (NUNES *et al.* 2005; SCHAHEEN *et al.* 2006; SCHROEDER *et al.* 2007), it is conceivable that alterations in ABC transporter function might simply affect cell physiology in a more general manner, with indirect effects on RNAi function. If we assume this possibility is correct, then we would

expect to observe RNAi defects in multiple tissues in most of the ABC transporter mutants, yet this is not what we observed. RNAi defects were observed in a limited subset of the mutants tested.

ABC$_{RNAi}$ transporters have specific effects on RNAi functions: Evidence of specificity with regard to RNAi phenotypes is provided by the following observations: only the ABC$_{RNAi}$ subset of transporters displays defects in RNAi, with the exception of *haf-9* mutants. While this is a functional definition that we applied on the basis of our results, this definition applies only to 30% of the 49 transporter mutants tested. Furthermore, only the ABC$_{RNAi}$ subset of transporter mutants displays SSNC interactions with *mut-7/rde-2*.

The fact that the RNAi functions of ABC$_{RNAi}$ transporters are required in germ-line tissue provides additional evidence of specificity. Even though several of the ABC$_{RNAi}$ genes are expressed in various somatic tissues (ZHAO *et al.* 2004), all ABC$_{RNAi}$ transporters are required for efficient RNAi in the germ line. Moreover, our extensive set of RNAi assays has not revealed an ABC transporter gene that is required for RNAi solely in somatic cells. *mut-7* and *rde-2*, which interact genetically with ABC$_{RNAi}$ mutants, are also required for efficient RNAi in the germ line, and the corresponding mutants display phenotypes indicative of germ-line defects (meiotic nondisjunction, cosuppression, and high rates of transposon mobilization). By contrast, some ABC transporters are expressed in the germ line, yet are not required for RNAi. For example, *ced-7* is required in the germ line for engulfment of apoptotic corpses (WU and HORVITZ 1998), yet *ced-7* mutants are wild type with respect to the RNAi activities assessed in this report. Finally, some ABC$_{RNAi}$ mutants display temperature-sensitive phenotypes in the germ line, phenotypes that are similar to those observed in *mut-7/rde-2* mutants. These temperature-sensitive phenotypes include Mutator activity, reductions in brood size, and RNAi defects in response to ingestion of *unc-22* food. While germ-line tissue is clearly the focus of ABC$_{RNAi}$ function, we cannot rule out additional trafficking roles between germ-line and somatic tissues.

Finally, within the RNAi network of mechanisms (LEE *et al.* 2006; YIGIT *et al.* 2006), evidence of specificity is observed with respect to ABC$_{RNAi}$ transporter function. This is revealed in SSNC assays using *mut-7/rde*-2, genes that are required in RNAi-related processes that affect chromosome as well as miRNA functions. By contrast, ABC$_{RNAi}$ transporters do not interact genetically with genes that are required in other RNAi-related processes, including genes that facilitate systemic silencing by dsRNA trafficking (*sid-1*) or genes that are required in dsRNA processing and RISC-based mechanisms (*rde-1* and *rde-4*) (TABARA *et al.* 2002; FEINBERG and HUNTER 2003; YIGIT *et al.* 2006). Additionally, even though ABC$_{RNAi}$ transporter mutants display temperature-sensitive defects in transposon silencing, they do not interact genetically

with other mutants that display temperature-sensitive transposon silencing defects (*rsd-6*) (HAN *et al.*, unpublished results). Similarly, while *mut-7/rde-2* mutants have increased rates of nondisjunction, ABC$_{RNAi}$ transporter mutants did not interact with other mutants that display dsRNA-inducible nondisjunction phenotypes (*rsd-2*) (HAN *et al.*, unpublished results). It may be the case that SSNC interactions are observed between pairs of genes that normally produce limited product. RSD-2, RSD-6, SID-1, RDE-1, and RDE-4 may not be limiting in quantity, and therefore the corresponding mutants would not be expected to display SSNC with ABC$_{RNAi}$ mutants. On the other hand, Him and Mutator phenotypes can be induced in *rsd-2* and *rsd-6* mutants (HAN *et al.*, unpublished results), which is consistent with the possibility that RSD-2 and RSD-6 proteins are limiting.

