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Title page

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Title: SID-1 functions in multiple roles to support parental RNAi in *Caenorhabditis elegans*

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Abstract

Systemic RNA interference (RNAi) in *Caenorhbaditis elegans* requires *sid-1*, *sid-3*, and *sid-5*. Injected, expressed, or ingested double-stranded RNA (dsRNA) is transported between cells, enabling RNAi in most tissues, including the germline and progeny (parental RNAi). A recent report claims that parental RNAi also requires the yolk receptor *rme-2*. Here, we characterize the role of the *sid* genes and *rme-2* in parental RNAi. We identify multiple independent paths for maternal dsRNA to reach embryos and initiate RNAi. We showed previously that maternal and embryonic *sid-1* contribute independently to parental RNAi. Here we demonstrate a role for embryonic *sid-5*, but not *sid-2* or *sid-3* in parental RNAi. We also find that maternal *rme-2* contributes to but is not required for parental RNAi. We determine that parental RNAi by feeding occurs nearly exclusively in adults. We also introduce 5-ethynyluridine to densely internally label dsRNA, avoiding complications associated with other labeling strategies such as inhibition of normal dsRNA trafficking and separation of label and RNA. Labeling shows that yolk and dsRNA do not co-localize following endocytosis, suggesting independent uptake, and furthermore dsRNA appears to rapidly progress through the RAB-7 endocytosis pathway independently of *sid-1* activity. Our results support the premise that although *sid-1* functions in multiple roles, it alone is central and absolutely required for inheritance of silencing RNAs.

Introduction

RNA interference (RNAi) is a powerful and well-conserved mechanism for sequence specific gene silencing (HUTVÁGNER AND ZAMORE 2002). Introduced double stranded RNA (dsRNA) triggers degradation of homologous transcripts (FIRE *et al.* 1998; HAMILTON AND BAULCOMBE 1999; ZAMORE *et al.* 2000; SHARP 2001) as well as subsequent transcriptional gene silencing (GUANG *et al.* 2008; GUANG *et al.* 2010; BUCKLEY *et al.* 2012). In some animals including the nematode *C. elegans*, RNAi is systemic; dsRNA introduced into the animal by any of several methods results in rapid spread of silencing throughout the animal (FIRE *et al.* 1998; IVASHUTA *et al.* 2015). This systemic silencing requires the dsRNA channel SID-1, which imports dsRNA into the cytoplasm (WINSTON *et al.* 2002; FEINBERG AND HUNTER 2003; SHIH AND HUNTER 2011). SID-1 supports particularly effective silencing in the progeny of dsRNA exposed mothers (FIRE *et al.* 1998; GRISHOK *et al.* 2000; ALCAZAR *et al.* 2008), implying transfer of dsRNA from mother to embryo.

Characterizing SID-1-dependent parental RNAi revealed the presence of a second, unexpected dsRNA transport pathway (WINSTON *et al.* 2002; WINSTON 2002). This second pathway does not require SID-1 in the mother if the progeny express SID-1. Recent results indicate that this second pathway requires the LDL-superfamily endocytosis receptor RME-2 (MARRÉ *et al.* 2016). Marré *et al*.'s results indicate that RME-2 dependent dsRNA uptake into oocytes is required for subsequent SID-1-dependent embryonic RNAi.

Here we report a detailed genetic and cytological investigation of dsRNA transport into oocytes and embryos. We identify three dsRNA transport processes that support inherited RNAi. First, germline injected dsRNA, apparently by SID-1-independent bulk flow, segregates to embryos resulting in silencing. Second, maternally

expressed SID-1 is required to transport extracellular dsRNA into the germline. Third, the LDL receptor superfamily homolog RME-2 enables likely endocytosis of dsRNA into oocytes, but to initiate RNAi in the resulting embryos zygotic *sid-1* and *sid-5* are required, presumably to release membrane encapsulated dsRNA into the cytosol. Marré *et al.* also identified RME-2 as important for this *sid-1* independent dsRNA transport (MARRÉ *et al.* 2016). In contrast to Marré *et al.*, our analysis shows that maternal RME-2 and SID-1 act independently, as neither single mutant prevents dsRNA transport to embryos. Our analysis of this discrepancy revealed a strong effect of maternal developmental stage on parental RNAi, which only strengthens the discrepancy. Our analysis of injected labeled dsRNA shows that although dsRNA and the yolk marker VIT-2::GFP co-localize in the PC space and even on the surface of oocytes, internalized VIT-2::GFP and dsRNA do not co-localize. Furthermore, labeling dsRNA with Cy5 interferes with dsRNA transport into oocytes. This indicates that non-specific interactions between yolk and dsRNA are unlikely to account for the RME-2 mediated uptake. Finally, our genetic analysis of post-endocytosis dsRNA trafficking shows that dsRNA transit through the endocytosis pathway in early embryos is independent of *sid-1*.

Materials and Methods

Strains

The following strains were used: N2 wild-type, HC977 *sid-1(qt101)*, HC970 *sid-1(qt78)*, *sid-1(qt78)*; *sid-2(qt40)*, HC306 *sid-2(qt40)*, HC770 *sid-3(tm342)*, HC302 *sid-5(qt24)*, HC975 *sid-1(qt78)*; *sid-3(tm342)*, HC976 *sid-1(qt78)*; *sid-5(qt24)*, DH1390 *rme-2(b1008)*, HC1064 *rme-2(b1008)*; *sid-1(qt101)*, HC17 bIs1[*vit-2::GFP* + *rol-6(su1006)*]; *emb-27(g48)*, HC1060 *sid-1(qt101)*; bIs1[*vit-2::GFP* + *rol-6(su1006)*], RT123 pwIs21[*unc-119*(+); P*pie-1*::*GFP*::*rab-7*], HC1099 *sid-1(qt78)*; pwIs21[*unc-119*(+); P*pie-1*::*GFP*::*rab-7*]

RNAi

Injections of *unc-22* dsRNA were done at a concentration of approximately ~2 mg/ml. For pseudocoelom injections, the needle was inserted beyond the bend of the gonad arm but before the pharynx or else in the tail beyond the gonad. Injections were done at ~13-20 psi, with successful injections appearing to briefly “highlight” tissues along the entire length of the animal under DIC. For RNAi experiments involving a cross, injected animals were recovered together on a single OP50 plate for ~12 h before the addition of triple the number of appropriate males. After ~36 h of mass mating, individual injected hermaphrodites were singled to new plates along with 3 males and allowed to lay eggs for ~48 h before all P_0 animals were removed. For the feeding RNAi timing experiment, a mixed population of N2 animals was bleached in a basic sodium hypochlorite solution until adult bodies had dissolved, and the released embryos were rinsed in M9. Embryos were allowed to hatch in shaking M9 for 10 h, and the hatched L1s were then roughly partitioned and transferred to *unc-22* RNAi food (TIMMONS *et al.* 2001) or OP50 plates as appropriate by pipetting. Each subsequent day, animals were washed off of the plates and washed four times in M9 and then transferred to new appropriate bacteria plates. Gravid adults were washed again, and 30 animals from each group were picked to individual OP50 plates for F_1 collection. Adult and L4 feeding assays were similarly washed before F_1 collection, but parents were simply picked from mixed populations.

Day 1 adults were prepared by isolating L4 larvae and maintaining for 12 h at 20°C. Scoring for the strong twitching phenotype characteristic of *unc-22* silencing was done in 10 mM levamisole in M9 buffer once the F_1 progeny were young adults.

Labeled dsRNA preparation

5EU and Cy5 labeled RNAs were synthesized using the Ampliscribe T7 Flash Transcription Kit (Epicentre) using a modified version of the manufacturer's protocol to include the substituted nucleotides.

