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REVIEW

Movement of Regulatory RNA Between Animal Cells

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Summary: Recent studies suggest that RNA can move from one cell to another and regulate genes through specific base-pairing. Mechanisms that modify or select RNA for secretion from a cell are unclear. Secreted RNA can be stable enough to be detected in the extracellular environment and can enter the cytosol of distant cells to regulate genes. Mechanisms that import RNA into the cytosol of an animal cell can enable uptake of RNA from many sources including other organisms. This role of RNA is akin to that of steroid hormones, which cross cell membranes to regulate genes. The potential diagnostic use of RNA in human extracellular fluids has ignited interest in understanding mechanisms that enable the movement of RNA between animal cells. Genetic model systems will be essential to gain more confidence in proposed mechanisms of RNA transport and to connect an extracellular RNA with a specific biological function. Studies in the worm *C. elegans* and in other animals have begun to reveal parts of this novel mechanism of cell-to-cell communication. Here, I summarize the current state of this nascent field, highlight the many unknowns, and suggest future directions. **genesis 53:395–416, 2015.**

Key words: epigenetics; mobile RNA; systemic RNAi; exosomes; hormones; *C. elegans*

The evolution of multicellularity was made possible by cooperation between cells through physical association or cell adhesion and chemical dependence or cell signaling. During cell signaling, a recipient cell responds to a signal from a donor cell. For example, when a hormone receptor in a recipient cell binds a steroid hormone from a donor cell, the hormone-bound receptor can regulate many genes through sequence-specific interactions with DNA. Recent studies suggest that RNA can move from donor cells and regulate genes in recipient cells through sequence-specific interactions with other RNA. Such RNAs that cross cell boundaries will be referred here as mobile RNAs. The similarities between steroid hormones and mobile RNAs are underscored by their shared ability to cross cell boundaries and to be used in sequence-specific gene regulation. While the sequence regulated by a steroid hormone is dictated by the binding-specificity of the hormone receptor, the sequence regulated by a mobile RNA is dictated by the base-pairing ability of the mobile RNA. Much of how signaling by a mobile RNA occurs and what it has been selected for during evolution is unclear.

Signaling by mobile RNAs can be conceptually divided into five steps (Fig. 1). First, RNA within a donor cell is recruited or modified for transport (biogenesis). Second, the mobile RNA crosses the plasma membrane of the donor cell to exit the cell (export). Third, mobile RNA in the extracellular space is stabilized to prevent degradation (stability). Fourth, extracellular mobile RNA is imported into recipient cells and gains access to the cytosol (import). Fifth, intracellular mobile RNA base-pairs with other RNA or DNA and engages gene regulatory mechanisms in the cytosol or in the nucleus (regulation). Not all five steps need to occur in every instance of gene regulation by RNAs that cross cell membranes. For example, a mobile RNA could cross membranes to enter cells but may not be the result of specific secretion from other cells. It is also possible that RNAs are secreted out of cells but do not

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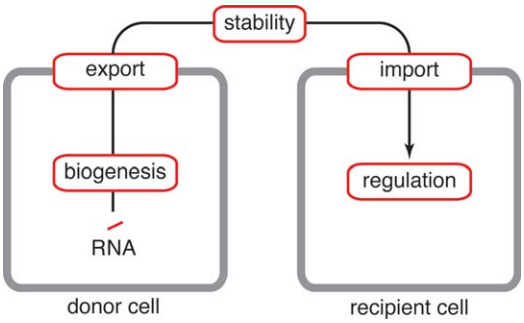

FIG. 1. Gene regulation by mobile RNA can be divided into five conceptual steps. Mobile RNA need to be made or recruited from intracellular RNA (biogenesis), secreted from donor cells (export), protected in extracellular space (stability), and imported into recipient cells (import) to regulate gene expression (regulation).

Table 1
C. elegans Proteins With Roles in RNA Transport

Protein	Function	Human homologs
SID-1	dsRNA-selective importer	SIDT1, SIDT2
SID-2	dsRNA receptor	TLR3?
SID-3	Tyrosine kinase	ACK
SID-5	Endosomal trafficking	Unknown
MUT-2	Nucleotidyltransferase	TUT2/GLD2/PAPD4
RSD-3	Endocytosis	CLINT1

enter any cell and such RNAs are not referred to as mobile RNAs in this review.

This review focuses on mobile RNAs that enter animal cells from many sources including other animal cells. Unlike in animals, in plants, most cells are connected by intercellular bridges called plasmodesmata that enable the transport of large macromolecules (reviewed in Brunkard *et al.*, 2015), which suggests that mechanisms of mobile RNA transport differ between plants and animals (see Mlotshwa *et al.*, 2002 for an early review and Pyott and Molnar, 2015 for the latest on RNA transport in plants). Different animal models have been informative for different aspects of RNA transport between cells. Studies in the worm *Caenorhabditis elegans* (see Table 1 for a summary of *C. elegans* proteins with roles in RNA transport) and in the fly *Drosophila melanogaster* have yielded the most mechanistic insights thus far. Here, I describe these insights and discuss how they could relate to instances of RNA transport across membranes observed in other animals.

BIOGENESIS

The selection along with possible modification of specific RNAs for transport between cells could be considered as the biogenesis of mobile RNAs. However, it is conceivable that RNAs exit cells upon cell lysis or cell damage and such extracellular RNAs are then imported into cells to regulate gene expression. Considerations of biogenesis would not apply to mobile RNAs that end up in the extracellular space through such nonspecific mechanisms. Although some hints toward specific biogenesis of mobile RNAs are available from studies in *C. elegans* and in mammals, evidence for RNAs being specifically selected or modified for secretion out of cells is currently lacking.

The expression of base-paired RNA in one tissue in *C. elegans* can generate mobile RNAs that cause specific gene silencing of matching sequence in other tissues (Briese *et al.*, 2006; Devanapally *et al.*, 2015; Jose *et al.*, 2009, 2011, 2012; Timmons *et al.*, 2003; Winston *et al.*, 2002). Typically, >100 bp double-stranded RNA or hairpin RNA (together referred to as dsRNA in this review for simplicity) is expressed within a tissue to generate mobile RNAs. Such long dsRNA is expected to be processed by the RNA interference (RNAi) pathway within the tissue (see Fire *et al.*, 1998 for initial discovery and Billi *et al.*, 2014 and Grishok, 2013 for reviews). Therefore, a basic understanding of RNAi is necessary to consider possible RNAs derived from dsRNA that could act as mobile RNAs in *C. elegans*. According to the current model of RNAi in *C. elegans*, long dsRNA is recruited by the dsRNA-binding protein RDE-4 to be processed into double-stranded short interfering (ds-siRNA) by the conserved endonuclease Dicer. One strand of this ds-siRNA is then cleaved by the Argonaute protein RDE-1. The single-stranded short interfering RNA (ss-siRNA) is then used by RDE-1 as a guide to find mRNAs of complementary sequence. After the target RNA is identified, the RNA-directed RNA polymerase (RdRP) RRF-1 is recruited to generate numerous short single-stranded secondary siRNAs. Subsequently, these secondary siRNAs are bound by other Argonaute proteins and can cause posttranscriptional silencing by degrading mRNA in the cytoplasm or initiate co-transcriptional silencing by binding nascent pre-mRNA in the nucleus. In addition to this canonical pathway of RNAi for exogenously delivered dsRNA, many RNAi-related mechanisms have been identified through the analysis of endogenous small RNAs in *C. elegans*. Conceivably, any of the RNAs generated by RNAi or RNAs derived from such RNAs could act as mobile RNAs. An early candidate for RNAs that move between cells were the amplified secondary siRNAs because of the potency of RNA silencing (Fire *et al.*, 1998), which results in silencing throughout the organism (systemic RNAi) (Winston *et al.*, 2002). However, genetic mosaic analyses suggest that long dsRNA and processed double-stranded siRNA (ds-siRNA) are either transported between cells or are precursors for mobile RNAs made independent of gene silencing by RNAi (Fig. 2 and Jose *et al.*, 2011). Intriguingly, a putative nucleotidyltransferase MUT-2 is sufficient in donor cells for gene silencing in recipient cells, suggesting

