Artificial and natural RNA interactions between bacteria and *C. elegans*

Fabian Braukmann[1,2], David Jordan[1,2] & Eric Miska[1,2,3]

[1] Gurdon Institute, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QN, United Kingdom

[2] Department of Genetics, University of Cambridge, Downing Street, Cambridge, CB2 3EH, United Kingdom

[3] Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Cambridge, CB10 1SA, United Kingdom

Abstract

19 years after Lisa Timmons and Andy Fire first described RNA transfer from bacteria to *C. elegans* in an experimental setting [Timmons and Fire, 1998] the biological role of this trans-kingdom RNA-based communication remains unknown. Here we summarize our current understanding on the mechanism and potential role of such social RNA.

1 Introduction

Caenorhabditis elegans is a small (≈ 1mm) free-living nematode found in microbe rich rotting fruits and vegetation in temperate climates. It was chosen by Sydney Brenner as a model organism due to its numerous advantages for genetics and cell biological analysis. It is easy to maintain in the laboratory, living on a diet of *Escherichia coli* bacteria. It is a self-fertilizing hermaphrodite with a relatively short generation time (three days), and a large brood size (≈300), facilitating the generation of large isogenic populations of worms. In addition, it is transparent, an advantage for microscopy and cell biology. Through the painstaking work of John Sulston, together with Robert Horvitz, the entire cell lineage from egg to adult was mapped, including the 302 neuron nervous system [Sulston and Horvitz, 1977][Sulston, 1976].

1.1 Discovery of RNA interference (RNAi) in an animal

The use of RNA sequences which are complementary (antisense) to some region of the mRNA of a gene of interest, in hopes that through Watson-Crick base pairing, the two molecules will hybridize and lead to misregulation of the expression of that gene, was first described by Izant and Weintraub in 1984 [Izant and Weintraub, 1984] as an alternative to classical genetic analysis of mutants. Antisense RNA continued to be developed and became a common tool in the molecular biology toolkit [Fire et al., 1991]. However, antisense gene knockdowns were generally not robust, and the mechanism by which silencing occurred was not known. It was not until a decade later that the first tantalizing hints of the pathway, later to become known as the RNA

interference, or RNAi pathway, were uncovered. In 1995, Guo and Kemphures, in an attempt to inject antisense RNA to knockdown par-1 in *C. elegans*, reported similar knockdown efficiency for both the antisense and the sense strands alone, as well as the co-injection of both the sense and antisense strands corresponding to the par-1 mRNA [Guo and Kemphues, 1995]. While previous reports of so-called co-suppression, or quelling, had been reported in petunias [Napoli et al., 1990], and in *Neospora crassa* [Romano and Macino, 1992], this was the first example in an animal, and the inspiration for the seminal work of Fire and Mello, who, in 1998, hypothesized that contaminants in the single-stranded RNA (ssRNA) preparation led to double-stranded RNA (dsRNA) and that these dsRNAs were responsible for the silencing. Their work showed that targets could be efficiently silenced by injection of dsRNAs with sequence complementarity to the target [Fire et al., 1998].

1.2 RNAi Mechanism in *C. elegans*

The RNAi pathway is used to regulate gene expression [Grishok, 2013]. The pathway begins with the processing of either an endogenously generated or an environmentally supplied dsRNA. The dsRNA binding protein RDE-4 interacts with the dsRNA and initiates cleavage of the RNA by the conserved endonuclease DCR-1 [Tabara et al., 2002] [Parker et al., 2006]. DCR-1 produces double stranded-short interfering RNAs (ds-siRNAs) which are subsequently loaded into the Argonaute protein RDE-1 [Knight and Bass, 2001] [Yigit et al., 2006]. The passenger strand is degraded and the remaining strand guides RDE-1 via Watson and Crick base pairing to a target mRNA [Steiner et al., 2009]. The mRNA-RDE-1 complex is thought to recruit the RNA-dependent RNA polymerase (RdRP) RRF-1 which leads to generation of secondary siRNAs [Pak and Fire, 2007] [Sijen et al., 2007]. The secondary siRNAs are loaded into other proteins of the Argonaute family and initiate post-transcriptional gene silencing in the cytoplasm via mRNA degradation and co-transcriptional gene silencing in the nucleus via modification of the chromatin [Guang et al., 2008][Guang et al., 2010].