Immunohistochemistry

Slides for microscopy were prepared as in (HINAS *et al.* 2012), with some modifications for click labeling of 5EU. For mounted adults, 5EU was conjugated to an Alexa 594-azide using the Click-iT RNA Imaging Kit (Invitrogen). For embryos, 5EU was first conjugated to biotin-azide (Lumiprobe) using the same Click-iT kit, followed by the Alexa Fluor 594 Tyramide SuperBoost Kit with streptavidin (Invitrogen) for enhanced signal.

Microscopy

Most images were captured using a Zeiss LSM880 microscope using the ZEN software (Zeiss) at the Harvard Center for Biological Imaging. Embryo images from Cy5/5EU dual labeling experiments were captured using a Zeiss Axiovert 200 spinning disk confocal microscope with Axiovision (Zeiss).

Data Availability

Strains are available upon request. Detailed descriptions of labeled dsRNA preparation and immunohistochemistry methods are found in File S1.

Results

Temporally restricted autonomous RNAi by germline injected dsRNA

SID-1 is required to transport dsRNA to the germline, as shown by injecting dsRNA into specific tissues or the pseudocoelom (PC) (WINSTON 2002; WINSTON *et al.* 2002). However, while *unc-22* dsRNA injection into any tissue in wild-type animals resulted in *unc-22* silenced twitching progeny, *unc-22* dsRNA injected into the syncytial germline of a *sid-1* mutant, unexpectedly, did not result in any affected progeny (WINSTON 2002; WINSTON *et al.* 2002). To further investigate this result, we injected *unc-22* dsRNA directly into the syncytial germline of wild-type and *sid-1* mutant adult hermaphrodites and every two hours after injection collected their self-progeny. As expected, and consistent with previous observations of systemic silencing, the proportion of twitching progeny from injected wild-type animals quickly rose to 100% and was sustained for the duration of the experiment (Figure 1B). In contrast to previous observations, we found that injecting dsRNA directly into the syncytial gonads of *sid-1*$^{-/-}$ animals produced strongly twitching progeny. However, this was only true of embryos laid within approximately the first 18 hours after injection (Figure 1A). The timing of peak silencing varied between injected P_0 animals, but always reached 100% (Figure S1 in File S1). Furthermore, when a single gonad arm was injected in *sid-1* mutant animals, only a maximum of 50% twitching progeny was produced (Figure 1C and Figure S1 in File S1). The subsequent decrease in fraction of silenced F_1 embryos to zero and the animal-to-animal variability in when production of silenced progeny ceased suggests that the injected *unc-22* dsRNA is rapidly depleted. In previous experiments (WINSTON 2002;

WINSTON *et al.* 2002), the injected hermaphrodites were allowed to recover for up to 24 hours before progeny were collected and scored for silencing. The apparent rapid depletion of syncytial germline injected dsRNA explains the past failure to detect *sid-1*-independent silencing.

Maternal SID-1-independent systemic spread and perdurance of parental RNAi

DsRNA injections into a single gonad of wild-type hermaphrodites produced 100% twitching progeny, clearly showing that gonad injected dsRNA is mobile between gonad arms (Figure 1D). Additionally, in wild type animals, dsRNA-induced silencing is not limited by the bolus of injected dsRNA, but persists indefinitely in the injected hermaphrodites. Interestingly, injecting *unc-22* dsRNA into a single gonad arm of *sid-1* mutant hermaphrodites and then crossing them to wild-type males produced nearly 100% twitching heterozygous cross progeny (Figure 1E). This indicates that maternal *sid-1* activity is not required for gonad to gonad transfer of dsRNA. It also indicates that maternal *sid-1* activity is not required for long-term persistence of the silencing signal. The gonad to gonad dsRNA transfer likely occurs via the PC fluid, which may also be the conduit to the reservoir of dsRNA that persists indefinitely after injection. However, both *sid-1*-independent gonad to gonad dsRNA transfer and long-term persistence of silencing are only apparent when SID-1 is expressed in the embryo. These new observations, like previous observations following injection into both gonad arms or the PC space, indicate the presence of a maternally active, *sid-1*-independent dsRNA transport pathway from the PC to the germline that subsequently requires *sid-1* in the progeny to realize silencing (WINSTON 2002; WINSTON *et al.* 2002).

***sid-1*-independent dsRNA Transmission Requires RME-2**

The above results, showing *sid-1* independent delivery of dsRNA from mothers to embryos and a *sid-1*-dependent step in the embryos for effective RNAi is similar to feeding RNAi, where the uptake and transport of ingested dsRNA is separable. In feeding RNAi, the intestinal lumen-localized transmembrane protein SID-2 is first required for endocytosis of ingested dsRNA into gut cells, and SID-1 subsequently is required for effective RNAi within intestinal cells, likely to release dsRNA from endosomes (WINSTON *et al.* 2007; MCEWAN *et al.* 2012). A candidate receptor for dsRNA endocytosis in the germline is RME-2, an LDL receptor superfamily homolog that functions in oocytes as a yolk/lipoprotein receptor. In *C. elegans,* yolk is synthesized in the intestine, exported to the PC, and then taken up by oocytes via receptor-mediated endocytosis (SHARROCK *et al.* 1990; GRANT AND HIRSH 1999). Thus, we hypothesized that dsRNA that exits injected gonads or is injected into the PC space may bind to either yolk protein, another RME-2 substrate, or directly to RME-2 for endocytosis into oocytes. To test this hypothesis, we examined *rme-2* mutants, which do not take up any yolk (GRANT AND HIRSH 1999). We injected *unc-22* dsRNA into the PC of *rme-2*$^{-/-}$*; sid-1*$^{-/-}$ double mutant hermaphrodites and then crossed these animals to wild-type males. The heterozygous cross progeny did not twitch, showing that, in the absence of maternal *sid-1*, RME-2 is required for parental RNAi in response to PC injected dsRNA (Figure 2A). While this work was in progress, it was reported that parental RNAi by feeding RNAi also requires RME-2 (MARRÉ *et al.* 2016). Both of these findings support the hypothesis that RME-2 may endocytose dsRNA into oocytes.

SID-1 is sufficient in the germline to transmit maternal dsRNA to progeny

The results reported by Marré *et al*. suggest that RME-2 is essential for parental RNAi, (MARRÉ *et al.* 2016). We tested this explicitly by injecting *unc-22* dsRNA into the PC of *rme-2* single mutants. We found that the progeny twitched (Figure 2A). This result indicates that RME-2 is not required for dsRNA uptake in *sid-1*+ mothers, and further suggest that SID-1 may directly transport PC dsRNA to oocytes and embryos.

This finding of asymmetric redundancy between maternal SID-1 and RME-2, where *sid-1* is required and *rme-2* is only required in the absence of *sid-1*, contrasts with the report that *rme-2* single mutants are defective for inherited RNAi initiated by ingesting *unc-22* dsRNA (MARRÉ *et al.* 2016). Although RME-2 is most abundantly expressed in oocytes, it is possible that *rme-2* is also required for feeding RNAi. It is also possible that their negative result may be explained by the difference in potency between PC injection of concentrated *in vitro* transcribed dsRNA as described above and the feeding RNAi used by Marré *et al*. To address these possibilities, we repeated the feeding RNAi assay as described, *i.e.* exposing L4 hermaphrodites to *unc-22* dsRNA-expressing bacteria for one day before washing and transferring to control bacteria. By these methods, we detected robust twitching in the fed animals, but failed to detect inherited RNAi among the progeny of even wild-type parents. This indicates that *rme-2* is not required for feeding RNAi, but the lack of parental RNAi was perplexing. Further analysis revealed a striking dependency on maternal developmental stage for inherited silencing. We split a batch of freshly hatched wild-type larvae into 3 populations and exposed each to *unc*-22 RNAi food on either day 1 (L1/L2), day 2 (L3/L4), or day 3 (adult) after hatching, thoroughly washing off residual bacteria each day. After a final wash, animals were allowed to lay F_1 progeny on non-RNAi food. We found that only animals exposed to *unc-22* food as adults efficiently produced twitching progeny (Figure 2B), even though the P_0 animals fed on days 1 and 2 still exhibited a strong Unc-22 twitching phenotype immediately after RNAi exposure. We then used these modified feeding RNAi conditions, which are effective for parental RNAi in wild type animals, to determine whether *rme-2* is required for parental RNAi. We found that the progeny of wild-type and *rme-2* adults placed on *unc-22* food show similar proportions of twitching progeny (Figure 2C). Note that *rme-2* hermaphrodites are less fecund that wild-type hermaphrodites (GRANT AND HIRSH 1999). This result shows that *rme-2* is not required in *sid-1*+ hermaphrodites for parental RNAi, even when the dsRNA is introduced by ingestion. Because inherited RNAi by dsRNA feeding requires adult exposure (Figure 2B and 2C), it is reasonable to assume that the slightly slower development of *rme-2* mutants differentially limited the period of adult exposure compared to wild-type animals when initially placed on the RNAi food as L4 larvae. Thus, the slowed development of *rme-2* mutants may have led Marré *et al.* to the erroneous conclusion that *rme-2* is required for parental RNAi (MARRÉ *et al.* 2016). Indeed, exposing wild-type or *rme-2* L4 animals to *unc-22* RNAi food for 24 hours produces few twitching progeny (Figure 2B and 2C). In summary, independent of the means of dsRNA delivery, in *sid-1*+ animals *rme-2* is not required for transport of dsRNA into the germline.

