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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 RNAi¹. 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 pathway⁸. Since animals transcribe dsRNA from numerous loci⁹, 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

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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-1¹³, 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 RNAs⁸, 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 silencing of the target gene *unc-22* in mutant 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-siRNA¹⁹, 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-2¹² 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 RNA²³. 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 the numerous downstream secondary siRNAs, is not required for the generation of

mobile RNAs (Fig. 4b); (2) Rescuing a partial loss-of-function *dcr-1* mutant²⁴ 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-siRNA²⁵, 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 mRNAs²⁶.

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 dsRNA²⁷. 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 cells⁷, 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 cells²⁸, 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 Dicer²⁹) 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 tissues³¹. 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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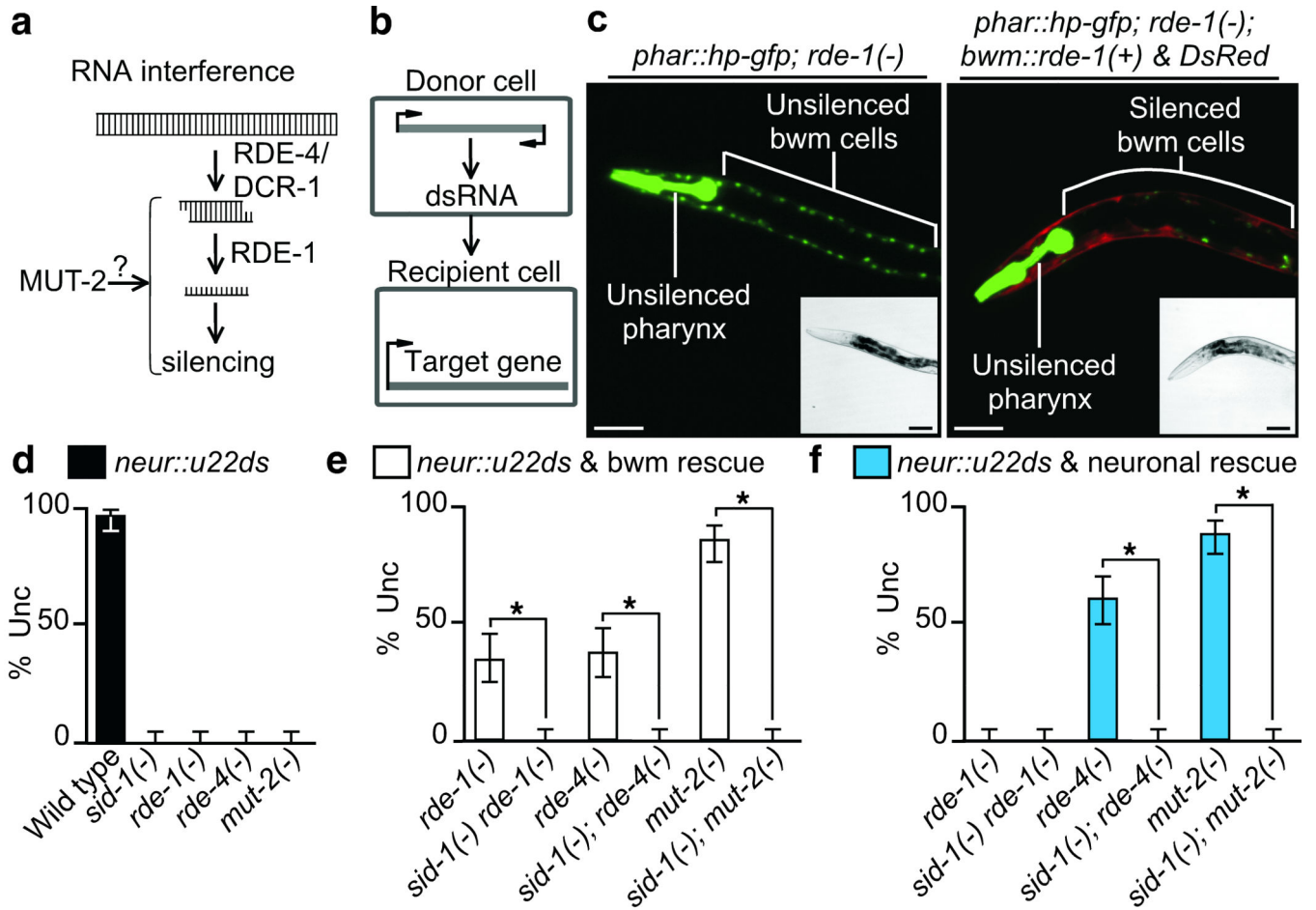