2 Systemic and Environmental RNAi

While it took many years for the details described above to be worked out, soon after RNAi was first described in 1998, Tabara and Mello showed that RNAi could be initiated by soaking worms in a solution of dsRNA [Tabara et al., 1998], and Timmons and Fire demonstrated that by expressing dsRNAs in the *E. coli* food of *C. elegans*, silencing could be induced by feeding [Timmons et al., 2001]. This has become known as environmental RNAi. The RNAi by feeding technique was optimized a few years later, using RNase deficient bacteria [Kamath et al., 2000], and a few years after that, the first genome wide RNAi screen was reported [Kamath and Ahringer, 2003]. In the intervening year, working to understand how dsRNA injected into one tissue could lead to silencing in other tissues, Winston and Hunter reported the results of the first genetic screens for this so-called systemic RNAi, uncovering systemic RNAi deficient (Sid) genes, including

sid-1 [Winston et al., 2002], which encodes a dsRNA specific trans-membrane channel [Feinberg and Hunter, 2003]. A second Sid protein, SID-2, which is a single pass trans-membrane protein, was found to be essential for environmental RNAi (See detailed review [Jose, 2015]). The systemic RNAi genes are distinct from the genes involved in the canonical RNAi pathway described above and are not required for RNAi itself. In other words, in the absence of systemic RNAi genes, silencing still occurs, but it is localized and must be initiated by endogenously produced or injected dsRNA. The genes involved in systemic RNAi can be divided into two classes. One set of genes are important for the uptake of dsRNA from the environment into the worm, while a second set of genes are important for the transport of RNA, which is already in the worm, from one tissue to another.

2.1 Environmental RNAi

In the current understanding of the environmental RNAi pathway, dsRNA from the environment is initially internalized from the gut lumen. Subsequently, the internalized dsRNA is either transported through the intestinal cell from the apical to the basal surface and released into the pseudocoelom, or is first released into the gut cytoplasm and from there exported into the pseudocoelom. The dsRNA is later imported into the recipient tissue, where it enters the RNAi pathway [Jose, 2015].

2.1.1 SID-2

In *C. elegans,* the only known role of the single-pass trans-membrane protein SID-2 is the uptake of dsRNA from the environment into the gut. *Sid-2* mutants do not show any sign of RNAi by feeding, but are able to down-regulate GFP in the body wall via pharyngeal expressed RNA hairpins which target GFP (hpGFP) (Winston et al., 2007). Therefore SID-2 is not required for intercellular transport of RNA within a *C. elegans*. However, SID-2 is required for dsRNA uptake from the gut lumen into the worm as indicated by the failure to detect fluorescently labeled dsRNA inside *sid-2* mutant worms after soaking (McEwan et al., 2012). In addition, SID-2 localizes in the gut as indicated by expression analysis of SID-2::GFP fusion protein (Winston et al., 2007). Expression of *sid-2* in a heterologous system showed specificity for the uptake of ≥ 50 bp dsRNA (McEwan et al., 2012). These findings indicate SID-2's important role in environmental RNAi, and in the future, studies on naturally occurring RNA uptake should focus on dsRNA uptake in the gut. However, it cannot be ruled out that another mechanism of RNA uptake exists that does not require SID-2. Such a system would have different properties and if it exists, its function may be independent of the RNAi pathway. Here, we focus on the potential of environmental dsRNA as a mechanism of communication.

2.2 Systemic RNAi

2.2.1 SID-1

The first gene identified to be important for dsRNA transport was *sid-1*. (Winston et al., 2002). SID-1 is a multispan trans-membrane protein and putative dsRNA channel. Expression of a SID-1::GFP fusion protein showed localization at the plasma membrane of almost all *C. elegans* cells, but appeared to be excluded from most neurons [Winston et al., 2002]. The use of *sid-1* mutant worms expressing SID-1 in a mosaic fashion indicated that SID-1 is required for the import of mobile RNA [Jose et al., 2011, Jose et al., 2009]. Recent experiments have shed further light on the precise mechanism of dsRNA import via SID-1. This year, Marré and colleagues showed that fluorescently labeled dsRNA enters a cell via RME-2 (Receptor Mediated Endocytosis), a member of the low-density lipoprotein receptor superfamily, but that SID-1 is required for the release of the dsRNA into the cytoplasm [Marré et al., 2016]. Work in *Drosophila melanogaster* S2 cells indicated that SID-1 enhances the uptake of dsRNA longer than 50 bp, but that complete sequence complementarity is not required. Thus primary miRNAs, which include a hairpin, can potentially be transported by SID-1 [Shih and Hunter, 2011]. Surprisingly, neurons are deficient in RNAi by feeding, however over-expression of SID-1 from a neuronal promoter renders these cells RNAi competent indicating that SID-1 is required to take up dsRNA into neurons [Calixto et al., 2010]. These findings suggest that naturally occurring dsRNA can regulate the function of most tissues except for neurons. Therefore, an environmental dsRNA can have a systemic effect on gene expression or even a transgenerational effect via the germline. Current evidence suggests that SID-1 and SID-2 have similar physical requirements for the transport of RNA.