***sid-5* functions with *sid-1* in the embryo**

Pseudocoelomic *unc-22* dsRNA transported into oocytes or embryos in the absence of maternal *sid-1* requires embryonic *sid-1* to silence *unc-22*. We next asked whether *sid-*

2, *sid-3*, or *sid-5* are also required in the embryo. We first crossed *sid-1; sid-2* double mutant hermaphrodites injected with *unc-22* dsRNA to either wild type or *sid-2* males and scored silencing in the cross progeny. If *sid-2* activity is required in the embryo, then the cross progeny of only the wild-type males will twitch. If *sid-2* activity is not required in the embryo, then the cross progeny of both wild-type and *sid-2* males will twitch. Using this method we determined that neither *sid-2* nor *sid-3* are required for either maternal uptake or embryonic release (Figure 3A and 3B), but we found a striking requirement for *sid-5* for embryonic release.

SID-5 is an endosome-associated protein required for efficient systemic RNAi (HINAS *et al.* 2012). In our double mutant rescue experiments, almost no silencing was observed in the next generation if *sid-5* is not rescued (Figure 3C), the same effect as if *sid-1* is not rescued. Because *sid-5* is located on the X-chromosome, crossing a *sid-1*$^{-/-}$*; sid-5*$^{-/-}$ hermaphrodite to a wild-type male results in heterozygous *sid-5*$^{+/-}$ hermaphrodite progeny and hemizygous *sid-5*$^{0/-}$ mutant male progeny. When performing the *unc-22* dsRNA PC injection and rescue in this context, we saw that 100% of hermaphrodite progeny but few of the male progeny showed the *unc-22* silencing phenotype (Figure 3C). The importance of SID-5 in the context of *sid-1*-independent transmission of RNA is especially surprising given the previous reports of only weak systemic RNAi defects for *sid-5* mutants (HINAS *et al.* 2012). However, those experiments were performed in the context of fully functional and normally expressed SID-1, in which case there may be alternative routes for RNA to reach the cytoplasm that are less dependent on SID-5.

Labeled Nucleotides Can Be Used to Visualize Functional Transported dsRNA

Efforts to visualize labeled dsRNA to date have been hampered by various technical challenges, primarily the observation that large bulky fluorescent moieties interfere with RNAi and dsRNA transport, and methods that either fail to label 100% of the dsRNA molecules or only label the ends produce false positive results. Further, it is essential to demonstrate that fluorescence remains associated with dsRNA. To address these issues, we synthesized *unc-22* dsRNA (500 base pair [bp]) using 5-ethynyluridine (5EU) nucleotides, replacing a significant fraction of the normal uridine nucleotides with nucleotides carrying the modified base. 5EU carries a small alkyne modification that allows the RNA to be easily visualized after fixation and labeling through click chemistry (JAO AND SALIC 2008). 5EU-labeled *unc-22* dsRNA injected into a single gonad arm of a wild-type worm results in >85% twitching progeny, indicating that, like unmodified dsRNAs, 5EU dsRNA is both mobile and capable of silencing target genes (Figure 4A).

To determine whether fluorescently labeled 5EU signal represents intact dsRNA and not degraded nucleotides or other irrelevant species, we used a second label and synthesized dsRNA composed of one strand labeled with 5EU and the other strand internally labeled with Cy5-uridine. Although Cy5-labeled dsRNA is not capable of systemic RNAi, it suffices for demonstrating the properties of labeled dsRNA. We injected the Cy5:5EU duplex dsRNA into the PC of adult hermaphrodites and several hours later fixed the animals to visualize both labels (Figure 4B). Much of the signal appears in the pseudocoelom as pools surrounding other tissues. Importantly, both labels were detected in the same locations and at similar intensities. Coinciding labeled RNA was also detected in punctate foci in coelomocytes, providing further evidence for the structure and stability of the injected RNA.

In addition to the Cy5:5EU duplex, we also injected a 1:1 mixture of Cy5-labeled dsRNA with 5EU-labeled dsRNA. We found that although these mixed dsRNAs were both readily detectable in the PC, their apparent relative abundances were more disparate than the co-labeled duplex dsRNA, and more frequently one label could be seen without the other (Figure 4C). That is, when not physically bound together, each label appears free to vary independently. Together, these data suggest that 5EU fluorescence represents *bona fide* dsRNA as opposed to disassociated strands or degraded nucleotides or labels.

We also attempted to detect labeled RNA in embryos from PC-injected hermaphrodites. The Cy5:5EU heteroduplex was not detected above background in embryos (Figure 4D). Furthermore, while we could detect 5EU-labeled RNA punctae in embryos, the co-injected Cy5-labeled RNA was only detected in the PC and coelomocytes (Figure 4E). These observations are consistent with Cy5-containing RNA being impaired for normal trafficking while 5EU-labeled RNA behaves as expected for unmodified RNA. The inability of either the Cy5:5EU heteroduplex or Cy5-RNA to enter embryos suggests there is selectivity in the SID-1 and RME-2-dependent transmission processes. Such selectivity via RME-2 mediated uptake is not expected if dsRNA is simply hitchhiking on yolk proteins.

Labeled dsRNA and VIT-2::GFP largely fail to co-localize

RME-2 was initially described as a yolk receptor (GRANT AND HIRSH 1999), thus the observation that RME-2 may function in parallel to SID-1 to transport dsRNA into oocytes and embryos suggests an association between RNA and yolk (Marré *et al.,* 2016; Fig. 2). We used 5EU-labeled dsRNA to examine interactions between yolk and dsRNA. The Marré *et al*. study also examined interactions between yolk and a 50 bp end-labeled dsRNA (MARRÉ *et al.* 2016). However, these studies did not control for interactions between yolk and the dye or control *in vivo* for integrity of the dye and dsRNA.