The common phenotypes and genetic interactions between ABC$_{RNAi}$ transporters and *mut-7/rde-2* imply common functions that affect chromosome function and transcriptional silencing: In addition to roles in chromosome function and integrity, *mut-7* and *rde-2* are also required for cosuppression and silencing of transgenes in the germ line (TABARA *et al.* 1999; DERNBURG *et al.* 2000; KETTING and PLASTERK 2000; KIM *et al.* 2005). Both these phenomena are observed when transgenes are introduced into *C. elegans*, and both phenomena have similar features that are suggestive of transcriptional-level silencing. Cosuppression refers to an ability of a transgene to silence gene expression from the transgene itself, as well as from endogenous genes with sequence homology to the transgene. While specific sequences may be more prone to cosuppression, the exact nature of the triggering molecule is not known. Silencing of transgene expression is frequently observed for sequences that express in the germ line. Both cosuppression and transgene silencing require chromatin factors and RNAi, and the transgenes that are silenced in the germ line display histone methylation patterns reminiscent of heterochromatin (KELLY *et al.* 2002; ROBERT *et al.* 2005). Both transgene silencing and cosuppression require *mut-7* and *rde-2*, but not *rde-1* (DERNBURG *et al.* 2000).

Here, we described an unusual temperature-sensitive and method-of-delivery-dependent RNAi defect that we observe in *mut-7*, *rde-2*, and *haf-6* mutants, and we postulate that this phenotype also reflects impairments in transcriptional silencing. Upon ingestion of *unc-22* food, these mutants display RNAi defects when the experiments are conducted at elevated temperatures. Normally, the *unc-22* gene is an effective RNAi target in RNAi experiments using wild-type animals, and *unc-22* sequences have been noted to induce cosuppression as well (FIRE *et al.* 1991). Taken together, we postulate that *unc-22* sequences have a tendency to trigger transcriptional silencing responses in these assays and that *unc-22* is a particularly amenable RNAi target due to

the combined activities of RISC and transcriptional silencing mechanisms. We further hypothesize that *mut-7*, *rde-2*, and *haf-6* are required to maintain transcription-level silencing at elevated temperatures. On a related note, we recently reported temperature-sensitive defects in transposon silencing in *rsd-6* mutants, and *rsd-6* also displays temperature-sensitive RNAi defects when reared on *unc-22* food (W. Han, unpublished results). Finally, we point out that chromosome-related events such as chromosome disjunction, transposon mobilization, and recombination are intrinsically sensitive to environmental conditions (Hildreth and Ulrichs 1969; Rose and Baillie 1979; Hashida *et al.* 2006). Thus the temperature-sensitive response to *unc-22* food that we observe in mutants is likely a hallmark for genes with RNAi activities that may ultimately affect transcriptional silencing.

Precisely how ABC_{RNAi} transporter and *mut-7/rde-2* functions are integrated is not known; however, we can invoke at least two models on the basis of the genetic and transgene rescue data (Figure 4). In a parallel pathways model, HAF-6 and RDE-2/MUT-7 activities are predicted to reside in different pathways that converge to a common RNAi function, with a threshold level of activity from both pathways required for full RNAi effect. Alternatively, in a protein complex model, both ABC_{RNAi} and RDE-2/MUT-7 function as a multiprotein complex that is effectual only when threshold numbers of complexes are complete. Because overexpression of one protein can rescue the RNAi defect in a mutant that is deficient for the second protein (Figure 2B), the former model is strongly favored; however, the latter model cannot be confidently eliminated. Indeed, RDE-2 and MUT-7 proteins physically associate (Tops *et al.* 2005).

Thus, ABC_{RNAi} proteins increase RNAi efficiency in the *mut-7/rde-2* branch of RNAi mechanisms where they ultimately affect silencing mechanisms that act at the level of the chromosome. An important consequence of this observation resides in the potential for environmental signals to produce changes in gene expression via RNAi mechanisms that are regulated or influenced by ABC_{RNAi}-dependent transport of specific substrates.

The maternally deposited MUT-7/RDE-2 products are particularly long lived: We noted that maternally deposited MUT-7/RDE-2 products were particularly long lived, as revealed in the SSNC tests, and the maternal requirement for MUT-7/RDE-2 is observed only when animals are heterozygous for ABC_{RNAi} (Table 3). We propose that the set-aside nature of germ-line progenitors and asymmetric cell divisions during embryogenesis allows for preferential segregation of maternal MUT-7/RDE-2 gene products into germ-line progenitor cells. We further speculate that the RNAi defects observed in adult *trans*-heterozygotes provides evidence of a long-lived nature of maternal MUT-7/RDE-2.
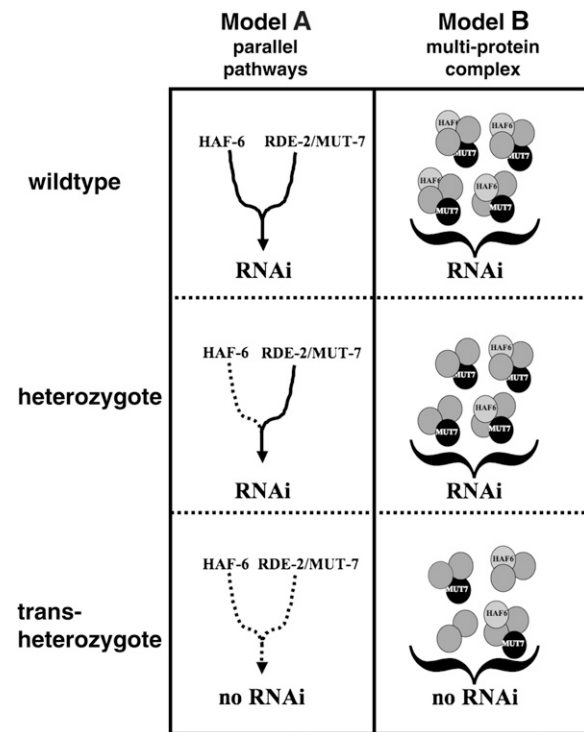