2.2.2 SID-3

SID-3 is a conserved tyrosine kinase required for the import of mobile RNAs [Jose et al., 2012]. SID-3 is expressed in many tissues in the cytoplasm in punctuated foci as indicated by the analysis of the expression of *sid-3::gfp* and *sid- 3::mCherry* transgenes. It is speculated that these foci are related to endocytosis, since the human homologue of SID-3, the activated CDC42-associated kinase (ACK) localizes to endocytic vesicles [Harris and Tepass, 2010]. The use of *sid-3* mutant worms with tissue specific rescue of *sid-3* revealed that SID-3 is required for the import of mobile RNA [Jose et al., 2012]. It is believed that SID-3 is an important protein in a signaling pathway for efficient RNA import, since the human CDC42 is implicated in many signaling pathways [Melendez et al., 2011]. Interestingly, in the absence of SID-3, local RNAi is enhanced.

2.2.3 SID-5

SID-5 is a predicted single-pass trans membrane protein important for efficient systemic RNAi. SID-5 is an important member of the pathway because it links RNA mobility to vesicle transport. Reports previously indicated that endosomal proteins are required for efficient RNAi but not for RNA mobility [Lee et al., 2009]. SID-5 is the first protein of the Sid-pathway, which co-localises with endosome markers and has a demonstrated role in RNA transport [Hinas et al., 2012]. SID-5 is required for RNAi in the gut via RNAi by feeding, suggesting that SID-5 has a similar role as SID-2. However, immunofluorescent

stains show SID-5 expression in a range of tissues, in contrast to SID-2 which is expressed only in the gut. In addition, SID-5 is relevant for RNA silencing in the body wall muscle (bwm) initiated from the pharynx in contrast to SID-2. This indicates that SID-5 has a different role than SID-2. In addition, SID-5's role must differ from the role of SID-1 and SID-3, as a rescue of *sid-5* in the recipient tissue (bwm) does not restore RNA silencing [Hinas et al., 2012]. This indicates that it is important for the export of RNA. Further experiments are required to determine the exact role of SID-5 in the systemic RNAi pathway.

2.2.4 SEC-22

SEC-22 is a SNARE (Soluble NSF Attachment Protein Receptor) protein. SNAREs are a superfamily of proteins involved in membrane fusion and therefore important for vesicle trafficking [Ungar and Hughson, 2003]. In *C. elegans,* SEC-22 suppresses small RNA mediated silencing in a *sid-5* dependent manner. The use of *sid-5* mutant worms with tissue specific rescue of *sec-22* indicates that SEC-22 either inhibits trafficking or the RNAi machinery in the cell. In addition, mCherry::SEC-22 fusion proteins localize to the late endosome similar to SID-5. Furthermore, SEC-22 interacts with SID-5 in a yeast two-hybrid system [Zhao et al., 2016]. Therefore, it is clear that SEC-22 is a negative regulator of RNAi and one may speculate that SEC-22 does so by promoting late endosome degradation. However, further experiments are required to untangle the role of SEC-22 in systemic RNAi or regulation of the RNAi factors within the cell.

2.2.5 RSD-3

RNAi spreading defective-3 (*rsd-3*) is required for RNA uptake in somatic and germline cells [Imae et al., 2016]. Similar to SID-1 and SID-3, RSD-3 functions in RNA import. RSD-3 encodes for a homolog of epsinR and has a conserved ENTH (epsin N-terminal homology) domain. In mammalian systems, the ENTH domain is important for endomembrane trafficking [Saint-Pol et al., 2004]. Interestingly, the ENTH domain of RSD-3 is sufficient in *C. elegans* for mobile RNAi. This suggests that the ENTH domain is able to mediate on its own downstream activities for RNA uptake or alternatively the ENTH domain is sufficient to down-regulate a RNA uptake suppressing signal. This provides another piece of evidence for the importance of the vesicle transport pathways for mobile RNA. Transgene expression experiments shows that RSD-3 is expressed in many tissues and co-localizes with factors of the trans-Golgi network and the endosome [Imae et al., 2016]. While the exact role of RSD-3 remains unclear, it is tempting to speculate about its role, and the role of its ENTH domain is to function as cargo adapter. However, the ENTH domain has not been reported to bind RNA, therefore it is unlikely that RSD-3 directly interacts with RNA. More likely, the role of *rsd-3* could be to sort other SID pathway members to their correct locations in vesicle trafficking.

