Review

Environmental RNA interference

Jennifer S. Whangbo[1,2,3] and Craig P. Hunter[1]

[1] Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA 02138, USA
[2] Division of Hematology/Oncology, Children's Hospital Boston, Boston, MA, 02115, USA
[3] Department of Pediatric Oncology, Dana-Farber Cancer Institute, Boston, MA 02115, USA

The discovery of RNA interference (RNAi), the process of sequence-specific gene silencing initiated by double-stranded RNA (dsRNA), has broadened our understanding of gene regulation and has revolutionized methods for genetic analysis. A remarkable property of RNAi in the nematode *Caenorhabditis elegans* and in some other multicellular organisms is its systemic nature: silencing signals can cross cellular boundaries and spread between cells and tissues. Furthermore, *C. elegans* and some other organisms can also perform environmental RNAi: sequence-specific gene silencing in response to environmentally encountered dsRNA. This phenomenon has facilitated significant technological advances in diverse fields including functional genomics and agricultural pest control. Here, we describe the characterization and current understanding of environmental RNAi and discuss its potential applications.

Overview of cell autonomous versus non–cell autonomous RNA interference

Exposure of eukaryotic cells to double-stranded RNA (dsRNA) can lead to post-transcriptional degradation of homologous mRNA sequences. Known as RNA interference (RNAi), this process was initially described in the nematode *Caenorhabditis elegans* and subsequently demonstrated in fungi, plants, insects and animals [1,2]. In many organisms, RNAi seems to be exclusively cell autonomous (i.e. affecting only the cells that generate dsRNA or that are directly exposed to experimentally introduced dsRNA). The dsRNA molecules that trigger RNAi vary in length and can be delivered to the cell by microinjection, transfection or expression of hairpin transcripts from endogenous genes or transgenes. These long dsRNA precursors are processed by a member of the Dicer family of RNase-III-like enzymes into short-interfering RNA (siRNA) duplexes of ~21 nucleotides in length [3]. The siRNAs serve as sequence-specific guides for the RNA-induced silencing complex (RISC), leading to target mRNA cleavage [4]. Cell autonomous RNAi uses broadly conserved machinery and similar strategies in a wide range of organisms [5–7]. In this review, we will focus on the more recently described process of non-cell autonomous RNAi (Figure 1; Box 1).

A fascinating aspect of RNAi in plants and some animals is that locally initiated gene silencing can spread to distant parts of the organism, presumably via dsRNA movement from cell to cell. In plants, the introduction of a transgene encoding part or the entire coding sequence of a host gene can lead to post-transcriptional silencing of both the transgene and homologous host sequences (co-suppression) [8]. A clear demonstration of the systemic spread of sequence-specific gene silencing was performed in transgenic tobacco plants expressing green fluorescent protein (GFP) by infiltrating leaves with strains of *Agrobacterium tumefaciens* carrying a GFP reporter gene [9]. Initially, only a small region of the infiltrated leaves silenced GFP expression; however, by 18 days after infiltration, silencing of GFP fluorescence had spread to the upper leaves. Further evidence for the systemic spread of RNAi in plants was shown by grafting experiments in tobacco plants in which the silenced state of one plant could be transmitted with 100% efficiency to a grafted bud from another plant [10].

Non–cell autonomous RNAi was first noted in *C. elegans* when injection of dsRNA into the body cavity of either the head or tail led to targeted gene silencing throughout the injected animal and its progeny [1]. Experimentation with alternative dsRNA delivery methods in *C. elegans* soon revealed that RNAi could be triggered by soaking animals in a solution containing dsRNA or by feeding on bacteria expressing dsRNAs [11,12]. dsRNA ingestion produces a specific and durable interference effect, although generally not as potent as the effect seen after dsRNA injection [13,14]. In addition to *C. elegans*, RNAi triggered by environmental exposure to dsRNA has now been documented in some other invertebrates including planaria, hydra, honey bee and parasitic nematodes [15–19].

In the decade since its discovery, roles for endogenous RNAi pathways have been demonstrated in diverse biological processes including gene regulation during development, defense against viral infection and tumor suppression [20,21]. Additionally, there has been much progress in elucidating the mechanics of cell autonomous RNAi. Newer directions for inquiry include the mechanism of cell to cell movement of silencing, or systemic RNAi, in the context of multicellular organisms [22]. We are also beginning to recognize that animals can trigger RNAi in response to dsRNA molecules encountered in their environment. For example, the ingestion of bacteria or plants expressing dsRNAs can lead to targeted gene silencing in nematodes and insects, respectively [12,23,24]. The phenomenon of environmental RNAi poses many questions that challenge established notions of gene regulation: Do naturally encountered (e.g. from the diet) dsRNAs regulate endogenous gene expression? Is RNAi transmitted between organisms? What is the evolutionary significance of gene regulation via environmental RNAi? Here, we review the current understanding of environmental RNAi

Corresponding author: Hunter, C.P. (hunter@mcb.harvard.edu).

0168-9525/$ – see front matter doi:10.1016/j.tig.2008.03.007 Available online 29 April 2008

