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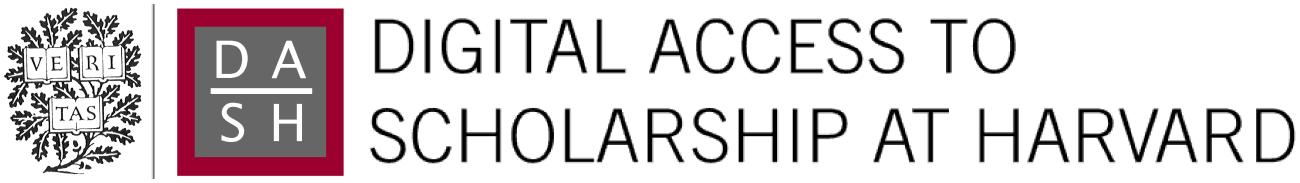

Two Classes of Silencing RNAs Move between Caenorhabditis elegans Tissues

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Two classes of silencing RNAs move between *C. elegans* tissues.

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Summary: Organism-wide RNA interference (RNAi) is due to the transport of mobile silencing RNA throughout the organism but the identities of these mobile RNA species in animals are unknown. Here we present genetic evidence that both the initial double-stranded RNA (dsRNA), which triggers RNAi, and at least one dsRNA intermediate produced during RNAi can act as or generate mobile silencing RNA in *Caenorhabditis elegans*. This dsRNA intermediate requires the long dsRNA-binding protein RDE-4, the endonuclease DCR-1, which cleaves long dsRNA into double-stranded short-interfering RNA (ds-siRNA), and the putative nucleotidyltransferase MUT-2 (RDE-3). However, single-stranded siRNA and downstream secondary siRNA produced upon amplification by the RNA-dependent RNA Polymerase RRF-1 do not generate mobile silencing RNA. Restricting inter-tissue transport to long dsRNA and directly processed siRNA intermediates rather than amplified siRNA may serve to modulate the extent of systemic silencing in proportion to available dsRNA.

Intercellular transport of RNA has been inferred in plants and animals undergoing gene silencing by RNAi[1]. In plants, short-interfering RNAs (siRNA) processed from long dsRNA move between cells through intercellular bridges called plasmodesmata and travel long distances via the phloem to convey gene-specific silencing information[2,3,4]. Although the nature of mobile silencing signals in animals is unknown, the conserved RNA transporter SID-1 is required for their import in *Caenorhabditis elegans* and has been implicated in RNA transport in other animals[5,6,7]. In addition, dsRNA expressed in multiple tissues can generate *sid-1*-dependent mobile silencing RNA through an as yet unknown pathway[8]. Since animals transcribe dsRNA from numerous loci[9], understanding how mobile RNA is produced from dsRNA has broad implications for systemic control of gene expression.

Multiple distinct RNA species are produced during RNAi in *C. elegans*, but, it is unclear which of these are mobile (**Fig. 1a**)[10,11,12,13]. These RNA species include transcribed sense and anti-sense duplexes (dsRNA), double-stranded short-interfering RNA (ds-siRNA) generated upon cleavage of long dsRNA by the RDE-4–Dicer(DCR-1) complex, primary single-stranded siRNA generated upon cleavage of ds-siRNA by the Argonaute RDE-1[13], and the subsequent numerous secondary siRNAs generated by RNA-dependent RNA polymerases (RdRP) that are responsible for potent silencing of the target gene. In addition, enzymes that can modify RNA such as the putative nucleotidyltransferase MUT-2[14,15,16], which is required for efficient RNAi (**Supplementary Fig. 1**), may also generate RNA species that act as mobile RNA. Early studies using dsRNA injected into the cytoplasm of gut cells suggested that RNA silencing in gut cells is not required to transport a mobile silencing signal to the germline[17,18]. However, whether this signal is the injected exogenous dsRNA itself or a dsRNA-derived mobile RNA or both is unclear and how endogenously transcribed dsRNA leads to the production of mobile RNA is unknown.

Here we determine the genetic requirements for silencing due to mobile RNAs using well-characterized promoters to restrict the expression of dsRNA or RNAi pathway genes to specific tissues and examining target gene silencing in other tissues. In most experimental systems that use similar approaches, it is difficult to control for low levels of misexpression in the target tissues. Since SID-1 is strictly required for the import of mobile silencing RNAs[8], SID-1-dependence of silencing serves to clearly distinguish silencing due to mobile RNA from silencing due to misexpression in the target tissues.

RESULTS

Long dsRNA is mobile in *C. elegans*

We examined how endogenously transcribed dsRNA produces mobile silencing RNA using mosaic animals (i.e. animals that have some mutant cells and some wild-type cells) in which a mutant donor tissue expresses dsRNA that targets a gene in a wild-type recipient tissue (**Fig. 1b**). To determine whether the activity of the primary Argonaute RDE-1 is required to produce a mobile silencing signal, we first expressed dsRNA targeting the green fluorescent protein (*gfp*) in the pharynx of *rde-1(-)* animals. We then coexpressed *gfp* and *rde-1(+)* in the body-wall muscle (bwm) cells, making bwm a wild-type recipient tissue (**Fig. 1c** and **Supplementary Fig. 2**). We observed *gfp* silencing in anterior *rde-1(+)* bwm cells. Thus, RNAi-mediated silencing in the pharynx is not required to produce and transport mobile RNA to the bwm cells. To determine whether RNAi pathway genes upstream of RDE-1 are required to produce a mobile silencing signal from expressed dsRNA, we developed a sensitive assay that measures silencing of an endogenous gene due to mobile RNA (**Fig. 1d** and **Supplementary Fig. 2**). Specifically, we introduced a neuronally expressed transgene that produces a ~560 bp dsRNA that targets the muscle gene *unc-22* (*neur::u22ds*). All *unc-22* silencing detected

in animals with the *neur::u22ds* transgene required the RNAi pathway genes and the RNA transporter SID-1, showing that all silencing occurred through RNAi in these animals and was due to mobile RNA enabled RNAi (**Fig. 1d**). Using this source of mobile RNA, we detected *unc-22* silencing in *rde-4(-)* animals that expressed *rde-4(+)* in bwm cells and in *mut-2(-)* animals that expressed *mut-2(+)* in bwm cells (**Fig. 1e**). Thus, neither dsRNA cleavage through RDE-4 recruitment of Dicer nor modification by the nucleotidyltransferase MUT-2 is required in neurons that express dsRNA for the generation and export of mobile RNA. Together, these results show that a mobile RNA is generated from transcribed long dsRNA, independent of processing by the canonical RNAi pathway, can generate a mobile silencing RNA.

A processed dsRNA also moves between cells

To determine whether products of dsRNA processing by the canonical RNAi pathway are also mobile, we expressed dsRNA in a wild-type, RNAi proficient donor tissue and examined silencing in RNAi defective recipient tissues. If a processed RNA produced in the wild-type donor tissue can act as or generate a mobile silencing RNA, that RNA may bypass the lack of the earlier-acting RNAi pathway gene in the recipient tissue and cause silencing. Note that using this approach we cannot infer anything about RNAs that move between tissues but that fail to cause gene silencing.

To detect silencing triggered by mobile processed RNAs we rescued RNAi pathway mutants only in neurons of animals that contain the neur::u22ds transgene and measured *unc-22* silencing in the mutant target muscle cells. We detected *unc-22* silencing in *rde-4(-)* animals that expressed *rde-4(+)* in neurons. Consistent with silencing due to mobile RNAs, SID-1 is required for the observed silencing (**Fig. 1f**). Since RDE-4 is required for DCR-1 cleavage of long dsRNA into ds-siRNA[19], these mobile RNAs are either ds-siRNA or downstream RNAi products. To distinguish between these two possibilities, we similarly examined the role of the primary Argonaute RDE-1 in the production of mobile RNA. In contrast to the analogous experiment with RDE-4, we observed no detectable *unc-22* silencing in *rde-1(-)* animals that express *rde-1(+)* in neurons. This observation suggests that primary siRNA and downstream RNAi products such as RdRP-dependent secondary siRNA are not mobile. Finally, we detected *unc-22* silencing in *mut-2(-)* animals that expressed *mut-2(+)* in neurons (**Fig. 1f**) and this silencing was due to mobile RNA since it required SID-1 (**Fig. 1d**). Therefore, we infer that like RDE-4, MUT-2 functions upstream of RDE-1 to generate a species of mobile RNA that can bypass the need for MUT-2 activity in the recipient tissue.

