Endomembrane-associated RSD-3 is important for RNAi induced by extracellular silencing RNA in both somatic and germ cells of Caenorhabditis elegans

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RNA silencing signals in C. elegans spread among cells, leading to RNAi throughout the body. During systemic spread of RNAi, membrane trafficking is thought to play important roles. Here, we show that RNAi Spreading Defective-3 (rsd-3), which encodes a homolog of epsinR, a conserved ENTH (epsin N-terminal homology) domain protein, generally participates in cellular uptake of silencing RNA. RSD-3 is previously thought to be involved in systemic RNAi only in germ cells, but we isolated several deletion alleles of rsd-3, and found that these mutants are defective in the spread of silencing RNA not only into germ cells but also into somatic cells. RSD-3 is ubiquitously expressed, and intracellularly localized to the trans-Golgi network (TGN) and endosomes. Tissue-specific rescue experiments indicate that RSD-3 is required for importing silencing RNA into cells rather than exporting from cells. Structure/function analysis showed that the ENTH domain alone is sufficient, and membrane association of the ENTH domain is required, for RSD-3 function in systemic RNAi. Our results suggest that endomembrane trafficking through the TGN and endosomes generally plays an important role in cellular uptake of silencing RNA.

In multicellular organisms, cells communicate with each other through various signaling molecules, including proteins and lipids, to maintain tissue homeostasis and regulate growth and differentiation. Growing evidence indicates that RNA also function as an intercellular signaling molecule1,2. For example, extracellular RNA has been detected in various body fluids of mammals3, and some microRNAs are actually transported between cells and exert gene silencing4,5. Currently, the molecular mechanism of RNA transport between cells and the biological significance of extracellular RNA remain largely unknown.

In some animals, including C. elegans, introduction of double-stranded RNA (dsRNA) into cells induces RNA interference (RNAi) not only in the cells, but also in cells distant from the cells where dsRNA is initially introduced6–7. This phenomenon is referred to as systemic RNAi, and its underlying mechanism may involve intercellular transport of silencing RNA. Although many questions remain to be resolved about the mechanism of systemic RNAi, it is well known that, in C. elegans, intercellular RNAi signal, which is assumed to dsRNA, is imported into the cell via a conserved transmembrane protein SID-1, a putative dsRNA transporter8,10. Meanwhile, endocytosis also plays an important role in cell entry of dsRNA in drosophila S2 cells11–13, and the same may be true in C. elegans11,14. In addition, endosome-associated protein, SID-5, is suggested to promote the export of RNAi silencing signals out of the cell15. Thus, the importance of membrane traffic in systemic spread of silencing RNA is suggested in C. elegans, but little is known about how membrane traffic regulates the phenomenon and which trafficking pathways are involved.

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Figure 1. RSD-3 is required for systemic RNAi not only in germ cells but also in somatic cells. (a) Upper: genomic structure of rsd-3. Black boxes indicate coding exons. Deletion regions of tm9006, tm6416 and tm6420, which were isolated in this study, and the Tc1 insertion site in pk2013 are indicated. Lower: Schematic representation of human epsinR and RSD-3 proteins. Protein domains and motifs are indicated. γ-ear/GAE indicates ear domain of the γ-subunit of AP-1 (adaptor protein-1) and the γ-adaptin ear (GAE) domain of GGA (Golgi-localized, Gamma-ear-containing, Arf-binding protein). AP-1 and GGA are both clathrin adaptors. Corresponding deletion regions of tm9006, tm6416 and tm6420 are indicated. (b) Newly isolated rsd-3 mutants show resistance to germline RNAi induced by pos-1 dsRNA feeding as rsd-3(pk2013). Bars represent the percentage of hatched progeny. (c,d) rsd-3 mutants show resistance to bli-3 feeding RNAi. (c) L1 larvae were placed on plates seeded with bacteria without dsRNA (mock) or bacteria expressing bli-3 dsRNA, and 48 hr later
photographed. Wild type animals that were fed on food with bli-3 dsRNA showed severe melting defects and resulted in larval arrest, whereas almost all rsd-3(tm9006) worms reached adulthood. A portion of rsd-3(tm9006) worms showed mild cuticle blister formation (inset, arrowhead). Scale bars, 500 μm. (d) Bars represent the percentage of animals reached adulthood. Expression of RSD-3::GFP under the control of the rsd-3 promoter (see Fig. 2a for a schematic representation of the construct) rescues the phenotype of rsd-3(tm9006). (e) rsd-3 mutants show resistance to unc-22 feeding RNAi. Bars represent the percentage of twitching animals. (f) rsd-3 mutants show resistance to elt-2 feeding RNAi. Bars represent the percentage of animals reached adulthood. (g) rsd-3(tm9006) worms show resistance to transgene-induced systemic RNAi. Expression of gfp hairpin RNA and mCherry in the neurons induces silencing of GFP signal (LET-858::GFP) in most cells of wild type animals, but not in rsd-3(tm9006) background (upper panels). snb-1::mCherry indicates the neurons that express gfp hairpin RNA (lower panels). Scale bars, 100 μm. Data are shown as mean ± SEM of three separate experiments. *P < 0.001 (Student’s t-test, two-tailed).