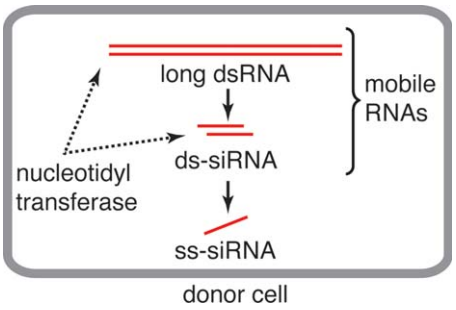

FIG. 2. Biogenesis of mobile RNAs is largely unknown. Genetic mosaic analyses in *C. elegans* suggest that unlike single-stranded short interfering RNA (ss-siRNA), long dsRNA and double-stranded short interfering RNA (ds-siRNA), potentially modified by a nucleotidyltransferase (dashed arrows), may be exported from donor cells as mobile RNAs.

that the biogenesis of some mobile RNAs includes an as yet unidentified modification of dsRNA (Fig. 2). Finally, the RdRP RRF-1 that is required for the generation of secondary siRNA in somatic tissues is not required for the export of some mobile RNAs (Jose *et al.*, 2011). Thus, organisms that do not have RdRPs (e.g. *Drosophila*) are not necessarily incapable of making and exporting mobile RNAs. In principle, amplification of RNA is not required for a systemic response because a signal that is used within a cell to instruct gene silencing and then reused for transmission to other cells can account for systemic RNAi. The direct detection of mobile RNAs and identification of modifications on mobile RNAs, if any, will clarify the essential steps in the biogenesis of mobile RNAs derived from dsRNA.

A second class of RNAs that have been proposed to act as mobile RNAs in animals is microRNAs (miRNAs)—conserved RNAs that bind Argonaute proteins and play important roles in animal development (see, Ambros, 2011; Hammond, 2015; Posadas and Carthew, 2014 for reviews). This proposal is supported chiefly by studies in mammals that report detection of miRNAs in the extracellular environment (see section on "Export from cells" below for references) but, in most cases, it is not known if or how specific miRNAs are selected or modified for secretion.

EXPORT FROM CELLS

Mobile RNA can exit a cell either through direct release into the extracellular space or as cargo within secreted vesicles. Although clear dissection of export mechanisms await a more extensive set of reagents that can specifically block the process in intact animals (e.g., genetic mutants, small molecule inhibitors), some support is available for both modes of mobile RNA export from cells.

The striking demonstration that extracellular vesicles secreted from mast cells contain miRNA and mRNA that can enter the cytosol of cells raised the possibility that such vesicles are carriers of mobile RNAs between cells (Valadi *et al.*, 2007). Numerous subsequent studies also detected miRNAs and other small RNAs within extracellular vesicles (Aucher *et al.*, 2013; Bayer-Santos *et al.*, 2014; Bellingham *et al.*, 2012; Bronisz *et al.*, 2014; Buck *et al.*, 2014; Collino *et al.*, 2010; Crescitelli *et al.*, 2013; Fernandez-Calero *et al.*, 2015; Figliolini *et al.*, 2014; Fong *et al.*, 2015; Guduric-Fuchs *et al.*, 2012; Hansen *et al.*, 2015; Hunter *et al.*, 2008; Ismail *et al.*, 2013; Kosaka *et al.*, 2010a; Lee *et al.*, 2013; Mittelbrunn *et al.*, 2011; Montecalvo *et al.*, 2012; Morel *et al.*, 2013; Njock *et al.*, 2015; Nolte-'t Hoen *et al.*, 2012; Ostenfeld *et al.*, 2014; Pegtel *et al.*, 2010; Pope and Lasser, 2013; Roberts *et al.*, 2013; Singh *et al.*, 2015; Skog *et al.*, 2008; Tominaga *et al.*, 2015; Umezu *et al.*, 2014; Villarroya-Beltri *et al.*, 2013; Wang *et al.*, 2010; Yuan *et al.*, 2009; Zhang *et al.*, 2010). Differences between the composition of RNA within the donor cell and that within the extracellular vesicles (e.g., in Mittelbrunn *et al.*, 2011; Montecalvo *et al.*, 2012; Nolte-'t Hoen *et al.*, 2012; Valadi *et al.*, 2007; Villarroya-Beltri *et al.*, 2013) suggest that specific mechanisms enrich RNAs within secreted vesicles. In support of this idea, miRNAs with a motif that is bound by sumoylated hnRNPA2B1 are specifically enriched in vesicles secreted from T cells (Villarroya-Beltri *et al.*, 2013), RNAs with a miR-1289 binding site and a sequence motif (CUGCC presented in a stem-loop structure) are recruited into secreted vesicles in human primary glioblastoma cells (Bolukbasi *et al.*, 2012), miRNA with non-templated 3' uridylation are specifically enriched within vesicles secreted from human B cells (Koppers-Lalic *et al.*, 2014), and endogenous RNAs can modulate the sorting of miRNAs into extracellular vesicles (Squadrito *et al.*, 2014). However, RNAs within extracellular vesicles are typically detected along with many other macromolecules including proteins and a recent estimate suggests that miRNAs can be present on average at <10 miRNA for every 1000 extracellular vesicle (Chevillet *et al.*, 2014). The isolation of extracellular vesicles relies on the use of differential centrifugation, filtration, and/or density gradients, but these procedures can result in substantial heterogeneity in the population of extracellular vesicles that are isolated (Witwer *et al.*, 2013). Furthermore, apparent enrichment or depletion of miRNA can also occur due to difficulties in the quantitative recovery of small amounts of miRNAs with low GC content from a small number of cells (Kim *et al.*, 2012). A recent systematic survey of small RNAs within cells and within extracellular vesicles did not find evidence for specific mechanisms for the secretion of most small RNAs (Tosar *et al.*, 2015). Furthermore, extracellular vesicles (e.g. exosomes) are currently loosely defined and could include a diverse set of distinct vesicles. Blocking vesicle secretion can require the

inhibition of different molecules in different cell types, suggesting that different cell types have different mechanisms to secrete vesicles. For example, blocking Rab7 affects secretion of exosomes from MCF-7 cells (Baietti *et al*., 2012) but not from HeLa cells (Ostrowski *et al*., 2010). Even when secreted by the same cells, two types of extracellular vesicles that can both be described as exosomes can differ in composition. For example, the vertebrate hedgehog protein is secreted from HEK293T cells on two types of extracellular vesicles that both share some exosome markers (TSG101 and CD9) but not others (Flotillins) (Vyas *et al*., 2014). The difficulty in comparing RNA-containing vesicles isolated by different groups has been recognized by the community of researchers working on extracellular vesicles (see, Colombo *et al*., 2014 for a review of the problem and Yáñez-Mó *et al*. (2015) for a comprehensive review on extracellular vesicles) and efforts to standardize preparation procedures are underway (Witwer *et al*., 2013). Improvements in methods for the reproducible and selective isolation of different extracellular vesicles will enable us to better evaluate the importance of RNA within vesicles.

Extracellular RNAs have also been detected outside vesicles bound to protein complexes in human plasma. Immunoprecipitation of the Argonaute protein Ago2 from human plasma recovered most of the circulating miRNAs suggesting that a large portion of the miRNA population in plasma is bound to the Ago2 protein (Arroyo *et al*., 2011, Turchinovich *et al*., 2011). Some miRNAs are found associated with high-density lipoproteins (HDL) and when bound by HDL, miRNAs can be delivered into the cytosol of cultured hepatocytes (Vickers *et al*., 2011). In addition, cultured human cells secrete nucleophosmin 1-bound miRNAs upon serum starvation (Wang *et al*., 2010). The precise mechanism for the export of these protein-bound RNAs is not known but is likely to be different from that for the export of RNA as cargo within vesicles. Consistently, production of ceramide promotes the secretion of miRNA-containing exosome vesicles (Trajkovic *et al*., 2008) and inhibits the export of HDL-bound miRNAs (Vickers *et al*., 2011).

In addition to secreted RNAs that can enable communication between distant cells, limited transport of RNA between physically associated cells has also been reported. Gap junctions such as those formed by Connexin-43 can transport siRNAs between cells (Valiunas *et al*., 2005) and transport of miRNAs between cardiac cells (Hosoda *et al*., 2011; Kizana *et al*., 2009) can be inhibited by a dominant-negative Connexin-43 mutant (Kizana *et al*., 2009). Bone-marrow stromal cells (Lim *et al*., 2011) and glioma cells (Katakowski *et al*., 2010) can also transport miRNAs between cells through gap junctions. Vesicle-mediated and gap junction-mediated transport between cells may be combined at specialized contacts between cells called synapses. For example, T cells can acquire small RNAs through a cell-contact dependent mechanism from B cells (Rechavi *et al*., 2009). Finally, RNA and RNA-bound proteins can be transported through intercellular bridges in mouse male germ cells (Morales *et al*., 2002). These special cases highlight the importance of local anatomy that could result in the use of different mechanisms to transport RNA between cells.