Mobile RNAs are similarly made from other sources of dsRNA.

We next tested whether other sources of silencing RNAs also rely on the same genes to produce mobile RNAs. Multicopy transgenes such as *sur-5::gfp* (which express nuclear-localized GFP in all somatic tissues) can generate mobile RNAs, presumably from trace amounts of dsRNA produced from the transgene[8,20]. We therefore generated *rde-4(-);sur-5::gfp* animals and moved a representative transgene that expresses *rde-4* in bwm cells (*bwm::rde-4(+)*) into these animals. Significant silencing ($P<0.05$) was detected in non-muscle tissues in the resultant mosaic animals and was most easily observed in the prominent gut nuclei (**Fig. 2a,b**). Consistent with silencing due to mobile RNAs, SID-1 is required for the observed silencing of gut nuclei (**Fig. 2c,d**). However, when we moved a representative transgene that expressed *rde-1(+)* in bwm cells (*bwm::rde-1(+)*) into *rde-1(-); sur-5::gfp* animals, we observed no detectable silencing of GFP expression in the gut. In contrast, moving a representative transgene that expressed *mut-2(+)* in bwm cells (*bwm::mut-2(+)*) into *mut-2(-); sur-5::gfp* animals resulted in the silencing of GFP expression in the gut (**Fig. 2d**). The observed silencing was dependent on SID-1,

showing that mobile RNA triggered the silencing in *mut-2(-)* gut cells (**Fig. 2d**). Therefore, as in the case of expressed dsRNA, multicopy transgenes also generate mobile RNAs that are upstream of RDE-1 and include those that are processed by RDE-4 and MUT-2.

We next tested whether RDE-4 and MUT-2 but not RDE-1 can similarly process exogenously supplied dsRNA to produce mobile RNA. We fed bacteria that express *gfp*-dsRNA (feeding RNAi)[21] to the above *rde-1(-)*, *rde-4(-)*, and *mut-2(-)* mutants that contain the *sur-5::gfp* transgene and that are rescued in muscle cells and examined silencing in the respective mutant gut cells. Consistent with our results with endogenously transcribed dsRNA, we found that *gfp* feeding RNAi increased silencing of GFP in the non-muscle cells of muscle-rescued *rde-4(-)* and *mut-2(-)* animals but not of muscle-rescued *rde-1(-)* animals (**Fig. 3a**). To assay silencing due to feeding RNAi targeting endogenous genes, we removed the *sur-5::gfp* transgenes from the transgenic bwm rescue lines and then fed these muscle-rescued animals bacteria that express dsRNA targeting the muscle gene *unc-22* or that express dsRNA targeting the skin gene *dpy-7* or that express dsRNA targeting the intestinal gene *act-5*. Silencing due to feeding RNAi of these endogenous genes was consistent with our results using *gfp* feeding RNAi and using endogenously transcribed dsRNA. Specifically, while we observed robust silencing of the muscle gene in all three strains of muscle-rescued animals, silencing of the skin and intestinal genes was detectable in muscle-rescued *rde-4(-)* and *mut-2(-)* animals (**Fig. 3b**) but not in muscle-rescued *rde-1(-)* animals (**Fig. 3b**, ref. 22). Thus, the silencing observed in these *rde-4* and *mut-2* mosaic animals by feeding RNAi is likely due to import of ingested long dsRNA into the rescued muscle cells followed by export of a processed mobile RNA that can silence the target genes in *rde-4(-)* and *mut-2(-)* cells. Therefore, both multicopy transgenes and ingested dsRNA use the same genetic pathway to produce short mobile silencing RNA.

Two classes of upstream dsRNAs are mobile RNAs

Taken together, our results suggest a model whereby upstream dsRNA species such as long dsRNA and ds-siRNA act as or generate mobile RNA, while all silencing RNAs produced after cleavage of ds-siRNA by RDE-1 cannot cause silencing in *rde-1(-)* cells (**Fig. 4a**). Since Dicer can cleave long dsRNA in the absence of MUT-2[12] and since MUT-2 acts upstream of RDE-1 to generate mobile RNA, one possible role for MUT-2 in RNAi is to modify ds-siRNA. Despite MUT-2 having the required catalytic residues, a systematic test of putative nucleotidyltransferases using *in vitro* assays failed to reveal how MUT-2 might modify RNA[23]. Nevertheless, consistent with our model, overexpression of neither *mut-2(+)* nor *rde-4(+)* in bwm cells of *rde-1(-); sur-5::gfp* animals resulted in detectable silencing (**Supplementary Table 1**). Further, neither overexpression of *mut-2(+)* in bwm cells of *rde-4(-); sur-5::gfp* animals nor overexpression of *rde-4(+)* in the bwm cells of *mut-2(-);sur-5::gfp* animals resulted in detectable silencing (**Supplementary Table 1**), suggesting that RDE-4 and MUT-2 act in the same pathway to generate mobile RNA.

The following tests provide additional support for the model that long and short dsRNAs, but not single-stranded siRNA act as mobile silencing RNA: (1) The RdRP RRF-1, which makes numerous downstream secondary siRNAs, is not required for the generation of mobile RNAs (**Fig. 4b**); (2) Rescuing a partial loss-of-function *dcr-1* mutant[24] in the recipient tissue (which increases processing of imported Dicer precursors [long dsRNA]) improves silencing, presumably of imported long dsRNA (**Fig. 4b**); (3) Rescuing the *dcr-1* mutant in donor tissues also increased silencing in recipient cells, presumably by increased transport of ds-siRNA (**Fig. 4b**); and (4) expression of

Inhibitors of RNAi in recipient cells, including the conserved exonuclease ERI-1 that can degrade ds-siRNA[25], inhibited silencing (**Supplementary Fig. 3**).

DISCUSSION

We provide evidence for the existence of at least two distinct species of mobile RNA in *C. elegans*: one that is produced from long dsRNA independent of RNAi genes in donor tissues but requires all tested RNAi genes for silencing in recipient tissues and one that requires RDE-4, DCR-1, and MUT-2 for production in donor tissues but not for silencing in recipient tissues.

Animal mobile silencing RNAs differ from plant mobile RNAs

In plants, mobile RNAs move between cells through relatively non-selective intercellular bridges called plasmodesmata[2,3]. In the plant *Arabidopsis*, grafting experiments between genetically distinct source and target tissues have enabled the molecular identification of mobile RNAs. These studies have identified both single-stranded siRNA and ds-siRNA whose movement to distant tissues correlates with mobile RNAs[2,3]. In addition, accumulating evidence supports the intercellular movement of microRNAs, tasiRNAs, and mRNAs[26].

Our results indicate that in *C. elegans*, long dsRNA and a form of ds-siRNA can move between cells (**Fig. 4a**). Unexpectedly, and in contrast to what is observed in plants, single-stranded siRNAs produced by RdRP amplification are either not mobile, or if mobile, are incapable of causing detectable silencing in recipient cells. Consistent with mobile silencing signals being restricted to double-stranded forms of RNA, most systemic RNAi silencing observed in *C. elegans* is dependent on SID-1[5,8], which is exquisitely selective for dsRNA[27]. This restriction couples the extent of RNAi spreading to the amount of primary dsRNA produced within cells or imported from the environment.

A conserved pathway to make animal mobile RNAs

Since a mammalian SID-1 homolog can transport ds-siRNAs into mammalian cells[7], ds-siRNA, perhaps modified by a nucleotidyltransferase, may move between mammalian cells. Importantly, short dsRNAs can escape the interferon response that results in non-specific effects in differentiated mammalian cells[28], thus their transport between differentiated tissues should be tolerated. In contrast, the transport of long dsRNA will result in specific gene silencing only in undifferentiated mammalian cells. Furthermore, the protein activities required to make short mobile RNA in worms are found in most animals: dsRNA-binding proteins, such as RDE-4, that act with Dicer (e.g. PACT and TRBP with human Dicer[29]) and β-nucleotidyltransferases, such as MUT-2, that play a role in RNA silencing[14,15,16]. Modulation of such conserved biochemical pathways may contribute to the tissue- and environment-dependent differences in silencing due to mobile RNA that are observed in *C. elegans*[30,8]. Regulated transport of mobile RNA is evident in plants, where mobile RNA produced in metabolic source tissues control gene expression in distant metabolic sink tissues[31]. Similarly, *C. elegans* mobile RNAs are preferentially imported into cells that express SID-1 at high levels[32,8], suggesting that SID-1 expression produces a sink for mobile RNA. Therefore, short dsRNAs produced from endogenous loci in a mammalian cell may control gene expression in another cell type that expresses a SID-1 homolog.

