RNA interference in *Caenorhabditis elegans*: Uptake, mechanism, and regulation

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(*Received 17 August 2011; revised 8 September 2011; accepted 18 September 2011; first published online 11 November 2011*)

SUMMARY

RNA interference (RNAi) is a powerful research tool that has enabled molecular insights into gene activity, pathway analysis, partial loss-of-function phenotypes, and large-scale genomic discovery of gene function. While RNAi works extremely well in the non-parasitic nematode *C. elegans*, it is also especially useful in organisms that lack facile genetic analysis. Extensive genetic analysis of the mechanisms, delivery and regulation of RNAi in *C. elegans* has provided mechanistic and phenomenological insights into why RNAi is so effective in this species. These insights are useful for the testing and development of RNAi in other nematodes, including parasitic nematodes where more effective RNAi would be extremely useful. Here, we review the current advances in *C. elegans* for RNA delivery methods, regulation of cell autonomous and systemic RNAi phenomena, and implications of enhanced RNAi mutants. These discussions, with a focus on mechanism and cross-species application, provide new perspectives for optimizing RNAi in other species.

Key words: *Caenorhabditis elegans* RNA interference, reverse genetics methods, RNAi transport, RNAi regulation, systemic RNAi, autonomous RNAi.

INTRODUCTION

RNA interference (RNAi) is triggered by double-stranded RNA (dsRNA) (Fire *et al*. 1998). This dsRNA is processed into single-stranded small interfering RNAs (siRNAs) that act as guide sequences to target homologous mRNAs and nascent transcripts for post-transcriptional gene silencing (PTGS) (Chekulaeva and Filipowicz, 2009) and transcriptional gene silencing (TGS) (Moazed, 2009), respectively. A broad array of endogenous RNAi-related mechanisms is used to control gene expression (White and Allshire, 2008; Mochizuki, 2010; Teixeira and Colot, 2010). Likely because it accesses these endogenous gene activities, experimentally induced RNAi is potent and specific (Sharp, 1999), leading to its popular and wide use as a genetic tool (Sioud, 2011). However, many challenges remain. For many organisms, intracellular delivery of dsRNA presents a significant experimental obstacle; coupled to this is variable or low potency. In contrast, RNAi works very well in *C. elegans* because of ease of delivery coupled to efficient RNA-directed RNA polymerase (RdRP) amplification of effector siRNAs. Here we review what is known about RNA delivery and genetic control of RNAi efficacy in *C. elegans* with the goal of using this knowledge to enable RNAi in other organisms.

Reverse genetics via RNAi has become extremely popular over the past decade (Silva *et al*. 2004). This is particularly true in organisms like *C. elegans*, *Planaria* and Apidae that readily take up and apparently spread the triggering dsRNA and/or derived silencing signals. However, biological and methodological diversity in dsRNA delivery can lead to variability in RNAi efficacy (Echeverri *et al*. 2006). Therefore, to maximize RNAi silencing, it is important to understand the organism-specific limitations as well as advantages of dsRNA uptake (Geldhof *et al*. 2007; Knox *et al*. 2007). The ease of both classic genetics and RNAi has made *C. elegans* the exemplary model organism for this analysis.

The discovery and subsequent in-depth mechanistic characterization of RNAi in *C. elegans* helped established the entire RNAi field (Hannon, 2002). RNAi in *C. elegans* is both easy and remarkably potent. The ease of dsRNA delivery is unmatched, including most notably by ingestion – so called environmental RNAi. However, a variety of enhanced RNAi (Eri) mutants show that even in *C. elegans*, RNAi can become even more potent (Kennedy *et al*. 2004). Although not all members of the nematode genus *Caenorhabditis* are equally accessible for dsRNA delivery, most are capable of RNAi (Felix, 2008). Interestingly, the identified *eri* genes are conserved across *Caenorhabditis* and, in many instances, widely conserved across evolution. This indicates independent selection for delivery and regulation of potency. Similar observations have been made more broadly in the phylum Nematoda, as in the genera *Haemonchus*, *Heterohabditis*, *Ostertagia*, *Heterodera*, *Globodera*, *Meloidogyne*, *Panagrolaimus* and *Brugia* which have all been shown to respond to