2.2.6 MUT-2

Mut-2, known also as *rde-3*, is a putative nucleotidyltransferase important for mobile

RNA. *Mut-2* was first identified to play a role in transposon regulation [Collins and Anderson, 1994]. Subsequently, it became evident that *mut-2* is essential for RNAi induced by feeding, but is not required for RNAi induced by transgene-driven expression of dsRNA [Chen et al. 2005]. A more detailed analysis by Jose et al. 2011 found that *mut-2* is required for silencing by transgene-driven expression of dsRNA that is spatially separated from the tissue where the silencing occurs. Surprisingly, *mut-2* can be rescued by the expression of *mut-2* in only the tissue producing the mobile RNA (donor), or in only the tissue in which the silencing occurs (recipient). This indicates that mut-2 has a dual role (1) exporting the RNAi signal from the donor tissue, and (2) generating efficient RNAi in the recipient tissue. Therefore, in the donor tissue, the role of MUT-2 could be marking the mobile RNA by nucleotide addition for efficient export, and in the recipient, to generate efficiently the secondary siRNA.

2.3 Summary of Environmental RNAi

Overall, environmental RNAi is an intriguing biological process. In the last 20 years, we have gained insights into the mechanism via genetic analysis and have come to understand some of the physical requirements for RNA mobility in *C. elegans*. RNA transport is not as simple as diffusion and uptake in a recipient tissue. Many cellular processes, such as vesicle transport, are involved to regulate export and import of mobile RNAs. However, we still do not understand how RNA transport is regulated and how an RNA is chosen for delivery. Furthermore, our understanding of RNA transport remains very limited to its function in relation to RNAi, additional functions remain to be explored.

3 From Artificial to Natural Context

RNAi by feeding has become a simple and powerful tool for the specific knockdown of genes. That it works so well, however, is intriguing because so far, it is only known in the artificial context of bacteria genetically engineered to express dsRNA with sequence complementarity to endogenous *C. elegans* genes. This leads one to speculate whether there may be a natural context in which the uptake of RNA for silencing may be important. While there are clear examples of trans-kingdom communication via RNA intermediates in a natural context, e.g. between humans and some of their parasites, and between plants and fungi [Knip et al., 2014], it is odd that no natural context has yet been discovered for *C. elegans*, where RNAi by feeding is such a powerful tool. Some recent experiments by Melo and Ruvkun may provide a conceptual bridge between artificial and natural RNAi by feeding. In these experiments, Melo and colleagues showed that *E. coli* artificially expressing dsRNAs against conserved genes in *C. elegans* could induce an avoidance response in the nematodes [Melo and Ruvkun, 2012]. The next step, then, was to look for naturally occurring RNAs in *E. coli* with sequence complementarity to *C. elegans* genes. Liu and colleagues reported on two such naturally occurring non-coding RNAs in *E. coli*, OxyS and DsrA, which have sequence complementarity to the

C. elegans genes *che-2* and F42G9.6, respectively [Liu et al., 2012]. In these experiments, Liu et al. used a variety of behavioral and genetic techniques to assess whether these bacteria which had altered expression of these non-coding RNAs had any effect on *C. elegans* which fed on them. Their paper reports avoidance of *E. coli* over-expressing the OxyS ncRNA, as well as a variety of other chemosensory deficiencies in worms fed on OxyS expressing bacteria. Further, they report increased lifespan in worms fed on DsrA deficient bacteria. However, there is no direct evidence in this paper that these physiological changes are the direct result of ingested ncRNAs, in fact, they show that these effects are still observed in mutants of *sid-1* and *sid-2*, which are required for canonical environmental RNAi. In a follow up to this work, Akay and colleagues [Akay et al., 2015], showed that there is no activation of the canonical RNAi pathway by showing the absence of primary and secondary siRNAs mapping to the OxyS RNA via small RNA sequencing.