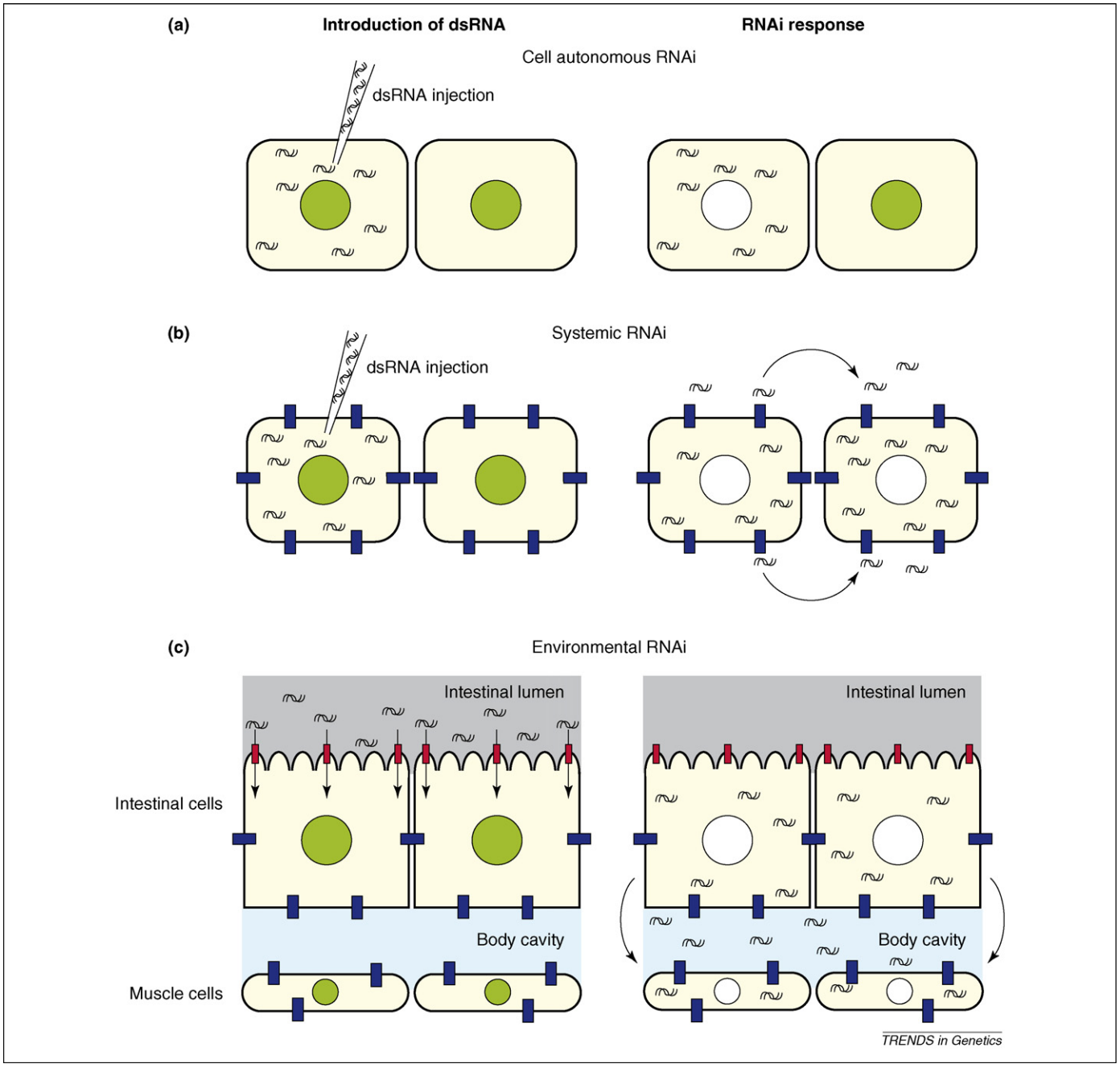

Figure 1. Cell autonomous, systemic and environmental RNA interference (RNAi). Depictions of cells expressing green fluorescent protein (GFP) are shown in the left column. Cells in the left column are presented with double-stranded RNA (dsRNA) targeting *gfp* expression. Cells in the right column show an RNAi response to dsRNA targeting *gfp* expression. **(a)** Cell autonomous RNAi: GFP expression is silenced only in the cell that is directly exposed to dsRNA. The neighboring cell continues to express GFP. **(b)** Systemic RNAi: GFP expression is silenced in the cell that is directly exposed to dsRNA. In addition, dsRNA and dsRNA-derived silencing signals can spread by dsRNA-transporting channels (depicted by blue rectangles) and lead to silencing of GFP expression in the neighboring cell. **(c)** Environmental RNAi: dsRNA molecules present in the intestinal lumen are taken up at the apical membrane of intestinal cells by dsRNA importers (depicted by red rectangles) and lead to silencing of GFP expression in the intestinal cells. The dsRNA and dsRNA-derived silencing signals are exported from the intestinal cells and spread to cells throughout the animal by systemic RNAi and lead to the spread of GFP silencing in distant cells.

and propose future directions for inquiry in this emerging field.

Genetic analysis of environmental RNAi in *C. elegans*

Studies in *C. elegans* have provided insight into how dsRNA molecules enter an organism from the environment to trigger RNAi. With an impermeable cuticle covering nearly its entire surface, *C. elegans* is thought to take up dsRNA through the intestinal lumen while feeding. The ingested dsRNAs and resulting silencing signals spread systemically to other cells in the animal. Importantly, these silencing signals can be generated in the absence of target gene expression in the intestine [25]. Thus, environmental RNAi in *C. elegans* minimally requires the following steps: (i) dsRNA uptake by the intestinal cells, (ii) export of either the dsRNA or of dsRNA-derived silencing signals from the intestinal cells, (iii) import of the silencing signals into other tissues (e.g. muscle, epidermis, germline) and (iv) targeted gene silencing via the cell autonomous RNAi machinery. Genetic screens in *C. elegans* have begun to identify

Box 1. Environmental RNA interference in multicellular organisms

Gene silencing by cell autonomous RNA interference (RNAi) is restricted to cells that generate double-stranded RNA (dsRNA) or that are directly exposed to experimentally introduced dsRNA. Non–cell autonomous RNAi, which encompasses both systemic and environmental RNAi, refers to the ability of dsRNA to trigger RNAi in cells distant from the site of initiation. By definition, systemic RNAi occurs exclusively in multicellular organisms and describes the process by which dsRNA-derived silencing signals cross cellular boundaries and spread from one cell to another. In *Caenorhabditis elegans*, injection of dsRNA into one tissue can lead to the spreading of targeted gene silencing in tissues throughout the injected animal and in the animal's progeny [1]. Systemic RNAi is widespread in plants and is important in restricting viral infection [8]. Environmental RNAi refers to the process by which sequence-specific gene silencing occurs in response to environmentally encountered dsRNA. In *C. elegans* and some other animals, an RNAi effect is observed after the presumed ingestion of dsRNA by feeding and soaking [11,12]. Thus, environmental RNAi in multicellular organisms involves the uptake of dsRNA by a primary group of cells (e.g. intestinal lumen cells) followed by the systemic spread of gene silencing into a secondary group of cells and tissues. Although environmental and systemic RNAi are likely to share some overlapping machinery, the processes are distinct because environmental RNAi has been observed in unicellular organisms such as protozoans (Box 2). Furthermore, results from recent genetic screens in *C. elegans* imply that genes such as *sid-2* function exclusively in the uptake of dsRNA from the environment and are not required for the subsequent systemic spread of RNAi [25].

The machinery required for the uptake of environmental dsRNA or for the spread of RNAi is distinct from the cell autonomous RNAi machinery. Among the *C. elegans* genes required for autonomous RNAi are the RNAi defective mutants *rde-1* and *rde-4*, which have no readily detectable mutant phenotype under laboratory conditions other than resistance to RNAi [57]. *rde-1* encodes a conserved member of the Argonaute family of proteins [58], and *rde-4* encodes a dsRNA binding protein [58]. Homozygous *rde-1* or *rde-4* mutant animals injected with dsRNA in the intestine are capable of efficiently transporting the RNAi effect to heterozygous progeny, implying that the transport mechanisms for systemic RNAi remain intact in these animals [57]. Likewise, genes required for systemic RNAi or environmental RNAi do not disrupt cell autonomous RNAi [26].

components that participate in each of these steps (Table 1) [26–29].