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doi:10.1017/S0031182011001788

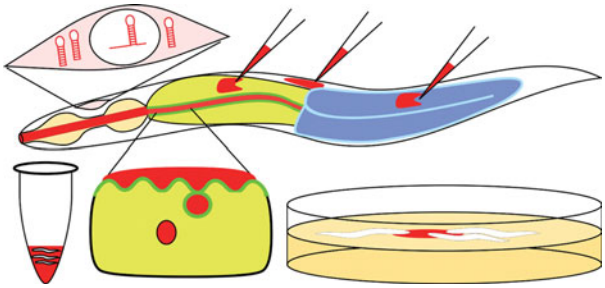

Fig. 1. Double-stranded RNA delivery in *C. elegans*. Microinjection of concentrated dsRNA (red) into the large gut cells (yellow), the syncytial germline (blue), or the body cavity (white) affords the greatest control over delivery and the most potent response; however, throughput is limited. Throughput is improved by soaking whole animals in dsRNA, or feeding worms bacteria engineered to express dsRNA. Both soaking and feeding results in ingested dsRNA that requires the intestinal transmembrane protein SID-2 (green) for delivery into the animal. Finally, transgenic expression of double-stranded RNA or hairpin constructs can target dsRNA delivery to specific cell types not accessible by microinjection, and in *sid-1* mutant backgrounds, can limit the RNAi knock-down effect to the targeted cells.

at least some forms of RNAi delivery, though with different apparent potencies (Felix, 2008). These observations indicate that comparative analysis of RNAi in nematodes is likely to reveal much about the selective pressures that modify small RNA pathways. In this review of *C. elegans* RNAi genetics and methods, we pay particular attention to conserved genetic networks with the goal of leveraging the wealth of mechanistic information available in *C. elegans* to the application of RNAi in less accessible nematodes.

METHODS

The robustness of RNAi in *C. elegans* is likely due to both RdRP activity that amplifies silencing signals and the systemic nature of *C. elegans* RNAi that enables silencing signals to move between cells, tissues and generations. Thus, small amounts of locally delivered dsRNA can cause robust silencing in any tissue in the treated animal as well as its progeny. Here, we compare the relative silencing potency of the three principal dsRNA delivery methods: microinjection, ingestion, and transgene expression (Fig. 1).

Microinjection is the most direct and potent way to introduce RNAi triggers. Microinjection also provides control over dsRNA concentration and the cell or tissue to score for knockdown. Control of concentration is critical to maximize the effective dose while simultaneously avoiding non-specific toxicity or off-target effects. The concentration of dsRNA to inject will vary from organism to organism (Kuwabara and Coulson, 2000; Nasevicius and Ekker, 2000; Svoboda and Stein, 2009), from cell type to cell type (Grishok and Mello, 2002; Wang *et al*. 2005), and even from gene target to gene target (Krueger *et al*. 2007). Although in *C. elegans* silencing signals can spread from the injected cell or tissue, this is not true of other, even closely related species (Winston *et al*. 2007). Therefore, initial analysis of RNAi effectiveness should be limited to scoring the injected cell or syncytial tissue.

In some organisms, long dsRNA is toxic. For example, in vertebrates, long dsRNA triggers a non-sequence specific interferon response that leads to cell death (Cullen, 2006). Whether long dsRNA is toxic to invertebrates is largely unexplored. These toxic effects are avoided in mammalian cells by using siRNA to trigger RNAi; siRNAs are too short to trigger the non-specific effect (Mittal, 2004). Microinjection of siRNAs is effective in *C. elegans*, but the response is attenuated compared to long dsRNA (Yang *et al*. 2000; Carpenter and Sabatini, 2004).