Note: *Supplementary Information is available on the Nature Structural & Molecular Biology website.*

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Author contributions A.M.J. performed the experiments and G.G. generated most of the DNA constructs; A.M.J. and C.P.H. designed the study, analyzed the data, and wrote the paper. All authors discussed the results and commented on the manuscript.

Figure Legends

Figure 1 RNAi-independent biogenesis of mobile RNA from expressed dsRNA. (**a**) Schematic of RNAi within a cell. See text for details. (**b**) Schematic of assay to measure silencing due to mobile RNA. dsRNA that targets a gene in recipient cells are expressed only in other donor cells. (**c**) A representative *rde-1(-)* L4 animal that expresses *gfp*-hairpin RNA only in the pharynx (*phar::hp-gfp*) but *gfp* in pharynx and bwm cells (left panel) and one that in addition coexpress *rde-1(+)* and DsRed only in bwm cells (right panel). 100% of animals from three independent *bwm::rde-1(+) & DsRed* lines showed silencing as above. Scale bar, 50 μm. (**d-f**) A representative transgenic line that expresses *unc-22*-dsRNA under the control of the neuronal *rgef-1* promoter (*neur::u22ds*) was generated in wild-type animals and crossed into the genetic backgrounds indicated. The uncoordinated twitching (Unc) due to the silencing of *unc-22* was measured in these animals (black) and in animals that in addition had the corresponding RNAi gene rescued in body-wall muscles (white) or in neurons (blue). n=100 animals; error bars indicate 95% confidence intervals; and asterisks indicate significant differences (P<0.05). Partial silencing in rescued transgenic lines likely indicate that levels of the rescuing genes are inadequate for complete silencing in response to the low levels of neuronal *unc-22* dsRNA. Consistently, feeding *unc-22* dsRNA to animals with the same muscle-rescued transgenic lines above results in complete silencing (**Fig. 3**). See **Supplementary Fig. 2** for details of constructs used.

Figure 2 Restricted expression of RDE-4 and MUT-2 but not RDE-1 enables non-cell autonomous RNA silencing. (**a-c)** Representative animals that express nuclear-localized GFP in all cells (*sur-5::gfp*). (**a**) *rde-4(-)*. (**b**) *rde-4(-)* animals with *rde-4(+)* and DsRed expressed in bwm cells (*qtEx[bwm::rde-4(+) & DsRed]*). (**c**) *rde-4(-)* animals that only express DsRed in bwm cells (*qtEx[bwm::DsRed]*). Brackets indicate silencing in gut nuclei. Insets are widefield (**a**) or red channel (**b**, **c**) images. Scale bars, 50 μm. Note that unlike overexpression of *rde-4(+)*, overexpression of the coinjection marker DsRed did not result in any silencing of *gfp* expression [compare (**b)** and (**c)**]. When coexpressed with *rde-4(+)*, DsRed expression was lower (enhanced in (**b)** inset to clearly indicate expression in the bwm), which likely reflects enhanced silencing of the DsRed transgene. (**d**) The number of brightly fluorescent gut nuclei that show *sur-5::gfp* expression were counted in *rde-1(-)*, *rde-4(-)*, and *mut-2(-)* mutant backgrounds as well as in mutant animals with a corresponding representative bwm rescue transgenes from **Fig. 1e**. Similar experiments done with *rde-4(-); sid-1(-)* and *mut-2(-); sid-1(-)* double mutant backgrounds are also shown. n=25 L4 animals. Averages (red bars), significant differences (brackets and *, P<0.05) and similar values (brackets) are indicated. Minor variations in the average number of nuclei (± 2 nuclei) observed between animals were not due to silencing of *gfp* expression but rather due to small changes in the number of intestinal nuclei (see **Supplementary Fig. 4** and the discussion therein).

Figure 3 RDE-4 and MUT-2 but not RDE-1 processed ingested dsRNA is mobile. (**a**) Feeding RNAi of *rde-1(-)*, *rde-4(-),* and *mut-2(-)* animals with *sur-5::gfp* and their corresponding representative bwm rescue transgenic lines used in **Fig. 2d**. The number of brightly fluorescent gut nuclei that show *sur-5::gfp* expression were counted in L4 animals that were fed either control bacteria (brown, re-plotted from **Fig. 2d**) or bacteria expressing *gfp*-dsRNA (blue). n=25 animals. Averages (red bars), significant differences (brackets and *, P<0.05) and similar values (brackets) are indicated. (**b**) Feeding RNAi of strains in (**a)** after removal of *sur-5::gfp*. L4 animals were fed L4440 (control) or dsRNA

targeting the muscle gene *unc-22* (bwm) or the skin gene *dpy-7* (skin) or the gut gene *act-5* (gut) and the percentage of L4 progeny that showed the corresponding defects were determined. n=100 L4 animals; error bars indicate 95% confidence intervals.

Figure 4 Biogenesis of mobile RNA in *C. elegans*. (**a**) Schematic of the biogenesis pathway. Double-stranded forms of RNA produced during the early steps of RNA interference act as or generate mobile RNAs. RNAs produced after the Argonaute RDE-1 cleaves ds-siRNA to release single stranded RNA[13] are restricted to intracellular silencing. MUT-2 expression enables the generation and export of mobile RNA possibly through enzymatic modification of dsRNA. Since expressed as well as ingested dsRNA generate mobile RNA, additional regulation in response to the environment and selection of specific endogenous loci to make mobile RNAs is likely. (**b**) RdRP activity is not required for mobile RNA production and dsRNAs both upstream and downstream of Dicer generate mobile RNAs. The representative transgene used in **Fig. 1** to express *unc-22*-dsRNA under the control of the neuronal *rgef-1* promoter (*neur::u22ds*) was crossed into the genetic backgrounds indicated. Two deletion alleles of *rrf-1* (*pk1417* & *ok589*) were rescued with *rrf-1(+)* in bwm and the missense allele *dcr-1(bp132)* was rescued with *dcr-1(+)* in the bwm and in neurons. Silencing of *unc-22* was measured (% Unc) in the mutant animals (black) and in animals with the corresponding RNAi gene rescued in bwm (white) and in neurons (blue). n=100 animals. 95% confidence intervals (error bars) and significant differences (brackets and *, P<0.05) are indicated. See **Supplementary Fig. 2** for details of constructs used.

ONLINE METHODS

Strains Used. N2 wild-type, HC196 *sid-1(qt9)*, WM30 *mut-2 or rde-3(ne298)*, WM27 *rde-1(ne219)*, WM49 *rde-4(ne301)*, NL2099 *rrf-3(pk1426)*, GR1373 *eri-1(mg366)*, HC70 *rde-1(ne219); mIs11[Pmyo-2::gfp]; ccIs4251[Pmyo-3::gfp]; qtIs3[Pmyo-2::hp-gfp]*, PD4792 *mIs11[myo-2::GFP, gut::GFP, pes-10::GFP]*, HC195 *nrIs20[sur-5::gfp]*, HC731 *sid-1(qt9); eri-1(mg366)*, HC732 *sid-1(qt9); rrf-3(pk1426)*, HC733 *mut-2(ne298); sid-1(qt9); nrIs20*, HC734 *sid-1(qt9); rde-4(ne301); nrIs20*, HC735 *mut-2(ne298); mIs11*, HC736 *qtEx136[Prgef-1(F25B3.3)::unc-22sense; Prgef-1::unc-22antisense; Prgef-1::DsRed line 8],* HC737 *rde-4(ne301); nrIs20,* HC738 *rde-1(ne219); nrIs20,* HC739 *mut-2(ne298); nrIs20*, HZ202 *dcr-1(bp132); wIs51[scm-1::GFP]*[24], RB798 *rrf-1(ok589),* NL2098 *rrf-1(pk1417),* HC779 *dcr-1(bp132)* [outcrosssed with N2 twice], HC780 *rrf-1(ok589)* [outcrossed with N2 twice], HC781 *rrf-1(pk1417)* [outcrossed with N2 twice], HC782 *sid-1(qt9) rde-1(ne219)*, HC783 *sid-1(qt9); rde-4(ne301)*, HC784 *sid-1(qt9); mut-2(ne298)*.