Identification of sid-1 and sid-2

The *sid-1* and *sid-2* genes were initially identified in a forward genetic screen designed to isolate *C. elegans* mutants defective in systemic RNAi [26]. Subsequently, both genes were also found to have a role in environmental RNAi. The screen used a worm strain that enabled the simultaneous monitoring of autonomous and systemic RNAi. Mutant worms that were defective in RNAi spreading between tissues but competent in the initiation or maintenance of a cell autonomous RNAi response were selected for further analysis [26]. Two hundred systemic RNAi defective (Sid) mutants were isolated, comprising three large complementation groups (20–100 alleles each) and several smaller uncharacterized complementation groups. *sid-1* and *sid-2* have been cloned [25,26], whereas *sid-3,* although mapped to a small genetic interval, remains uncloned.

sid-1 encodes a widely conserved multipass transmembrane protein with homologs in most animals, including mammals [26]. *sid-1* mutant worms remain competent in cell autonomous RNAi, but cannot perform systemic RNAi in response to feeding, soaking, injection of dsRNA or *in vivo* expression of dsRNA from transgenes. In addition to their defect in systemic RNAi, *sid-1* mutants cannot perform environmental RNAi: they are unable to trigger RNAi in intestinal cells in RNAi feeding assays [25]. *sid-1* mutant worms appear otherwise healthy and fertile and do not exhibit any other overt phenotypes. Heterologous SID-1 expression in *Drosophila melanogaster* S2 cells, which lack a SID-1 homolog, confers rapid dsRNA import from the growth media by an apparently passive mechanism [30]. Together, these data suggest that SID-1 functions as a dsRNA channel, although we cannot rule out the possibilities that SID-1 might modify the activity of a channel or that it might be a component of a multi-subunit channel.

SID-2 characterization

SID-2 is necessary for the initial import of dsRNA into the animal from the gut lumen but is not required for the systemic spread of silencing signals between cells and tissues [25]. *sid-2* encodes a 311 amino acid single-pass transmembrane protein with homologs identified only in *Caenorhabditis briggsae* and *Caenorhabditis remanei* [25]. A SID-2–GFP fusion protein localizes to intestinal cell apical membranes, which are densely packed with microvilli for the absorption of nutrients. This expression pattern suggests that SID-2 facilitates the import of ingested dsRNA from the intestinal lumen [25]. However, SID-2 alone is not sufficient for dsRNA import from the intestinal lumen because *sid-1* mutants are also unable to uptake environmental dsRNA.

Expression data and genetic studies have suggested several models for SID-2 function. SID-2 might modify or accessorize SID-1 at the lumen or might function in series with SID-1, internalizing dsRNA for SID-1 transport across the membrane (Figure 2). Further studies are necessary to distinguish between these models. In particular, it will be informative to determine whether SID-2 has dsRNA binding activity. Using the *D. melanogaster* S2 cell system, the ability of SID-2 to facilitate import of dsRNA can be tested under various conditions such as in the presence or absence of SID-1 or in the presence of drugs that block endocytosis.

Further gene discovery in C. elegans

Two forward genetic screens in *C. elegans* have identified genes required for environmental RNAi: the feeding defective *(fed)* mutants (*fed-1* and *fed-2*) [27], and the RNA spreading defective (*rsd*) mutants (*rsd-2, -3, -4, -6 and -8*) [28]. Although further characterization or cloning of the *fed* mutants has not yet been reported, *fed-1* is likely to be allelic with *sid-2* based on phenotype and genetic map position. In addition, *fed-2* is likely to be allelic with *rsd-2* (www.wormbase.org, release 188).

The *rsd* mutants are divided into two phenotypic classes: (i) class I, resistant to RNAi by feeding on dsRNA directed against both germline and somatic genes (*rsd-4* and *rsd-8*); (ii) class II, resistant to feeding on dsRNA of germline-expressed genes but sensitive to dsRNA directed against somatic genes (*rsd-2*, *rsd-3* and *rsd-6*).

Table 1. Genes involved in environmental RNAi[a] in *Caenorhabditis elegans*

Caenorhabditis elegans Gene name	Gene product	Expression pattern	Mutant phenotype	Refs
Genes identified in forward genetic screens				
sid-1 (rsd-8)	Multipass transmembrane protein (likely dsRNA channel)	All non-neuronal cell types	Defective in systemic and environmental RNAi	[26]
sid-2 (fed-1 or rsd-4)	Singlepass transmembrane protein (likely dsRNA receptor)	Apical membrane of intestinal cells	Defective in RNAi by feeding and soaking; sensitive to RNAi by injection	[25,27,28]
fed-2 (rsd-2)	Novel protein	Not known	Defective in RNAi by feeding; sensitive to RNAi by injection	[27,28]
rsd-3	Protein with epsin N-terminal homology (ENTH) domain found commonly in vesicle trafficking proteins	Coelomocytes (endocytic scavenger cells)	Defective in RNAi by feeding; sensitive to RNAi by injection	[28]
rsd-6	Protein with a Tudor domain, a motif found frequently in RNA binding proteins	Not known	Defective in RNAi by feeding; sensitive to RNAi by injection	[28]
***Caenorhabditis elegans* Gene name**	**Gene product**	**RNAi phenotype[b]**		**Refs**
Genes identified by RNAi screen				
arl-1	Homolog of the mammalian *ARF-like* protein 1 (small GTPase); involved in vesicle-mediated transport	Defective in RNAi by feeding		[29]
F22G12.5	Homolog of *D. melanogaster* ninaC.5 protein; involved in intracellular transport	Defective in RNAi by feeding		[29]
cgoc-2	Homolog of mammalian COG-2, a subunit the *c*onserved oligomeric *Go*lgi *c*omplex; involved in intracellular transport	Defective in RNAi by feeding		[29]
ZK1098.5	Transport protein particle (TRAPP) complex subunit; involved in intracellular transport	Defective in RNAi by feeding		[29]
vps-41	Homolog of the *S. cerevisiae* *v*acuolar *p*rotein *s*orting factor Vps41p; involved in intracellular transport	Defective in RNAi by feeding		[29]
vps-34	Homolog of the phosphoinositide 3-kinase VPS34 in *S. cerevisiae*, a protein that regulates multiple steps in endocytosis	Defective in RNAi by feeding		[29]
ger-1	*G*DP-keto-6-deoxymannose 3,5-*E*pimerase/4-*R*eductase; involved in lipid metabolism	Defective in RNAi by feeding		[29]
bre-3[c]	Beta-glycosyltransferase	Defective in RNAi by feeding		[29]
sedl-1	Human SEDL (*s*pondylo*e*piphyseal *d*ysp*l*asia tarda) related; Transport protein particle (TRAPP) complex subunit of the cis-Golgi	Defective in RNAi by feeding		[29]
sym-3[d]	Novel protein with unknown function	Defective in RNAi by feeding		[29]

[a]RNAi, RNA interference; dsRNA, double-stranded RNA.
[b]Genes have not been tested for a role in cell autonomous or systemic RNAi.
[c]Bt toxin resistant.
[d]Synthetic lethal with Mec.