Figure 1. RNAi-independent biogenesis of mobile RNA from expressed dsRNA. **(a)** Schematic of RNAi within a cell. See text for details. **(b)** Schematic of assay to measure silencing due to mobile RNA. dsRNA that targets a gene in recipient cells are expressed only in other donor cells. **(c)** A representative *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 μ m. **(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.

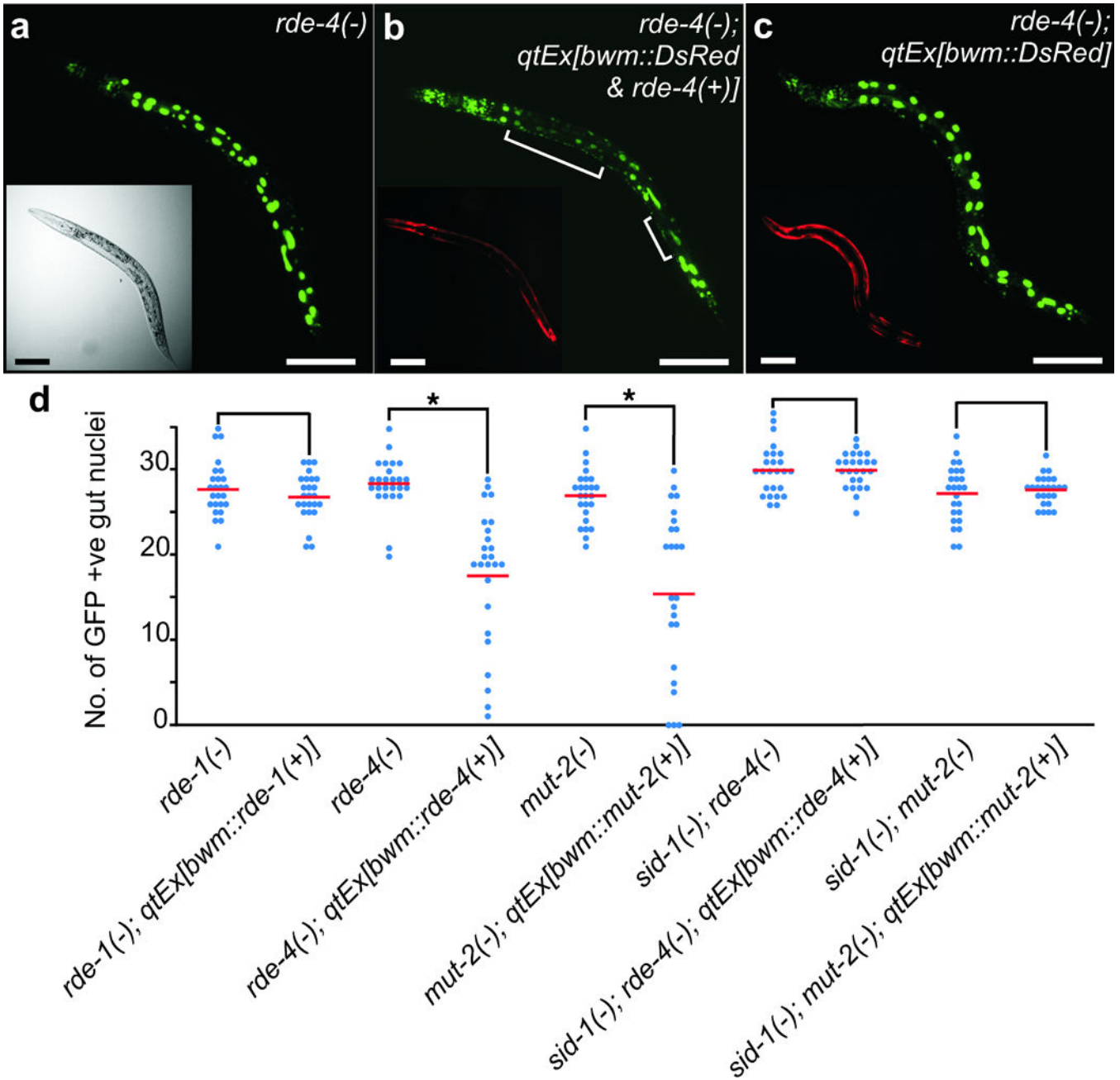