We injected our 5EU-labeled dsRNA into the PC of adults expressing GFP-labeled yolk (*vit-2::GFP*) and imaged the injected adults as well as isolated embryos. We detected 5EU-labeled dsRNA co-localized with VIT-2::GFP granules at the surface of developing oocytes in adult gonads (Figure 5A). This co-localization is consistent with co-dependence on oocyte-expressed RME-2 for the uptake of yolk and RNA, as reported previously (MARRÉ *et al.* 2016). However, interior optical sections contain 5EU punctae not associated with any yolk, suggesting that although yolk and dsRNA may share the same endocytosis receptor, they are either separately endocytosed or sorted to different endosomes soon after import (Figure 5A). Analysis of 5EU-labeled dsRNA and VIT-2::GFP in embryos is consistent with this view. The majority of detectable 5EU fluorescence in 1-2 cell embryos does not co-localize with GFP fluorescence (Figure 5B). The lack of co-localization might reflect *sid-1*-dependent release of dsRNA from endosomes. However, in *sid-1*$^{-/-}$ mutant embryos labeled dsRNA also fails to co-localize with VIT-2::GFP (Figure 5B). This further supports the idea that although dsRNA and yolk share a common mechanism for endocytosis into oocytes, they likely do not share common endosomal compartments. This contrasts with the observations and conclusions of Marré *et al.*, who observed extensive co-localization between VIT-2::GFP and their end-labeled dsRNA in oocytes and early embryos, but segregation during later embryogenesis. These observations led them to conclude that mechanisms exist to

separate the two components from within a common vesicle (MARRÉ *et al.* 2016). The difference between the results presented here and those previously reported is most likely related to differing dsRNA labeling strategies (see discussion).

dsRNA Transits RAB-7-Containing Vesicles Independent of SID-1

Since internalized dsRNA appears to be physically separate from yolk granules, we wondered whether the 5EU punctae might co-localize with the late endosome marker RAB-7 (FENG *et al.* 1995; GRANT AND HIRSH 1999). We injected adult hermaphrodites in the PC with 5EU-labeled dsRNA and visualized the resulting embryos in conjunction with antibody staining against GFP-labeled RAB-7. In wild-type embryos, we occasionally detected dsRNA together with RAB-7-positive vesicles, but the majority of the 5EU punctae were not associated with GFP-RAB-7 (Figure 6A, upper row). In *sid-1* mutant embryos, 5EU punctae are also readily detected outside of RAB-7 vesicles, and there does not appear to be significantly more 5EU retained within RAB-7 vesicles (Figure 6A, lower row). Thus, although silencing by inherited dsRNA involves early steps in the endocytotic pathway, the dsRNA appears to transit this system rapidly. Although *sid-1* is required for inherited silencing, its activity is not apparent in the localization of 5EU-labeled dsRNA in oocytes or early embryos.

Discussion

Intercellular dsRNA transport in *C. elegans* via the dsRNA channel SID-1 supports systemic gene silencing throughout the animal and its progeny. Here, we have described and characterized the maternal and embryonic processes that support intergenerational dsRNA transport, as summarized in Figure 6B.

We more fully described the result of dsRNA injection into the gonad. In wild-type animals, dsRNA injection into a single gonad arm results in robust silencing in nearly all progeny, while similar injection in *sid-1* mutant results in transitory silencing and only in progeny derived from the injected gonad arm (Figure 1; (WINSTON 2002; WINSTON *et al.* 2002). The systemic silencing in response to a single-gonad arm injections likely reflects dsRNA leakage into the PC via the injection site. All injections, whether into the intestine, gonad, or PC result in the long-term persistence of silenced *sid-1*+ progeny, suggesting a reservoir of dsRNA that can be accessed by either a *sid-1*-dependent process or a *sid-1*-independent but *rme-2*-dependent process (Figure 2). Coelomocytes are PC-localized phagocytic cells that accumulate 5EU labeled dsRNA (Figure 4B), and could thus act as the dsRNA reservoir if small amounts of dsRNA are constitutively released to produce persistent low levels of PC dsRNA. Consistent with the role of RME-2 as an endocytosis receptor, *sid-1* activity is required subsequent to RME-2-dependent dsRNA uptake, presumably to release the dsRNA from a membrane enclosed compartment (Figure 2). Because a recent report concluding that *rme-2* is required for parental RNAi conflicts with our results (MARRÉ *et al.* 2016), we repeated their analysis, discovering that progeny silencing in response to parental feeding RNAi is developmentally restricted, being most effective in adults. We found that the progeny of *rme-2* adults are efficiently silenced by feeding RNAi, which implies that SID-1 expression in oocytes is sufficient for import of PC dsRNA directly into the germline. (Figure 2). It remains unclear whether SID-1 is subsequently required in the embryo if maternal SID-1 is active in the oocyte.

The asymmetric redundancy between *sid-1* and *rme-2* for uptake and transport of dsRNAs into the germline contrasts with the necessity of both *sid-1* and *sid-2* for uptake and import of ingested dsRNAs into the intestine (WINSTON *et al.* 2007). Presumably, oocyte expressed SID-1 localizes to the plasma membrane adjacent to the PC space, whereas intestinal expressed SID-1 does not localize the lumenal plasma membrane. This difference may reflect the privileged environment of the PC space, relative to the ingested intestinal milieu. The redundancy between *sid-1* and *rme-2* is significant in light of reports of *sid-1*-independent RNAi-dependent transgenerational inheritance (SCHOTT *et al.* 2014). It will be interesting to determine whether this alternative RNA uptake mechanism mediates transgenerational inheritance and inheritance of acquired traits.

Our cytological analysis of dsRNA within oocytes and embryos showed that despite sharing an endocytosis receptor, yolk protein and dsRNA travel independent intracellular routes (Figure 5). While dsRNA and yolk may interact in the PC, our results indicate that they are either endocytosed independently or are quickly separated and independently sorted upon entering the oocyte. The observation that 5EU- but not Cy5-labeled dsRNA injected into the PC is internalized within oocytes (Figure 4) suggest specificity in dsRNA uptake. Indeed, the result reported by Marré *et al.* may actually represent a non-specific interaction between yolk protein and the bulky dye attached to a relatively short dsRNA, which may artificially prolong transient interactions with yolk, while the small single carbon modification of EU is less likely to interfere with the normal trafficking of RNA. Terminal labels can also potentially be hydrolyzed and separated from their targets. It is also not surprising that *sid-1*-dependent uptake was not observed (MARRÉ *et al.* 2016), as published results indicate that SID-1 transport is sensitive to minor dsRNA structural modifications (SHIH AND HUNTER 2011).

Our genetic analysis of dsRNA uptake in oocytes and embryos revealed a role for *sid-5* alongside *sid-1* in dsRNA import. SID-5 is a late endosomal/multivesicular body localized protein required for efficient systemic RNAi (HINAS *et al.* 2012). Tissue-specific rescue experiments showed that *sid-5* expression in the intestine, but not the muscle, was sufficient for RNAi silencing of a muscle gene (HINAS *et al.* 2012). The results presented here, showing a role for SID-5 in dsRNA import, suggest that efficient release of endocytosed ingested dsRNA to the intestinal cytoplasm may be important for subsequent export to muscle cells. This conclusion is supported by analysis of partial-loss-of-function *sid-1* alleles (WHANGBO *et al.* 2017). While *sid-1* and *sid-5* are required for this endocytosed dsRNA to trigger RNAi silencing, *sid-1* activity is apparently not required for transit to post-RAB-7 compartments (Figure 5).

Our results support a model where SID-1 is required to facilitate dsRNA transport into the cytoplasm directly from extracellular spaces as well as from endocytotic vesicles. Indeed, other organisms capable of systemic RNAi may be more reliant on endocytosis of dsRNA, including organisms that lack SID-1 (SALEH *et al.* 2006), and at least one insect species has been shown to use both SID-1 and endocytosis (CAPPELLE *et al.* 2016). SID-1 appears to be the ultimate gatekeeper for entry of dsRNA into cells, but endocytosis may facilitate SID-1 activity. A better understanding of the mechanisms by which circulating RNA reaches the next generation will help us identify endogenous inherited RNAs.

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References

Alcazar, R. M., R. Lin and A. Z. Fire, 2008 Transmission dynamics of heritable silencing induced by double-stranded RNA in Caenorhabditis elegans. Genetics 180**:** 1275-1288.

Buckley, B. A., K. B. Burkhart, S. G. Gu, G. Spracklin, A. Kershner *et al.*, 2012 A nuclear Argonaute promotes multigenerational epigenetic inheritance and germline immortality. Nature 489**:** 447-451.

