A

hjf41 Q262stop
qt133 Q263stop
qt135 W339stop
qt136 Q573stop
qt134 Q657stop
qt131 Q695stop
qt137 Q920stop

500bp

DEAD-box RNA Helicase

FG domain

B

α-RDE-12

α-tubulin

WT   rde-12(hj41)

C

vha-6 promoter   atgl-1b cDNA   gfp   unc-54 3'UTR

hjIs21 L1

hjIs21 L4

rde-12(-);hjIs21

rde-12(-);hjSi394;hjIs21

D

Fraction RNAi sensitive

WT
rde-12

unc-45   pos-1   fkh-6   unc-22   bli-1

E

Fraction of animals

arrested
WT
bagged

wild-type   rde-1   rde-12

F

DCR-1   RDE-4   dsRNA trigger

RDE-1

m7G   RDE-1   AAAAAAAA   target mRNA

P bodies   ?

m7G   RDE-1   RDE-12   AAAAAAAA

m7G   RDE-12   AAAAAAAA   P granules

m7G   RDE-12   RSD-2   RDE-10   RSD-6   RDE-11   R2 bodies

RRF-1/RdRP recruitment
secondary siRNA synthesis

Mutator foci

A

	WT	rde-12(-)				
	1	2	3	4	5	6

α-RDE-12 — GFP::RDE-12 — RDE-12

α-GFP

α-Tubulin

Si[rde-12(+)] Si[rde-12(ΔFG)] Si[rde-12(DQAD)] Si[rde-12(AAA)]

GFP   RFP   merge   merge

B RDE-12   DCAP-1

C RDE-12   RSD-6

D RDE-12 (ΔFG)   RSD-6

A

letm-1p::gfp::rde-12

intestinal cells

germline

B

germline and intestinal cells

RDE-12 (ΔFG)

DCAP-1

Legend: small RNAs in coding genes | miRNA | piRNA | rRNA | other

A, B — *sel-1* RNAi treated wild-type

A: other 32.7%, small RNAs in coding genes 37.9%, piRNA 0.2%, rRNA 3.4%, miRNA 25.8%

B: other 31.9%, small RNAs in coding genes 36.4%, piRNA 0.2%, rRNA 3.7%, miRNA 27.8%

C, D — *sel-1* RNAi treated *rde-12* mutant

C: other 29.1%, small RNAs in coding genes 35.1%, piRNA 0.3%, rRNA 3.7%, miRNA 31.9%

D: other 31.3%, small RNAs in coding genes 35.9%, piRNA 0.3%, rRNA 3.8%, miRNA 28.8%

E, F — OP50 wild-type

E: other 30.3%, small RNAs in coding genes 33.6%, piRNA 0.2%, rRNA 4.1%, miRNA 31.8%

F: other 30.9%, small RNAs in coding genes 35.0%, piRNA 0.3%, rRNA 3.8%, miRNA 30.0%

G, H — OP50 *rde-12* mutant

G: other 28.0%, small RNAs in coding genes 36.1%, piRNA 0.2%, rRNA 4.1%, miRNA 31.6%

H: other 29.4%, small RNAs in coding genes 37.1%, piRNA 0.2%, rRNA 3.9%, miRNA 29.3%

I — relative small RNA abundance (*rde-12* mutant/WT)

Legend: 26G RNAs | total small RNAs

26G >1 RPM: C44B11.6, Y37E11B.2, F39E9.7, ZK380.5, W04B5.1

26G <1 RPM: H16D19.4, H09G03.1, W05H12.2, F52D2.6, K02E2.6, Y43F8B.9, Y17D7B.4, K06B9.6, W04B5.2, E01G4.5, C36A4.11, F14F7.5, Y17D7C.1

Supplemental Figure Legends

Figure S1. *rde-12* **is required for transgene silencing and RNAi.** (A) Gene structure of *rde-12*. Exons are depicted in boxes. Mutant alleles and corresponding changes in the protein sequence are indicated. (B) RDE-12 was undetectable in *rde-12(hj41)* mutant animals. The expression levels of RDE-12 in whole worm lysates from animals of the indicated genotypes were determined by Western blotting using an antibody raised against *C. elegans* RDE-12. (C) Schematic representation of the *vha-6p::atgl-1b::gfp* transgene (*hjIs21*). Images of larval stage L1 or L4 wild-type animals carrying *hjIs21* are shown, together with *rde-12(hj41)* and *rde-12(hj41);hjSi394[rde-12(+)]* L4 animals carrying the same transgene. Scale bar, 100μm. (D) *rde-12(qt131)* animals were resistant to feeding RNAi against the indicated genes. Number of animals scored n=70-280. Mean ± standard deviation. (E) *rde-12(qt131)* animals were partially sensitive to feeding RNAi against the *act-5* gene. Fraction of animals that arrested as larvae, bagged as adults or showed no phenotype (WT: wild-type) for each genotype is shown. n=60-148. Mean ± standard deviation. (F) A model for RDE-12 in the exogenous RNAi pathway in *C. elegans*.