Results

RSD-3 is required for systemic RNAi in both germ cells and somatic cells. To understand the role of membrane trafficking in the intercellular transport of silencing RNA in C. elegans, we searched for mutants of membrane trafficking-related genes exhibiting defects in systemic RNAi spreading. We assayed various mutants of endocytosis, exocytosis or intracellular membrane trafficking-related genes for their sensitivity to RNAi induced by feeding transformed bacteria expressing dsRNA (feeding RNAi). Among 61 strains examined (Supplementary Table S1), we found that the RB733 strain showed resistance to feeding RNAi. The RB733 strain contains a deletion (ok498) in the ctpb-1 gene, a homolog of C-terminal binding protein 1 (CtBP1), which is involved in macropinocytosis in mammals19. We found that the RB733 strain showed resistance to RNAi when fed on dsRNA against both germline expressed gene pos-1 and soma expressed gene bli-3, an epithelial gene (Supplementary Fig. S1). However, other ctpb-1 mutants (tm6130 and tm6188) did not phenocopy the RB733 strain (Supplementary Fig. S1) and the defects of the RB733 strain were not rescued by expression of ctpb-1, suggesting the existence of a mutation other than ctpb-1(ok498) in the RB733 strain. We performed whole-genome sequencing of the RB733 strain and identified a large deletion in the rsd-3 gene. This mutation was confirmed by Sanger sequencing and revealed a 7809 bp deletion and 7 bp insertion located in the genomic region of rsd-3 suggesting the existence of a mutation other than rsd-3, suggesting the existence of a mutation other than rsd-3 (Supplementary Fig. S1). However, other ctpb-1 mutants (tm6130 and tm6188) did not phenocopy the RB733 strain (Supplementary Fig. S1) and the defects of the RB733 strain were not rescued by expression of ctpb-1, suggesting the existence of a mutation other than ctpb-1(ok498) in the RB733 strain. We performed whole-genome sequencing of the RB733 strain and identified a large deletion in the rsd-3 gene. This mutation was confirmed by Sanger sequencing and revealed a 7809 bp deletion and 7 bp insertion located in the genomic region of rsd-3. We named this mutation rsd-3(tm9006) (Fig. 1a). rsd-3 and ctpb-1 are both located on chromosome X, 10.9 cM apart from each other. By crossing the RB733 strain with the wild type, we obtained segregants that contained only the ctpb-1(ok498) mutation or only the rsd-3(tm9006) mutation. Mutants that contained only rsd-3(tm9006) were resistant to both pos-1 and bli-3 feeding RNAi (Fig. 1b–d), but mutants that contained only ctpb-1(ok498) showed a normal response to feeding RNAi (Supplementary Fig. S1b,c), suggesting that the rsd-3(tm9006) mutation is essentially responsible for the systemic RNAi defects observed in the RB733 strain.

To examine whether rsd-3(tm9006) shows systemic RNAi defects in somatic tissues other than epithelia, where bli-3 is expressed, we fed rsd-3(tm9006) bacteria that express dsRNA targeting the muscle-expressed gene (unc-22) and intestine-expressed gene (elt-2), and found that rsd-3(tm9006) was resistant to unc-22 and elt-2 feeding RNAi (Fig. 1e,f). These results suggest that rsd-3(tm9006) is resistant to feeding RNAi throughout the organism. However, unlike sid-1 mutants, that were fully resistant to systemic RNAi, rsd-3 mutants were partially resistant to silencing soma-expressed genes (Fig. 1c–f).

rsd-3 encodes a homolog of epsinR, an evolutionarily conserved epsin family protein20,21. RSD-3/epsinR contains an N-terminal ENTH domain, a well-characterized phosphoinositide binding module20,21, and motifs for binding clathrin and clathrin adaptor proteins in the C-terminal region (Fig. 1a). In mammalian cells, epsinR has been implicated in clathrin-mediated membrane trafficking between the trans-Golgi network (TGN) and endosomes, and membrane association of the ENTH domain of RSD-3 is required for systemic RNAi in both germ cells and somatic cells. We also confirmed the phenotypes of rsd-3(pk2013); i.e., rsd-3(pk2013) showed no RNAi phenotype when fed pos-1 dsRNA, but displayed RNAi phenotype when fed bli-3 dsRNA (Fig. 1b and Supplementary Fig. S2e). Because rsd-3(tm9006) exhibits systemic RNAi defects in both the germline and the soma, we examined whether RSD-3 actually plays a role in systemic RNAi in somatic tissue. We generated a transgene containing rsd-3 genomic sequences including 4 kb of upstream promoter sequences, the complete ORF and introns, fused in frame to GFP (see Fig. 2a for a schematic representation of the construct), and introduced this transgene into rsd-3(tm9006). We found that expression of RSD-3::GFP completely rescued the silencing defects in the
Figure 2. Expression pattern of RSD-3. (a) Schematic representation of the genomic rsd-3::gfp expression construct. The genomic region of wild type rsd-3 including 4 kb of upstream promoter sequences and the full-length rsd-3 was C-terminally fused to gfp. Black boxes indicate coding exons of rsd-3. Gray box indicates gfp sequence. (b–g) RSD-3::GFP is ubiquitously expressed. The expression pattern of genomic rsd-3::gfp was observed. Arrowheads indicate cytoplasmic puncta. (b) Pharynx, head neurons and excretory canal (arrows). (c) Head neurons (dotted bracket). (d) Coelomocyte. (e) Intestine. (f) Body wall muscle (dotted brackets).
(g) Hypodermis and seam cell. Arrows indicate seam cell nuclei. Scale bars in (b,c,e–g), 20 μm and scale bar in (d), 5 μm. (h) Schematic representation of the hypodermis-specific rsd-3::ecfp expression construct. dpy-7 promoter (431 bp) was used as hypodermis-specific promoter. Black box indicates rsd-3 cDNA. Gray box indicates ecfp sequence. (i–n) RSD-3::ECFP is associated with the TGN and endosomes in hypodermal cells. ECFP-tagged RSD-3 and Venus-tagged organelle markers were coexpressed under the dpy-7 promoter and observed in late L4 stage worms. ECFP fluorescence is pseudocolored green, and Venus fluorescence is pseudocolored red. (i,j) RSD-3::ECFP does not substantially overlap with the early endosome marker 2xFYVE::Venus both near the apical (i) and basolateral (j) plasma membranes, but structures labeled by both RSD-3::ECFP and 2xFYVE::Venus are occasionally observed (arrowheads). (k) RSD-3::ECFP shows no significant overlap with the late endosome marker Venus::RAB-7, but structures labeled by both RSD-3::ECFP and Venus::RAB-7 are occasionally observed (arrowheads). (l) RSD-3::ECFP closely associates with the medial Golgi marker AMAN-2::Venus, but not substantially overlaps each other. Most RSD-3::ECFP forms somewhat ring-like structures, and surrounds the AMAN-2::Venus-positive puncta. (m,n) RSD-3::ECFP colocalizes extensively with the TGN marker, Venus::SYN-16 (m) and Venus::CHC-1 (n). Enlarged images of the boxed areas are shown in the inset (l–n). Scale bars, 10 μm.