Figure 2. Restricted expression of RDE-4 and MUT-2 but not RDE-1 enables non-cell autonomous RNA silencing. **(a–c)** Representative animals that express nuclear-localized GFP in all cells (*sur-5::gfp*). **(a)** *rde-4(-)*. **(b)** *rde-4(-)* animals with *rde-4(+)* and DsRed expressed in bwm cells (*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 μ m. 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).

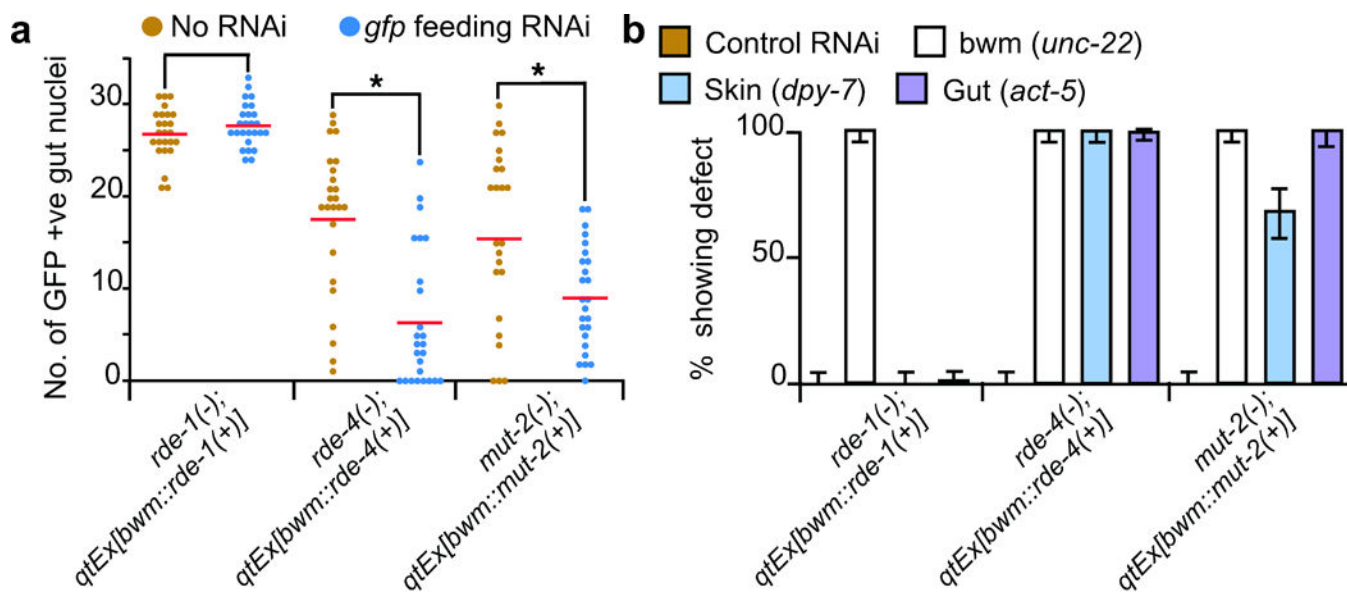


Figure 3.

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.