Transgene-expressed dsRNA can also initiate RNAi and allows introduction of dsRNA into cells and tissues that are not accessible to microinjection, including neurons and muscle cells (Schepers, 2005). Another advantage is that transgenic lines can be maintained indefinitely and expanded to large populations that are not accessible by microinjection. In *C. elegans*, RNAi can be effectively triggered by either expressed hairpin RNA constructs or co-expressed sense and antisense RNA. However, the production of dsRNA-expressing transgenic animals is more complicated and less controllable than injecting dsRNA. First, it is difficult to avoid non-specific expression of the transgene; the promoter may be active in unintended cells (Grove *et al*. 2009). Second, it is difficult to assess the quality or quantity of RNAi trigger; unlike loading a microinjection needle with known concentrations of precisely defined dsRNA, endogenously expressed dsRNA does not come with easily quantifiable measures. As a consequence, RNAi potency can vary between independent lines (Praitis *et al*. 2001) and from simple structural changes to the same hairpin construct (Boudreau *et al*. 2008). Third, transgenes in *C. elegans* are subject to spontaneous silencing via a mechanism that is at least in part dependent on RNAi-silencing genes. Since RNAi silencing is saturable, expressed dsRNA may interfere with such silencing in a dose-dependent and variable way (Kim *et al*. 2005*b*) which adds a confounding factor when evaluating the presence, absence or penetrance of RNAi silencing.

Ingestion of dsRNA is the third principal means of introducing RNAi triggers into *C. elegans*. Ingestion can be accomplished either by soaking worms in a concentrated solution of purified dsRNA (Maeda *et al*. 2001) or more simply by feeding worms bacteria engineered to expressed dsRNA (Timmons and Fire, 1998; Timmons *et al*. 2001). This mechanism of

inducing effective RNAi is entirely dependent on systemic RNAi. However, systemic RNAi is not sufficient as specialized dsRNA uptake machinery is also required (Winston *et al*. 2007). In *C. elegans*, the transmembrane proteins SID-1 and SID-2 are required independently for ingestion-mediated RNAi. SID-1 is required for the uptake of silencing signals into all cells, while SID-2 is required only for silencing initiated by ingested dsRNA. SID-2 is expressed exclusively in the intestine and localizes primarily to the apical membrane, suggesting that SID-2 may directly interact with ingested dsRNA for internalization (Winston *et al*. 2007).

SID-2 homologues are highly divergent, recognizable in only *Caenorhabditis* nematodes, and even among these, ingested dsRNA induces RNAi in only a few species (Winston *et al*. 2007). This molecular and functional divergence is consistent with the unpredictable distribution of organisms that are susceptible to ingested dsRNA-mediated RNAi (Whangbo and Hunter, 2008). Consequently, absence of ingestion-mediated RNAi should not be interpreted as absence of RNAi or even systemic RNAi.

In organisms that are susceptible to ingestion-mediated RNAi, the ability to easily subject animals to a large variety of dsRNA sequences has many advantages. In *C. elegans*, the construction and availability of libraries of engineered 'RNAi foods' targeting the entire genome has made "feeding RNAi" an extremely powerful genetic tool (Kamath and Ahringer, 2003; Kamath *et al*. 2003; Rual *et al*. 2004). Furthermore, feeding worms dsRNA-expressing bacteria, like transgene-expressed dsRNA, enables large numbers of RNAi knockdown worms to be produced for genetic screens or biochemical assays; however, this conditional feeding RNAi has an advantage over transgene-expressed dsRNA when targeting genes important for growth, fertility and viability. The apparent delivered dose of ingested dsRNA, however, is less than is achieved by microinjection, causing less penetrant phenotypes, which makes it often necessary to expose animals to ingested dsRNA for multiple generations (Timmons and Fire, 1998). Furthermore, different tissues respond differently to RNAi triggers, making it difficult to score the relative efficacy of RNAi (Calixto *et al*. 2010).

Other less frequently used means to introduce RNAi triggers into small metazoans include electroporation transfection, and soaking in liposome-encapsulated dsRNA (Issa *et al*. 2005; Geldhof *et al*. 2006; Krautz-Peterson *et al*. 2007). These methods are not used in *C. elegans*.