Cappelle, K., C. F. R. de Oliveira, V. B. Eynde, O. Christiaens and G. Smagghe, 2016 The involvement of clathrin-mediated endocytosis and two Sid-1-like transmembrane proteins in double-stranded RNA uptake in the Colorado potato beetle midgut. Insect Molecular Biology 25**:** 315-323.

Feinberg, E. H., and C. P. Hunter, 2003 Transport of dsRNA into cells by the transmembrane protein SID-1. Science 301**:** 1545-1547.

Feng, Y., B. Press and A. Wandinger-Ness, 1995 Rab 7: an important regulator of late endocytic membrane traffic. The Journal of Cell Biology 131**:** 1435-1452.

Fire, A., S. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver *et al.*, 1998 Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 391**:** 806-811.

Grant, B., and D. Hirsh, 1999 Receptor-mediated endocytosis in the Caenorhabditis elegans oocyte. Molecular biology of the cell 10**:** 4311-4326.

Grishok, A., H. Tabara and C. C. Mello, 2000 Genetic requirements for inheritance of RNAi in C. elegans. Science 287**:** 2494-2497.

Guang, S., A. F. Bochner, K. B. Burkhart, N. Burton, D. M. Pavelec *et al.*, 2010 Small regulatory RNAs inhibit RNA polymerase II during the elongation phase of transcription. Nature 465**:** 1097-1102.

Guang, S., A. F. Bochner, D. M. Pavelec, K. B. Burkhart, S. Harding *et al.*, 2008 An Argonaute transports siRNAs from the cytoplasm to the nucleus. Science 321**:** 537-541.

Hamilton, A. J., and D. C. Baulcombe, 1999 A species of small antisense RNA in posttranscriptional gene silencing in plants. Science 286**:** 950-952.

Hinas, A., A. J. Wright and C. P. Hunter, 2012 SID-5 is an endosome-associated protein required for efficient systemic RNAi in C. elegans. Current biology 22**:** 1938-1943.

Hutvágner, G., and P. D. Zamore, 2002 RNAi: nature abhors a double-strand. Current opinion in genetics & development 12**:** 225-232.

Ivashuta, S., Y. Zhang, B. E. Wiggins, P. Ramaseshadri, G. C. Segers *et al.*, 2015 Environmental RNAi in herbivorous insects. RNA 21**:** 840-850.

Jao, C. Y., and A. Salic, 2008 Exploring RNA transcription and turnover in vivo by using click chemistry. Proc Natl Acad Sci USA 105**:** 15779-15784.

Marré, J., E. C. Traver and A. M. Jose, 2016 Extracellular RNA is transported from one generation to the next in Caenorhabditis elegans. Proc Natl Acad Sci USA 113**:** 12496-12501.

McEwan, D. L., A. S. Weisman and C. P. Hunter, 2012 Uptake of extracellular double-stranded RNA by SID-2. Molecular Cell 47**:** 746-754.

Saleh, M.-C. C., R. P. van Rij, A. Hekele, A. Gillis, E. Foley *et al.*, 2006 The endocytic pathway mediates cell entry of dsRNA to induce RNAi silencing. Nature Cell Biology 8**:** 793-802.
Schott, D., I. Yanai and C. P. Hunter, 2014 Natural RNA interference directs a heritable response to the environment. Scientific Reports 4**:** 7387.
Sharp, P. A., 2001 RNA interference—2001. Genes & development 15**:** 485-490.
Sharrock, W. J., M. E. Sutherlin, K. Leske, T. K. Cheng and T. Y. Kim, 1990 Two distinct yolk lipoprotein complexes from Caenorhabditis elegans. The Journal of biological chemistry 265**:** 14422-14431.
Shih, J. D., and C. P. Hunter, 2011 SID-1 is a dsRNA-selective dsRNA-gated channel. RNA 17**:** 1057-1065.
Timmons, L., D. L. Court and A. Fire, 2001 Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in Caenorhabditis elegans. Gene 263**:** 103-112.
Whangbo, J. S., A. S. Weisman, J. Lu, J. Chae and C. P. Hunter, 2017 SID-1 domains important for dsRNA import in C. elegans. bioRxiv.
Winston, W. M., 2002 Systemic RNAi Defective Mutants in the Nematode *C. elegans*. Harvard University.
Winston, W. M., C. Molodowitch and C. P. Hunter, 2002 Systemic RNAi in C. elegans requires the putative transmembrane protein SID-1. Science 295**:** 2456-2459.
Winston, W. M., M. Sutherlin, A. J. Wright, E. H. Feinberg and C. P. Hunter, 2007 Caenorhabditis elegans SID-2 is required for environmental RNA interference. Proc Natl Acad Sci USA 104**:** 10565-10570.
Zamore, P. D., T. Tuschl, P. A. Sharp and D. P. Bartel, 2000 RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell 101**:** 25-33.