soma of rsd-3(tm9006) (Fig. 1d,e). To provide further proof for the gene identity of rsd-3, we isolated other rsd-3 mutants, tm6416 and tm6420 (Fig. 1a), and found that these mutants showed systemic RNAi defects not only in the germline (Fig. 1b) but also in the soma (Fig. 1d–f) as was the case with rsd-3(tm9006). From these results, we conclude that RSD-3 is required for systemic RNAi in somatic cells as well as in germ cells.

Because transgene-induced silencing information is also transported between cells,7 we also examined the response of rsd-3(tm9006) to RNAi triggered by a transgene. We generated an RNAi-inducing transgene expressing gfp hairpin RNA and mCherry under the control of the panneuronal mb-1 promoter and introduced this transgene into let-688gfp transgenic animals, which express nuclear-localized GFP in all somatic cells.24 In the wild type, neuronally expressed gfp hairpin RNA spread to other tissues and potently silenced the GFP signal in most cells, although it did not affect GFP expression in the neurons as reported previously.26 (Fig. 1g, upper left panel). In contrast, GFP expression was not prominently silenced in any of the somatic cells with the rsd-3(tm9006) background (Fig. 1g, upper right panel). These results indicate that RSD-3 is involved in transport of both environmentally- and transgene-derived silencing signals between cells.

We also investigated why the originally identified rsd-3(pk2013) exhibited no defects in systemic RNAi in somatic tissues. In pk2013, Tc1 transposon (a 1.6-kb repeated DNA sequence) is inserted within the first exon of rsd-3, immediately after the start codon. To determine whether the pk2013 mutation affects the expression or function of RSD-3 in the soma, we constructed a pk2013 mutation-containing rsd-3, amplified from the rsd-3(pk2013) genome, with a C-terminal fusion to GFP under the control of the rsd-3 promoter (genomic rsd-3(pk2013)::gfp) (see Supplementary Fig. 2a for a schematic representation of the construct), and generated an extrachromosomal array in rsd-3(tm9006). We detected GFP expression in all somatic tissues, and found that the subcellular localization of the GFP signal was indistinguishable from that of normal RSD-3::GFP (Supplementary Fig. 2b–d and see below). Furthermore, expression of genomic rsd-3(pk2013)::gfp completely rescued the systemic RNAi defects in the soma of rsd-3(tm9006) (Supplementary Fig. 2e). These results indicate that the pk2013 mutation did not strongly affect RSD-3 expression and function in the soma. As reported for some Tc1 insertions,27,28 in somatic cells of rsd-3(pk2013), Tc1 may be excised from the genome, or may be removed from pre-mRNA by splicing, resulting in the production of an in-frame message to produce RSD-3 protein that is functional in vivo.

We tested this hypothesis by analyzing the sequence of rsd-3 cDNA derived from the Tc1-containing allele of rsd-3 in somatic cells. We used an extrachromosomal array of genomic rsd-3(pk2013)::gfp described above, because transgene expression from an extrachromosomal array is typically restricted to somatic cells.22 mRNA from the wild type animals, rsd-3(tm9006) and rsd-3(tm9006);Ex[genomic rsd-3(pk2013)::gfp] were reverse transcribed, and rsd-3 cDNAs around the Tc1 insertion site were PCR amplified (see Supplementary Fig. S3a,b). As shown in Supplementary Fig. S3b, no PCR product was observed from rsd-3(tm9006) as expected. The size of the PCR product from rsd-3(tm9006);Ex[genomic rsd-3(pk2013)::gfp] was almost the same as that from wild type, suggesting that most of Tc1 is spliced from mature mRNA of genomic rsd-3(pk2013)::gfp or imprecisely excised from the extrachromosomal array (Supplementary Fig. S3b). Then, we cloned the PCR product from rsd-3(tm9006);Ex[genomic rsd-3(pk2013)::gfp] and sequenced five independent cDNA clones (#1–5). As shown in Supplementary Fig. S3c, of the five cDNA clones, three clones (#1–3) have 48 bp insertion sequences at the site corresponding to genomic Tc1 insertion site. These insertions do not lead to premature stop codons, and thus, the three clones maintain the normal rsd-3 translational reading frame. The predicted amino acid sequences translated from such mRNAs contain extra 16 amino acids at the site 11 amino acids downstream from the N-terminus. This insertion site is located upstream of the functionally important region of the ENTH domain (see below), and therefore, such insertion may not significantly affect the function of RSD-3. Meanwhile, two clones (#4 and #5) have a small deletion and/or insertion, resulting in a frameshift with a premature stop codon (Supplementary Fig. S3c). These results suggest that the Tc1-containing allele of rsd-3 produces functional RSD-3 in addition to some non-functional RSD-3 in somatic cells.