MECHANISMS OF dsRNA TRANSPORT BY SID-1

Intercellular transport of dsRNA-silencing signals in *C. elegans* requires the highly conserved dsRNA channel SID-1 (Jose and Hunter, 2007). SID-1 is a transmembrane protein with 11 predicted transmembrane domains, a 400+ amino acid extracellular N-terminal domain and a short cytosolic C-terminal domain (Feinberg and Hunter, 2003). Many recovered *sid-1* mutants have missense mutations in the transmembrane domains, suggesting that these sequences are essential for function. SID-1 is autonomously required for the import but not the export of RNAi triggers (Jose *et al*. 2009). A *sid-1* promoter *gfp* construct was found to be expressed from the late embryo throughout adulthood in all non-neuronal tissues (Winston *et al*. 2002). Interestingly, neuronal cells are resistant to RNAi triggered by ingested or injected dsRNA, but sensitive to neuronally expressed dsRNA, indicating the defect is in delivery of dsRNA to neurons, not RNAi effectiveness in neurons; consistent with this, transgenic expression of SID-1 in neurons enables efficient systemic RNAi (Calixto *et al*. 2010). Furthermore, such expression enhances RNAi efficacy in these cells at the expense of wild-type cells (Calixto *et al*. 2010). These results suggest that SID-1 expression is limiting for systemic RNAi in *C. elegans*.

Mechanistic studies performed in *Drosophila* S2 cells indicate that SID-1 functions as a dsRNA-gated channel. *Drosophila* lacks a SID-1 homologue and endogenous mechanisms of dsRNA uptake in S2 cells are relatively inefficient, making these cells an ideal 'blank slate' system to investigate SID-1 dsRNA transport properties. SID-1 activity in S2 cells has been primarily measured by uptake of radio-labeled dsRNA and by RNAi silencing of reporter genes. Recent studies have also used whole-cell patch-clamp analysis to characterize SID-1 channel properties. ^{32}P-labeled dsRNA added to the culture media of SID-1-expressing S2 cells is rapidly taken up, showing that SID-1 enables dsRNA transport (Feinberg and Hunter, 2003; Shih *et al*. 2009). To distinguish between active transport mechanisms that require continuous energy input (ATP) for dsRNA transport – i.e. pumps or receptors that require vesicle transport – versus passive transport mechanisms that could transport dsRNA without additional energy input – i.e. channels or pores – the uptake assays were repeated in either ATP-depleted cells or in cells maintained at 4 °C. For both treatments, the endogenous S2 cell RNA uptake was eliminated, while SID-1-dependent uptake was still very productive (Feinberg and Hunter, 2003). These results indicate that SID-1 acts as a passive transporter, likely a channel or pore. Consistent with SID-1 functioning as a channel, whole-cell patch-clamp analysis showed that adding dsRNA to the cell media increased the conductance (opened channels) of SID-1-expressing cells and that washing the dsRNA away led to a return to baseline conductance (Shih and Hunter, 2011). Together these results indicate that SID-1 is a dsRNA-gated channel.

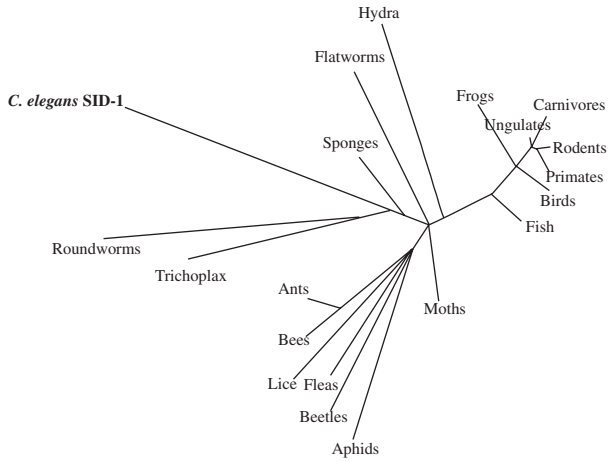

Fig. 2. *C. elegans* SID-1 is widely conserved. SID-1 homologues are present in many taxonomic groups, suggesting widespread conservation of a protein, which may support systemic RNAi in these other species. The taxonomic tree of *C. elegans* SID-1 was created using Grishin (protein) distance, with a max sequence difference of 0·85, a fast minimum evolution parameter, and with radial display representing inferred evolutionary distance.