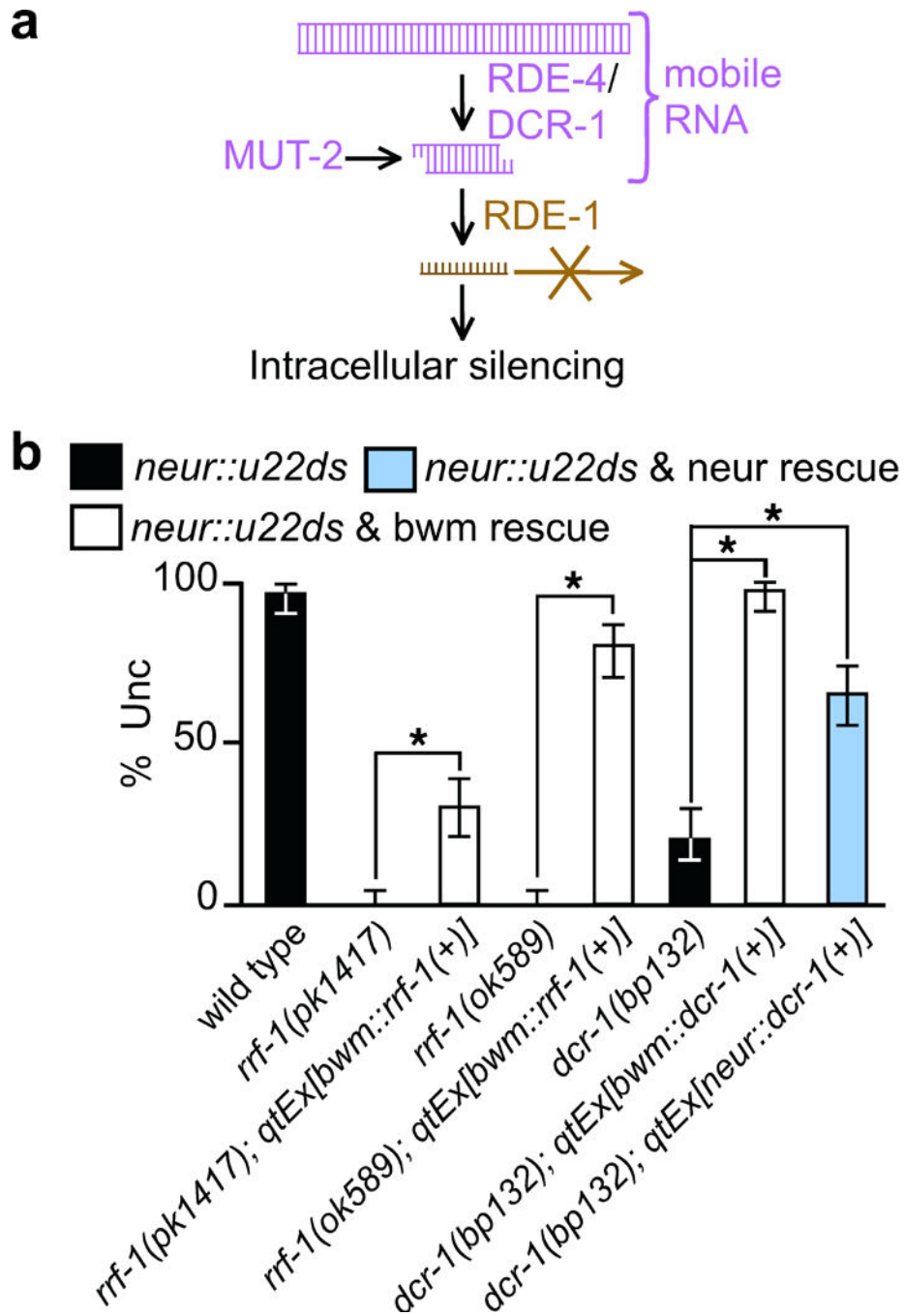


Figure 4. Biogenesis of mobile RNA in *C. elegans*. (a) Schematic of the biogenesis pathway. Double-stranded forms of RNA produced during the early steps of RNA interference act as or generate mobile RNAs. RNAs produced after the Argonaute RDE-1 cleaves ds-siRNA to release single stranded RNA¹³ are restricted 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 *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.