Strain constructions and transgenic analyses. Double mutants were made using standard genetic approaches and were verified by genotyping using DNA sequencing or PCR analysis. Additional strains were constructed by crossing representative transgenes into various genetic backgrounds. These include strains generated by crossing HC736 into either single mutants (WM27, HC196, WM27, WM49, NL2099, GR1373, HC779, HC780, HC781) or double mutants (HC731, HC732, HC782, HC783, HC784; by crossing a representative line that coexpresses *rde-4(+)* and DsRed2 in bwm cells of WM49 into HC734 and HC737; by crossing a representative line that coexpresses *rde-1(+)* and DsRed2 in bwm cells of WM27 into HC738; by crossing a representative line that coexpresses *mut-2(+)* and DsRed2 in the bwm cells of WM30 into HC733 and HC739; and by crossing a representative line that coexpresses *gfp*-dsRNA and DsRed2

in the pharynx of HC195 and PD4792 into HC739 and HC735, respectively. To avoid bias due to observed phenotypic defects, cross progeny or re-homozygosed progeny were either selected using the DsRed2 co-injection markers or selected randomly and the genotype was determined subsequently by PCR.

30-35 animals from three independent transgenic rescue lines were analyzed (n=100) in all cases except for the rescue of *rde-4(+)* in neurons of *rde-4(-)* animals, where a representative rescue line was crossed into *rde-4(-)* animals with the *neur::u22ds* transgene and 100 double-transgenic animals were analyzed.

Microscopy. Fluorescent images shown are projections of Z-series that were acquired using a Ziess spinning-disc confocal microscope except in **Supplemental Fig. 1a**, where widefield fluorescent images taken using a dissecting fluorescent microscope are shown. Images for strains that are being compared in all figures were acquired under the same non-saturating exposure conditions and, with the exception of the DsRed inset in **Fig. 2b**, then adjusted identically using Image J (NIH) and Photoshop (Adobe) software for display.

RNAi assays. To measure the extent of GFP silencing, we used a dissecting fluorescent microscope to count the number of brightly fluorescent gut nuclei in animals of the fourth larval stage (L4 stage) that are visible at a fixed magnification. The 2 nuclei that are located below 2 other nuclei in the first segment of the intestine (Int 1) are not easily resolved at this level of magnification and were not counted in this assay. Silencing in **Fig. 1c** was measured at 25°C, since some silencing of pharyngeal GFP is observed at lower temperatures, which is consistent with previous reports of RDE-1-independent silencing[20]. For feeding RNAi, L4-staged animals were fed bacteria that express L4440 control dsRNA or dsRNA matching a target gene on agar plates that contain 1mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The percentage of the resultant L4 progeny that showed the corresponding defects was determined. For *act-5* silencing, the number of animals that survive beyond L4 on day 5 for each genotype are expressed as a percentage of L4 and older animals on day 5 of the same genotype on L4440. To measure *unc-22* silencing in response to expressed *unc-22*-dsRNA or *unc-22* feeding RNAi, we determined the percentage of L4-staged animals that twitched within 3 minutes in 3 mM levamisole (Sigma Aldrich) or on RNAi feeding plates without levamisole, respectively.

DNA constructs and transgenic animals. PCR fragments for transgenic expression[33] and transgenic animals[34] were generated using standard methods as in ref. 8. Briefly, PCR fragments corresponding to the coding sequences and 3′UTR were amplified and fused to promoter sequences using overlap extension PCR[32]. These fragments were then purified using a PCR clean-up column (Qiagen) and injected along with appropriate co-injection markers into *C. elegans* to generate transgenic lines. The specific primers used for PCR (**Supplementary Table 2**) and the specific concentrations and co-injection markers used for injections are detailed in **Supplementary Information**.

Statistical Analysis. Statistical significance of differences in average numbers of gut nuclei was calculated using Student's t-test. For all other assays, 95% confidence intervals for single proportions were calculated using Wilson's estimates with continuity correction[35] and significant differences were determined using Wilson's pooled estimates.

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Figure 1

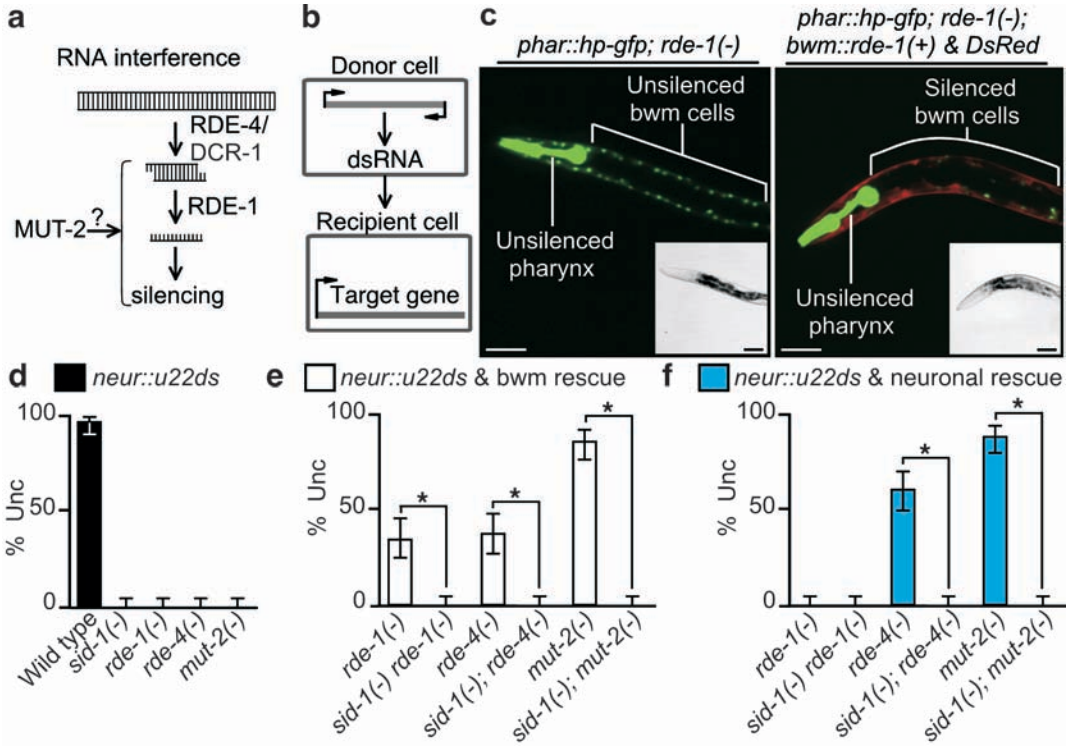

a
RNA interference
RDE-4/
DCR-1
RDE-1
MUT-2?
silencing
b
Donor cell
dsRNA
Recipient cell
Target gene
c
phar::hp-gfp; rde-1(-)
phar::hp-gfp; rde-1(-);
bwm::rde-1(+) & DsRed
Unsilenced
bwm cells
Unsilenced
pharynx
Silenced
bwm cells
Unsilenced
pharynx
d
neur::u22ds
% Unc
100
50
0
Wild type
sid-1(-)
rde-1(-)
rde-4(-)
mut-2(-)
e
neur::u22ds & bwm rescue
% Unc
100
50
0
rde-1(-)
sid-1(-) rde-1(-)
rde-4(-)
sid-1(-); rde-4(-)
mut-2(-)
sid-1(-); mut-2(-)
f
neur::u22ds & neuronal rescue
% Unc
100
50
0
rde-1(-)
sid-1(-) rde-1(-)
rde-4(-)
sid-1(-); rde-4(-)
mut-2(-)
sid-1(-); mut-2(-)