The class I mutants likely have a general defect in either environmental dsRNA uptake or systemic spreading of the silencing signal. Consistent with this hypothesis, *rsd-8* was found to be an allele of *sid-1* [28], and the map position of *rsd-4* indicates that it is likely to be an allele of *sid-2* (*fed-1*). The three class II genes likely play a role in the spread of silencing signals from the intestine into the germline, indicating the presence of specific pathways that transport silencing signals between tissues. The molecular identity of the RSD-2 protein does not reveal any functional information because there are no discernible motifs or close homologs outside the genus *Caenorhabditis*. By contrast, RSD-6 contains a Tudor domain, a motif frequently found in RNA-binding proteins. A yeast two-hybrid screen using RSD-6 as bait identified RSD-2 as the most frequent interacting protein, but further genetic interactions or expression patterns of these genes have not been reported. *rsd-3* encodes a homolog of human Enthoprotin and is highly expressed in coelomocytes, scavenger cells that continuously and nonspecifically endocytose fluid from the worm pseudocoelom (body cavity). The Enthoprotin family is widely conserved in eukaryotes and is required for the clathrin-dependent membrane budding that occurs during the internalization step of endocytosis. This finding suggests a role for endocytosis in the uptake or spreading of silencing signals from the intestine. The *rsd* mutants do not exhibit generalized endocytosis defects, and several mutants in the clathrin endocytosis pathway and receptor-mediated endocytosis pathway remain competent for systemic RNAi [28]. However, it is difficult to assay environmental or systemic RNAi in mutants of the general endocytic pathway because these genes are essential. It is

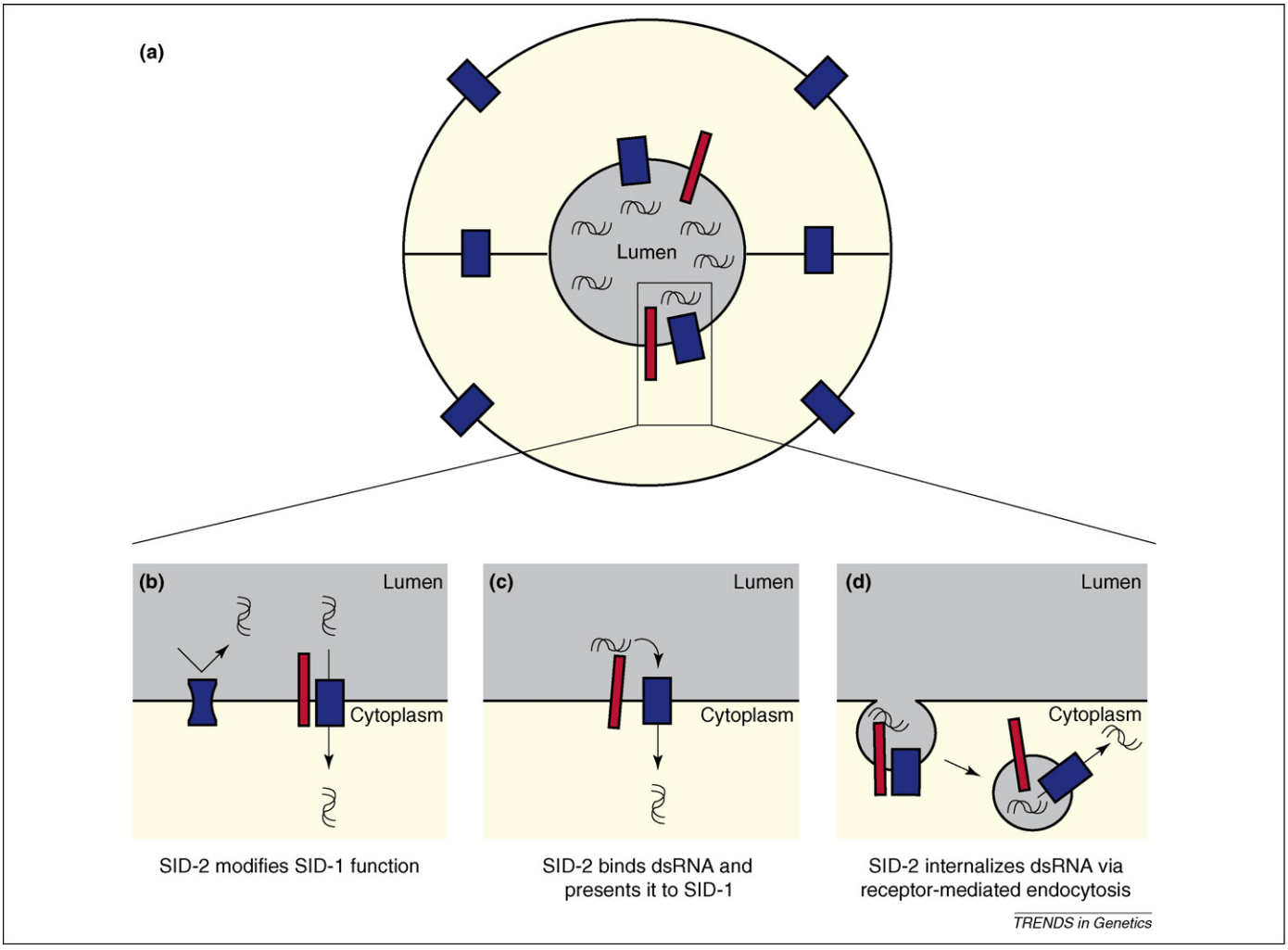

Figure 2. Models of SID-1 and SID-2 function in double-stranded RNA (dsRNA) uptake from the *Caenorhabditis elegans* intestinal lumen. The first step in environmental RNA interference (RNAi) involves the uptake of ingested dsRNA from the intestinal lumen. Based on current genetic and expression data for *sid-1* and *sid-2*, two genes required for this process, we propose several models of SID-1 and SID-2 function. **(a)** Depiction of the intestinal cross-section in *C. elegans*. SID-2 (red) localizes to the luminal membrane of the intestine, whereas SID-1 (blue) is present at cell membranes. **(b)** SID-2 modifies the properties of SID-1 to allow it to function efficiently in the intestinal lumen. Once modified by SID-2, SID-1 serves as a channel for the import of dsRNA into the intestinal cell. **(c)** SID-2 acts as a receptor that first binds dsRNA in the intestinal lumen and subsequently presents it to the SID-1 channel for transport into the intestinal cell. **(d)** SID-2 binds and internalizes the dsRNA from the intestinal lumen by receptor-mediated endocytosis. The dsRNA is exported into the intestinal cell cytoplasm via the SID-1 channel.

possible that RSD-3 might affect vesicle trafficking pathways that are specific for the uptake or spread of RNAi or that are tissue specific.

A role for endocytosis in environmental RNAi is further supported by the findings of a reverse genetic screen performed in *D. melanogaster* and *C. elegans* [29]. Of note, the screen was designed to identify any gene involved in the response to exogenous dsRNA; thus, it is possible that these genes are not exclusively involved in environmental RNAi but are also required for the cell autonomous RNAi machinery or for the systemic spread of silencing. Using a dsRNA library targeting ~50% of the *D. melanogaster* genome, the first step of the screen identified 23 genes required for RNAi triggered by dsRNA added to the growth medium of cultured *D. melanogaster* S2 cells.. Many of these genes encode components of the endocytic pathway involved in vesicle trafficking and protein sorting. Subsequently, orthologs of these genes were knocked down by RNAi in *C. elegans* to test for a requirement in the uptake of environmental dsRNA. Worms were first fed bacteria expressing dsRNA targeting the candidate gene. To assay for sensitivity to environmental RNAi, the progeny of these worms were transferred to bacteria expressing dsRNA targeting *unc-52*. RNAi of the *unc-52* gene phenocopies the *unc-52* mutant phenotype (defects in myofilament assembly and subsequent paralysis) [31]. Knockdown of 10 of the 23 genes identified in *D. melanogaster* disrupted the ability to trigger an RNAi response to *unc-52* dsRNA delivered by feeding in *C. elegans*. Four of these genes encode components of intracellular vesicle transport, two encode lipid modifying enzymes, one encodes a glycosyltransferase and three encode proteins of unknown function (Table 1). Further studies with genetic mutants of these genes are necessary to determine whether their function is required for cell autonomous RNAi, systemic RNAi or environmental RNAi.