There appear to be differences between animals in mechanisms that export mobile RNAs into the extracellular environment. The expression of dsRNA within a tissue can generate mobile RNAs that cause gene silencing in other tissues in *C. elegans* (Briese *et al*., 2006; Devanapally *et al*., 2015; Jose *et al*., 2009, 2011, 2012; Timmons *et al*., 2003; Winston *et al*., 2002) but not in *Drosophila* (Roignant *et al*., 2003). This is particularly surprising because multiple *C. elegans* cell types, including pharyngeal cells (Winston *et al*., 2002), intestinal cells (Jose *et al*., 2009), body-wall muscle cells (Jose *et al*., 2009), and neurons (Briese *et al*., 2006, Jose *et al*., 2009) can export mobile RNAs. This difference between organisms likely reflects differences in the mechanisms of biogenesis and/or export of mobile RNA because both organisms can import dsRNA from the extracellular environment into cells (see section on "Import into cells").

In summary, the export of mobile RNAs may occur through multiple mechanisms (the most general modes are summarized in Fig. 3) and these mechanisms may differ between animals as well as between cell types within an animal.

EXTRACELLULAR STABILITY

RNA has been detected in diverse extracellular fluids in humans including blood (Arroyo *et al*., 2011), saliva (Ogawa *et al*., 2013), breast milk (Kosaka *et al*., 2010b), placenta (Chim *et al*., 2008), and semen (Vojtech *et al*., 2014). The ease of clinical access to such RNA has sparked interest in their diagnostic use and a large effort to understand the biology of extracellular RNAs is underway (Leslie, 2013). The presence of detectable amounts of RNA in these extracellular fluids suggests that at least some secreted RNAs are protected from the many RNases that are present in extracellular fluids in humans (reviewed in Sorrentino, 1998). Mechanisms of RNA export out of mammalian cells, through binding to RNA-binding proteins or through sequestration within vesicles or both, may also protect mobile RNAs from RNases in the extracellular environment. In addition, inferences about the export and import of mobile RNA in *C. elegans* (see below) suggest that naked dsRNA is stable in the extracellular environment. Furthermore, some extracellular dsRNAs that accumulate when *C. elegans* ingest dsRNAs expressed in bacteria (see section

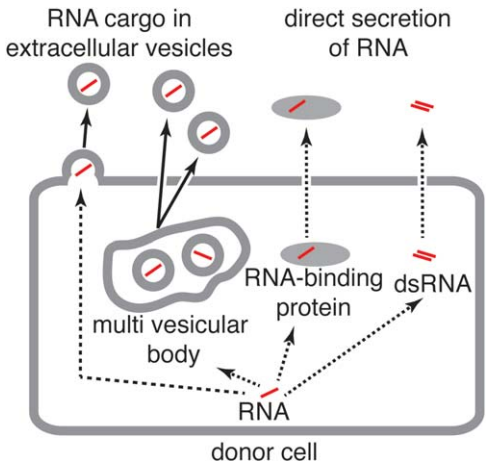

FIG. 3. Multiple mechanisms may enable the export and extracellular stability of mobile RNAs. RNA can be secreted (solid arrows) as cargo within vesicles when vesicles pinch from the plasma membrane or as cargo within intraluminal vesicles when multivesicular bodies fuse with the plasma membrane. Alternatively, RNA may be directly secreted in complex with an RNA-binding protein or as naked dsRNA. How RNA is recruited for secretion through each of these mechanisms and how dsRNA or protein-bound RNA are secreted into the extracellular space are unknown (dashed arrows).

on "Import into cells") can be isolated from the extracellular space of adult animals (Banse and Hunter, 2012). Thus, the stability of secreted RNA in the extracellular environment could be attributed to sequestration within vesicles, binding to RNA-binding proteins, or presence of RNase-resistant base-paired structures.

IMPORT INTO CELLS

A remarkable feature of some animals is that their genes can be silenced by dsRNA of matching sequence that they ingest (reviewed in Whangbo and Hunter, 2008). This phenomenon called environmental RNAi or feeding RNAi was first discovered in *C. elegans* (Timmons and Fire, 1998) and has enabled genome-wide RNAi screens (Kamath *et al*., 2003) that have been applied widely to discover genes that control many biological processes. Some aspects of how ingested dsRNA enters the cytosol and gains access to most tissues in animals are discussed here along with our current understanding of the import of dsRNA and mobile RNAs into cells. Studies, chiefly in *C. elegans* and in *Drosophila*, suggest that import requires receptor-mediated endocytosis in combination with RNA transport across the membrane through a transmembrane protein or through other unknown mechanisms (Fig. 4). Analyses that led to this view of RNA import into cells are detailed below.

Four *C. elegans* proteins with relatively well-characterized roles in the import of RNA into cells were identified through the **s**ystemic RNA**i** **d**efective (*sid*) screen, which looked for animals that fail to silence GFP expression in body-wall muscles in response to *gfp*-dsRNA expressed in pharyngeal cells and in bacteria supplied as their only food source (Winston *et al*., 2002). Additional alleles of the same genes were identified in two other screens - the *fed* (**fe**eding RNAi **d**efective) screen (Timmons *et al*., 2003) and the *rsd* (**R**NAi **s**preading **d**efective) screen (Tijsterman *et al*., 2004). Below I detail insights from the SID proteins that have been analyzed thus far – SID-1, SID-2, SID-3, and SID-5.

SID-1 is a conserved transmembrane domain protein with homologs present in all sequenced vertebrates, the social amoeba *Dictyostelium discoedium*, hydra, and many invertebrates but not in two-winged insects (Winston *et al*., 2002 and see Zhuang and Hunter, 2012 for a taxonomic tree). Expression of *C. elegans* SID-1 in *Drosophila* S2 cells (Feinberg and Hunter, 2003), in cells of the silkmoth *Bombyx mori* (Kobayashi *et al*., 2012, Mon *et al*., 2012, 2013; Xu *et al*., 2013a, 2014), in a cell line from the fall armyworm moth *Spodoptera frugiperda* (Xu *et al*., 2013b) or in mouse embryonic stem cells (Tsang *et al*., 2007) can enhance import of dsRNA into cells. This ability of *C. elegans* SID-1 to enhance dsRNA import in a variety of cellular contexts suggests that either SID-1 is sufficient for the transport of dsRNA across membranes or that additional machinery required for such import is broadly conserved and functional.

SID-1 is required in *C. elegans* cells for the import of extracellular dsRNA into the cytosol (Winston *et al*., 2002). A SID-1::GFP fusion protein is enriched on the plasma membrane of *C. elegans* cells and excluded from most neurons (Winston *et al*., 2002) – a tissue that is typically refractory to silencing by ingested dsRNA. Cells that overexpress SID-1 near the source of mobile RNAs showed enhanced silencing to the exclusion of silencing in other cells (Calixto *et al*., 2010; Jose *et al*., 2009), suggesting that SID-1 acts as a sink for diffusing extracellular RNA. Consistently, ectopic expression of SID-1 in *C. elegans* neurons can enable silencing by ingested dsRNA within neurons (Calixto *et al*., 2010). However, SID-1 is not required for the export of mobile RNAs made from dsRNA expressed in neurons, muscles, or intestinal cells (Jose *et al*., 2009). Furthermore, ingested dsRNA can be transported across intestinal cells independent of cytosolic entry within intestinal cells to cause silencing in other tissues that express SID-1 (Calixto *et al*., 2010; Jose *et al*., 2009). When expressed in *Drosophila* S2 cells, SID-1 enables substantial import of dsRNA under conditions of reduced membrane fluidity and ATP levels, suggesting that entry into the cytosol through SID-1 is not strongly dependent on endocytosis or energy (Feinberg and Hunter, 2003). In this heterologous system, a SID-1-dependent current can be detected in patch clamp experiments in response to the addition of dsRNA but not dsDNA or RNA/DNA hybrids (Shih and Hunter, 2011), suggesting