Figure 2

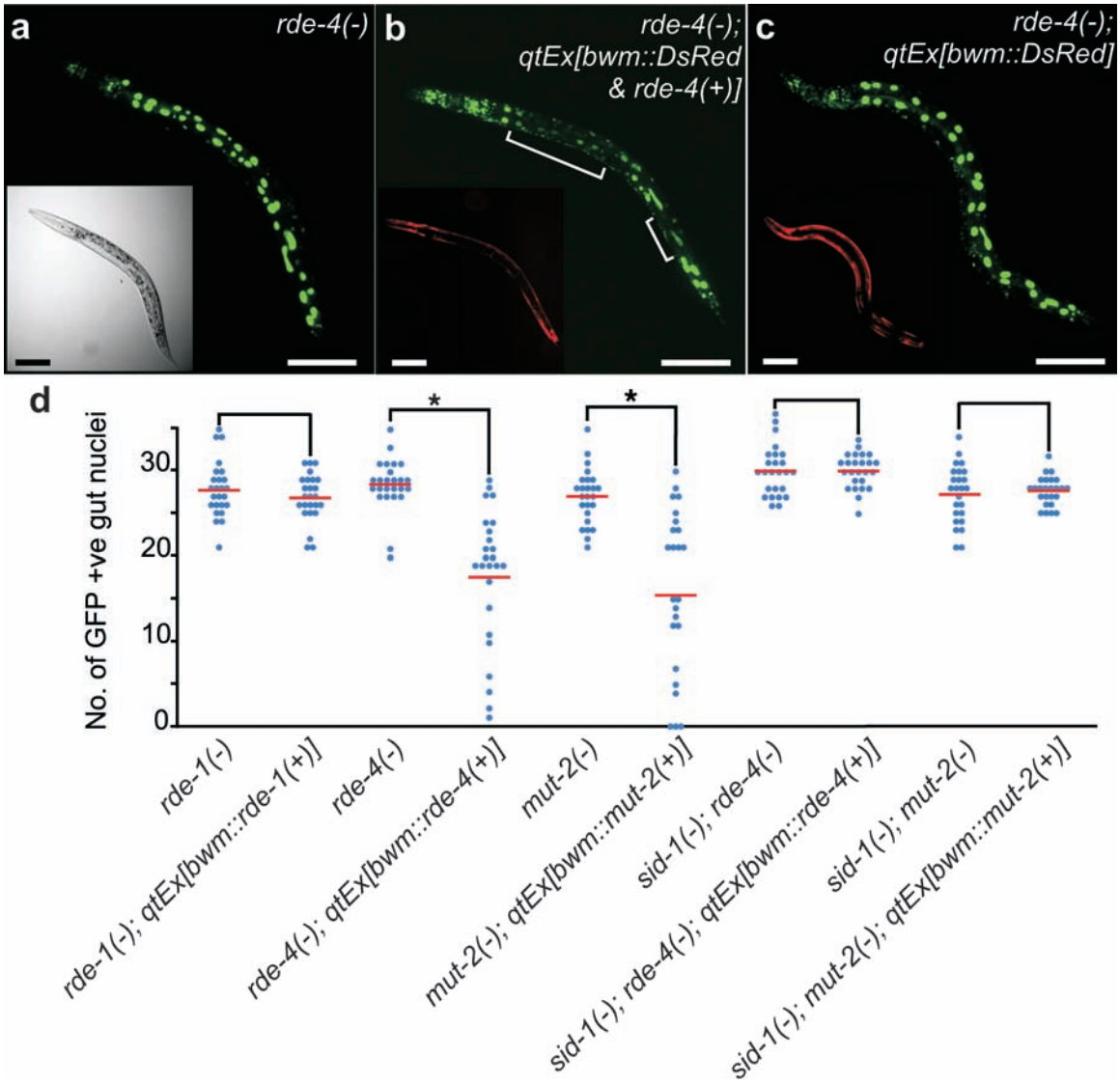

a
rde-4(-)
b
rde-4(-);
qtEx[bwm::DsRed
& rde-4(+)]
c
rde-4(-);
qtEx[bwm::DsRed]
d
No. of GFP +ve gut nuclei
30
20
10
0
*
*
rde-1(-)
rde-1(-); qtEx[bwm::rde-1(+)]
rde-4(-)
rde-4(-); qtEx[bwm::rde-4(+)]
mut-2(-)
mut-2(-); qtEx[bwm::mut-2(+)]
sid-1(-); rde-4(-)
sid-1(-); rde-4(-); qtEx[bwm::rde-4(+)]
sid-1(-); mut-2(-)
sid-1(-); mut-2(-); qtEx[bwm::mut-2(+)]

Figure 3

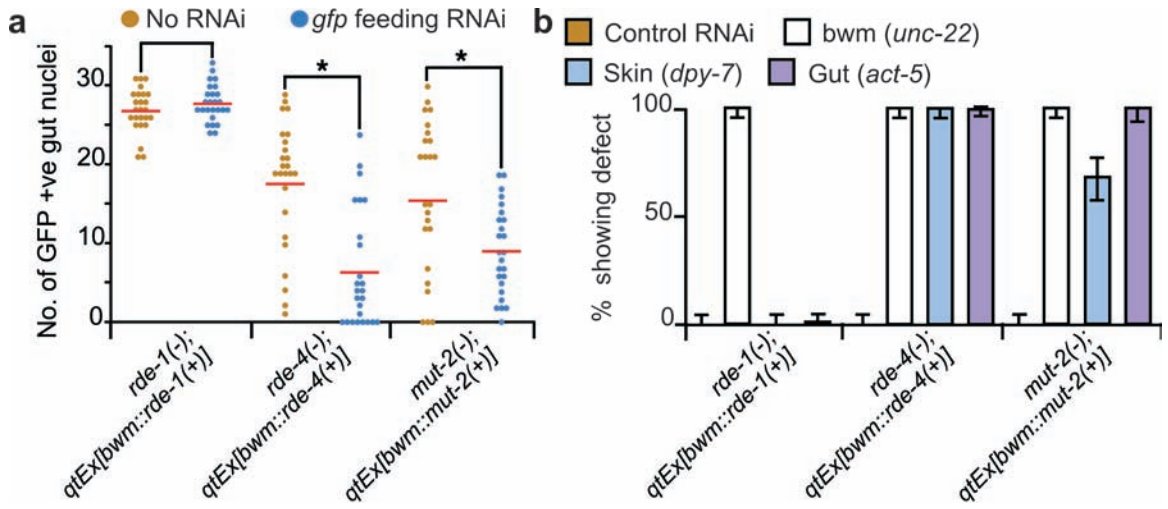
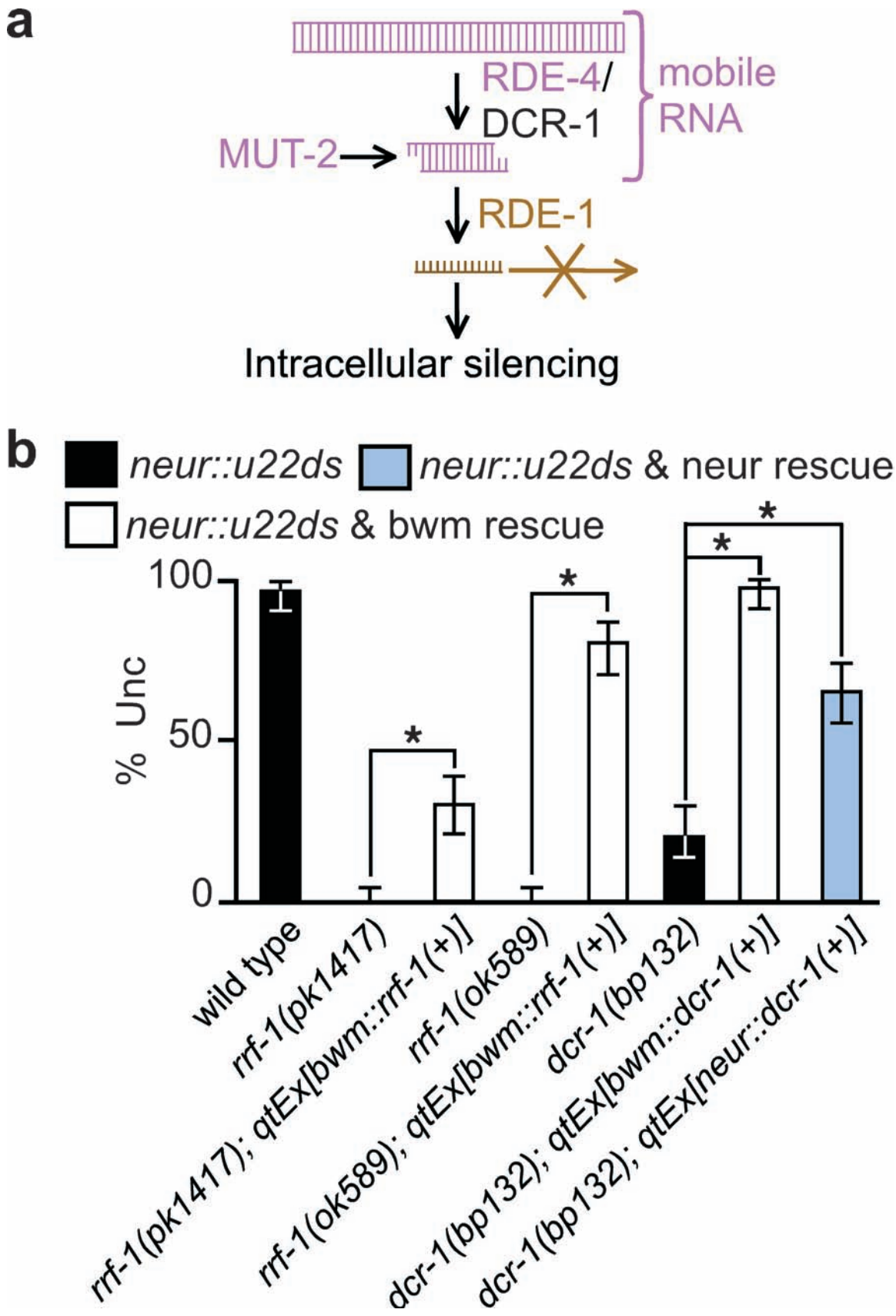
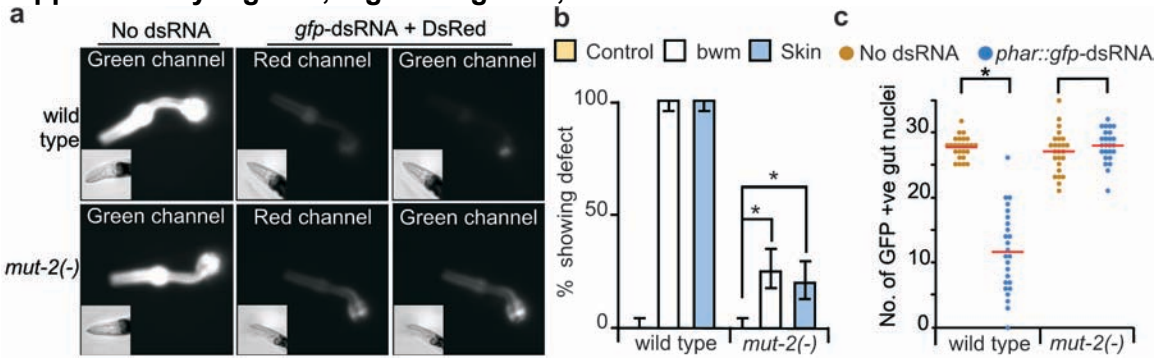