RSD-3 is ubiquitously expressed. RSD-3 was reported to be widely expressed in transgenic lines expressing RSD-3::GFP under its own promoter, but the detailed expression pattern was not described.4 To determine the precise tissue expression, we observed the transgenic animals described above, expressing RSD-3::GFP driven by 4 kb of rsd-3 upstream sequences (Fig. 2a). Considering the rsd-3 rescue activity (Fig. 1d,e), expression
pattern of the tagged RSD-3 protein very likely reflects those of endogenous protein. RSD-3::GFP fusion protein was expressed in all somatic tissues including the pharynx, neurons, excretory canal, coelomocytes, intestine, body-wall muscles, hypodermis and seam cells (Fig. 2b–g). The RSD-3::GFP fusion protein was diffusely distributed in the cytoplasm and also localized in a punctate pattern in most tissues, indicating that RSD-3 associates with some organelles or vesicles (see the next section). Expression of RSD-3 was previously reported to be high in the coelomocytes16, but in our observation, the expression level of RSD-3::GFP in coelomocytes was comparable with those in other tissues. The discrepancy may be due to a difference in the length of rsd-3 upstream sequences used to generate the transgenes.

**RSD-3 is associated with the TGN and endosomes.** To examine the subcellular localization of RSD-3, we performed a series of colocalization studies in the hypodermis of transgenic animals expressing ECFP-tagged RSD-3 and a set of Venus-tagged organelle markers under the control of the dpy-7 promoter (see Fig. 2h for a schematic representation of rsd-3::ecfp expression construct). To identify early endosomes, we used phosphatidylinositol-3-phosphate (PI3P)-binding construct 2xFYVE::Venus. Near the apical membrane of hypodermal cells, most 2xFYVE::Venus formed ring-like structures (Fig. 2i, middle panel), whereas near the basolateral membrane, most 2xFYVE::Venus was mainly found in small punctate structures (Fig. 2j, middle panel), suggesting that apical early endosomes and basolateral early endosomes are morphologically different. RSD-3::ECFP did not substantially overlap with 2xFYVE::Venus near either the apical or basolateral membranes (Fig. 2j), although RSD-3::ECFP occasionally overlapped with, or juxtaposed to the 2xFYVE::Venus-positive puncta, especially near the basolateral membrane (Fig. 2j, arrowheads). Likewise, RSD-3::ECFP did not significantly overlap with a late endosome marker, Venus::RAB-7, but occasionally these proteins were partially colocalized, or localized to adjacent domains in close proximity (Fig. 2k, arrowheads). We next examined RSD-3 for Golgi localization. In the hypodermal cells, the medial Golgi marker alpha-mannosidase II::Venus (AMAN-2::Venus) was targeted to puncta scattered throughout the cell, a pattern consistent with localization of Golgi ministacks in most invertebrate cells (Fig. 2l, middle panel)29. Most RSD-3::ECFP-positive structures were close to, but did not substantially overlap with, AMAN-2::Venus signals (Fig. 2l). More specifically, most RSD-3::ECFP formed somewhat ring-like structures, and surrounded the AMAN-2::Venus-positive puncta (Fig. 2l, insets). As for the trans-Golgi network (TGN), almost all RSD-3::ECFP-positive structures colocalized extensively with the TGN marker, Venus::SYN-16 (Fig. 2m). RSD-3::ECFP also strongly colocalized with the Venus-tagged clathrin heavy chain (Venus::CHC-1) (Fig. 2n). A major population of intracellular clathrin has been reported to localize to the TGN31, confirming RSD-3 localization to the TGN. Taken together, these results indicate that RSD-3 is mainly localized to the TGN, with a minor portion localized to endosomes.

The subcellular localization of RSD-3 in the coelomocytes was also checked with double labeling of RSD-3::mCherry (see Supplementary Fig. S4a for a schematic representation of the expression construct) with endocytic compartment GFP-markers. RSD-3::mCherry partially overlapped with, or was juxtaposed to the early endosome marker 2xFYVE::GFP, the late endosome marker GFP::RAB-7 and the recycling endosome marker GFP::RME-1 (Supplementary Fig. S4b–d, arrowheads). RSD-3::mCherry also formed ring-like structures surrounding the medial Golgi marker AMAN-2::GFP-positive puncta (Supplementary Fig. S4e, arrowheads), but RSD-3::mCherry did not overlap with the lysosome marker LMP-1::GFP (Supplementary Fig. S4f). These results indicate that the localization pattern of RSD-3 in the coelomocytes is similar to that in the hypodermis. In cultured mammalian cells, human epsinR also localizes primarily to the TGN with a minor portion to the endosomal compartment17,21. Thus, in addition to the sequence similarity, the subcellular localization of RSD-3 is very similar to that of human epsinR.