Figure S2. RDE-12 is localized to discrete cytoplasmic foci in the soma. (A) Expression levels of endogenous RDE-12 and GFP::RDE-12 fusion proteins. Lane 1: wild-type (WT) animals without GFP::RDE-12, Lane 2: *rde-12(hj41)* animals without GFP::RDE-12, Lane 3: *rde-12(hj41);hjSi395[rde-12(+)]*, Lane 4: *rde-12(hj41);hjSi426[rde-12(ΔFG)]*, Lane 5:*rde-12(hj41);hjSi428[rde-12(DQAD)]*, Lane 6: *rde-12(hj41);hjSi429[rde-12(AAA)]*. The GFP::RDE-12(ΔFG) protein could not be detected in lane 4 by the anti-RDE-12 antibody because the epitope has been deleted. (B-D) Live embryos expressing GFP and RFP fusion proteins were imaged by confocal microscopy. The border of the eggshell is depicted by dotted lines. (B) GFP::RDE-12 and mRuby::DCAP-1. (C) GFP::RDE-12 and tagRFP::RSD-6. (D) GFP::RDE-12(ΔFG) and tagRFP::RSD-6. Solid arrowheads indicate co-localization of GFP with RFP signals. Open arrowheads indicate the positions of GFP signals that do not overlap with RFP signals. Boxed areas were magnified 3x and shown in the far right panels. Scale bars, 6.5μm.

Figure S3. Localization of wild-type and mutant GFP::RDE-12. (A) The GFP::RDE-12 protein was expressed from a single copy transgene, driven by the endogenous *rde-12* promoter *letm-1p*. *rde-12* is the second gene in an operon whose expression is driven by *letm-1p*. (B) FG Domain deleted GFP::RDE-12 was not localized to P bodies in the germline. Scale bars, 6.5μm.

Figure S4. RDE-12 promotes endogenous secondary siRNA synthesis in the somatic ERGO-1 pathway (A-H) Composition of small RNA libraries. Pie charts showing the relative abundance of indicated small RNAs in each of the eight libraries. (A, B) Wild-type animals subjected to *sel-1* RNAi. (C, D) *rde-12(hj41)* mutant animals subjected to *sel-1* RNAi. (E, F) Wild-type animals without RNAi. (G, H) *rde-12(hj41)* mutant animals without RNAi. (I) The relative abundance of 26G RNAs and total endo-siRNAs in target genes of the somatic ERGO-1 pathway (wild-type (WT)=1.0).

	pos-1	mex-3	unc-15	nhr-23	elt-2	flr-1
	germline	germline	muscle	hypodermis	intestine	intestine
Wild type(N2)	+++	+++	+++	+++	+++	+++
rde-1(ne300)	-	-	-	-	-	-
rde-12(hj41)	-	-	-	-	-	-
rde-12(hj41);hjSi394[rde-12(+)]	+++	+++	+++	+++	++	+++
rde-12(hj41);hjSi425[rde-12(+)]	+++	+++	+++	+++	++	+++
rde-12(hj41);hjSi395[rde-12(+)]	+++	+++	+++	+++	++	+++
rde-12(hj41);hjSi426[rde-12(ΔFG)]	-	-	-	-	-	-
rde-12(hj41);hjSi428[rde-12(DQAD)]	-	-	+	++	+	+
rde-12(hj41);hjSi429[rde-12(AAA)]	-	-	++	+++	++	++

Table S1. RDE-12 is required for RNAi in the soma and the germline. Animals of the indicated genotype were grown on *E. coli* (HT115) expressing double stranded RNA at 20°C. Strong phenotype of high penetrance was indicated by (+++) and resistance to RNAi was indicated by (-). Observations were made from at least three independent experiments.