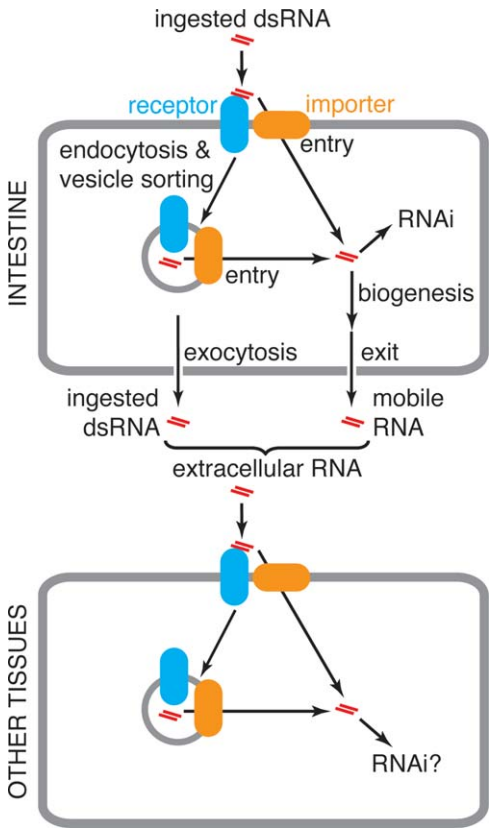

FIG. 4. The import of RNA into cells is the most understood step in gene regulation by mobile RNAs. Integration of results from *C. elegans* and *Drosophila* suggest that ingested dsRNA (red) is bound by a receptor (blue, e.g., SID-2, scavenger receptors) and imported into the cytosol (entry) through an importer protein (orange, e.g., SID-1) either at the plasma membrane or at intracellular vesicles. Formation of these intracellular vesicles relies on proteins that play a role in endocytosis (e.g. Clathrin heavy chain and SID-3) and vesicle sorting (e.g., Rab7 and SID-5). Entry of dsRNA into the intestinal cytosol can result in gene silencing (RNAi) and independently result in the biogenesis and export (exit) of mobile RNA. Exocytosis of dsRNA without entry into the intestinal cytosol can also occur. As a result, both ingested dsRNA and mobile RNA can be present in the extracellular space (extracellular RNA) or circulatory system along with mobile RNAs derived from expressed dsRNA (not depicted). The entry of extracellular RNA into the cytosol of non-intestinal cells likely relies on similar mechanisms as that required for dsRNA entry into the cytosol of intestinal cells. While some proteins (e.g., SID-1 and SID-3) have been demonstrated to be required for entry into the cytosol of nonintestinal cells, many additional proteins required for this process (e.g., receptor, if any) and the mechanisms of gene regulation by mobile RNAs (RNAi?, see section on "Gene regulation") remain to be identified.

that the pore formed by SID-1 to transport dsRNA across the membrane can be selectively opened by dsRNA. Import does not require complete base-pairing of dsRNA because RNA with internal bulges such as miRNA precursors can be imported through SID-1 (Shih and Hunter, 2011). The ability of a mutant SID-1 to interfere with the function of a wild-type SID-1 protein suggests that SID-1 functions as a multimer (Shih and Hunter, 2009). The extracellular domain of SID-1 and its human homologs selectively bind dsRNA (Li *et al.*, 2015) and preliminary structural analyses of the extracellular domain of human SID-1 suggest a tetrameric structure that could enable the end-on entry of dsRNA into cells (Pratt *et al.*, 2012). Together, these observations could explain the ability of SID-1 to discriminate between dsRNA and RNA/DNA hybrids (Shih and Hunter, 2011). The molecular structure of SID-1, potentially in complex with dsRNA, is needed to clarify the precise mechanism of RNA entry through this dsRNA-selective importer.

In addition to the SID-1-dependent mechanism described above for the entry of dsRNA into the cytosol, there are likely additional mechanisms for the uptake of dsRNA into cells. For example, *Drosophila* and mosquitoes can take up dsRNA despite the loss of SID-1 homologs in the dipteran lineage (Tomoyasu *et al.*, 2008). The 12 sequenced *Drosophila* species (Tomoyasu *et al.*, 2008), the mosquitoes *Anopheles gambiae* and *Ades aegypti* (Tomoyasu *et al.*, 2008), and the barley midge *Mayetiola destructor* (Shreve *et al.*, 2013) all lack obvious SID-1 homologs. Genetic screens for the import of dsRNA into cells in cultured *Drosophila* cells implicate scavenger receptors (SR-CI and Eater) and clathrin-mediated endocytosis in the uptake of extracellular RNA (Saleh *et al.*, 2006; Ulvila *et al.*, 2006), but the precise mechanism by which RNA crosses cellular membranes to enter the cytosol of *Drosophila* cells remains unknown. When siRNAs (Vickers *et al.*, 2011) or miRNAs (Wolfrum *et al.*, 2007) are delivered in complex with HDL, the scavenger receptor SR-BI is required for uptake into mammalian cells, suggesting that the requirement for a receptor is likely to be a conserved feature of RNA import into cells. Consistently, clathrin-dependent endocytosis is required for the uptake of dsRNA in *Tribolium* (Xiao *et al.*, 2015). The genes identified as required for endocytosis in *Drosophila* that have clear homologs in *C. elegans* are also required for the uptake of dsRNA in *C. elegans* (Saleh *et al.*, 2006). Interestingly, the *rsd* screen identified the *C. elegans* homolog of human Clathrin interactor 1 (CLINT1) called RSD-3 (Tijsterman *et al.*, 2004), but its role in RNA silencing has not yet been analyzed in detail.

The presence of SID-1-like genes in an organism is not necessarily an indicator of dsRNA uptake into that organism through a SID-1-dependent mechanism. The single SID-1 present in the brown planthopper *Nilaparvata lugens* was required for efficient RNAi (Xu *et al.*, 2013c), a SID-1 homolog from the mandarin fish *Siniperca chuatsi* enables improved uptake of dsRNA in heterologous systems (Ren *et al.*, 2011), and SID-1 has been implicated in RNAi in the honeybee *Apis melifera* (Aronstein *et al.*, 2006). Furthermore, overexpression of human SID-1 in human cells enables enhanced uptake of siRNA (Duxbury *et al.*, 2005), import of

cholesterol-conjugated siRNA into cells requires a mammalian homolog of SID-1 (Wolfrum *et al*., 2007), and contact-dependent acquisition of small RNA into cultured mammalian cells requires SID-1 (Elhassan *et al*., 2012). However, many insect homologs of SID-1 and the human homologs of SID-1 have sequences that are more similar to a paralog of SID-1 called CHUP-1 (Tomoyasu *et al*., 2008). CHUP-1 is required for cholesterol uptake rather than for dsRNA uptake (Valdes *et al*., 2012), suggesting that homologs of SID-1 in some organisms may not be required for the uptake of dsRNA. Consistent with this possibility, knockdown of the SID-1-like protein in the migratory locust *Locusta migratoria* do not result in a defect in systemic RNAi (Luo *et al*., 2012). Nor do SID-1-like proteins in the diamondback moth *Plutella xylostella* (Wang *et al*., 2014) or in the red flour beetle *Tribolium castaneum* (Miller *et al*., 2012) appear to be required for RNAi. In planaria, silencing in response to ingested dsRNA can occur in most tissues (Rouhana *et al*., 2013) and weak homologs of SID-1 are present (Zayas *et al*., 2005) but the roles of these homologs in the uptake of dsRNA are yet to be evaluated. Finally, a mammalian homolog of SID-1, SidT2, is a lysosomal membrane protein (Jialin *et al*., 2010), which is in contrast to the reported plasma membrane localization of *C. elegans* SID-1 (Winston *et al*., 2002), and mouse knockouts of SidT2 have impaired glucose tolerance (Gao *et al*., 2013), which is in contrast to the lack of obvious defects in *C. elegans* that lack SID-1 (Winston *et al*., 2002), suggesting that SID-1 and its mammalian homologs also have alternative function(s). An intriguing clue to such alternative functions comes from a close inspection of sequence similarity, which suggests that both SID-1 and CHUP-1 have a domain with weak similarity to hydrolases (Pei *et al*., 2011). Thus, specific tests in each animal and cell type will be required to determine if the SID-1 homolog present is required for or facilitates the entry of dsRNA into the cytosol.