**RSD-3 is not an integral part of the RNAi machinery.** Because the observed phenotypes of rsd-3 mutants might be due to disruption of cellular RNAi machinery itself rather than to a systemic RNAi defect, we examined whether RSD-3 plays a role in cell-autonomous RNAi. Tijsterman et al. showed that, by injection of dsRNA into one gonad arm, cell-autonomous RNAi machinery is intact in germ cells of rsd-3(pk201316). We also injected various concentrations of pos-1 dsRNA into both gonad arms of rsd-3(tm9006) and found that RNAi efficiency in germ cells of rsd-3(tm9006) is comparable to that in the wild type (Supplementary Fig. S5). This result confirmed that RSD-3 is not involved in cell-autonomous RNAi machinery in germ cells, in contrast to RSD-2 and RSD-6, which were also identified in the rsd screen16 but turned out to be involved in cell-autonomous RNAi machinery, in particular, secondary siRNA amplification39.

To examine the involvement of RSD-3 in the RNAi mechanism in somatic cells, we generated an extrachromosomal array expressing gfp hairpin and mCherry under the control of the muscle-specific myo-3 promoter. This transgene was introduced into myo-3::gfp transgenic animals (ccls4251), which express GFP in the muscle nuclei and mitochondria. In the wild type background, GFP was largely silenced in red muscle cells expressing the GFP-RNAi construct (Supplementary Fig. S6). GFP expression was reduced equally in red muscle cells with an rsd-3::gfp(m9006) background (Supplementary Fig. S6), suggesting that the RNAi pathway is not compromised in rsd-3(m9006) muscle cells. These results indicate that RSD-3 is not involved in the RNAi machinery itself, but rather required for the systemic spread of silencing RNA in both somatic and germ cells.

**RSD-3 is involved in the import but not the export of silencing RNA.** When *C. elegans* is fed with dsRNA, the dsRNA is first taken up by intestinal cells from the intestinal lumen. The silencing RNA is then exported from intestinal cells to the pseudocoelom and subsequently imported into the germ and somatic cells32. To determine which step of feeding RNAi is impaired in rsd-3(tm9006), we performed tissue-specific rescue experiments. We used myo-3::gfp transgenic animals (ccls4251) and fed them with bacteria expressing GFP dsRNA. In the wild type background, GFP expression in muscle cells was significantly decreased, but in
muscle cells with an rsd-3(tm9006) background. GFP was not silenced prominently after exposure to GFP dsRNA (Fig. 3a). We examined whether expression of rsd-3 cDNA in the body wall muscle rescues the feeding RNAi defect caused by rsd-3(tm9006). We found that expression of RSD-3 and mCherry under the control of the muscle-specific myo-3 promoter efficiently restored GFP feeding RNAi sensitivity in body wall muscles of rsd-3(tm9006);ccIs4251;Ex[myo-3p::rsd-3 & myo-3p::mCherry] worms fed with mock or GFP RNAi food. myo-3p::mCherry indicates the cells expressing RSD-3. Arrows indicate the cells in which RSD-3 is expressed and GFP RNAi is restored. Arrowhead indicates the cell in which RSD-3 is not expressed and GFP RNAi defect is not rescued. Scale bars, 20 μm. (c) Intestine-specific expression of RSD-3 does not restore GFP feeding RNAi sensitivity in body wall muscles of rsd-3(tm9006);ccIs4251;Ex[ges-1p::rsd-3 & ges-1p::DsRed] worms fed with mock or GFP RNAi food. DsRed fluorescence confirms the expression of rsd-3 in the intestine (lower panels). Scale bars, 20 μm. (d) rsd-3(tm9006) animals show partial resistance to pseudocoelomic injection of pos-1 dsRNA. pos-1 dsRNA (10 ng/μl) were injected into the pseudocoelom of more than twenty wild type and rsd-3(tm9006) animals, and percentage of hatched progeny was scored for each injected animal. Data is shown as mean ± SEM. *P < 0.05 (Student’s t-test, two-tailed).

The ENTH domain is necessary and sufficient to rescue the systemic RNAi defects of rsd-3 mutants. Human epsinR possesses an N-terminal ENTH domain with affinity for membrane phosphoinositides, especially for phosphatidylinositol 4-phosphate (PI4P) that is mainly generated on the TGN membrane (Fig. 1a)37,24. The unstructured C-terminal region of human epsinR contains motifs for binding clathrin and clathrin adaptor proteins, such as AP-1 (adaptor protein-1) and GGAs (Golgi-localized, Gamma-ear-containing, Arf-binding proteins), functioning at the TGN and endosomes (Fig. 1a)33–35. These domain/motifs have been proposed to target human epsinR to the TGN and endosomes. In addition, the ENTH domain of human or rat epsinR interacts with specific endosomal soluble NSF attachment protein receptors (SNAREs)36–38. Thus, mammalian epsinR has been implicated in clathrin-mediated membrane trafficking between endosomes and the TGN, and particularly functions in sorting of cargoes into transport vesicles36,37,39. RSD-3 possesses domain and motif structures similar to those of human epsinR, except for the methionine-rich domain of unknown function at the