In summary, genetic screens in *C. elegans* are beginning to elucidate the mechanisms underlying environmental RNAi. An essential step in environmental RNAi is the ability to internalize dsRNA encountered in the environment (i.e. while feeding). SID-2 is present on the apical membrane of intestinal cells and is likely to have a role in

transporting dsRNA from the intestinal lumen. It might function together with the SID-1 dsRNA channel or with endocytosis pathway components to facilitate dsRNA uptake. It is appealing to invoke the endocytic pathway in the uptake of dsRNA from the intestinal lumen as endocytosis is a major mechanism by which cells absorb material from their external environment. The implication of endocytic pathway genes in RNAi in both *D. melanogaster* and *C. elegans* might also indicate a more general role for endocytosis in RNAi in other organisms.

Environmental RNAi in other organisms

In addition to *C. elegans*, environmental RNAi is observed in several multicellular organisms, including other nematodes, arthropods and cnidarians (e.g. hydra, jellyfish, anemones). Surprisingly, a related nematode *C. briggsae* cannot perform environmental RNAi, although it can trigger a robust systemic RNAi response to dsRNA injection. Investigating the similarities and differences between these organisms will help to elucidate the mechanisms, function and regulation of environmental RNAi. Furthermore, recent studies show that environmental RNAi is not limited to multicellular organisms or even to the animal kingdom: environmental dsRNA can trigger RNAi in the protozoa *Paramecium* and *Entamoeba histolytica* (Box 2).

Caenorhabditis briggsae is deficient in environmental RNAi

Although *C. briggsae* is competent for systemic RNAi in response to injected dsRNA, it is completely resistant to RNAi by feeding or soaking [25]. Why is *C. briggsae* proficient for systemic RNAi but insensitive to environmental RNAi? The difference in sensitivity to environmental RNAi cannot be accounted for by changes in expression or localization of *C. briggsae* SID-2. Similar to *C. elegans* SID-2–GFP, a *C. briggsae* SID-2–GFP fusion protein is also expressed in the intestine and localizes to the apical membrane. However, the sequence divergence between *C. elegans* and *C. briggsae* SID-2 proteins might explain the lack of environmental RNAi in *C. briggsae*. Sequence alignment shows 23% identity in the N terminus (190 amino acids), 86% identity in the transmembrane domain (21 amino acids) and 53% identity in the C terminus (100 amino acids) [25]. Strikingly, expression of the *C. elegans sid-2–gfp* transgene in *C. briggsae* confers sensitivity to environmental RNAi, suggesting that *C. briggsae* has the supporting machinery required to perform environmental RNAi [25]. It is possible that the nearly 80% divergence of the SID-2 extracellular domain has resulted in either *C. elegans* SID-2 gaining or *C. briggsae* SID-2 losing the ability to support environmental RNAi.

The finding that *C. briggsae* is deficient in environmental RNAi prompted a survey of environmental RNAi in the eight available *Caenorhabditis* species [25]. Only one of these tested species, *Caenorhabditis* species 1, was proficient in environmental RNAi. Thus, the ability to perform environmental RNAi might be uncommon, or the process might be regulated by factors that are not present in standard laboratory growth conditions.

Environmental RNAi in other multicellular organisms

With the exception of vertebrates, environmental RNAi has been observed in a wide range of species. Although apparently rare among *Caenorhabditis* nematodes, environmental RNAi is observed in many animal- and plant-parasitic nematodes [19]. Because genome sequence information is not readily available for the majority of these parasitic nematode species, it is not known whether the same machinery observed in *C. elegans* is present in these nematodes. Interestingly, the draft genome of the filarial nematode *Brugia malayi* was recently published [32], and a comparative analysis with the *C. elegans* genome revealed an apparent absence of *sid-1* and *sid-2* despite competence for environmental RNAi. It is possible that these homologs might be contained in the yet unfinished sequences. Conversely, these genes might be rapidly evolving, or organisms might have evolved alternative pathways for the transport of dsRNA.

Environmental RNAi has been well described in flatworms. In the planarian *Dugesia japonica*, gene knockdown occurs after the soaking of animals in a dsRNA solution [15]. The soaking method is effective not only for genes expressed in cells in direct contact with the environment but also for a gene expressed in the eye, which is located in the mesenchyme inside the body. The silencing effect of the dsRNA also occurs in newly regenerated tissues, indicating that planaria are capable of performing both systemic and environmental RNAi. dsRNA delivery via bacterial feeding can also trigger RNAi in the planarian *Schmidtea mediterranea* [16]. Gene silencing was observed as early as 1–2 days after the third feeding, and the effects were observed up to 24 days after feeding. The inhibition was specific and could target genes in a variety of tissue types.

Box 2. Environmental RNA interference in unicellular organisms

RNA interference (RNAi) is conserved across eukaryotic phyla. Environmental RNAi is a process by which an organism generates an RNAi response to double-stranded RNA (dsRNA) encountered in the environment (i.e. by ingestion). Since its initial observation in *Caenorhabditis elegans,* environmental RNAi has been demonstrated in only a limited number of animals. Surprisingly, the ability to perform environmental RNAi also exists outside of the animal kingdom in at least two protozoans: *Paramecium* [59] and *Entamoeba histolytica* [60]. Initial studies in *Paramecium* established that RNAi triggered by transgene transformation or by direct dsRNA injection could lead to loss-of-function phenotypes [61]. Subsequently, feeding dsRNA-expressing *E. coli* to *Paramecium* also generated complete loss-of-function phenotypes for several different target genes [59]. For all genes tested, the phenotypes obtained by feeding were reportedly equivalent to the phenotypes generated by dsRNA injection.

Environmental RNAi by soaking has been demonstrated in the human pathogen *E. histolytica* [60]. siRNAs targeting the γ-tubulin gene were added to a growing *E. histolytica* culture, resulting in highly specific and efficient silencing of the γ-tubulin gene and the disruption of microtubule organization. The components required for the uptake of environmental dsRNA are not known in either *Paramecium* or *E. histolytica*. A search of the *E. histolytica* genome database identified an RNase III and an Argonaute family member, two conserved protein families involved in cell autonomous RNAi [60]. However, the genome does not appear to contain *sid-1* or *sid-2* homologs, suggesting that these organisms have evolved an independent mechanism for environmental RNAi.

RNAi triggered by feeding and soaking has also been demonstrated in a variety of arthropods including ticks, honey bee (*Apis mellifera*) and western corn rootworm (WCR; *Diabrotica virgifera virgifera* LeConte) [18,23]. In the well-studied dipteran, *D. melanogaster*, environmental RNAi seems to function under some conditions. Soaking *D. melanogaster* embryos in dsRNA solutions can also initiate RNAi [33]. However, RNAi in response to dsRNA feeding has not been reported in *D. melanogaster* larvae or adults.