Figure 4.—Alternative models are predicted to help explain the genetic interactions. In a parallel pathways model, wild-type activity requires input from two pathways that converge to an RNAi function (left). In animals heterozygous for both genes, activities in multiple pathways may be reduced (left, bottom). In a protein complex model, a threshold number of complete multiprotein complexes is required for robust RNAi activity (right), while in *trans*-heterozygotes, a subthreshold number of complete complexes limits full activity (right, bottom). The models are not mutually exclusive; multiprotein complexes may act in pathways, and transported substrates may affect complex activity.

The presence of functional, plasma membrane-associated ABC transporters has important applications in stem cell biology, as ABC transporters serve as phenotypic markers for stem cell populations, and expression of particular transporters can influence developmental decisions of stem cells (Bunting 2002; Lin *et al.* 2006). It is interesting to speculate that specific transcriptional or chromosome-related functions that could affect differentiation are afforded by ABC transporters acting through specific RNAi mechanisms in germ-line progenitors and in cultured stem cells.

A network of activity is revealed through analysis of noncomplementing pairs of ABC_{RNAi} mutants: A few pairs of ABC_{RNAi} mutants were noncomplementing: some pairs of mutants failed to complement in reciprocal crosses, whereas SSNC in other sets of mutants was dependent upon the parental configuration of alleles (Table 3 and Figure 5, left section). In examples where SSNC is observed in reciprocal crosses, the results may reflect requirements for both transporters in one germ line. Alternatively, one transporter may be required in the male germ line. A SSNC that displays a
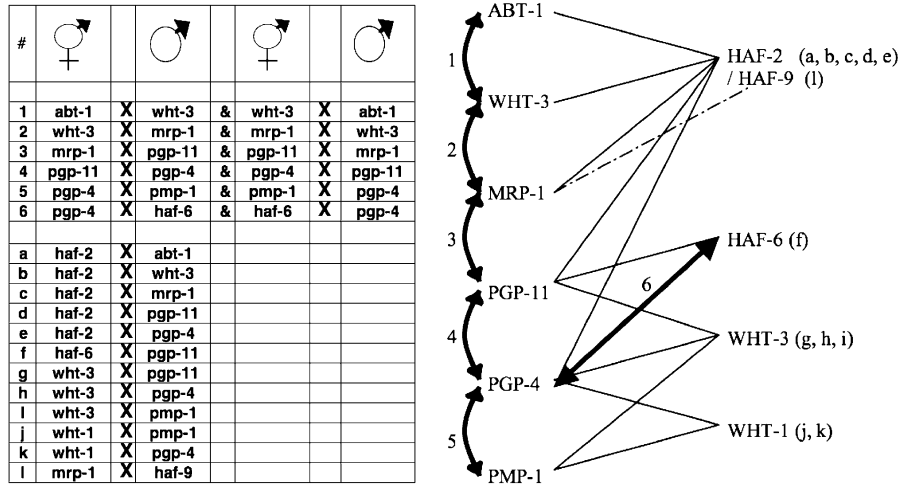
#	♀ X ♂	♀ X ♂	♀ X ♂	♂ X ♀
1	abt-1 X wht-3	& wht-3	X wht-3	X abt-1
2	wht-3 X mrp-1	& mrp-1		X wht-3
3	mrp-1 X pgp-11	& pgp-11		X mrp-1
4	pgp-11 X pgp-4	& pgp-4		X pgp-11
5	pgp-4 X pmp-1	& pmp-1		X pgp-4
6	pgp-4 X haf-6	& haf-6		X pgp-4
a	haf-2 X abt-1			
b	haf-2 X wht-3			
c	haf-2 X mrp-1			
d	haf-2 X pgp-11			
e	haf-2 X pgp-4			
f	haf-6 X pgp-11			
g	wht-3 X pgp-11			
h	wht-3 X pgp-4			
i	wht-3 X pmp-1			
j	wht-1 X pmp-1			
k	wht-1 X pgp-4			
l	mrp-1 X haf-9			

Network diagram nodes: ABT-1, WHT-3, MRP-1, PGP-11, PGP-4, PMP-1 (left column, numbered 1–5) connecting to HAF-2 (a, b, c, d, e) / HAF-9 (l), HAF-6 (f), WHT-3 (g, h, i), WHT-1 (j, k), with connection 6 between PGP-11/PGP-4 and HAF-6.