Figure 3. RSD-3 is required for importing silencing RNA into cells. (a) rsd-3(tm9006);ccIs4251 (myo-3p::gfp) animals show resistance to GFP feeding RNAi. Body wall muscle GFP fluorescence in ccIs4251 animals is efficiently reduced by GFP RNAi food (lower left panel), but not in rsd-3(tm9006);ccIs4251 (lower right panel). Scale bars, 50 μm. (b) Body wall muscle-specific expression of RSD-3 restores GFP feeding RNAi sensitivity in body wall muscles of rsd-3(tm9006);ccIs4251. rsd-3(tm9006);ccIs4251;Ex[myo-3p::rsd-3 & myo-3p::mCherry] worms were fed with mock or GFP RNAi food. myo-3p::mCherry indicates the cells expressing RSD-3. Arrows indicate the cells in which RSD-3 is expressed and GFP RNAi is restored. Arrowhead indicates the cell in which RSD-3 is not expressed and GFP RNAi defect is not rescued. Scale bars, 20 μm. (c) Intestine-specific expression of RSD-3 does not restore GFP feeding RNAi sensitivity in body wall muscles of rsd-3(tm9006);ccIs4251. rsd-3(tm9006);ccIs4251;Ex[ges-1p::rsd-3 & ges-1p::DsRed] worms were fed with mock or GFP RNAi food. DsRed fluorescence confirms the expression of rsd-3 in the intestine (lower panels). Scale bars, 20 μm. (d) rsd-3(tm9006) animals show partial resistance to pseudocoelomic injection of pos-1 dsRNA. pos-1 dsRNA (10 ng/μl) were injected into the pseudocoelom of more than twenty wild type and rsd-3(tm9006) animals, and percentage of hatched progeny was scored for each injected animal. Data is shown as mean ± SEM. *P < 0.05 (Student’s t-test, two-tailed).
Figure 4. ENTH domain is necessary and sufficient for the function of RSD-3. (a) Alignment of the amino acid sequences of RSD-3 and their homologs. The sequences of RSD-3, human epsinR, Xenopus epsinR and Drosophila lqfR (liquid facets-Related) were aligned using the program ClustalW. Identical amino acids are shown on a black background, and similar amino acids are on a gray background. The solid underline indicates the region corresponding to the ENTH domain of RSD-3 (RSD-3_ENTH), and the dotted underline indicates the region corresponding to the C-terminal region of RSD-3 (RSD-3_Cterm). The region corresponding to putative helix 0 (α0) of human epsinR is indicated with a bracket. Blue boxes indicate conserved hydrophobic...
residues on the outer surface of α0 helix. Green boxes indicate clathrin-binding motifs of human epsinR and RSD-3. Red boxes indicate γ-ear/GAE-binding motifs of human epsinR and RSD-3. Accession numbers for the sequences used were as follows: human epsinR: NP_055481; Xenopus epsinR: NP_001088404; Drosophila IqR: NP_732734; RSD-3: NP_509973. (b) Left: schematic representations of RSD-3 truncation constructs. The region containing putative α0 is indicated with a bracket. Amino acid numbers are indicated. Right: ECFP-tagged each construct was expressed in the hypodermis of rsd-3(tm9006) and examined the ability to restore sensitivity to bli-3 feeding RNAi. Bars represent the percentage of animals reached adulthood. Expression of full-length RSD-3 or RSD-3_ENTH fully restores the phenotype in rsd-3(tm9006) but RSD-3_Cterm or RSD-3_ENTHΔα0 doesn't have rescue activity. Data is shown as mean ± SEM of three separate experiments. *P < 0.001 (Student's t-test, two-tailed). N.S.: not statistically different. (c–f) Intracellular distribution of ECFP-tagged each construct in the hypodermis. N indicates the nucleus. Full-length RSD-3::ECFP and RSD-3_ENTH::ECFP are localized to puncta (c,d). Nuclear localization of RSD-3_ENTH::ECFP is also observed (d). RSD-3_Cterm::ECFP is very weakly localized to puncta, and nuclear localization is observed (e). RSD-3_ENTHΔα0::ECFP rarely associates with puncta, but nuclear localization is observed (f). Scale bars, 5 μm.

Discussion

Our data indicates that endomembrane-associated RSD-3 generally plays an important role in cellular uptake of silencing RNA. It is known that plasma membrane-localized ENTH domain proteins, such as epsin1/EPN-1, regulate clathrin-mediated endocytosis21,24, whereas the TGN/endosome-localized epsinR regulates clathrin-mediated intracellular membrane trafficking. Because RSD-3 appears to be an ortholog of human epsinR, it is conceivable that RSD-3 is involved in endocytosis. However, RSD-3 appears to be an ortholog of human epsinR, and RSD-3 may also function in systemic RNAi. Therefore, RSD-3 may be involved in systemic RNAi, suggesting that RSD-3 function is not mediated by formation of clathrin-coated vesicles. Instead, because the ENTH domain of mammalian epsinR/Ent3p interacts with some SNAREs such as Vti1b/Vti1p and functions as a cargo adaptor36–39,46, RSD-3 may also function as a cargo adaptor for transporting some cargoes to the appropriate compartment. We generated mutants of vti-1, a homolog of Vti1b/Vti1p, but vti-1 mutants did not show systemic RNAi defects (unpublished observation). Other SNAREs may function redundantly with vti-1.
The mechanism by which RSD-3 is involved in the uptake of silencing RNA into cells remains unclear. At present, we suggest the following two possibilities. First, RSD-3 may regulate SID-1 localization. Several plasma membrane transporters, such as GLUT4 (glucose transporter) and Fet3p/Ftr1p (iron transporter), are known to cycle between the plasma membrane and the TGN, via endosomes\(^\text{47,48}\). SID-1, which is considered as a plasma membrane-localized dRNA transporter, may also cycle between the plasma membrane and intracellular compartments via retrograde transport from endosomes to the TGN, and RSD-3 may be involved in this transport pathway and required for the proper localization of SID-1. Second, RSD-3 may regulate the intracellular transport of endocytosed silencing RNA. In this case, SID-1 may transport silencing RNA into the cytosol at an intracellular compartment but not at the plasma membrane. In fact, SID-1::GFP was observed at intracellular compartments as well as in the cell periphery\(^\text{9}\). In addition, endomembrane localization of SITD1 and SIDT2, mammalian homologs of SID-1, was reported\(^\text{49,50}\). Growing evidence indicates that RNA silencing is connected to the endomembrane system\(^\text{2}\). Particularly, exogenous siRNAs are loaded into RNA-induced silencing complexes (RISCs) at the cytosolic membrane surface of the rough endoplasmic reticulum (ER)\(^\text{31}\). Thus, it is conceivable that endocytosed silencing RNA arrives at the appropriate compartment, such as ER, by RSD-3-mediated retrograde trafficking, then enters the cytosol through SID-1, and is immediately incorporated to the RNAi machinery. Such a mechanism may be important for the efficient transfer of exogenously delivered silencing RNA into the intracellular RNAi machinery.