a
No RNAi
gfp feeding RNAi
No. of GFP +ve gut nuclei
30
20
10
0
*
*
rde-1(-); qtEx[bwm::rde-1(+)]
rde-4(-); qtEx[bwm::rde-4(+)]
mut-2(-); qtEx[bwm::mut-2(+)]
b
Control RNAi
bwm (unc-22)
Skin (dpy-7)
Gut (act-5)
% showing defect
100
50
0
rde-1(-); qtEx[bwm::rde-1(+)]
rde-4(-); qtEx[bwm::rde-4(+)]
mut-2(-); qtEx[bwm::mut-2(+)]

Figure 4

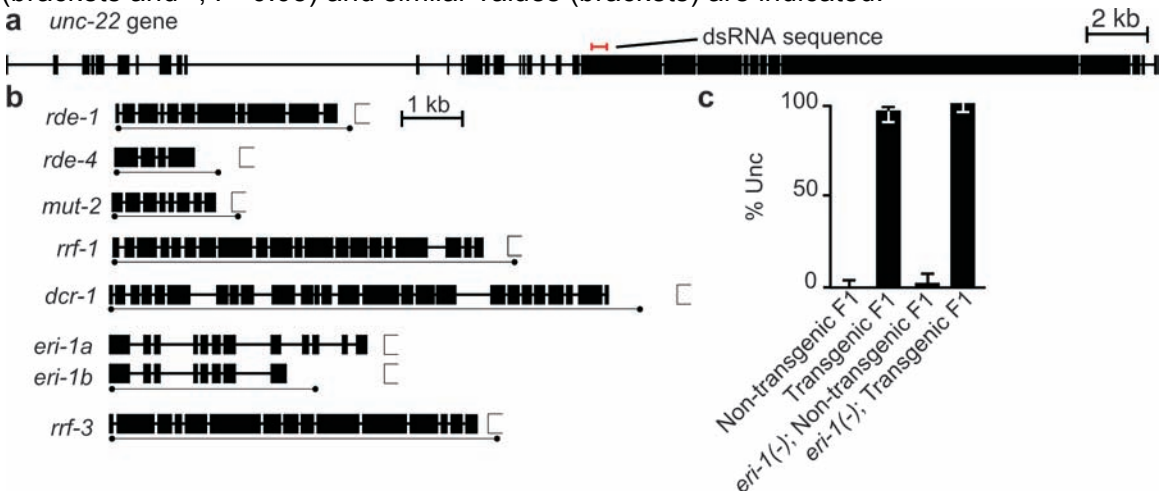

a
RDE-4/
DCR-1
mobile
RNA
MUT-2
RDE-1
Intracellular silencing
b
neur::u22ds
neur::u22ds & neur rescue
neur::u22ds & bwm rescue
% Unc
100
50
0
*
*
*
*
wild type
rrf-1(pk1417)
rrf-1(pk1417); qtEx[bwm::rrf-1(+)]
rrf-1(ok589)
rrf-1(ok589); qtEx[bwm::rrf-1(+)]
dcr-1(bp132)
dcr-1(bp132); qtEx[bwm::dcr-1(+)]
dcr-1(bp132); qtEx[neur::dcr-1(+)]

Two classes of silencing RNAs move between *C. elegans* tissues.
Antony M Jose, Giancarlo Garcia, and Craig P Hunter.

Supplementary Information
Supplementary Figures, Figure Legends, and Tables.

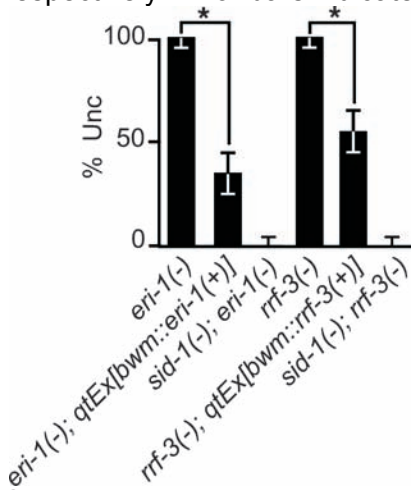


Supplementary Figure 1 MUT-2 is required for efficient RNAi. (**a**) Representative images of the pharynx of wild-type and *mut-2(-)* animals that express GFP in the pharynx (left panels) and that in addition coexpress *gfp*-dsRNA and DsRed in the pharynx (middle and right panels). The representative animals presented in these grey scale images show that wild-type animals silence *gfp* expression (green channel) more potently than *mut-2(-)* animals even in response to lower levels of *gfp*-dsRNA (red channel). (**b**) Feeding RNAi of wild-type and *mut-2(-)* animals. L4 animals were fed either L4440 (control) or dsRNA targeting the body-wall muscle gene *unc-22* (bwm) or the skin gene *dpy-7* (skin) and the percentage of L4 progeny that showed the corresponding defects were determined. Error bars indicate 95% confidence intervals and asterisks indicate significant differences (P>0.05). (**c**) A representative transgene that expresses *gfp*-dsRNA in the pharynx (*phar::gfp-dsRNA*) in *sur-5::gfp* animals was crossed into *mut-2(-); sur-5::gfp* animals and the number of brightly fluorescent gut nuclei were counted. n=25 L4 animals. Averages (red bars), significant differences (brackets and *, P<0.05) and similar values (brackets) are indicated.