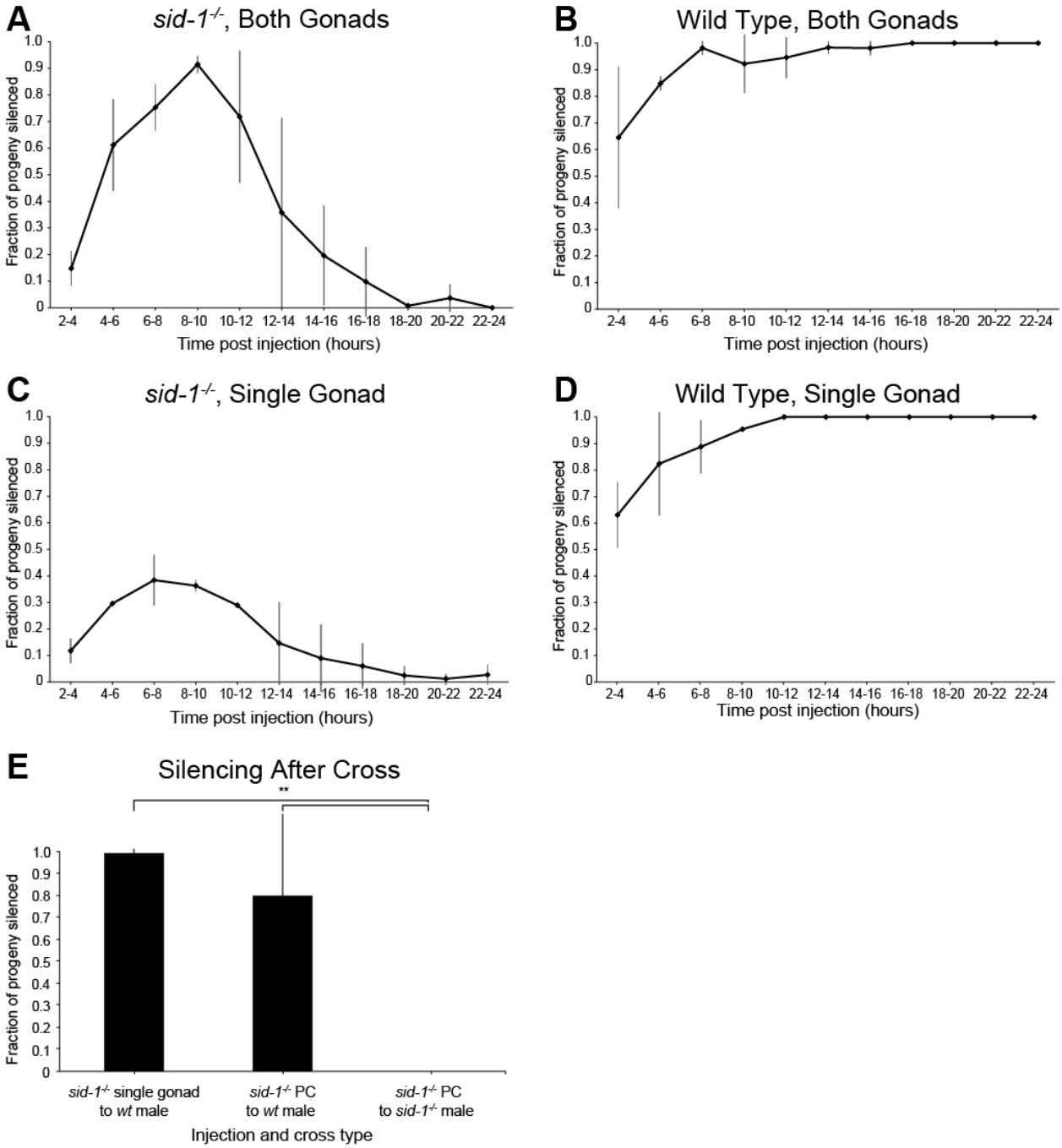

Fig. 1. *sid-1*-dependent and -independent silencing in progeny of dsRNA injected parents. (*A-D*) Time course of fraction of progeny with the Unc-22 phenotype laid after *unc-22* dsRNA gonad injection into wild-type or *sid-1* mutant hermaphrodites. (*E*) Fraction of progeny with Unc-22 phenotype following *unc-22* dsRNA gonad or PC injection into *sid-1*$^{-/-}$ mutant hermaphrodites crossed to wild-type or *sid-1* mutant males. Error bars in (*A-D*) represent standard error from two experiments with 10 injected hermaphrodites each. Error bars in (*E*) represent standard deviation from 4, 6, and 3 injected hermaphrodites, left to right. $^{**}p < 0.01$ by Welch's *t* test.

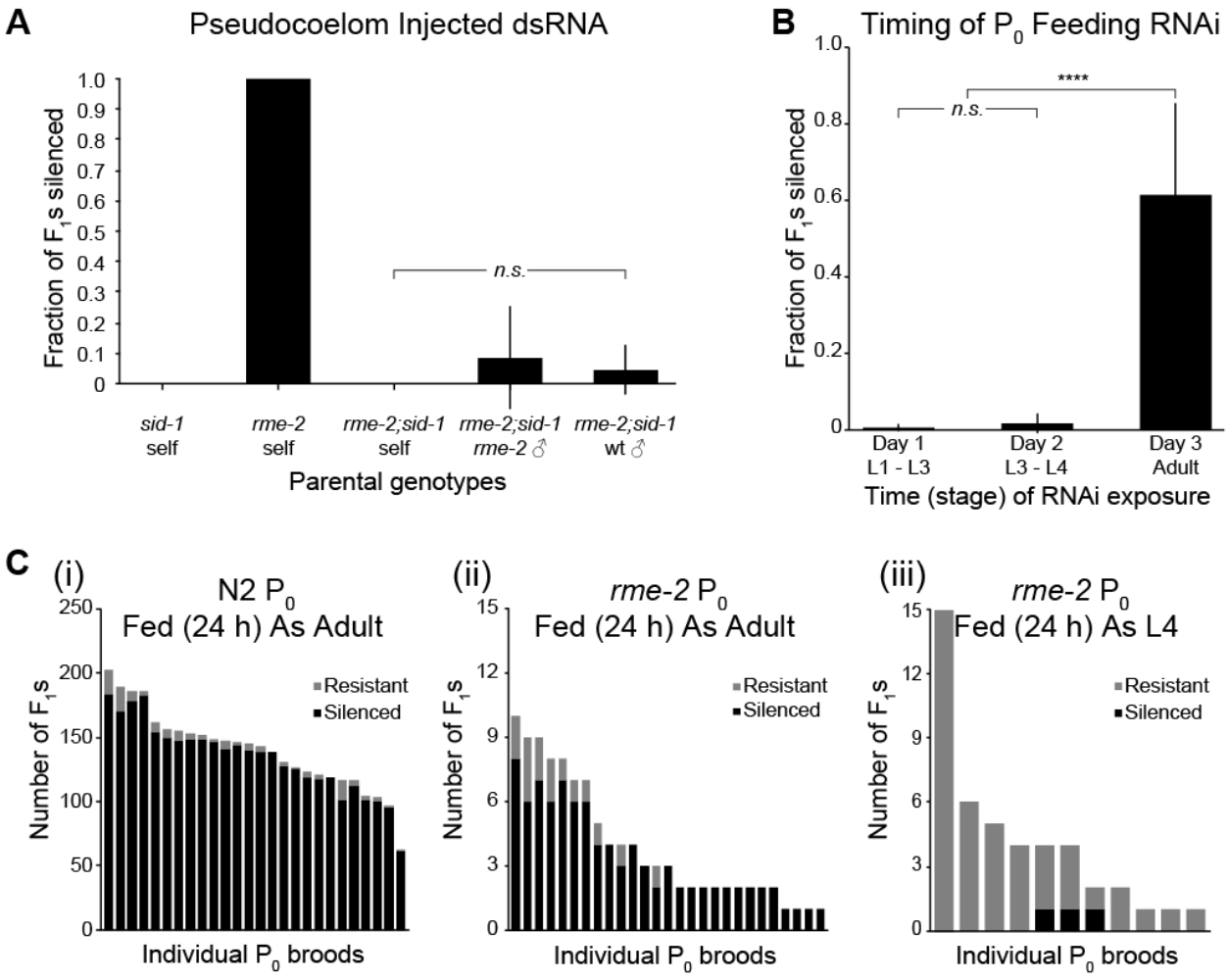

Fig. 2. Maternal RME-2-dependent inherited silencing. (*A*) Fraction of progeny sensitive to *unc-22* silencing among the self-progeny or indicated cross-progeny of hermaphrodites PC-injected with *unc*-22 dsRNA. *n* = 6, 5, 3, 4, and 12 injected hermaphrodites respectively. (*B*) Fraction of progeny sensitive to *unc-22* silencing after wild-type parents were exposed to feeding RNAi at the given periods of time after hatching. *n* = 30 treated parents for each group. (*C*) Sensitivity to *unc-22* feeding RNAi in progeny after treating (*i*) wild-type parents as adults, (*ii*) *rme-2* mutant parents as adults, or (*iii*) *rme-2* mutant parents as L4 larvae. Because *rme-2* mutants have severely reduced fecundity, the results from each individual parent are presented separately for clarity, with silenced progeny represented in black bars and non-silenced progeny in grey bars. All error bars represent standard deviation. ****$p < 0.00001$ by *t* test. *n.s.* = not significant.