Observations in lepidopterans (e.g. moths, butterflies) indicate that environmental RNAi does not occur uniformly in insects. For example, oral dsRNA delivery leads to effective systemic gene silencing in apple moth (*Epiphyas postvittana*) larvae [34] but not in the oriental leafworm moth (*Spodoptera litura*) [35]. Failure to perform environmental RNAi in *S. litura* could be explained by physiological differences in the gut environment between species or by variations in feeding techniques or dsRNA amounts.

Environmental RNAi has also been observed in cnidarians (hydra), a sister group to the bilaterians (vertebrates, insects, nematodes). Repeated feedings of dsRNA-expressing bacteria specifically suppressed target gene expression in hydra below the *in situ* hybridization detection limit, and importantly, also phenocopied the expected mutant phenotype [17].

Applications of environmental RNAi

Although the mechanisms underlying environmental RNAi remain poorly understood, the process of environmental dsRNA uptake has already been harnessed for important biological applications. Environmental RNAi provides an inexpensive and technically simple method of delivering dsRNA. In particular, this method has revolutionized large-scale RNAi screening and has prompted innovative approaches to agricultural pest control. In the future, we can envision the oral delivery of dsRNA therapeutics in humans or animals (e.g. livestock) via transgenic crops or genetically modified bacteria (Box 3).

High-throughput RNAi screens

Feeding or soaking methodologies for dsRNA delivery enable large-scale RNAi screens to be performed that would be otherwise prohibitive using the more technically demanding and time-consuming microinjection technique. With the creation of an RNAi library of bacterial clones covering ~90% of the predicted genes in *C. elegans*, it is possible to perform high-throughput genome-wide RNAi screens at low cost [36–38]. The capacity to perform large-scale functional RNAi screens *in vivo* makes *C. elegans* an extremely powerful model system for the study of development and disease pathways in higher organisms. Indeed, genome-wide feeding RNAi screens have rapidly identified conserved genes involved in important biological processes including fat regulation, aging, RNAi, and neuromuscular junction development [39–44]. The success of these RNAi screening strategies in *C. elegans* has inspired similar approaches in numerous organisms.

Susceptibility to environmental RNAi has enabled significant advances in gene discovery and analysis, particularly in organisms that are not amenable to traditional genetic analysis. For example, the ability to perform large-scale feeding RNAi screens in the freshwater planarian *S. mediterranea* established this organism as an invaluable model for the molecular analyses of tissue regeneration and stem cell biology [45]. Furthermore, SID-1 expression in *D. melanogaster* cell lines that do not exhibit efficient dsRNA uptake by soaking has facilitated the completion of cell-based assays and large-scale screening [30] [46,47]. Similarly, overexpression of a human SID-1 homolog enhances siRNA uptake in a human pancreatic cancer cell line, and heterologous expression of *C. elegans* SID-1 enables exogenous dsRNA uptake in cultured mouse embryonic stem cells [48,49]. Thus SID-1 and its homologs might facilitate large-scale RNAi soaking screens in mammalian cell culture.

Pest control in agricultural crops

The annual cost of damage caused by insects and plant-parasitic nematodes to the world's major crops is estimated to be in the billions of dollars. Although the use of chemical

Box 3. Delivery of RNA interference–based therapies in mammals by environmental RNA interference?

Although there is no demonstrated evidence for naturally occurring environmental RNA interference (RNAi) in mammals, recent work in mice has shown the potential for therapeutic RNAi delivery by oral administration [62]. Nonpathogenic *E. coli* engineered to produce short hairpin RNAs (shRNAs) targeting an oncogene, *CTNNB1* (*Catenin β-1*), induced specific gene silencing in both *in vitro* and *in vivo* assays. For example, co-culturing of the bacteria expressing shRNA against *CTNNB1* with human colon cancer cells led to potent *CTNNB1* downregulation at the mRNA and protein level. The gene silencing observed in the human colon cancer cell line relied on *E. coli* cellular entry but not on persistent bacterial replication. After this *in vitro* demonstration of intercellular RNAi, oral administration of the shRNA-expressing *E. coli* to mice five times per week produced specific *CTNNB1* silencing in the intestinal epithelium after 4 weeks of treatment. Do mechanisms for gene regulation via environmental RNAi exist in mammals? Does systemic spread of silencing from the intestine occur following ingestion of the shRNA-expressing bacteria? Further studies using this model system might provide an entry point for the investigation of environmental and systemic RNAi in mammals. In addition, this method could be developed further as a delivery for RNAi-based therapeutics in humans and other animals, including livestock.

Homologs of the putative double-stranded RNA (dsRNA) channel protein SID-1 have been found in all sequenced vertebrate genomes [22]. Mice and humans have two *sid-1* homologs, SidT1 and SidT2, and a recent study suggests a role for human SidT1 in dsRNA uptake [63]. SidT1 knockdown in primary human hepatocytes blocked internalization of cholesterol-conjugated siRNA in an *in vitro* uptake assay. Additionally, an anti-SidT1 antibody introduced into the culture medium blocked hepatocyte uptake of siRNA. It will be interesting to determine whether either of the mouse SID-1 homologs participates in the uptake of dsRNA from ingested bacteria.

Another intriguing observation involves the oral administration of tumor-targeting bacteria in mice. Some facultative anaerobic bacteria such as *Salmonella typhimurium* replicate preferentially in the hypoxic and necrotic regions of tumors when delivered intravenously in animal models [64]. Recent findings in mice demonstrated that oral administration of *S. typhimurium* leads to preferential replication of the bacteria in tumor tissue and subsequently elicits an antitumor effect by causing tumor cell apoptosis [65]. The oral administration of tumor-targeting bacteria engineered to express shRNAs targeting oncogenes might provide a new approach to anticancer therapeutics.

pesticides is effective, the cost and toxicity to humans and the environment has motivated the search for alternative pest control strategies. The observation that ingested dsRNA can silence endogenous genes in plant-parasitic nematodes and insects suggested the possibility of applying RNAi technology to agricultural pest control [50,51]. This strategy, known as host-delivered RNAi or *in planta* RNAi, was first reported for the root knot nematode, *Meloidogyne incognita*. Several studies showed that ingestion of plant-generated dsRNA could trigger sequence-specific gene silencing in *M. incognita* and could confer effective nematode resistance to the plant [52,53].

Similarly, two recent studies have shown the potential of harnessing environmental RNAi to protect plants from herbivorous insects by transgenic expression of dsRNAs targeting essential insect genes. In one study, a feeding RNAi screen was first carried out to identify gene targets that caused lethality in the western corn rootworm [23]. Next, transgenic corn plants were generated that expressed one of the lethal insect dsRNAs. After 3 weeks of infestation by WCR larvae, the transgenic plants expressing the lethal insect dsRNAs showed significant root protection compared with the nontransgenic control plants.