| sel-1 RNAi | | | reads per million mapped reads (rpm) | | | | | |
| | | | 5' to trigger | 5' to trigger | trigger | trigger | 3' to trigger | 3' to trigger |
			sense	antisense	sense	antisense	sense	antisense
+	wild type	sample 1	0.91	5380.84	118.75	18029.09	0.32	1266.57
+		sample 2	0.78	5461.92	127.14	17166.78	0.50	1259.59
+	rde-12(hj41)	sample 1	1.05	85.44	208.53	441.33	0.58	230.82
+		sample 2	0.65	84.91	196.55	457.65	0.74	239.80
-	wild type	sample 1	0.04	8.13	0.33	10.77	0.18	14.82
-		sample 2	0.00	8.37	0.22	11.60	0.36	16.27
-	rde-12(hj41)	sample 1	0.00	9.67	0.27	11.73	0.09	15.89
-		sample 2	0.08	7.82	0.35	9.93	0.12	16.60

| sel-1 RNAi | | | reads per kilobase per million mapped reads (rpkm) | | | | | |
| | | | 5' to trigger | 5' to trigger | trigger | trigger | 3' to trigger | 3' to trigger |
			sense	antisense	sense	antisense	sense	antisense
+	wild type	sample 1	1.73	10229.73	259.28	39364.84	0.30	1179.31
+		sample 2	1.48	10383.88	277.60	37482.06	0.46	1172.80
+	rde-12(hj41)	sample 1	2.00	162.44	455.31	963.60	0.54	214.91
+		sample 2	1.23	161.43	429.14	999.23	0.69	223.28
-	wild type	sample 1	0.07	15.46	0.71	23.52	0.17	13.80
-		sample 2	0.00	15.91	0.47	25.33	0.33	15.15
-	rde-12(hj41)	sample 1	0.00	18.39	0.58	25.62	0.08	14.80
-		sample 2	0.15	14.87	0.75	21.68	0.11	15.46

Table S2. Deep sequencing results of 21-26nt small RNAs that mapped to the *sel-1* mRNA in wild-type and *rde-12* mutant animals undergoing *sel-1* RNAi or no RNAi.

Experimental Procedures

Strains and transgenes
The wild-type strain was Bristol N2. All animals were raised at 20°C unless specified. The following alleles and transgenes were used:

LGI: *rde-10(hj20), rrf-1(pk1417)*
LGV: *rde-1(ne300), rde-12(hj41), rde-12(hj57), rde-12(hj58), rde-12(hj59), rde-12(qt131)*

hjSi10[rde-10p::3xFLAG-TEV-GFP::rde-10]
     Generated by MosSCI (Chro II), outcrossed 2 times with N2.
hjSi394[letm-1p::3xFLAG-TEV::rde-12]
     Generated by MosSCI (Chro II), outcrossed 2 times with N2.
hjSi395[dpy-30p::gfp::rde-12]
     Generated by MosSCI (Chro II), outcrossed 2 times with N2.
hjSi396[dpy-30p::mRuby::pgl-1]
     Generated by MosSCI (Chro I), outcrossed 2 times with N2.
hjSi397[dpy-30p::mRuby::dcap-1]
     Generated by MosSCI (Chro I), outcrossed 2 times with N2.
hjSi425[letm-1p::gfp::rde-12]
     Generated by MosSCI (Chro II), outcrossed 2 times with N2.
hjSi426[dpy-30p::gfp::rde-12(AA875-959 deleted)]
     Generated by MosSCI (Chro II), outcrossed 2 times with N2.
hjSi428[dpy-30p::gfp::rde-12(DQAD)]
     Generated by MosSCI (Chro II), outcrossed 2 times with N2.
hjSi429[dpy-30p::gfp::rde-12(AAA)]
     Generated by MosSCI (Chro II), outcrossed 2 times with N2.
hjSi430[dpy-30p::rsd-6::tagRFP-TEV-3xFLAG]
     Generated by MosSCI (Chro I), outcrossed 2 times with N2.
hjSi431[dpy-30p:: tagRFP::rsd-6]
     Generated by MosSCI (Chro I), outcrossed 2 times with N2.
hjIs21[vha-6p::atgl-1b cDNA::GFP]
     Generated by UV irradiation, outcrossed 5 times with N2.