These same transport and activity assays indicate that SID-1 nucleic acid transport is efficient, specific and selective for dsRNA. SID-1 expression in S2 cells enabled detectable RNAi silencing at a 10^7-fold lower dsRNA concentration than in control cells (Feinberg and Hunter, 2003; Shih *et al*. 2009); this translates into less than one molecule of dsRNA per cell, indicating very efficient uptake. Similar results were obtained with cultured *C. elegans* cells (Shih *et al*. 2009). Although initial studies using RNAi silencing of luciferase reporters indicated that *sid-1*-dependent uptake efficiency is sensitive to dsRNA length (Feinberg and Hunter, 2003), subsequent studies using radio-labeled 50 bp, 100 bp and 500 bp dsRNAs showed indistinguishable results (Shih *et al*. 2009). Similarly sized dsRNAs also indistinguishably open channels on whole-cell patched SID-1-expressing cells (Shih and Hunter, 2011). Since size does not affect activation or transport, it is thought that longer dsRNA, when delivered systemically, is a more efficient silencing trigger. The whole-cell patch-clamp analysis also indicates that nucleic acid transport by SID-1 is specific to dsRNA-containing molecules. First, neither dsDNA nor a DNA-RNA heteroduplex can activate SID-1 expressing cells. Nucleotide substitution experiments indicate a requirement for the ribose 2′-OH. Although dsRNA is required for transport, molecules that contain single-stranded regions can be transported. Transport of hairpin molecules containing greater than 300 nucleotide single-stranded loops as well as pre-microRNA precursors were also detected. These results dramatically expand the possible repertoire of molecules transported by SID-1.

SID-1 homologues are present in nematodes, diverse invertebrate phyla and all sequenced vertebrate genomes (Altschul *et al*. 1997; Grimson *et al*. 2008) (Fig. 2). These proteins are highly conserved, which indicates a strongly selected function. Although *C. elegans* SID-1 has a demonstrated long-dsRNA transport activity, an activity or function remains unknown for all other homologues. *C. elegans* contains five SID-1 homologues (Fig. 3) (Gille, 2006), some of which are more similar to vertebrate homologues than SID-1. However, alleles for none of these were recovered in the Sid screen. There are many reasons why mutations in these were not recovered in the Sid screen: these genes may not function in dsRNA transport, their dsRNA transport function may be redundant with another gene(s), or they may have additional essential functions such that mutations that disrupted dsRNA transport may be lethal. Because mutations in these genes have not yet been recovered, only RNAi is available to study their possible role in systemic RNAi or other functions. A serious limitation of this approach is illustrated by our analysis of *sid-1* by RNAi: repeated early attempts to produce an RNAi defect by *sid-1(RNAi)* failed. However, our certainty of the phenotype led us to continue pursuing RNAi of *sid-1* by injection of dsRNA, which ultimately caused a reduction in RNAi sensitivity in up to 50% of the progeny of an injected animal. Our difficulty producing a *sid-1* (RNAi) Sid phenotype likely reflects the tremendous efficiency of dsRNA transport by SID-1 (Shih *et al*. 2009), thus animals that retain even a modicum of SID-1 will be capable of a potent systemic RNAi response.

The vertebrate SID-1 homologues are unlikely to transport long dsRNA due to the interferon response (Bridge *et al*. 2003). This raises the possibility that because the *C. elegans* SID-1 paralogues are more similar to the vertebrate proteins than is SID-1, they may share a function and/or nucleic acid specificity different than that of SID-1. These considerations, along with the possibility of functional redundancy, challenge the mirror assumptions that the presence of a SID-1 homologue is evidence for systemic RNAi capacity and the absence of systemic defect when a SID-1 homologue is knocked-down or knocked-out demonstrates lack of dsRNA transport activity for that homologue.