It is also important to note that *E. coli* is not a significant natural food source for *C. elegans* [Samuel et al., 2016]. In this paper, Samuel and colleagues collected likely *C. elegans* microbiomes from a variety of decaying vegetation in which these worms are often found. These were classified using 16S sequencing and, while no sequences matching Escherichia were found, many isolates contained other *Enterobacteriaceae* in low abundance. Samuel and colleagues also measure the time to adulthood of worms fed on different natural bacteria and found that the different bacteria can accelerate or delay development. In addition, they showed that the effect was not purely nutritional. While activation of pathogenicity responses to toxic bacteria are likely the cause of these effects, it will be interesting to see if environmental RNAi may also play a role.

It may be however, that despite the effectiveness of bacterially produced RNAs to silence *C. elegans* genes in the laboratory, that this simply does not occur naturally. It would require the co-evolution of the sequences across kingdoms, which, while not unprecedented, requires repeated interaction of the species over evolutionary time scales. One alternative may be that environmental RNAi is a mechanism of communication between worms. One could imagine that feeding on the corpses of worms from the previous generation could provide information via RNA which could be interpreted using the RNAi machinery. Interestingly, *C. elegans*, under stressful conditions such as starvation, exhibit a so-called bag-of-worms phenotype, where embryos hatch inside the cuticle of the mother [Fay D. 2006]. This may provide one mechanism for the transmission of information vertically via environmental RNAi. One can speculate that this might be useful for relaying very recent information from the parental generation.

Initially proposed by Lisa Timmons and Andy Fire, environmental RNAi could also function in antiviral immunity [Timmons and Fire, 1998]. The recent discovery of a first *C. elegans* virus, the Orsay RNA virus, [Felix et al., 2011] allowed this to be tested. Indeed, *C. elegans* uses RNAi in antiviral defense [Felix et al., 2011][Ashe et al., 2013] and delivery of dsRNA against the viral genome through bacterial feeding immunizes

C. elegans against the virus [Ashe et al.,2015]. However, the generation of an antiviral RNAi response seems to be localized to the infected cells, with no evidence that it is either systemic or transgenerational [Ashe et al., 2015].

4 Perspectives

RNAi in *C. elegans* is potent and specific. Using sequence complementarity, information about a specific gene to silence is encoded into RNA molecules. A specific pathway exists for these molecules to be taken up from the environment and initiate systemic gene silencing. This system has a unique property in that it is a sort of universal translator. Any system capable of generating a dsRNA molecule can use this system as a communication channel. Thus, in contrast to a small molecule based system, which requires the evolution of a signaling pathway to respond to it, RNAi can be used to affect the expression of specific genes for which no pre-existing pathway yet exists. This system provides a way for information to be passed vertically, from one organism to its offspring, and horizontally, from the environment, and thus, in theory, between organisms [Sarkies and Miska, 2013]. Furthermore, this provides a mechanism for information acquired from the environment of the parent to be transmitted and to influence the phenotype of the offspring. Physicist Murray Gell-Mann stated, regarding particle physics, that anything not forbidden is compulsory. Taking this to heart, surely inappropriately, we look forward to the first conclusive evidence of RNA mediated communication in a natural context in *C. elegans*. The questions to follow, such as when is RNA used as a signaling medium in contrast to other modalities, are surely to be of interest not just to RNA biologists, but to biologists in general.

Figure Legends

Figure 1. **Working model of the Sid pathway**.

A) dsRNA is taken up via SID-2 mediated endocytosis from the gut into the intestinal epithelium. There are two competing theories for how the dsRNA exits the vesicle.

B) First is immediate exit into the cytoplasm via SID-1.

C) The second is that the dsRNA is transported via SID-5 through the membrane into the body cavity, and subsequently, re-enters the cell via a complex mechanism involving RSD-3, SID-3, SEC-22 and SID-1.

D) Once the dsRNA entered the cytoplasm silencing requires MUT-2. Additionally, MUT-2 has been proposed to have a role in the Sid pathway. MUT-2 may also mark dsRNA within a cell for export.

E) In the germline, an additional mechanism of RNA import exists. Genetic evidence demonstrates that dsRNA enters via the RME-2 receptor. A previously described role of the receptor is the import of the intestinally expressed yolk protein YP170 from the body cavity. The main role of the yolk protein is the delivery of nutrients for the newly forming oocytes. Therefore, it can be speculated that dsRNA associates with YP170 in the body cavity and/or in the intestinal epithelium, which can lead to silencing in the germline and in the offspring via a SID-1 dependent mechanism.