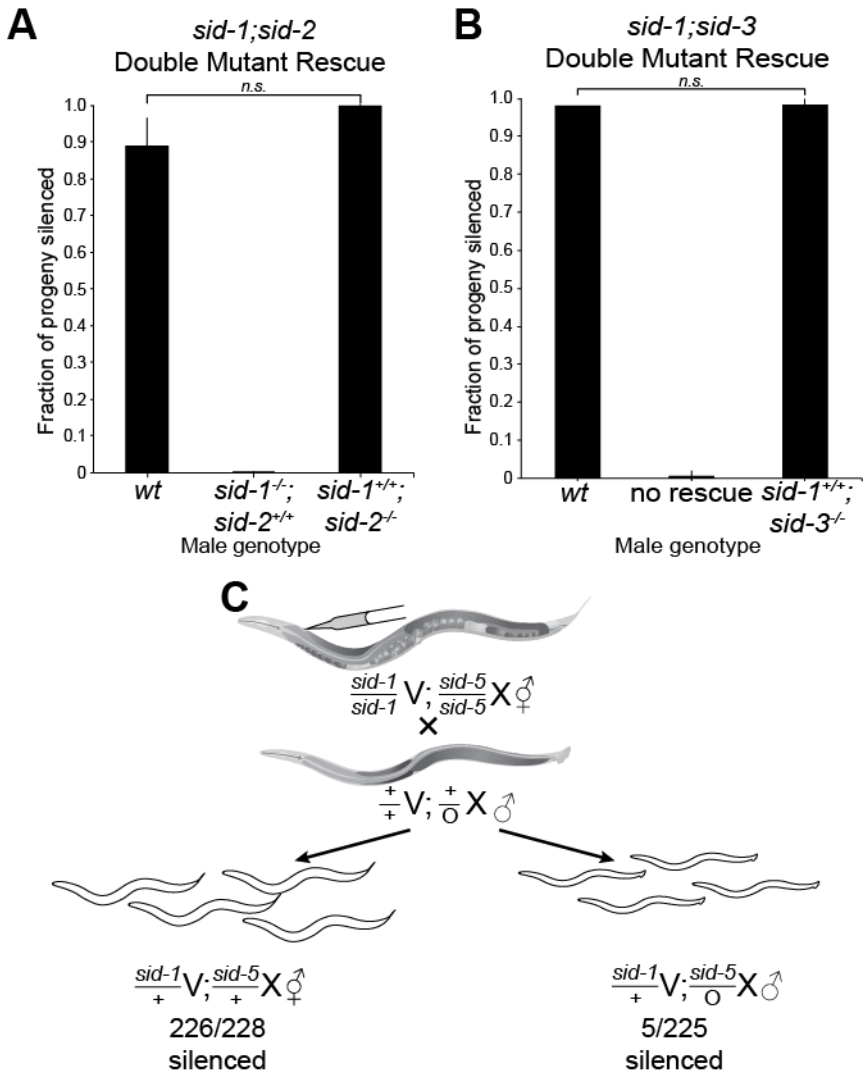

Fig. 3. Maternal and zygotic Sid-dependence of inherited silencing. (*A*) Fraction of *unc-22* silenced cross progeny from *sid-1; sid-2* double-mutant hermaphrodites first PC-injected with *unc-22* dsRNA and then crossed to either wild-type, *sid-1; sid-2* double mutant, or *sid-2* single mutant males. (*B*) Fraction of *unc-22* silenced cross progeny from *sid-1; sid-3* double-mutant hermaphrodites first PC-injected with *unc-22* dsRNA and then crossed to wild-type, *sid-1; sid-3* double mutant, or *sid-3* single mutant males. (*C*) Schematic of the injection, cross, and *unc-22* silencing scoring of cross progeny from *sid-1; sid-5* double mutant hermaphrodites first PC-injected with *unc-22* dsRNA and then crossed to wild-type males. *sid-5* is X-linked, thus hermaphrodite progeny are heterozygous and males are hemizygous. Error bars in (*A, B*) represent standard deviation from 4, 7, and 5 injected hermaphrodites in (*A*) and 1, 3, and 3 injected hermaphrodites in (*B*). 3 injected hermaphrodites in (*C*). *n.s.* = not significant.

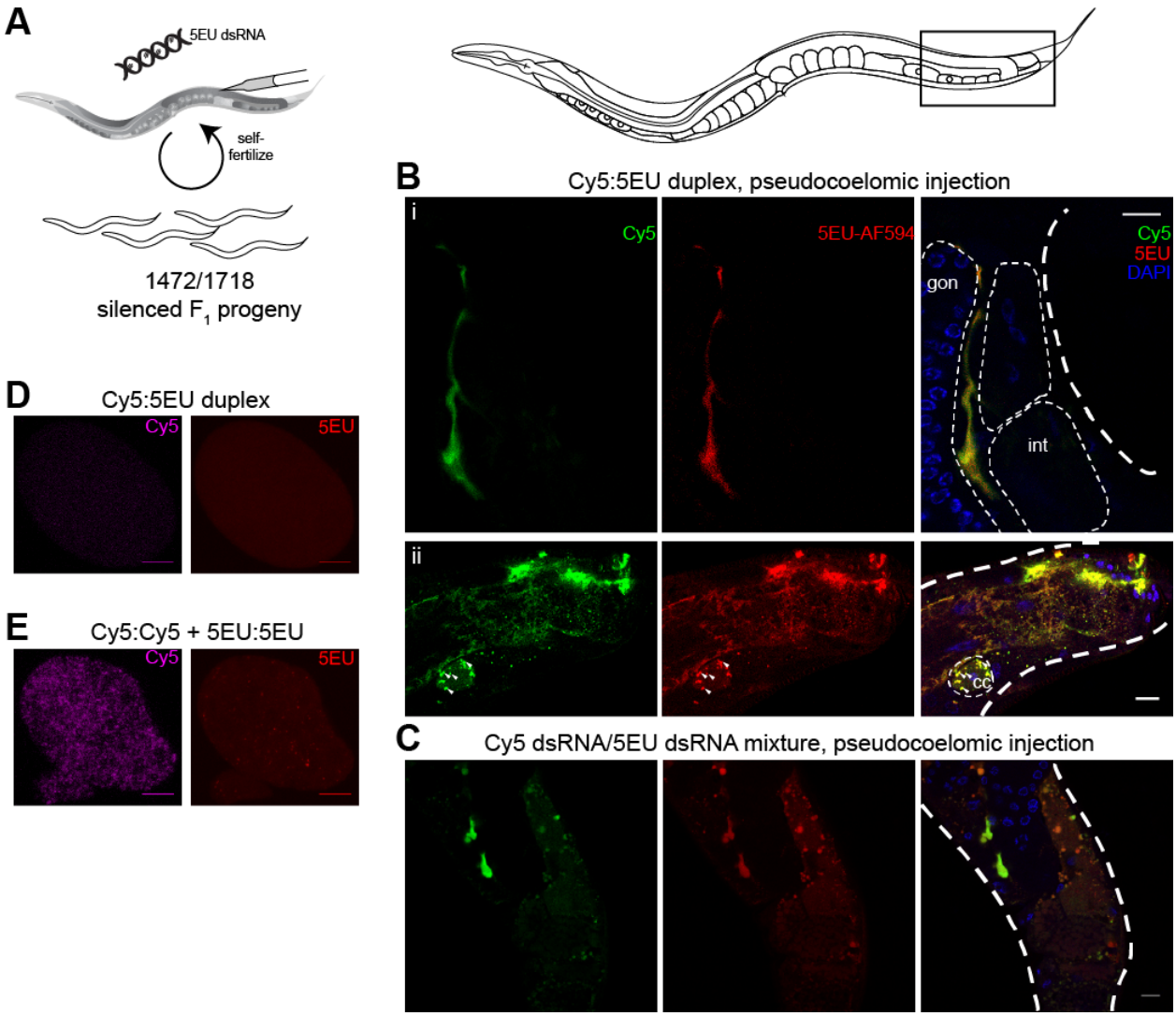

Fig. 4. Visualizing 5-Ethynyluridine (5EU) labeled functional dsRNA. (*A*) Injected 5EU dsRNA injected into only one gonad arm produces >50% affected progeny. *n* = 8 injected hermaphrodites. (*B*) Localization of PC injected Cy5::5EU heteroduplex dsRNA. Cy5 fluorescence and 5EU detection co-localize in the pseudocoelom (*i*) and a coelomocyte (*cc)* (ii; white arroweads). (*C*) Independent localization of PC injected 5EU and Cy5 labeled dsRNA. Images in (*B*) and (*C*) represent portions of dissected and partially flattened adult hermaphrodites. Thick dotted lines mark the boundary of the animal, and thinner dotted lines mark structures such as the gonad (*gon*) or intestinal cells (*int*) as landmarks for orientation. (*D, E*) Cy5 and 5EU signal in embryos collected from adults injected with the dsRNA species described in (*B*) and (*C*) respectively. The two Cy5 images are overexposed, revealing diffuse autofluorescence and no detectable RNA. Scale bars = 10 μm.