SID-2 is a single-pass transmembrane protein with recognizable homologs in some nematodes that is localized to the intestinal lumen (Winston *et al*., 2007) and acts as a receptor for ingested dsRNA (McEwan *et al*., 2012). Consistently, while animals that lack *sid-2* are completely resistant to silencing by ingested dsRNA, they do not have a detectable defect in silencing by mobile RNAs made in pharyngeal cells or delivered by injection. Fluorescently labeled ingested dsRNA fails to enter intestinal cells in *C. elegans* that lack SID-2 and the uptake of labeled dsRNA into cells is enhanced when *C. elegans* SID-2 is expressed in *Drosophila* S2 cells. Uptake through SID-2 requires at least 25-nt long dsRNA and endocytosis. Interaction of SID-2 with dsRNA requires an acidic pH, which is similar to the interaction of dsRNA with mammalian Toll-like receptor 3 (Fukuda *et al*., 2008; Liu *et al*., 2008,) and is in agreement with the measured pH of 5.0–3.6 in the intestinal lumen of *C. elegans* (Chauhan *et al*., 2013). Although a homolog of SID-2 is present in the related species *C. briggsae*, only ~30% of the extracellular domain of *C. briggsae* SID-2 is identical to that of *C. elegans* SID-2. This divergence of the extracellular domain of SID-2 is sufficient to explain the lack of environmental RNAi in *C. briggsae*. Replacing the extracellular domain of *C. briggsae* SID-2 with that of *C. elegans* SID-2 enabled environmental RNAi in *C. briggsae*, suggesting that other aspects of RNA transport are functional in *C. briggsae*. Understanding the ecological basis for this difference between *C. elegans* and *C. briggsae* could potentially reveal the evolutionarily selected function (if any) of environmental RNAi in *C. elegans*.

SID-3 is a conserved tyrosine kinase with clear homologs in most animals that is localized in intracellular puncta and likely promotes endocytosis (Jose *et al*., 2012). SID-3 is the *C. elegans* ortholog of human activated Cdc42-associated kinase (ACK), which promotes endocytosis in human cells (Harris and Tepass, 2010). Consistently, mosaic analyses show that SID-3 is required in recipient cells for silencing in response to ingested dsRNA and mobile RNAs derived from expressed dsRNA. This function of SID-3 requires an intact kinase domain suggesting that phosphorylation at a tyrosine of an unknown substrate (e.g., Cdc42, Dynamin) is required for SID-3 to promote the import of RNA into cells. Intriguingly, in the absence of SID-3, gene silencing within the pharynx caused by dsRNA expressed within the pharynx and within the germline caused by dsRNA injected into the germline appear to be enhanced. The basis of this enhanced silencing within cells is unknown and could be related to the role of SID-3 in the uptake of dsRNA. For example, lack of import in *sid-3* mutants might relieve competition for limiting RNAi factors and enhance the use of dsRNA generated within a cell for gene silencing. Additional analyses are needed to test this hypothesis, to identify the substrates of SID-3, and to determine the precise role of SID-3 in the import of RNA into cells.

SID-5 is a single-pass transmembrane protein with homologs in some nematodes that partially colocalizes with endosomes and could play a role in vesicle transport (Hinas *et al*., 2012). Expression of SID-5 in the intestine was required for ingested dsRNA to silence a gene in body-wall muscles. This silencing did not require SID-1 within intestinal cells suggesting that the silencing was due to dsRNA transport across intestinal cells without import into the cytosol of intestinal cells. However, SID-5 was also required for silencing of target genes within the intestine, suggesting that SID-5 is required for uptake from the intestinal lumen, subsequent release into the cytosol, and/or silencing within the intestine. Furthermore, silencing within cells has been proposed to occur in association with endosomal

membranes (Lee *et al*., 2009). Thus, the role of SID-5 in the uptake of dsRNA from the intestinal lumen is sufficient to explain its requirement in intestinal cells for silencing by ingested dsRNA in body-wall muscles. Animals that lack *sid-5* also have a defect in silencing in response to mobile RNAs made in the pharynx but where the SID-5 protein is needed for this silencing has not been determined. Additional experiments are needed to examine possible roles of SID-5 in the execution of RNA silencing within cells or in the export of RNA from cells.

Long dsRNA appear to be more efficient than short dsRNA in causing gene silencing when delivered through injection or ingestion in multiple organisms. This difference in efficiency is observed even when the long dsRNA and short dsRNA have the same length of sequences that match a target gene (Feinberg and Hunter, 2003). A similar preference for long dsRNA is observed in *Drosophila* (Saleh *et al*., 2006) and in the red flour beetle *Tribolium castaneum* (Miller *et al*., 2012). Recognition of this preference was crucial for the recent success in the efficient delivery of dsRNA into insect pests by expressing long dsRNA within plastids in plants (Zhang *et al*., 2015). This is consistent with either uptake or subsequent steps preferring long dsRNA. In *Drosophila*, labeled long dsRNA is taken up better than labeled short siRNAs (Saleh *et al*., 2006). Longer dsRNA results in more efficient silencing and/or uptake in *Drosophila* S2 cells that express SID-1 and in the *C. elegans* germline (Feinberg and Hunter, 2003). However, SID-1 has been reported to have dsRNA-selectivity (Shih and Hunter, 2011) but lack any length selectivity (Shih *et al*., 2009). One possible explanation for this discrepancy is that a conserved downstream factor like the dsRNA-binding protein RDE-4 that binds long dsRNA cooperatively (Parker *et al*., 2006) also imposes a preference for long dsRNA. Further studies are needed to understand this conserved preference for long dsRNA for gene silencing in invertebrates. In most mammalian cells, import of long dsRNA is expected to trigger a nonspecific block in translation and subsequent cell death through the interferon response (Alexopoulou *et al*., 2001 and reviewed in Gantier and Williams, 2007). In embryonal teratocarcinoma cell lines (Billy *et al*., 2001), stem cells, and oocytes (Svoboda *et al*., 2000), sequence-specific silencing is possible using long dsRNA. Such sequence-specific RNAi appears to occur independent of RdRP activity in mammalian oocytes (Stein *et al*., 2003). In some organisms, like the marine shrimp *Litopenaeus vannamei* (Robalino *et al*., 2005), both interferon response and sequence-specific silencing can occur. In this shrimp, both Ago and Sid1 genes are upregulated in response to dsRNA ingestion (Labreuche *et al*., 2010). In summary, both long dsRNA and short dsRNA can enter cells, but long dsRNA preferentially enters into cells in many organisms, the basis of which merits further analysis.

Numerous studies have demonstrated the uptake of dsRNA into insects (see Huvenne and Smagghe, 2010; Jose and Hunter, 2007; Katoch *et al*., 2013; Tomoyasu *et al*., 2008, Whyard *et al*., 2009 and for early references), although, in most cases, efficient approaches for dsRNA delivery have not yet been developed (Terenius et al., 2011) because of a limited understanding of underlying mechanisms. For example, in *Drosophila*, intra-abdominal injection in adult animals can result in silencing in many cell types, including the central nervous system (Dzitoyeva *et al*., 2001; Goto *et al*., 2003; Petruk *et al*., 2006), but delivery into larvae does not result in silencing in tissues other than hemocytes (Miller *et al*., 2008). Better understanding of silencing by mobile RNAs can help the field move beyond the empirical approaches that are currently available for efficient RNA silencing in insects (Scott *et al*., 2013). Given the practical applicability of dsRNA uptake in insects [e.g., controlling agricultural pests (Zhang *et al*., 2015) and combating honeybee colony collapse disorder (Cox-Foster *et al*., 2007; Hunter *et al*., 2010; Maori *et al*., 2009)], additional mechanistic studies are urgently needed. The advent of CRISPR-based genome editing (e.g., Ma *et al*., 2014) should now enable rigorous dissection of mechanism in many insects using genetic mutants.

GENE REGULATION

RNA that enters a cell carries sequence information that could be used in a variety of ways for gene regulation. The ability to base-pair with other RNA or DNA enables imported mobile RNAs to recognize specific sequences. The way in which this sequence recognition is coupled to downstream factors could dictate the gene regulatory outcome of a mobile RNA. This proposed role of imported mobile RNAs within a cell is analogous to the role of transcription factors within a cell: sequence recognition by DNA-binding domains of transcription factors result in changes in gene expression based on downstream factors.