A second group of researchers began by investigating how the cotton bollworm (*Helicoverpa armigera*) resists gossypol, a naturally occurring toxin produced by cotton plants [24]. The cytochrome P450 monooxygenase gene (*CYP6AE14*) was identified as one of the gossypol-inducible genes in the cotton bollworm. Subsequently, transgenic *Arabidopsis thaliana* and tobacco (*Nicotiana tobacum*) plants were generated that expressed dsRNA targeted against *CYP6AE14*. Cotton bollworm larvae reared on these transgenic plants showed significant larval growth retardation in the presence of gossypol. Of note, this approach has not yet been performed using cotton plants, the natural host.

The application of *in planta* RNAi has exciting potential as a widespread approach for the management of herbivorous insects and nematodes. However, before this technology can be applied on a large scale, many important concerns must be addressed. Will the expressed dsRNAs have off-target effects in either the crops, crop pests or other consumers of the transgenic crops (i.e. livestock and people)? Will the pest populations develop resistance through acquisition of point mutations in the target genes? It is also possible that viral infection of the transgenic crops could significantly decrease the efficacy of this strategy. Although the RNAi machinery in plants is thought to serve as an antiviral defense mechanism, many plant viruses encode counter-silencing RNAi suppressor proteins [54]. For example, the P19 protein of the *Tomato bushy stunt virus* can bind siRNAs and inhibit RNAi in HeLa cells [55].

Concluding remarks and future perspectives

The discovery of RNAi revolutionized our understanding of gene regulation. The more recent descriptions of systemic and environmental RNAi have revealed a novel means of communication between cells. The ability to trigger an RNAi response to dsRNAs in the environment is particularly intriguing as a means of communication between organisms and their environment. Since the initial discovery in *C. elegans*, environmental RNAi has been observed in a wide range of metazoans and even in some protozoans. Practical applications of this process have facilitated genetic analysis in several organisms. Current research efforts are focused on identifying components of the machinery required for environmental RNAi; thus far, there is a lack of conservation in the machinery among the different organisms that exhibit environmental RNAi. Perhaps this process is not essential and is subject to frequent mutation or perhaps environmental RNAi has arisen independently many times during evolution.

What is the natural function of environmental RNAi? Organisms naturally encounter extracellular nucleic acids in the environment. Extracellular DNA, prevalent in soil and aquatic environments, can provide an important source of DNA to be taken up and integrated by 'naturally competent' bacteria [56]. This process of horizontal gene transfer has been observed between organisms of the same or different species and provides an important means for the acquisition of new genes. Although the discovery of environmental RNAi suggests a role for extracellular RNAs in the communication between organisms, many questions remain to be answered. What are the sources of natural dsRNA in the environment? Why do organisms silence endogenous genes in a sequence-specific manner in response to environmental dsRNA? We speculate that environmental RNAi might direct communication between organisms. As discussed in this review, there are several experimental examples of RNAi transfer between different species. In nature, an organism might use environmental RNAi to sense viruses (a natural source of dsRNA) and to communicate the response to the infected state. Although this is an appealing hypothesis, we note that there are no known natural viruses of *C. elegans*. We also speculate that environmental RNAi might be used to cross-regulate gene expression between organisms in a symbiotic relationship. Another attractive possibility is that environmental RNAi can synchronize epigenetic gene regulation within a species in which members live in close proximity to each other. For example, if silencing of a particular gene provides increased fitness to an organism, this advantage could be rapidly transferred via environmental RNAi to other members of the community.

Acknowledgements

The authors thank Javier Apfeld, Andrea Hinas, Antony Jose, Katerina Ragkousi, Daniel Schott and Lucie Yang for critical comments on the manuscript. This work was supported by grants to C.P.H. from the National Science Foundation (MCB0417102) and the National Institutes of Health (GM069891).

References

1 Fire, A. *et al.* (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391, 806–811
2 Mello, C.C. and Conte, D., Jr (2004) Revealing the world of RNA interference. *Nature* 431, 338–342
3 Elbashir, S.M. *et al.* (2001) RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev.* 15, 188–200
4 Hammond, S.M. *et al.* (2001) Argonaute2, a link between genetic and biochemical analyses of RNAi. *Science* 293, 1146–1150