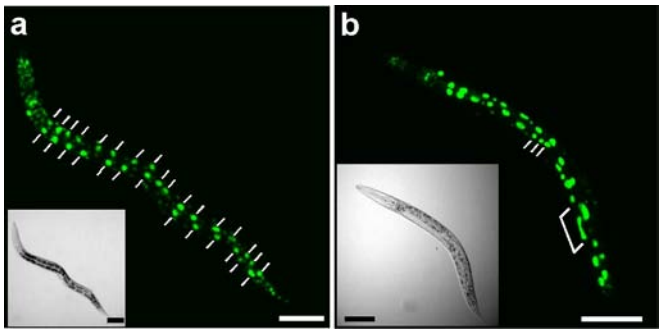


Supplementary Figure 2 Schematics of constructs used and inheritance of silencing in the transgenic strain used in **Fig 1**. (**a**-**b**) Structures of *unc-22* and RNAi pathway genes. Thin line, introns; thick line, exons; and red line, dsRNA sequence. Regions amplified to rescue each RNAi pathway gene are indicated below gene structures as thin lines with terminal circles (primer positions). Open brackets indicate start of the gene 3' to RNAi pathway gene. (**c**) L4 animals of the representative *neur::u22ds* transgenic line used in

Fig. 1 in wild-type and *eri-1(-)* background were allowed to have progeny. *Unc-22* silencing was measured as in **Fig. 1** in L4 staged progeny that either have or do not have the *neur::u22ds* transgene as indicated by presence or lack of DsRed expression, respectively. Error bars indicate 95% confidence intervals.



Supplemental Figure 3 Inhibitors of RNAi reduce silencing due to mobile RNAs. The representative transgene used in **Fig. 1** to express *unc-22*-dsRNA under the control of the neuronal *rgef-1* promoter (*neur::u22ds*) was crossed into the single- and double-mutant backgrounds indicated. Silencing of *unc-22* was measured (% Unc) in these animals and in animals with the corresponding RNAi gene rescued in bwm of single mutants. n=100 L4 animals. 95% confidence intervals (error bars) and significant differences (brackets and *, P<0.05) are indicated. See **Supplementary Fig. 2** for details of constructs used.



Supplemental Figure 4 Variation in number and morphology of gut nuclei as indicated by *sur-5::gfp* expression. (**a**) *sur-5::gfp* expression in a wild-type L4 animal with normal gut nuclei indicated by lines. (**b**) *sur-5::gfp* expression in a *rde-4(-)* L4 animal with regions of supernumerary (lines) and fewer (bracket) nuclei than normal. The reason for the variations in the number of *sur-5::gfp*-marked nuclei is currently unclear. Animals such as those in (**b**) occur in the case of various genotypes, including wild-type, suggesting that the reason for this variation is likely environmental. Interestingly, a role for RNAi-related genes and retinoblastoma genes in the control of nuclear divisions in the *C. elegans* gut has been demonstrated[36]. Scale bar, 50 μm.

Supplementary Table 1 Interdependence of RNAi pathway genes for mobile RNA production.

Genotype	% animals with gut silencing (n>100)[a]
rde-1(-); sur-5::gfp; qtEx[bwm::mut-2(+)]	0
rde-1(-); sur-5::gfp; qtEx[bwm::rde-4(+)]	0
rde-4(-); sur-5::gfp; qtEx[bwm::mut-2(+)]	0

mut-2(-); sur-5::gfp; qtEx[bwm::rde-4(+)]	0

[a]In each case, more than 20 animals from each of five transgenic lines were examined and the percentage of animals showing detectable silencing of GFP expression in the gut was measured.

Supplementary Table 2 Primers used for PCR

P1	CGAGGCATTTGAATTGGGGG
P2	CGTTCTCGGAGGAGGCCATCCGAATCGATAGGATCTCGG
P3	CCGAGATCCTATCGATTCGGATGGCCTCCTCCGAGAACG
P4	CGGTCATAAACTGAAACGTAAC
P5	GGTGGTGGACAGTAACTGTC
P6	CTGAAACGTAACATATGATAAGG
P7	CGATAATCTCGTGACACTCG
P8	CGTTCTCGGAGGAGGCCATCGTCGTCGTCGTCGATGC
P9	GCATCGACGACGACGACGATGGCCTCCTCCGAGAACG
P10	CGATAATCTCGTGACACTCG
P11	GAAAAGTTCTTCTCCTTTACTCATCGTCGTCGTCGTCGATGC
P12	GCATCGACGACGACGACGATGAGTAAAGGAGAAGAACTTTTC
P13	CAATGTTGCCAAATCACTTTCGCGTCGTCGTCGTCGATGC
P14	GCATCGACGACGACGACGCGAAAGTGATTTGGCAACATTG
P15	CTTGATTTGGAATGGAACCTTC
P16	GGAACCTTCACAACACATGG
P17	GAAGGTTCCATTCCAAATCAAGCGTCGTCGTCGTCGATGC
P18	GCATCGACGACGACGACGCTTGATTTGGAATGGAACCTTC
P19	CGAAAGTGATTTGGCAACATTG
P20	GGCAACATTGGAGACTGATG
P21	GGTCGGCTATAATAAGTTCTTG
P22	CGGGAAAATTCGAGGACATCAAGGGTCCTCCTGAAAATG
P23	CATTTTCAGGAGGACCCTTGATGTCCTCGAATTTTCCCG
P24	GTGAAATCACCTGCAGAGAG
P25	CCCGACAAAACATGAGTATTTC
P26	CACCTGCAGAGAGAAAACATTTT
P27	GATCTTTATTTGGTTGAGACATCAAGGGTCCTCCTGAAAATG
P28	CATTTTCAGGAGGACCCTTGATGTCTCAACCAAATAAAGATC
P29	CCTTGCTAGTTATCGTCTCC
P30	AGTTATCGTCTCCGTAATTCG
P31	CGTTAGTTTGGTTAAATCCATCAAGGGTCCTCCTGAAAATG
P32	CATTTTCAGGAGGACCCTTGATGGATTTAACCAAACTAACG
P33	CACTGCAGAGAATGAGTGTG
P34	GTAGAGGTCAGAGGCATAG
P35	ATCATTATCAAACGGGAGCATCAAGGGTCCTCCTGAAAATG
P36	CATTTTCAGGAGGACCCTTGATGCTCCCGTTTGATAATGAT
P37	CTGTGAGCAGTAGTACAAGTG
P38	GCAGTAGTACAAGTGAACCG
P39	CGGCTCATCTGCGCTCATCAAGGGTCCTCCTGAAAATG
P40	CATTTTCAGGAGGACCCTTGATGAGCGCAGATGAGCCG
P41	GCAAGACCGATAATAGAGGAT
P42	ACTGAAAACGCCAGAAACTAG
P43	CGGGAAAATTCGAGGACATCGTCGTCGTCGTCGATGC
P44	GCATCGACGACGACGACGATGTCCTCGAATTTTCCCG
P45	GATCTTTATTTGGTTGAGACATCGTCGTCGTCGTCGATGC

P46 GCATCGACGACGACGACGATGTCTCAACCAAATAAAGATC
P47 CGTTAGTTTGGTTAAATCCATCGTCGTCGTCGTCGATGC
P48 GCATCGACGACGACGACGATGGATTTAACCAAACTAACG
P49 CCATGACTTCGTTCCGACATCAAGGGTCCTCCTGAAAATG
P50 CATTTTCAGGAGGACCCTTGATGTCGGAACGAAGTCATGG
P51 GGCTTACCTGGTATCTTTGATC
P52 ACCTGGTATCTTTGATCTCTG
P53 CAGCTCTTACCCTGACCATCAAGGGTCCTCCTGAAAATG
P54 CATTTTCAGGAGGACCCTTGATGGTCAGGGTAAGAGCTG
P55 GCAGACTTCTTATCGGTGTG
P56 CAGCTCTTACCCTGACCATCGTCGTCGTCGTCGATGC
P57 GCATCGACGACGACGACGATGGTCAGGGTAAGAGCTG

Supplementary Methods

DNA constructs and transgenic animals. Co-injection markers: (a) pHC183[5]: Plasmid with the *myo-3* promoter cloned 5' of DsRed2 cDNA.