Genetic Screen

We mutagenized *hjIs21* animals with ethyl methane-sulfonate (EMS) using standard procedures for mutants that expressed ATGL-1::GFP in the adult stage. We screened ~20,000 haploid genomes and isolated ~20 transgene desilencing mutants. RNAi screen and candidate gene sequencing identified a C to T mutation that causes a pre-mature stop codon (Q262stop) in

F58G11.2 in *rde-12(hj41)*. A non-complementation screen was performed to identify additional mutant alleles of *rde-12*. We screened ~50,000 haploid genomes and isolated 3 more alleles of *rde-12*: *hj57*, *hj58* and *hj59*. Six additional *rde-12* mutant alleles were recovered from an independent genetic screen, originally designed to identify mutants that were defective in systemic RNAi (Fig. S1A). We used *hj41* as the reference allele because no RDE-12 protein was detectable in *rde-12(hj41)* mutant animals (Fig. S1B). The RNAi defective phenotype in *rde-12(hj41)* was rescued by single copy transgenes *hjSi394*, *hjSi395* and *hjSi425*.

RNAi experiments

RNAi was carried out as previously reported [S1]. Bacterial clones expressing dsRNA were obtained from the Ahringer RNAi library except for *dpy-28* (PCR product of 5'-cgaaacgtgcttcaactag-3' and 5'-atgtccatgtcgattattatcc-3'), *sel-1* (PCR product of 5'-tgcatggagccggaatcgga-3' and 5'-tgcatcggcacttcctgact-3') and *flr-1* (PCR product of 5'-ttggcagggaaaagctacat -3' and 5'-ctcctcctcagcaactgcat -3') RNAi clones. For *unc-15* RNAi experiments, 30 OD_{600} of bacteria was seeded on 100mm RNAi plates. Plates were left at room temperature overnight for dsRNA expression. 6000 L1 larvae were seeded on plates in triplicate for each strain. After 42 hrs at $20^{o}C$, worms were washed extensively with M9 and RNA was extracted with TRI Reagent (Molecular Research Center). Feeding RNAi assays in Fig. S1D and S1E were performed as described [S2], except that embryos, rather than L4s, were moved to the RNAi food. These embryos were then scored either four (*act-5* bagging) or three (for all other phenotypes) days later. Injection of dsRNA was performed essentially as described [S3]. Briefly, to obtain *pal-1* dsRNA, flanking T7 promoter sequences were added to the *pal-1* cDNA by PCR. Sense and antisense RNA was produced using the Ambion (Austin, TX) AmpliScribe T7 kit. RNA was purified by phenol/chloroform extraction, re-suspended in water, denatured at $70^{o}C$, and then allowed to re-anneal by cooling to $20^{o}C$ at .1°C/second in a PCR block. Concentration of dsRNA was determined by nanodrop. Injection into both gonads was performed as described [S4].

Real-time PCR

RNA was extracted using TRI reagent (Molecular Research Center) according to manufacturer's instructions. Genomic DNA contamination was removed using the TURBO DNA-free kit (AM1907, Ambion). cDNAs were synthesized using ImProm-II Reverse Transcription System (Promega). The cDNA was subject to real-time PCR analysis using the IQ SYBR Green supermix (Bio-Rad) on an iCycler (Bio-Rad).

Primer pairs (sequence from 5' to 3') for real-time PCR used in this study were:

unc-15, ccagagagtccgcagatacc and ccgtgacgaaaatcttggag
rpl-32, agggaattgataaccgtgtccgca and tgtaggactgcatgaggagcatgt
gpd-3, tggagccgactatgtcgttgag and gcagatggagcagagatgatgac
C44B11.6, acgtatagttgtttatggtgcatgc and gaactggaaatcttcacaacgat
E01G4.5, cggcaatttattctagagcacac and gtcaaacaacagctttccaacg
F39E9.7, cccagtggcccaattaaacg and cccacggcttgttctttgaca
H09G03.1, atgaagagcaggcccaaa and gcttcgattgcacaactgtc
K02E2.6, gacccaacctatcaactacaag and catttcggtagggcaatctttgtc
W04B5.1, ctaaatgaggccaaccaagc and gccaagtggaattttcttctc

elt-2
5' coding region: ctccaattgaacggcaaagt and cggttgcatggtagtttggt

dpy-28
5' coding region: caaagtcgtcgattatctcgtt and caccaattccacatcgacac

flr-1
5' coding region: aacggagacggaaagtgaaa and ccactacaacccagaaaatgc

sel-1
5' coding region: ctctggtatctgcggaagga and ctcatcttgctgtgatgtgacc

For quantitation of *elt-2* 22G-siRNA using Taqman assays, 7000 synchronized L1 worms of each strain were grown on 100mm *elt-2* RNAi plates. After 42 hrs at 20°C, worms were collected and washed with M9 for 4 times. Total RNA was extracted with Tri-reagent (Molecular Research Center). The Taqman small RNA assay was designed by Life Technologies using the sequence 5'-GUCCCAAAGAAGUGCUAUACGC and performed according to manufacturer's instructions. Three independent biological samples of each strain were assayed in triplicates.