ROLE OF SID-2 IN ENVIRONMENTAL RNAi

For ingested dsRNA to initiate RNAi, it must first be transported into the gut cell cytoplasm. Because SID-1 expressed in *Drosophila* S2 cells is sufficient to enable uptake, one possibility is that SID-1 functions at the luminal membrane to transport ingested dsRNA across this membrane. However, in the worm, SID-1 is not sufficient, because *sid-2* mutants are specifically defective for environmental RNAi.

Fig. 3. SID-1 has five homologues in *C. elegans*. Amino acid alignment of the six *C. elegans* genes homologous to human *SidT2*. *sid-1, tag-130, C08A9.3* and *Y37H2C.1* are similar in size and structure, while *C30E1.3* and *C30E1.4* are much more divergent. Yellow and orange indicate hydrophobic amino acids, green and purple indicate polar amino acids, red indicates acidic amino acids, cyan indicates basic amino acids, and brown indicates aromatic amino acids. Alignments are generated by Structure based Sequence Alignment Program (STRAP)'s built-in parameters (Gille, 2006).

Interestingly, SID-2 alone is also not sufficient, as *sid-1* mutants exposed to dsRNA fail to show silencing in gut cells. This indicates that these two proteins function together, either cooperatively or sequentially, to import ingested dsRNA (Winston *et al*. 2007).

SID-2 is a 311 amino acid single-pass transmembrane protein that is expressed in all gut cells and localizes strongly to the apical/luminal membrane. This indicates that SID-2 may be specialized to interact with ingested dsRNA. Curiously, the presumed dsRNA-interacting extracellular domain is much less conserved that the intracellular domain. The *C. briggsae* species is unable to initiate RNAi from ingested dsRNA. However, *C. briggsae* expresses and localizes Cb-SID-2 indistinguishably from Ce-SID-2. Transgenic expression of Ce-SID-2 in *C. briggsae* enables environmental RNAi, suggesting either expression and/or functional differences between these two genes homologues. In contrast, expressing Cb-SID-2 in a *sid-2* mutant *C. elegans* strain failed to rescue environmental RNAi. The functional difference between the two SID-2 proteins has been mapped by domain swap experiments to the extracellular domain (McEwan and Hunter, unpublished data). The *C. elegans* extracellular domain attached to the *C. briggsae* transmembrane and cytoplasmic domains functionally rescue *sid-2* mutants. The distribution of environmental RNAi-capable species within the known *Caenorhabditis* phylogeny is not consistent with either a simple loss or gain of ability. Furthermore, the linkage of environmental RNAi ability with SID-2 function has only been established for *C. elegans*, *C. briggsae*, and *C. remanei* (M. Felix, personal communication). These observations, combined with the non-predictable nature of which species are capable of taking up ingested dsRNA, suggest that gain and loss of this ability is rapid and likely encompasses many different proteins that can perform SID-2's function.

AUTONOMOUS VERSUS SYSTEMIC RNA INTERFERENCE

Systemic RNAi is the organism-wide spread of silencing either via distribution of the initial RNAi trigger or its effectors (Jose and Hunter, 2007). In contrast, cell autonomous RNAi silencing is restricted to the cells and their descendants that directly encounter dsRNA by injection, infection, transfection or expression. In *C. elegans*, cell autonomous RNAi is the activity that remains in a *sid-1* mutant. In *sid-1* mutants, transgene-expressed dsRNA and dsRNA injected directly into the syncytial germline or into single gut cells causes efficient silencing in the germline and injected cell respectively, but no detectable silencing in other cells.