Hints of the potential complexity of gene regulation by mobile RNAs come from the many small RNA-mediated processes that have been discovered thus far. In the 17 years since the discovery of RNAi (Fire *et al*., 1998), it has become clear that small RNAs direct a diversity of gene regulatory outcomes in *C. elegans* (see Billi *et al*., 2014; Grishok, 2013 for comprehensive reviews). These include post-transcriptional gene silencing by degrading mRNA of matching sequence upon binding to cytosolic Argonautes (e.g., RDE-1), co-transcriptional gene silencing by recruiting histone modifying enzymes to nascent transcripts upon binding to nuclear Argonautes (e.g. NRDE-3), and protection

from gene silencing, through mechanisms that are unclear, upon binding specific Argonautes (e.g., CSR-1). Within cells, the production of secondary small RNAs for silencing can occur in perinuclear foci called mutator foci (Phillips *et al*., 2012) or in cytoplasmic foci called R2 bodies (Yang *et al*., 2014). The specific protein factors and mechanisms that are engaged by a small RNA can vary depending on the source of the dsRNA that triggers small RNA production and the tissue where the function of the small RNA is interrogated. Thus far, studies examining the effects of mobile RNAs have only focused on gene silencing.

Import of mobile RNAs derived from expressed dsRNA results in the downregulation of genes of matching sequence through RNAi in *C. elegans*. Although multiple tissues can make and export mobile RNAs in response to dsRNA expression (Jose *et al*., 2009), only the mechanism of silencing by mobile RNAs exported from neurons has been examined to date. Neuronal mobile RNAs targeting the muscle gene *unc-22* require the Argonaute RDE-1 and the RdRP RRF-1 in muscle cells for silencing upon import (Jose *et al*., 2011). Furthermore, the dsRNA-binding protein RDE-4 and the putative nucleotidyltransferase MUT-2 are required in either the donor neurons or recipient muscles, suggesting that at least one class of neuronal mobile RNAs can silence independently of RDE-4 and MUT-2. Neuronal mobile RNAs targeting the green fluorescent protein GFP (made from *gfp*-dsRNA) requires RDE-1, an RNase D homolog MUT-7, and a nuclear Argonaute HRDE-1 but not RRF-1 for silencing GFP expression in the germline (Devanapally *et al*., 2015). The requirement for MUT-7 likely reflects the requirement of mutator foci for secondary small RNA synthesis by RdRPs (Phillips *et al*., 2012). The requirement for the nuclear Argonaute HRDE-1 despite the presence of many other secondary Argonautes (Yigit *et al*., 2006) could reflect a direct channeling of mobile RNAs to this nuclear silencing pathway in the germline. The lack of requirement for RRF-1 despite the requirement for MUT-7 likely reflects generation of secondary small RNAs by another RdRP, EGO-1, within the germline (Smardon *et al*., 2000). Additional analyses are required to test these possibilities and whether these genes are required within the germline or in neurons for silencing.

The situation is more complex in the case of mobile RNAs derived from ingested or injected dsRNA in *C. elegans* because the dsRNA can itself reach most tissues and potentially obscure any effects of dsRNA-derived mobile RNAs (Fig. 4). When dsRNA is delivered through injection, it is difficult to avoid spillage into the body cavity, which surrounds most tissues in *C. elegans*. When dsRNA is delivered through ingestion, the ingested dsRNA can be transported across intestinal cells to the body cavity, potentially through transcytosis, without SID-1-dependent cytosolic entry within the intestine (Calixto *et al*., 2010; Jose *et al*., 2009). Thus, if mobile RNAs were made from ingested or injected dsRNA, their effects would be mixed with the effects of the dsRNA present in the body cavity. Nevertheless, if no silencing is detected in the absence of a gene when silencing is triggered using ingested or injected dsRNA (e.g. RDE-1 – see Grishok, 2013 for comprehensive review), we can conclude that this gene is also required for silencing by mobile RNAs (if any) derived from ingested or injected dsRNA. Different genes can be required for ingested dsRNA to silence different targets (Zhuang *et al*., 2013). For example, the nuclear Argonaute NRDE-3 but not the P-granule component PGL-1 is required to silence *lir-1*, and PGL-1 but not NRDE-3 is required to silence *unc-73*. The reason for such differential requirements is not yet clear and it is unknown if there are target-dependent differences in silencing by mobile RNAs derived from ingested dsRNA. Furthermore, the ability to detect silencing by mobile RNAs could be obscured by other competing RNA silencing pathways that are active within a cell (Lee *et al*., 2006, Zhuang and Hunter, 2012). Mosaic analyses were used to characterize silencing by mobile RNAs derived from ingested dsRNA (Jose *et al*., 2011) and injected dsRNA (Blanchard *et al*., 2011), but limitations of this experimental approach weaken conclusions (see section on "Cautionary tales"). Finally, despite the ability to trigger gene silencing using dsRNA ingestion in many animals, we have minimal knowledge of the mechanism of gene silencing by mobile RNAs generated from such triggers because it is difficult to distinguish the effects of imported dsRNA from those of dsRNA-derived mobile RNAs. Therefore, additional experiments are needed to determine if ingested and injected dsRNAs even generate mobile RNAs in *C. elegans*.

SOMA TO GERMLINE

Charles Darwin envisioned a mechanism for inheritance that accommodated the transmission of acquired characters across generations in his "Theory of pangenesis" (Darwin, 1868). He postulated the transport of packets of information called "gemmules" from somatic tissues to germ cells to convey environment-dependent and use-dependent information to the next generation. Although Darwin's theory of inheritance was dismissed soon after (Weismann, 1883), the central idea of information transfer from somatic tissues to the germline may be alive as RNA transport from soma to germline.

When the germline nucleus and the somatic nucleus are present in a shared cytoplasm there is clear evidence for instructive RNA signals transported between the two nuclei. For example in the ciliated protozoan *Oxytricha trifallax* functional somatic nuclear DNA is constructed from scrambled sequences in the germline nucleus by using RNA from the parental somatic

nucleus as a template (Nowacki *et al*., 2008). In another ciliated protozoan, *Tetrahymena thermophila*, somatic nuclei are made after the elimination of segments of DNA from the germline nucleus using small RNA guides called scnRNAs (Mochizuki *et al*., 2002). Finally, small RNAs from the vegetative nucleus can move to the reproductive nucleus in the shared cytoplasm within plant gametes to silence transposons (Olmedo-Monfil *et al*., 2010; Slotkin *et al*., 2009).

The movement of regulatory RNA between animal cells could enable communication between somatic tissues and the germline despite the membranes and large distances separating them in animals. Except in the case of germline support cells (e.g., Sertoli cells in male and Granulosa cells in female mammalian gonads), most somatic tissues become well separated from the germline early during animal development. This is true for the most genetically tractable animal models in biology – mice, *Drosophila*, and *C. elegans* (see Extavour and Akam, 2003 for a survey across metazoa). This separation had led to the notion of a barrier between the soma and the germline - the Weismann Barrier - and supported the dismissal of the ability of somatic cells to send information back to the germline and therefore to subsequent generations. While the validity of this barrier has been debated for more than 100 years and plausible mechanisms for the transfer of information from the soma to the germline have been proposed (e.g. Steele, 1981), clear tests of this concept require a system where the communication between a somatic tissue and the germline can be initiated and disrupted at will. The movement of RNA between cells in *C. elegans* provides one such system because movement of specific RNA can be initiated by the expression of dsRNA within a tissue and the transport of RNA can be blocked using mutant animals that lack the dsRNA importer SID-1. Using this system, it was recently discovered that neurons can generate mobile RNAs that enter the germline in *C. elegans* (Devanapally *et al*., 2015). Amazingly, neuronal mobile RNAs that enter the germline cause transgenerational gene silencing within the germline. However, transgenerational gene silencing is not observed in somatic tissues (Devanapally *et al*., 2015), suggesting the presence of reprogramming mechanisms that counter inherited gene silencing and activate gene expression within the soma. Because other tissues such as muscle and intestine can also export mobile RNAs (Jose *et al*., 2009), it is reasonable to expect that many tissues will be able to transport mobile RNAs to the germline in *C. elegans*. Whether in all such cases transgenerational effects ensue is an open question.