5 Meister, G. and Tuschl, T. (2004) Mechanisms of gene silencing by double-stranded RNA. *Nature* 431, 343–349
6 Tomari, Y. and Zamore, P.D. (2005) Perspective: machines for RNAi. *Genes Dev.* 19, 517–529
7 Grishok, A. (2005) RNAi mechanisms in *Caenorhabditis elegans*. *FEBS Lett.* 579, 5932–5939
8 Baulcombe, D. (2004) RNA silencing in plants. *Nature* 431, 356–363
9 Voinnet, O. and Baulcombe, D.C. (1997) Systemic signalling in gene silencing. *Nature* 389, 553
10 Palauqui, J.C. *et al.* (1997) Systemic acquired silencing: transgene-specific post-transcriptional silencing is transmitted by grafting from silenced stocks to non-silenced scions. *EMBO J.* 16, 4738–4745
11 Tabara, H. *et al.* (1998) RNAi in *C. elegans*: soaking in the genome sequence. *Science* 282, 430–431
12 Timmons, L. and Fire, A. (1998) Specific interference by ingested dsRNA. *Nature* 395, 854
13 Timmons, L. *et al.* (2001) Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in *Caenorhabditis elegans*. *Gene* 263, 103–112
14 Vastenhouw, N.L. *et al.* (2006) Gene expression: long-term gene silencing by RNAi. *Nature* 442, 882
15 Orii, H. *et al.* (2003) A simple 'soaking method' for RNA interference in the planarian Dugesia japonica. *Dev. Genes Evol.* 213, 138–141
16 Newmark, P.A. *et al.* (2003) Ingestion of bacterially expressed double-stranded RNA inhibits gene expression in planarians. *Proc. Natl. Acad. Sci. U. S. A.* 100 (Suppl 1), 11861–11865
17 Chera, S. *et al.* (2006) Silencing of the hydra serine protease inhibitor Kazal1 gene mimics the human SPINK1 pancreatic phenotype. *J. Cell Sci.* 119, 846–857
18 Patel, A. *et al.* (2007) The making of a queen: TOR pathway is a key player in diphenic caste development. *PLoS ONE* 2, e509
19 Geldhof, P. *et al.* (2007) RNA interference in parasitic helminths: current situation, potential pitfalls and future prospects. *Parasitology* 134, 609–619
20 Novina, C.D. and Sharp, P.A. (2004) The RNAi revolution. *Nature* 430, 161–164
21 Hammond, S.M. (2006) MicroRNAs as oncogenes. *Curr. Opin. Genet. Dev.* 16, 4–9
22 Jose, A.M. and Hunter, C.P. (2007) Transport of sequence-specific RNA interference information between cells. *Annu. Rev. Genet.* 41, 305–330
23 Baum, J.A. *et al.* (2007) Control of coleopteran insect pests through RNA interference. *Nat. Biotechnol.* 25, 1322–1326
24 Mao, Y.B. *et al.* (2007) Silencing a cotton bollworm P450 monooxygenase gene by plant-mediated RNAi impairs larval tolerance of gossypol. *Nat. Biotechnol.* 25, 1307–1313
25 Winston, W.M. *et al.* (2007) *Caenorhabditis elegans* SID-2 is required for environmental RNA interference. *Proc. Natl. Acad. Sci. U. S. A.* 104, 10565–10570
26 Winston, W.M. *et al.* (2002) Systemic RNAi in *C. elegans* requires the putative transmembrane protein SID-1. *Science* 295, 2456–2459
27 Timmons, L. *et al.* (2003) Inducible systemic RNA silencing in *Caenorhabditis elegans*. *Mol. Biol. Cell* 14, 2972–2983
28 Tijsterman, M. *et al.* (2004) Genes required for systemic RNA interference in *Caenorhabditis elegans*. *Curr. Biol.* 14, 111–116
29 Saleh, M.C. *et al.* (2006) The endocytic pathway mediates cell entry of dsRNA to induce RNAi silencing. *Nat. Cell Biol.* 8, 793–802
30 Feinberg, E.H. and Hunter, C.P. (2003) Transport of dsRNA into cells by the transmembrane protein SID-1. *Science* 301, 1545–1547
31 Mackenzie, J.M., Jr *et al.* (1978) Muscle development in *Caenorhabditis elegans*: mutants exhibiting retarded sarcomere construction. *Cell* 15, 751–762
32 Ghedin, E. *et al.* (2007) Draft genome of the filarial nematode parasite Brugia malayi. *Science* 317, 1756–1760
33 Eaton, B.A. *et al.* (2002) Dynactin is necessary for synapse stabilization. *Neuron* 34, 729–741
34 Turner, C.T. *et al.* (2006) RNA interference in the light brown apple moth, Epiphyas postvittana (Walker) induced by double-stranded RNA feeding. *Insect Mol. Biol.* 15, 383–391
35 Rajagopal, R. *et al.* (2002) Silencing of midgut aminopeptidase N of Spodoptera litura by double-stranded RNA establishes its role as Bacillus thuringiensis toxin receptor. *J. Biol. Chem.* 277, 46849–46851
36 Fraser, A.G. *et al.* (2000) Functional genomic analysis of *C. elegans* chromosome I by systematic RNA interference. *Nature* 408, 325–330
37 Kamath, R.S. *et al.* (2001) Effectiveness of specific RNA-mediated interference through ingested double-stranded RNA in *Caenorhabditis elegans*. *Genome Biol.* 2, RESEARCH0002
38 Kamath, R.S. *et al.* (2003) Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. *Nature* 421, 231–237
39 Ashrafi, K. *et al.* (2003) Genome-wide RNAi analysis of *Caenorhabditis elegans* fat regulatory genes. *Nature* 421, 268–272
40 Lee, S.S. *et al.* (2003) A systematic RNAi screen identifies a critical role for mitochondria in *C. elegans* longevity. *Nat. Genet.* 33, 40–48
41 Hansen, M. *et al.* (2005) New genes tied to endocrine, metabolic, and dietary regulation of lifespan from a *Caenorhabditis elegans* genomic RNAi screen. *PLoS Genet.* 1, 119–128
42 Hamilton, B. *et al.* (2005) A systematic RNAi screen for longevity genes in *C. elegans*. *Genes Dev.* 19, 1544–1555
43 Kim, J.K. *et al.* (2005) Functional genomic analysis of RNA interference in *C. elegans*. *Science* 308, 1164–1167
44 Sieburth, D. *et al.* (2005) Systematic analysis of genes required for synapse structure and function. *Nature* 436, 510–517
45 Reddien, P.W. *et al.* (2005) Identification of genes needed for regeneration, stem cell function, and tissue homeostasis by systematic gene perturbation in planaria. *Dev. Cell* 8, 635–649
46 Muller, P. *et al.* (2005) Identification of JAK/STAT signalling components by genome-wide RNA interference. *Nature* 436, 871–875
47 Bartscherer, K. *et al.* (2006) Secretion of Wnt ligands requires Evi, a conserved transmembrane protein. *Cell* 125, 523–533
48 Duxbury, M.S. *et al.* (2005) RNA interference: a mammalian SID-1 homologue enhances siRNA uptake and gene silencing efficacy in human cells. *Biochem. Biophys. Res. Commun.* 331, 459–463
49 Tsang, S.Y. *et al.* (2007) Ectopic expression of systemic RNA interference defective protein in embryonic stem cells. *Biochem. Biophys. Res. Commun.* 357, 480–486
50 Bakhetia, M. *et al.* (2005) RNA interference and plant parasitic nematodes. *Trends Plant Sci.* 10, 362–367
51 Gordon, K.H. and Waterhouse, P.M. (2007) RNAi for insect-proof plants. *Nat. Biotechnol.* 25, 1231–1232
52 Yadav, B.C. *et al.* (2006) Host-generated double stranded RNA induces RNAi in plant-parasitic nematodes and protects the host from infection. *Mol. Biochem. Parasitol.* 148, 219–222
53 Huang, G. *et al.* (2006) Engineering broad root-knot resistance in transgenic plants by RNAi silencing of a conserved and essential root-knot nematode parasitism gene. *Proc. Natl. Acad. Sci. U. S. A.* 103, 14302–14306
54 Lecellier, C.H. and Voinnet, O. (2004) RNA silencing: no mercy for viruses? *Immunol. Rev.* 198, 285–303
55 Dunoyer, P. *et al.* (2004) Probing the microRNA and small interfering RNA pathways with virus-encoded suppressors of RNA silencing. *Plant Cell* 16, 1235–1250
56 Vlassov, V.V. *et al.* (2007) Extracellular nucleic acids. *Bioessays* 29, 654–667
57 Tabara, H. *et al.* (1999) The rde-1 gene, RNA interference, and transposon silencing in *C. elegans*. *Cell* 99, 123–132
58 Tabara, H. *et al.* (2002) The dsRNA binding protein RDE-4 interacts with RDE-1, DCR-1, and a DExH-box helicase to direct RNAi in *C. elegans*. *Cell* 109, 861–871
59 Galvani, A. and Sperling, L. (2002) RNA interference by feeding in Paramecium. *Trends Genet.* 18, 11–12
60 Vayssie, L. *et al.* (2004) Double-stranded RNA mediates homology-dependent gene silencing of gamma-tubulin in the human parasite Entamoeba histolytica. *Mol. Biochem. Parasitol.* 138, 21–28
61 Ruiz, F. *et al.* (1998) Homology-dependent gene silencing in Paramecium. *Mol. Biol. Cell* 9, 931–943
62 Xiang, S. *et al.* (2006) Short hairpin RNA-expressing bacteria elicit RNA interference in mammals. *Nat. Biotechnol.* 24, 697–702
63 Wolfrum, C. *et al.* (2007) Mechanisms and optimization of *in vivo* delivery of lipophilic siRNAs. *Nat. Biotechnol.* 25, 1149–1157
64 Sznol, M. *et al.* (2000) Use of preferentially replicating bacteria for the treatment of cancer. *J. Clin. Invest.* 105, 1027–1030
65 Jia, L.J. *et al.* (2007) Oral delivery of tumor-targeting Salmonella for cancer therapy in murine tumor models. *Cancer Sci.* 98, 1107–1112