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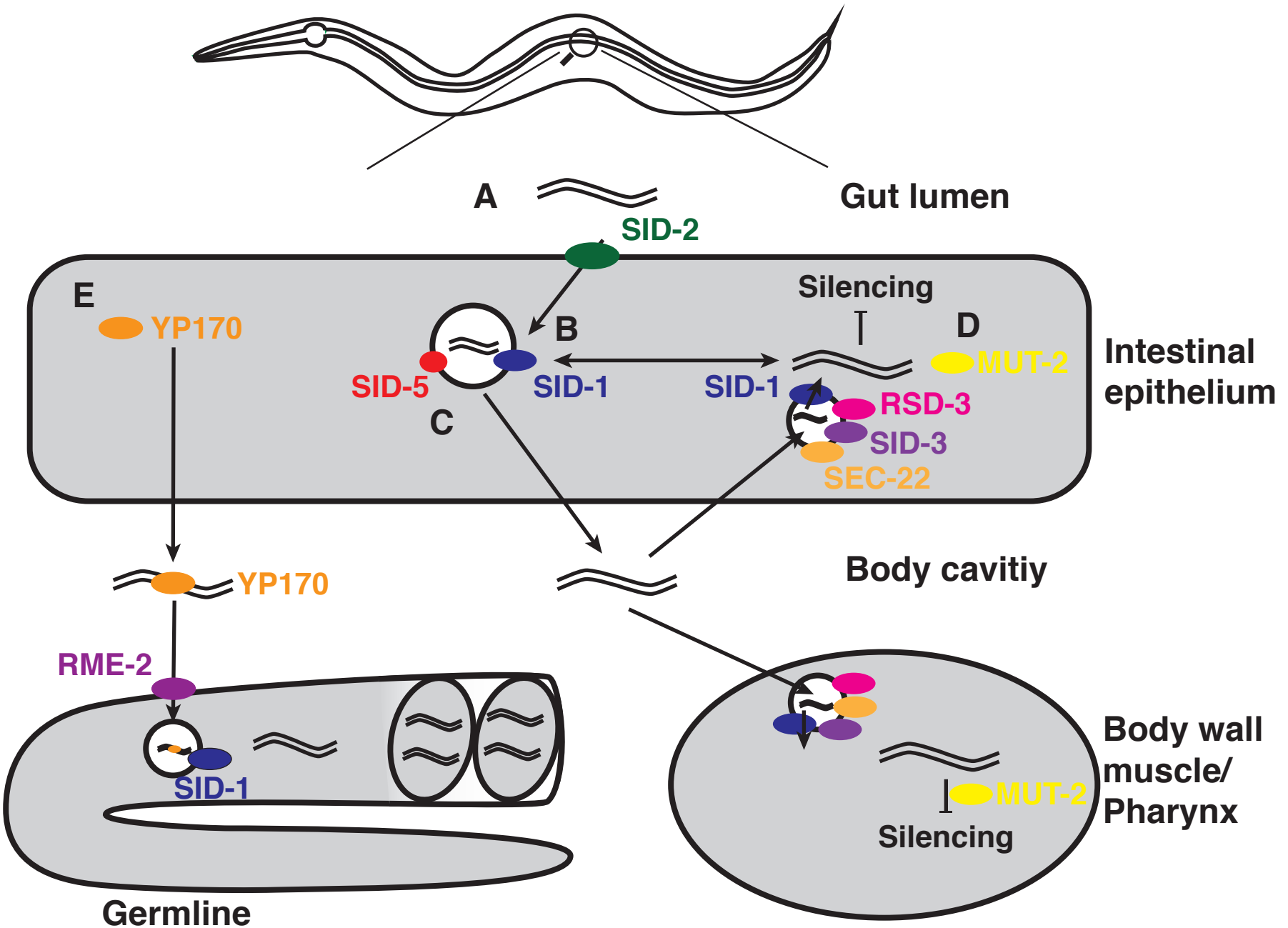
Figure 1


A
Gut lumen
SID-2
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Silencing
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MUT-2
RSD-3
SID-3
SEC-22
Intestinal epithelium
YP170
Body cavitiy
RME-2
SID-1
Germline
MUT-2
Silencing
Body wall muscle/ Pharynx