RNA Immuno-precipitation

RDE-12 RNA IP (RIP) was performed as previously described [S5]. To isolate RDE-12 associated total RNAs after RNAi, synchronized L1 animals were seeded onto 100mm RNAi plates and collected after ~42 hrs at 20°C. Animals were sonicated in lysis buffer (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 2.5 mM MgCl$_2$, 0.5% NP-40, and 10% glycerol, with proteinase inhibitor tablet EDTA-free (Roche) and RNaseOUT (Invitrogen)). 3xFLAG::RDE-12 was immunoprecipitated with Dynabeads M280 pre-coated with the anti-FLAG antibody (Sigma, F1804). After extensive washes, 3xFLAG::RDE-12 was eluted with 150 ug/mL 3xFLAG

peptide. Eluates were first incubated with Turbo DNase I (Ambion) at 37°C for 20 minutes. The RNAs were then purified with the TRI Reagent, followed by isopropanol precipitation. cDNAs were generated from RNAs with ImProm-II Reverse Transcription System using a mixture of oligo(dT) and random hexamer primers. Target mRNA enrichment was normalized by non-specific binding of *gpd-3* mRNA.

Live imaging of *C. elegans*

Confocal images of L4 larval animals were acquired on a spinning disk confocal microscope (Ultraview, Perkin Elmer) using a 100x, NA1.45 oil Plan-Apochromat objective, on a CCD camera (Orca-R2, Hamamatsu) controlled by the Volocity software (Perkin Elmer). For GFP, a 488nm laser was used for excitation and signals were collected with a 500-555nm emission filter. For mRuby or tagRFP, a 561nm laser was used for excitation and signals were collected with a (415-475nm) (580-650nm) dual-pass emission filter. For auto-fluorescence from lysosome related organelles, a 488nm laser was used for excitation and signals were collected with a (415-475nm) (580-650nm) dual-pass emission filter.

Antibodies

Monoclonal antibodies against the FLAG epitope (clone M2; Sigma), and polyclonal antibodies against RDE-12 (raised against AA940~959, YZ2004, Yenzym) and GFP (YZ769, Yenzym) were used for Western blotting. For immunoprecipitation, anti-FLAG M2 Affinity Gel (A2220, Sigma) was used.

Small RNA cloning and deep sequencing

Synchronized L1 worms were grown on RNAi plates seeded with HT115 *E. coli* expressing a *sel-1* RNAi trigger as previously described [S6] or OP50. After 50 hrs at 20°C, ~50000 worms were washed 5 times with 10ml M9, and then homogenized in Tri-reagent. Samples were kept at -80°C. Purified total RNA was processed with MirVana kit (Invitrogen) for small RNA enrichment. Equal amount of small RNAs (30~40ug) were resolved on 15% TBE-Urea Acrylamide gels. Gels containing small RNAs of 20-26nt were cut out and purified with a small-RNA PAGE Recovery Kit (Zymo Research, R1070). Each small RNA sample was treated with TAP and processed with the ScriptMiner Small RNA-Seq Library Preparation Kit (Epicentre, SMMP1012). Libraries from different samples were indexed with RNA-Seq Barcode Primers (Epicentre, RSBC10948). Two biological samples of each condition (wild type +*sel-1* RNAi, *rde-12*+*sel-1* RNAi, wild type+OP50 and *rde-12*+OP50) were used for library preparation. A total of 8 libraries were pooled together and subject to Illumina HiSeq 2000-50bp single read

deep sequencing. Sequencing reads were clipped with Fastx_clipper (v0.0.13) to remove adaptor sequence and aligned to the UCSC reference genome ce6 with Tophat (v1.4.1). The aligned reads ranged from 25.7 to 33.9 million for each library. The coverageBed component of BEDtools was used to count the reads of each region and small RNAs. RPMs were calculated by (reads_aligned_to_region / all_aligned_reads_for_sample) * 1000000. The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO). The sequences for 375 *C. elegans* miRNA were extracted from miRBase. The sequences for piRNA were based on previous study [S7]. The target genes of four classes endo-siRNA are listed in previous study [S8-11]. Sequences are available upon request.

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