The ability of neuronal mobile RNAs to enter the germline and cause transgenerational gene silencing—if general—has exciting implications and could underlie some intriguing phenomena that have been described recently. One study reported that when *C. elegans* is subjected to odorant imprints for five successive generations the imprinted preference is transmitted for more than 40 generations (Remy, 2010). F2 progeny of mice that were subjected to odor fear conditioning were reported to inherit epigenetic changes on the odorant receptor gene as well as changes in neuroanatomy and behavior (Dias and Ressler, 2014). However, it remains unclear if odors have direct effects on the germline or if information is transmitted from sensory neurons to the germline to initiate transgenerational effects. Nevertheless, a role for small RNA-mediated gene silencing mechanisms in neuronal function is emerging. Endogenous small RNA in the sea slug *Aplysia* can change neuronal activity to regulate the persistence of memory (Rajasethupathy *et al*., 2012). Cellular memory of odorant exposure in *C. elegans* forms through the downregulation of the odorant receptor via small RNA-directed mechanisms (Juang *et al*., 2013). Thus, it is possible that the transport of RNA from neurons to the germline is a mechanism by which changes in neuronal function can have transgenerational effects. Reasons that counter this view include the lack of clear demonstration of RNA transport from the soma to the germline in organisms other than *C. elegans* and the presence of robust reprogramming mechanisms as in the case of mammals (Feng *et al*., 2010). Intriguingly, the grafting of human tumor cells into mice was reported to result in the detectable accumulation of tumor-derived RNA within sperm (Cossetti *et al*., 2014) and small RNAs in sperm are associated with transgenerational effects of early trauma in mice (Gapp *et al*., 2014). Thus, whether the transport of RNA from somatic tissues to germline is possible in mammals and whether such transport can escape reprogramming mechanisms to cause transgenerational effects merits further study.

BETWEEN ORGANISMS

A few cases of the transfer of RNA into an animal from another organism have been recently documented. Two abundant small RNAs from *Wolbachia*, a genus of bacteria that infects many insects and some nematodes, accumulate within *Aedes aegypti*, *Drosophila melanogaster*, and *Drosophila simulans* (Mayoral *et al*., 2014). When western corn rootworm feed on corn or colorado potato beetle feed on tomato, plant-derived dsRNA are ingested by the insects and processed into small RNAs (Ivashuta *et al*., 2015). In a case of bidirectional transfer, dsRNA expressed in the honeybee *Apis mellifera* is transported into the mite *Varroa destructor* that feeds on the hemolymph of bees and subsequently transported back into bees to cause gene silencing (Garbian *et al*., 2012). Additional, albeit disputed, cases of RNA transfer into animals from another organism include the entry of endogenous RNA from *E. coli* into *C. elegans* and the entry of miRNAs from rice into

humans (see next section). RNA transfer between organisms also occurs outside the animal kingdom. For example, the transfer of fungal RNA into plants results in the suppression of plant immunity (Weiberg *et al*., 2013). More studies are needed to evaluate the extent of RNA transport between organisms across the tree of life.

A possible consequence of RNA exchange between organisms could be horizontal gene transfer (HGT) after reverse transcription and genomic integration in the recipient organism. Although the segregation of the germline is viewed as a barrier to HGT into animals, as described in the previous section, recent findings enable us to imagine a possible mechanism for the transfer of genetic information to animals despite early segregation of their germline. Indeed, HGT between viruses and vertebrate genomes appear to have occurred through LINE-element facilitated reverse transcription followed by genomic integration (Belyi *et al*., 2010; Horie *et al*., 2010). Many cases of HGT have also been observed between bacteria and eukaryotes (Dunning Hotopp *et al*., 2007 and reviewed in Dunning Hotopp, 2011). Recently discovered examples include the transfer of a lysozyme from proteobacteria to the pea aphid (Metcalf *et al*., 2014) and the repeated transfer of bacteriocidal enzymes to eukaryotes (Chou *et al*., 2015). A gene once acquired through HGT can confer beneficial functions on the eukaryote. For example, a bacteriocidal enzyme transferred to the deer tick *Ixodes scapularis* has evolved to limit the proliferation of Lyme disease-causing bacteria *Borrelia burgdorferi* within the tick (Chou *et al*., 2015). While the mechanism of horizontal gene transfer from bacteria remains unknown, in some cases, RNA transferred from one organism to another could be the potential carrier of genetic information that facilitates horizontal gene transfer.

CAUTIONARY TALES

Most studies on RNA transport have not yet been replicated, and the lack of appropriate genetic controls, in some cases, make it difficult to exclude key alternative interpretations. Cautious progress is needed in this nascent field, particularly as economical ways of identifying extracellular RNA and other contents of extracellular vesicles are fast becoming available for use as diagnostic tools (e.g., Chen *et al*., 2015) and clinical trials using extracellular vesicles have begun (e.g., Viaud *et al*., 2011). Cases of course correction during the brief history of the field of RNA transport between cells in *C. elegans* and extant difficulties in interpreting some results are instructive in this regard.

Before the discovery of the dsRNA-selective importer SID-1, unambiguous attribution of lack of silencing to a defect in transport of dsRNA between cells versus a defect in execution of RNA silencing within cells was difficult and led to erroneous interpretations. For example, an early genetic screen looked for **R**NAi **s**preading **d**efective (*rsd*) genes under the premise that mutants defective in genes required for the spread of RNA to the germline would be capable of silencing in response to dsRNA injected into the germline but not in response to dsRNA ingested from the environment (Tijsterman *et al*., 2004). While this screen identified alleles of *sid-1* (called *rsd-8* in the study), it identified additional genes such as *rsd-2* and *rsd-6* that satisfy the screen criteria but were nevertheless later found to be required for the efficient execution of RNA silencing within cells (Han *et al*., 2008; Sakaguchi *et al*., 2014). One possible explanation for the earlier misclassification is that the dosage of dsRNA delivered by injection is far greater than that delivered by ingestion, which makes a mutant with a partial defect in the execution of RNA silencing appear to have a defect in the transport of RNA between cells. The *rsd-2* and *rsd-6* genes are now classified as dosage-sensitive RNAi-defective genes (Zhang *et al*., 2012a).

The remarkable ability of *C. elegans* to respond to ingested dsRNA (Timmons and Fire, 1998) inspired similar studies in many other organisms and has led to a collection of organisms where this process called environmental RNAi has been reported (Whangbo and Hunter, 2008). While silencing by ingested RNA in some organisms such as planaria (Sánchez-Alvarado and Newmark, 1999) has been replicated by many studies (e.g. Rouhana *et al*., 2013), silencing in other organisms such as mammals (Zhang *et al*., 2012b) has not been replicated and remains controversial (Witwer and Hirschi, 2014; Zhang *et al*., 2012c). In the organisms where reliable ingestion and uptake of dsRNA has been reported, the search is ongoing to identify the ecologically relevant dsRNAs, if any, that are ingested by the organism and that result in gene regulation within the organism. Although one report suggested that RNAs from *E. coli* affect the physiology of *C. elegans* by apparently entering into cells independently of SID-1 and SID-2 (Liu *et al*., 2012), this result has been disputed (Akay *et al*., 2015). Nevertheless, the minimal conservation of environmental RNAi among *Caenorhabditis* species (Nuez and Felix, 2012) and the growing understanding of the ecology of *C. elegans* (Frézal and Félix, 2015) point to environmental RNAi being an adaptation in *C. elegans* driven by its food supply.

Well-controlled studies on the transport of RNA between cells in response to ingested dsRNA are difficult. For example, using dsRNA expression under the control of a tissue-specific promoter, apparent inducible transport of RNA between cells and apparently weak transport to the next generation was observed in response to the ingestion of dsRNA of unrelated sequence (Timmons *et al*., 2003). When tissue-specific promoters are used, one concern is the misexpression

of dsRNA within the germline (Sheth *et al*., 2010) or in other tissues. The *sid-1*-dependence of silencing serves as a reasonable control because misexpression from promoters is expected to lead to *sid-1*-independent silencing in the misexpressed tissue. In the above study of induced transport of RNA between cells (Timmons *et al*., 2003), such a control is simply not possible because the observed effect requires the import of unrelated dsRNA, which requires SID-1. In two other studies, when the *rde-4(+)* gene was expressed under the control of the muscle-specific *myo-3* promoter, apparently *rde-4*-independent silencing was observed in non-muscle cells in response to ingested dsRNA (Jose *et al*., 2011) or in the next generation in response to injected dsRNA (Blanchard *et al*., 2011). Similar inability to control for misexpression from promoters makes the interpretation of these observations provisional.