Oligonucleotides hold outstanding promise as potential therapeutic agents, but a major concern is their poor accessibility to the targets within the cells. Recently, some studies using mammalian cells suggested that the uptake and trafficking pathway of oligonucleotide could affect the ultimate pharmacological effectiveness\(^\text{52–54}\). Thus, future studies to elucidate the role of RSD-3 in the trafficking of silencing RNA may help to develop an efficient drug delivery system in oligonucleotide therapeutics.

**Methods**

**General methods and strains.** Bristol N2 was used as wild type strain. Worm cultures, genetic crosses, and other *C. elegans* methods were performed according to standard protocols\(^\text{55}\) except where otherwise indicated. All experiments were performed at 20 °C. The following deletion mutant strains were obtained by TnP (trimethylpsoralen)/UV method\(^\text{46}\): *abt-6(tm5404), aex-3(tm5659), arf-6(tm1447), arl-3(tm1703), arl-6(tm2622), cebp-1(tm6130), cebp-1(tm6188), mtcu-1(tm5041), rab-6(tm1214), rab-8(tm2526), rab-10(tm2992), rab-11.1(tm2287), rab-11.2(tm2081), rab-14(tm2095), rab-18(tm2121), rab-19(tm2629), rab-21(tm2999), rab-27(tm2270), rab-28(tm2636), rab-30(tm2653), rab-33(tm2641), rab-35(tm2058), rab-37(tm2089), rab-39(tm2466), rsd-3(tm6416), rsd-3(tm6420), scrn-2(tm650), sec-22(tm4552), sid-1(tm2700), smx-1(tm847), smx-3(tm1959), smx-6(tm3790), syx-17(tm3181), tat-1(tm3117), tat-2(tm2332), C52B11.5(tm3007), C56E6.2(tm3008), F11A5.3(tm2585), F11A5.4(tm2576), K02E10.1(tm2564), T04C9.1(tm5548), T28D6.6(tm5550), 4R79.2(tm2640).

The following mutants and transgenic animals were obtained from the *Caenorhabditis* Genetics Center (CGC, Minneapolis, MN): *arf-1(ok796), cav-1(ok2089), cav-2(hc191), cebp-1(ok498) (RB733), efc-1(ok2572), evl-20(ok1819), exoc-7(tm2006), exoc-8(ok2523), max-2(ok1904), pak-1(ok448), pak-2(ok332), pck-2(ok328), pak-7(tm511), rap-2(pk11), rsd-3(pk2013), sped-1(ok1667), sec-8(tm2187), sec-9(tm2189), sid-1(tm2700), smx-1(tm847), smx-3(tm1959), smx-6(tm3790), syx-17(tm3181), tat-1(tm3117), tat-2(tm2332), C52B11.5(tm3007), C56E6.2(tm3008), F11A5.3(tm2585), F11A5.4(tm2576), K02E10.1(tm2564), T04C9.1(tm5548), T28D6.6(tm5550), 4R79.2(tm2640). The following methods were performed according to standard protocols except where otherwise indicated.

**Identification of rsd-3(tm9006).** RB733 genomic DNA was purified by phenol and chloroform extraction including RNase A treatment. 1 μg DNA was subjected to fragmentation and adapter ligation using an Ion Xpress Plus Fragment Library Kit (Life Technologies), according to the manufacturer’s instructions. The library was subjected to emulsion PCR using the Ion OneTouch 200 Template Kit v2 DL (Life Technologies), followed by bead enrichment. Then, whole-genome sequencing was performed with an Ion Torrent PGM system using the Ion PGM 200 Sequencing Kit and Ion 318 chip (Life Technologies). Using IGV (Integrative Genomics Viewer), reads were mapped to *C. elegans* genome assembly CE10. All mutations were identified as described in “Identification of rsd-3(tm9006)” section and isolated by crossing RB733 strain with wild type. Mutations were outcrossed at least five times before further analysis except where otherwise indicated.