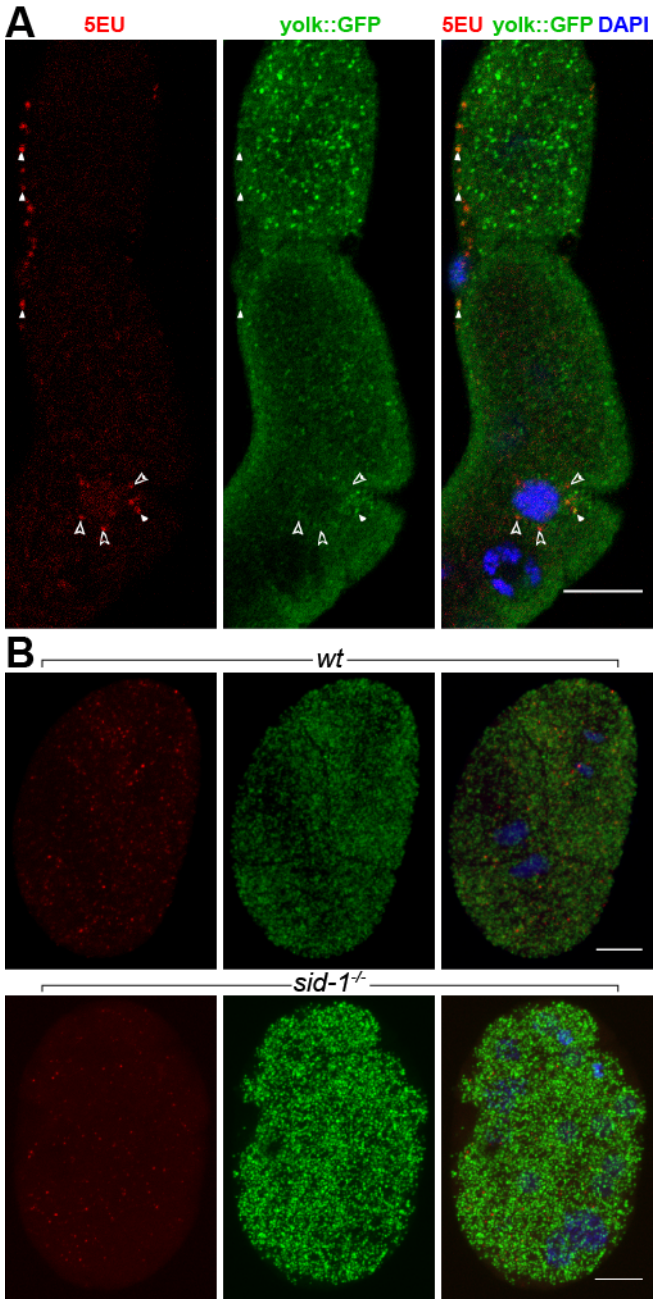

Fig. 5. Co-localization of 5EU-labeled dsRNA and VIT-2::GFP on and within oocytes. (*A*) PC-injected 5EU-labeled dsRNA co-localized with GFP-labeled yolk at the surface of developing oocytes (white arrowheads), but not intracellularly (notched arrowheads). More proximal oocyte (top) contains more VIT-2::GFP. (*B*) Maximum *z*-projections of VIT-2::GFP and 5EU-labeled dsRNA in wild-type and *sid-1*$^{-/-}$ embryos show little co-localization. Scale bars = 10 μm.

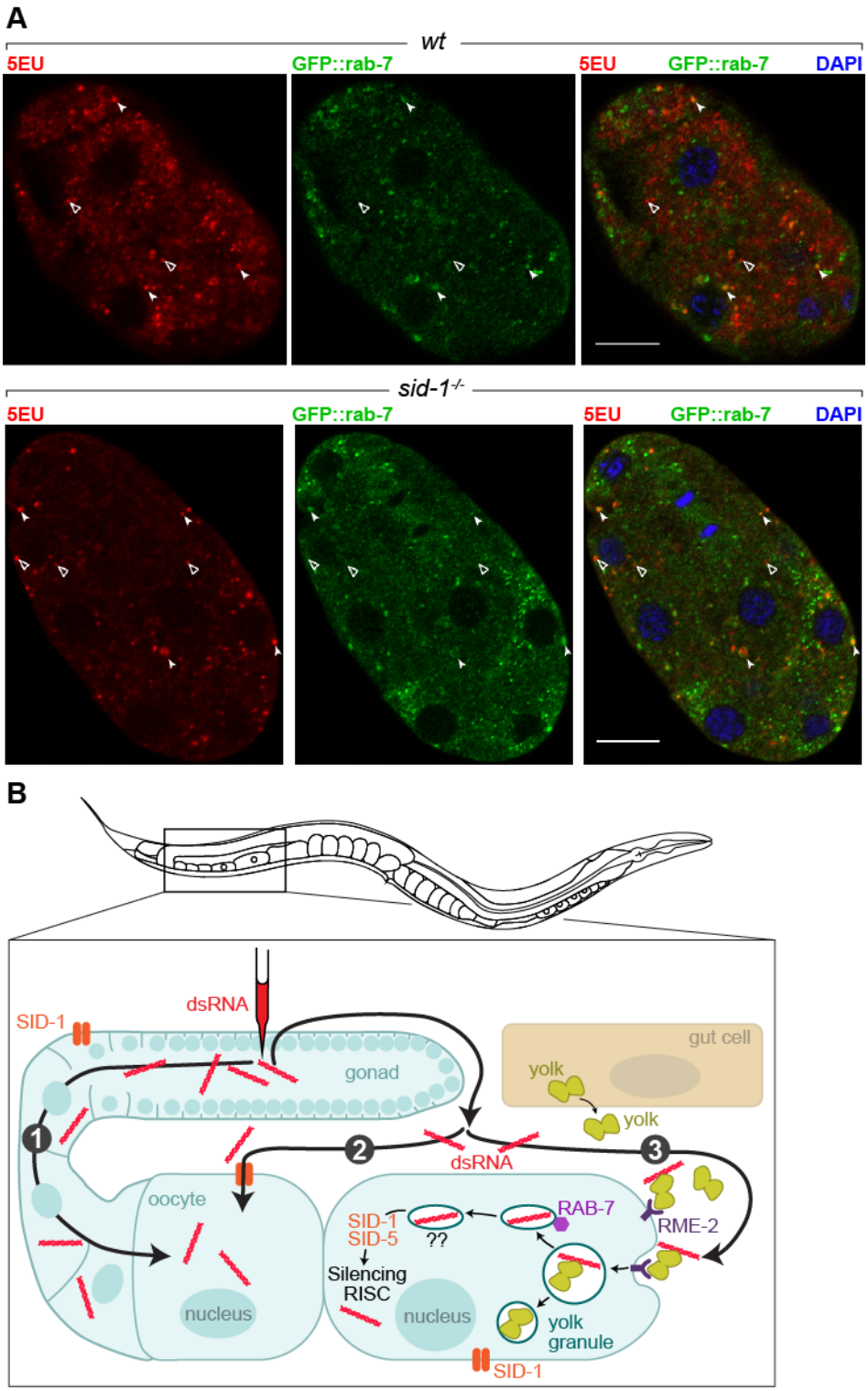

Fig. 6. Co-localization of dsRNA and GFP::RAB-7. (*A*) 5EU foci detected in embryos after pseudocoelomic 5EU-labeled dsRNA injection co-localizes with GFP:: RAB-7 (notched arrowheads) but is also found outside of RAB-7 structures (open arrowheads)

in both wild-type (upper row) and *sid-1* mutant (*Lower row*) embryos. Scale bars = 10 μm. (*B*) Model for three inherited dsRNA transport pathways. 1) DsRNA injected directly into the syncytial germline can silence the resulting progeny without SID-1. Some injected dsRNA exits the gonad to the PC and is then directly or indirectly via yolk 2) endocytosed into developing oocytes via LDL receptor super-family member RME-2, or 3) this PC dsRNA can also be directly transported into oocytes via SID-1.