FIGURE 5.—Noncomplementation patterns reveal a network of ABC$_{RNAi}$ transporter activity. Pairs of ABC$_{RNAi}$ transporters that failed to complement in reciprocal crosses are numbered; nonreciprocal, noncomplementing pairs are lettered. The diagram indicates the interconnected nature of noncomplementing pairs of ABC$_{RNAi}$ alleles. All 10 ABC$_{RNAi}$ transporter genes are represented in this diagram. *haf-9* has only one connection, but *haf-9* mutants do not display RNAi defects. RNAi defects in *abt-1*, *pgp-4*, and *wht-3* do not complement *mut-7/rde-2* when *mut-7/rde-2* are used as parental males, and these ABC$_{RNAi}$ transporters occupy pivotal positions in the network.

parent-of-origin effect in reciprocal crosses might reflect functional redundancy for ABC$_{RNAi}$ genes in one gonad. Because some transporters may function as homo- and/or heterodimers, and because ABC$_{RNAi}$ function may not be restricted to germ-line tissue, it is not possible to draw firm conclusions regarding functional redundancy, tissue-specific requirements, or dimerization partners using the existing data. Nonetheless, we did note a pattern emerging from these data (Figure 5, right section).

All 10 ABC$_{RNAi}$ transporters are interconnected in a noncomplementation network with the exception of *haf-9*, the only ABC$_{RNAi}$ that does not display RNAi defects. However, the *haf-9* interaction can be added to the network in a manner that is consistent with the assumption that HAF-9 functions as a heterodimer with another ABC$_{RNAi}$ protein. We note that both *haf-9* and *haf-2* fail to complement *mrp-1*; thus these two HAF proteins may function as heterodimers in the germ line. *abt-1*, *wht-3*, and *pgp-4* are the only mutants that display SSNC when *mut-7* or *rde-2* are used as the parental males; furthermore, *abt-1*, *wht-3*, and *pgp-4* are positioned at critical points in the noncomplementation network. *abt-1* is located at the end of the network, *pgp-4* has the greatest number of interacting partners, including strong interactions with *haf-6*, *pgp-11*, and *pmp-1*, and *wht-3* is located in two positions in the network (Figure 5, right section). While additional experiments are required to fully elucidate the meaning of the noncomplementation network, the fact that all 10 ABC$_{RNAi}$ genes can be represented in an interconnected manner lends validity to the results of our complementation testing. We speculate that the network may represent a trafficking route for one or more substrates that are required for efficient RNAi.

Environmental conditions may influence gene expression via two conserved and interrelated networks: Environmental conditions such as elevated temperature can influence chromosome-associated activities such as transcription, transgene silencing, recombination, mei-

otic chromosomal disjunction, and transposon mobilization. The additional phenotypes we observed when ABC$_{RNAi}$ mutants were reared at elevated temperatures (Mutator and *unc-22* RNAi defects) imply an ability for ABC$_{RNAi}$ transporters to protect chromosome-related functions and influence transcription in suboptimal environments. On a related note, but also mRNA regulation by miRNAs has been shown to be influenced by cellular stressors (BHATTACHARYYA *et al.* 2006a,b; PETERS and MEISTER 2007). Precisely how RNAi mechanisms respond to environmental influences is not known. Interestingly, in addition to a 3′–5′ exonuclease domain, the MUT-7 protein also has similarity to conserved proteins of unknown function that are found in several hyperthermophilic archaea and other bacteria, raising the possibility that this domain might have functional roles that are influenced by temperature. Regions similar to this conserved domain are also found in 3′–5′ exonuclease domain proteins from paramecium and humans (supplemental Figure 1 at http://www.genetics.org/supplemental/).

We speculate that a network of ABC$_{RNAi}$ functions may allow for multiple means of influencing *mut-7/rde-2*, and ultimately chromosome function and integrity, in various environmental conditions. ABC transporters and RNAi pathway components appear to be functionally connected as part of an ancient cellular mechanism that responds to the environment.

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