Thus, cautious revision of interpretations in light of new reagents that enable better-controlled experiments is needed.

FUTURE DIRECTIONS

The movement of regulatory RNA between animal cells has broad implications for organism-wide gene regulation in health and in disease. The many unknowns that remain in our understanding of this process promise fundamental discoveries. Below I discuss three directions that could lead to such discoveries.

1. Mobile RNAs: RNAs that move between cells can be viewed as RNA hormones because they originate in distant cells, cross cell membranes (albeit through importer proteins), and carry gene-specific regulatory information in their sequence. Studies in *C. elegans*, where genetic controls are available, have focused on mobile RNAs made from transgenically expressed dsRNA. Studies in other organisms, where genetic controls are as yet unavailable, have identified many putative mobile RNAs made from endogenously transcribed RNA. Further studies with appropriate genetic controls that identify endogenous mobile RNAs are needed to confidently link a mobile RNA to a specific function. The hunt for such RNA hormones produced within an organism can lead to examples of gene regulation by endogenous mobile RNAs in animals. Indeed, in plants, specific regulatory RNAs such as miRNAs that move between cells can act as morphogens to regulate development (reviewed in Benkovics and Timmermans, 2014).

   RNAs that enter an animal and are not encoded in its genome could be viewed as foreign mobile RNAs. These could include RNAs found in the environment, RNAs in organisms ingested as food, RNAs in organisms that infect, or RNAs in symbionts. It would be exciting to discover foreign mobile RNAs that "invade" animals to affect gene expression and to contemplate the evolutionary implications of such interactions.
2. Transport Mechanisms: Mobile RNAs are transported across membranes for entry into the cytosol through at least two mechanisms that are each incompletely understood. A basic model of the import of RNA into cells has emerged from analysis of dsRNA import in *C. elegans* and in *Drosophila*. While *C. elegans* cells require endocytosis for uptake and the dsRNA-selective importer SID-1 for subsequent entry into the cytosol, *Drosophila* cells require endocytosis for uptake but the subsequent entry into the cytosol occurs through an entirely unknown mechanism because *Drosophila* has no known homolog of SID-1. Except import, all other aspects of the movement of RNA between cells remain mysterious. While extracellular vesicles have been implicated in the export and stability of mobile RNA, the importance of such vesicles for the transport of RNA between cells in intact animals remains to be evaluated. Future work on identifying genes that play a role in specific steps to enable the transport of RNA between cells will lead to a mechanistic understanding of how RNA transport occurs. Analyzing knockouts of homologs of genes discovered in any one organism (e.g., SID-1) in other organisms using genome-editing approaches (e.g., CRISPR/Cas9) can reveal aspects of mechanisms that are conserved in different organisms.

   Uptake of foreign mobile RNAs has chiefly been studied using the ingestion of dsRNA, which also occurs through multiple mechanisms that are incompletely understood. Uptake of ingested dsRNA into the intestine in *C. elegans* and in insects appears to occur through receptor-mediated endocytosis. Once taken up into an organism, the subsequent transport of ingested RNA to other tissues for silencing remains poorly defined despite its recently demonstrated applicability in the control of agricultural pests (Zhang *et al*., 2015). Understanding the mechanism of transport between cells after ingestion would be particularly difficult until additional mutants that separate the uptake of dsRNA into intestinal cells from the subsequent spread to other tissues are discovered.
3. Selected functions: We do not yet know the evolutionarily selected function(s) for RNA transport into animal cells. An early idea was that mobile RNAs serve as warning signals against transposons and viruses of matching sequence (e.g., Plasterk, 2002). Consistently, in *Drosophila* dsRNA uptake into cells was reported to be required for antiviral immunity (Saleh *et al*., 2009). However, in *C. elegans*, although RNAi is indeed antiviral (Lu *et al*., 2005;

Schott *et al*., 2005; Wilkins *et al*., 2005), it is not yet clear if the transport of RNA between cells is required for anti-viral resistance. Some experiments do suggest the transmission across generations of RNA derived from transgenic copies of a virus genome (Rechavi *et al*., 2011) or from an infecting virus (Sterken *et al*., 2014) but do not exclude the origin of such RNAs within the *C. elegans* germline and thus their persistence across generations without the transport of RNA between cells. In mammals, an antiviral role for RNAi was recently proposed under some conditions and in some cell types (Li *et al*., 2013; Maillard *et al*., 2013), but in most cases RNAi appears dispensable for antiviral immunity (Backes *et al*., 2014; Cullen *et al*., 2013; see debate in Ding and Voinnet, 2014 and tenOever, 2014). Another idea, inspired by work in plants, is that mobile miRNAs form gradients that guide development by diffusing across cells from their site of synthesis (e.g., Carlsbecker *et al*., 2010; Chitwood *et al*., 2009; Miyashima *et al*., 2011). However, a systematic test for this possibility using the *lin-4* miRNA did not reveal such a role for this miRNA in *C. elegans* (Zhang and Fire, 2010). Finally, RNA could convey information about the environment from the soma to the germline to have transgenerational effects as was observed when dsRNA was expressed in *C. elegans* neurons (Devanapally *et al*., 2015). Chromatin modifications (Greer *et al*., 2011) and small RNAs (Rechavi *et al*., 2014) have been implicated in the heritable response to starvation and RNAi has been implicated in the heritable response to temperature (Schott *et al*., 2014) in *C. elegans*. While the heritable effects of temperature are independent of SID-1 (Schott *et al*., 2014), suggesting that the transport of RNA from somatic cells to the germline is not required for this effect, the role of RNA transport, if any, in mediating the effects of starvation in *C. elegans* is unknown. Thus, whether any environmental stimulus leads to the transport of RNA between cells remains an open question.

Defects in animals that lack RNA transport can provide clues to the selected functions of RNA transport. An intriguing recent report observed a decrease in misfolded protein turnover in *sid-1* and *rde-1* mutants suggesting an unexpected link between RNAi and the misfolded protein response (Long *et al*., 2014). Furthermore, knock-down of a mammalian homolog of SID-1 in HeLa cells resulted in the accumulation of a misfolded protein and overexpression of SID-1 homologs resulted in a decrease in such accumulation. Additional mechanistic studies that explain this defect could lead to the evolutionarily selected function for RNA transport between cells. Another clue to the possible function of RNA transport comes from the analysis of knockout mice lacking a mammalian homolog of SID-1 called Sidt2, which reveal that Sidt2 is required for the secretion of insulin (Gao *et al*., 2013). However, defects observed in mutants that lack a gene required for RNA transport do not necessarily have to be caused by a defect in RNA transport. For example in *C. elegans*, SID-1, SID-2, SID-3, SID-5, RDE-4, and MUT-2 have all been implicated in the transport of RNA between cells. While loss of MUT-2 leads to dramatic developmental defects because of increased transposon activity (e.g., Chen *et al*., 2005) and loss of RDE-4 leads to developmental defects only when animals are grown at high temperature (Blanchard *et al*., 2011), no defects in development have been reported upon loss of the SID proteins. Thus, beyond reports of a physiological defect in animals lacking any one of the above genes, the process of RNA transport itself needs to be linked to the physiological defect to infer that the defect was due to the loss of RNA transport into cells.

Overall, we are limited in our ability to deduce evolutionarily selected functions from experiments done under controlled lab conditions because organisms evolve in ecological niches, which remain poorly defined for most animals. Future work in different ecological settings and under non-standard experimental conditions that are potentially conditions of stress for the animal may reveal possible selected functions for the transport of RNA into cells.

CONCLUSION

The startling discovery that nucleotide sequence information can cross cell boundaries in the form of regulatory RNA necessitates a revision of our understanding of animals. The movement of mobile RNA between cells is minimally a new form of cell-to-cell communication within animals. The ability of foreign mobile RNA to enter and affect gene regulation in some animals hints at intimate communication between an animal and its environment. Extending this concept to an extreme, we can imagine a scenario where an animal cell responds to imported RNA that was exported from a cell in another organism. Much work remains to be done to discover how natural selection has favored organisms that can transport RNA across membranes to evolve gene regulatory interactions across the animal kingdom.

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