(b) pHC488: The *myo-2* promoter region was amplified from genomic DNA with the primers P1 and P2. The DsRed2 coding sequence along with *unc-54* 3´UTR sequence was amplified from pHC183 with primers P3 and P4. The fusion product was generated with P5 and P6 and then cloned into a T/A cloning vector (StrataClone, Stratagene) to make pHC488.

(c) *Prgef-1::DsRed*: The *rgef-1* promoter was amplified with P7 and P8. The DsRed2 coding sequence along with *unc-54* 3´UTR sequence was amplified from pHC183 using P9 and P4. The fusion product was generated with P10 and P6.

To express *gfp*-dsRNA in pharyngeal muscles*: Pmyo-2::gfp-sense*, and *Pmyo-2::gfp-antisense* were made as in ref. 8. A 1:1 mix of *Pmyo-2::gfp-sense* and *Pmyo-2::gfp-antisense* (0.01 mg/ml each) along with pHC488 (0.038 mg/ml) was injected into HC195 and PD4792 animals to generate transgenic lines.

To express *unc-22*-dsRNA in neurons (*neur::u22ds*)*:* (a) *Prgef-1::unc-22sense*: The *rgef-1* promoter was amplified with P7 and P13. An ~560 bp *unc-22* sequence was amplified from genomic DNA with P14 and P15. The fusion product was generated with P10 and P16. (b) *Prgef-1::unc-22antisense*: The *rgef-1* promoter was amplified with P7 and P17. The same ~560 bp *unc-22* sequence was amplified from genomic DNA with P18 and P19. The fusion product was generated with P10 and P20. A 1:1:1 mix of *Prgef-1::unc-22sense*, *Prgef-1::unc-22antisense*, and *Prgef-1::DsRed* (0.01 mg/ml each) was injected into N2 animals to generate transgenic lines.

To express *rde-1(+)* in bwm cells [*Pmyo-3::rde-1(+)*]: The *myo-3* promoter was amplified from pHC183 with P21 and P22. The *rde-1* coding and 3´UTR sequences were amplified from genomic DNA with P23 and P24. The fusion product was generated with P25 and P26. A mix of *Pmyo-3::rde-1* (0.01 mg/ml) and pHC183 (0.038 mg/ml) was injected into WM27, HC70, and HC782 animals with the *qtEx136* transgene to generate transgenic lines.

To express *mut-2(+)* in bwm cells [*Pmyo-3::mut-2(+)*]: The *myo-3* promoter was amplified from pHC183 with P21 and P27. The *mut-2* coding and 3´UTR sequences were amplified from genomic DNA with P28 and P29. The fusion product was generated using P25 and P30. A mix of *Pmyo-3::mut-2* (0.01 mg/ml) and pHC183 (0.038 mg/ml) was injected into WM30 and HC784 animals with the *qtEx136* transgene to generate transgenic lines.

To express *rde-4(+)* in bwm cells [*Pmyo-3::rde-4(+)*]: The *myo-3* promoter was amplified from pHC183 with P21 and P31. The *rde-4* coding and 3´UTR sequences were amplified from genomic DNA with P32 and P33. The fusion products were generated with P25 and P34. A mix of *Pmyo-3::rde-4* (0.01 mg/ml) and pHC183 (0.038 mg/ml) was

injected into WM49 and HC783 animals with the *qtEx136* transgene to generate transgenic lines.

To express *rrf-3(+)* in bwm cells [*Pmyo-3::rrf-3(+)*]: The *myo-3* promoter was amplified from pHC183 with P21 and P35. The *rrf-3* coding and 3´UTR sequences were amplified from genomic DNA with P36 and P37. The fusion product was generated with P25 and P38. A mix of *Pmyo-3::rrf-3* (0.01 mg/ml) and pHC183 (0.038 mg/ml) was injected into NL2099 animals with the *qtEx136* transgene to generate transgenic lines.

To express *eri-1(+)* in bwm cells [*Pmyo-3::eri-1(+)*]: The *myo-3* promoter was amplified from pHC183 with P21 and P39. The *eri-1* coding and 3´UTR sequences were amplified from genomic DNA with P40 and P41. The fusion product was generated with P25 and P42. A mix of *Pmyo-3::eri-1* (0.01 mg/ml) and pHC183 (0.038 mg/ml) was injected into GR1373 animals with the *qtEx136* transgene to generate transgenic lines.

To express *rde-1(+)* in neuronal cells [*Prgef-1::rde-1(+)*]: The *rgef-1* promoter was amplified from genomic DNA with P7 and P43. The *rde-1* coding and 3´UTR sequences were amplified from genomic DNA with P44 and P24. The fusion product was generated with P10 and P26. A mix of *Prgef-1::rde-1* (0.01 mg/ml) and pHC183 (0.038 mg/ml) was injected into WM27 animals with *qtEx136* and into HC782 animals with *qtEx136* to generate transgenic lines.

To express *mut-2(+)* in neuronal cells [*Prgef-1::mut-2(+)*]: The *rgef-1* promoter was amplified from genomic DNA with P7 and P45. The *mut-2* coding and 3´UTR sequences were amplified from genomic DNA with P46 and P29. The fusion product was generated using P10 and P30. A mix of *Prgef-1::mut-2* (0.01 mg/ml) and pHC183 (0.038 mg/ml) was injected into WM30 animals with *qtEx136* and into HC784 animals with *qtEx136* to generate transgenic lines.

To express *rde-4(+)* in neuronal cells [*Prgef-1::rde-4(+)*]: The *rgef-1* promoter was amplified from genomic DNA with P7 and P47. The *rde-4* coding and 3´UTR sequences were amplified from genomic DNA with P48 and P33. The fusion products were generated with P10 and P34. A mix of *Prgef-1::rde-4* (0.01 mg/ml) and pHC183 (0.038 mg/ml) was injected into WM49 animals with *qtEx136* and into HC783 animals with *qtEx136* to generate transgenic lines.

To express *rrf-1(+)* in bwm cells [*Pmyo-3::rrf-1(+)*]: The *myo-3* promoter was amplified from pHC183 with P21 and P49. The *rrf-1* coding and 3´UTR sequences were amplified from genomic DNA with P50 and P51. The fusion product was generated with P25 and P52. A mix of *Pmyo-3::rrf-1* (0.01 mg/ml) and pHC183 (0.038 mg/ml) was injected into HC780 animals with the *qtEx136* transgene and into HC781 animals with the *qtEx136* transgene to generate transgenic lines.

To express *dcr-1(+)* in bwm cells [*Pmyo-3::dcr-1(+)*]: The *myo-3* promoter was amplified from pHC183 with P21 and P53. The *dcr-1* coding and 3´UTR sequences were amplified from genomic DNA with P54 and P55. A 1:1 mix of the PCR products (0.01 mg/ml each) and pHC183 (0.038 mg/ml) was injected into HC779 animals with the *qtEx136* transgene to generate transgenic lines.

To express *dcr-1(+)* in neuronal cells [*Prgef-1::dcr-1(+)*]: The *rgef-1* promoter was amplified from genomic DNA with P7 and P56. The *dcr-1* coding and 3´UTR sequences were amplified from genomic DNA with P57 and P55. A 1:1 mix of the PCR products (0.01 mg/ml each) and pHC183 (0.038 mg/ml) was injected into HC779 animals with the *qtEx136* transgene to generate transgenic lines.

To express DsRed in bwm cells of HC737 animals, 0.038 mg/ml of pHC183 was injected to generate transgenic lines.

In most cases, transgenic lines were easily generated and transgenic animals were healthy, and appeared morphologically normal.

Supplementary Reference

36. Grishok, A. & Sharp, P. A. Negative regulation of nuclear divisions in *Caenorhabditis elegans* by retinoblastoma and RNA interference-related genes. *Proc. Natl. Acad. Sci. USA* **102**, 17360-17365 (2005).