**Constructs and transgenic worms.** For each construct, at least three independent transgenic lines were analyzed. To generate genomic *rsd-3::gfp*, the genomic region of *rsd-3* (3960 bp) upstream the ATG initiation codon and the full-length *rsd-3* was amplified by PCR from *N2* genomic DNA and inserted into pPD95.75 (a gift from Dr. A. Fire) at the SalI and SmaI sites. *snb-1p::gfp-hairpin* (a gift from Dr. A. Fire) at the SalI and SmaI sites. To generate genomic *rsd-3::GFP*, the genomic region of *rsd-3* (3960 bp) upstream the ATG initiation codon and the
full-length *rsd-3* containing Tc1 insertion) was amplified by PCR from *rsd-3*(pk.2013) genomic DNA and inserted into pPD95.75 at the Sall and Smal sites. To generate *dpy-7p::rsd-3::ECFP*, *rsd-3* cDNA was amplified by PCR from N2 cDNA and inserted into pPD95.75 at the Sall and Smal sites, yielding pPD95.75_rsd-3_dNA. The promoter region of *dpy-7* (431 bp) was amplified by PCR from N2 genomic DNA and inserted into pPD95.75_rsd-3_dNA at the HindIII and Sall sites, yielding pPD95.75_dpy-7_rsd-3_dNA. The ECFP sequence was amplified by PCR from pFX_ECFP57 and substituted GFP with ECFP using the Smal and EcoRI sites. To generate *dpy-7p::aman-2-Venus*, the Venus sequence was amplified by PCR from pFX_VenusUT59 and substituted GFP with Venus using the Smal and EcoRI sites, yielding pPD95.75_Venus. The *aman-2* cDNA fragment (1-84aa) was amplified by PCR from N2 cDNA and inserted into pPD95.75 at the Sall and Smal sites, yielding pPD95.75_aman-2_Venus. *dpy-7* promoter was subcloned into pPD95.75_aman-2_Venus at the HindIII and Sall sites. To generate *dpy-7p::2xFYVE::Venus*, a dimeric FYVE domain from Hrs (2xFYVE) sequence was amplified by PCR from cdl85 genomic DNA and inserted into pPD95.75_Venus at the Sall and Smal sites. Then, *dpy-7* promoter was subcloned at the HindIII and Sall sites. To generate *dpy-7p::Venus::RNAi assay, L1 larvae were placed on plates seeded with bacteria expressing dsRNA, reared to adulthood and allowed to lay eggs for several hours. Then adult animals were removed and 24 hours later, the percentage of animals that reached adulthood was scored. At least 100 animals were counted per strain. For *elt-2* RNAi assay, L4 larvae were placed on plates seeded with bacteria expressing dsRNA, reared to adulthood and allowed to lay eggs for several hours. Then adult animals were removed and 48 hours later, the percentage of animals that reached adulthood was scored. At least 100 animals were counted per strain. For *mex-3* RNAi assay, more than twenty L1 larvae were fed with bacteria expressing dsRNA, reared to adulthood and allowed to lay eggs for several hours. Then adult animals were removed and 54 hours later, the percentage of animals that reached adulthood was scored. At least 100 animals were counted per strain. For *rsd-3* RNAi experiments, *pos-1* dsRNA was synthesized as following: *pos-1* cDNA fragment attached with T7 promoter at both ends was amplified from N2 cDNA, and *pos-1* dsRNA was transcribed using T7 polymerase (Ambion). Synthesized *pos-1* dsRNA was treated with DNase I, purified by phenol and chloroform extraction, and then injected into both
gonad arms or the pseudocoelom of more than twenty day 1 adult hermaphrodites for each strain. The hatching rate of embryos laid between 12 to 24 hr post injection was scored for each injected animal. At least 300 progenies were counted per strain. For transgene-induced RNAi, gfp hairpin RNA was expressed from extrachromosomal arrays and GFP fluorescence was photographed at the L4 stage.

**Sequence analysis of cDNAs derived from genomic rsd-3 pk2013::gfp transgene.** Total RNA was extracted from mixed-stage tm9006;tmEx3681[genomic rsd-3_pk2013::gfp] animals using TRIzol reagent according to the protocol supplied by the manufacturer (Invitrogen). First-strand cDNA was reves transcribed from the total RNA with an oligo-dT primer using SuperScript IV (Invitrogen). rsd-3 cDNAs in the vicinity of the Tc1 insertion site were PCR amplified using primers shown in Supplementary Fig. S3a. The cDNA amplification products were cloned using TOPO TA Cloning Kit (ThermoFischer Scientific). Five cDNA clones were sequenced in the vicinity of the Tc1 insertion site.

**Microscopy.** Worms on NGM (nematode growth medium) plates were imaged with a Leica DFC280 camera using Leica IM500 imaging software. For confocal images, animals in 20 mM azide were mounted on a 5% agar pad on a glass slide. Micrographs were taken on a Zeiss LSM 710 inverted confocal microscope with 488 and 552 lasers and images were processed using ZEN software (Carl Zeiss) except for Supplementary Fig. S6. For Supplementary Fig. S6, micrographs were taken on a Leica TCS SP8 inverted confocal microscope with 488 and 552 lasers and HyD detectors, and images were processed using LAS AF software (Leica).

**Statistical analysis.** The standard error of the mean (SEM) was used as the error bar for bar charts plotted from the mean value of the data. All the data were compared using two-tailed Student’s t-test. Data were considered significantly different if P-values were lower than 0.05.

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Author Contributions
R.I. performed most of the experiments. E.K.-N. performed screening to find feeding RNAi defective mutants. K.D. performed sequence analysis of cDNAs derived from Tc1-containing allele of rsd-3. All authors discussed the results and designed the experimental approaches. R.I. and S.M. wrote the manuscript.

Additional Information
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