The RNAi silencing machinery is highly conserved, yet not all organisms have been shown to be RNAi-capable. One explanation may be that the machinery is used for TGS or RNA directed DNA elimination (Pal-Bhadra *et al*. 2002; Mochizuki, 2010). However, the lack of systemic RNAi may impede the detection of experimentally induced silencing phenotypes in many situations (Roignant *et al*. 2003). For *Caenorhabditis* spp. that have

systemic RNAi, either injection of dsRNA or transgenc expression of Ce-SID-2 enables whole-animal experimental RNAi and even transgenerational silencing. At least one species of *Caenorhabditis, C. brenneri* (*Caenorhabditis* sp. CB5161), apparently lacks systemic RNAi. This was discovered when dsRNA targeting the large subunit of RNA polymerase caused the expected early embryonic lethal phenotype when injected directly into the syncytial germline, but in contrast to all other tested species, failed to cause any detectable phenotype when injected into intestinal cells in *C. brenneri* (Winston *et al*. 2007); thus *C. brenneri* appears to be naturally systemic-RNAi-defective. Interestingly, the *C. brenneri* genomic sequence indicates that SID-1 is intact, indicating that addtional components required for systemic RNAi may be disabled or missing in this species. The apparent selection for an intact SID-1 in the absence of systemic RNAi indicates that SID-1 may have an additional function(s). While an ecologically important function for systemic RNAi in animals has not yet been reported, systemic RNAi appears to provide protection against viral spread in plants (Mourrain *et al*. 2000), but this will remain speculative until a mutant that specifically disrupts systemic RNAi is recovered.

The presence or absence of systemic RNAi in the target organism can have profound effects on both the determination of whether RNAi works in that organism and how dsRNA can be delivered effectively (Tomoyasu *et al*. 2008). The wide-spread use of RNAi in *Drosophila* is illustrative of the challenges and solutions. Microinjection of dsRNA into early syncytial *Drosophila* embryos provides access to all nuclei and their trancripts, but the lack of a robust RdRP-based amplification coupled with cellularization restricts effective RNAi to genes that function in the early embryo. This can be overcome by transgene-expressed dsRNA, which bypasses the complete lack of systemic RNAi in this organism (Perrimon *et al*. 2010). The lack of systemic RNAi is likely due to lack of a *sid-1* homologue as well as other components required for systemic RNAi (SID-1 expression in *Drosophila* has not yet been reported to enable systemic RNAi, despite many groups attempting this approach (personal communications). Complementing the *in vivo* approach, RNAi screens have been applied to a variety of *Drosophila*-derived cultured cell lines, like S2 cells, where dsRNA added to the culture medium is taken up via endogenous scavenging receptors that rely on the endocytosis machinery (Saleh *et al*. 2006; Ulvila *et al*. 2006) or via transgenic expression of *C. elegans* SID-1 (Bartscherer *et al*. 2006).

Organisms in which RNAi works very well have both systemic RNAi and RdRP-enabled amplification of RNAi triggers, leading to speculation that they may be mutually dependent. In some organisms, like *Arabidopsis*, it is these amplified products that become systemically mobile (Fagard and Vaucheret, 2000).

Viral defence has been proposed as an evolutionary explanation for systemic RNAi. In *Drosophila* and *C. elegans*, some RNAi-related genes have antiviral roles, reducing viral titres in infected cells and animals (Lu *et al*. 2005; Schott *et al*. 2005; Wilkins *et al*. 2005; Saleh *et al*. 2009; Ding, 2010). However, cultured *sid-1* mutant *C. elegans* cells were not more susceptible to viral infections than wild-type cells (Schott *et al*. 2005), suggesting that systemic RNAi may not play a vital role in viral defence. The recent identification of viruses that can naturally infect whole worms will provide an opportunity to test this hypothesis properly (Felix *et al*. 2011). However, the systemic antiviral interferon response in mammals, which is triggered in response to long dsRNA (Sledz *et al*. 2003), provides a contrapositive argument to this hypothesis. In plants, viral infection induces a strong anti-viral RNAi response, which includes RdRP amplification of RNAi triggers, which then spread systemically to provide viral immunity to as yet uninfected cells and tissues (Vance and Vaucheret, 2001).

Whatever the evolutionary roles of systemic RNAi may be, it is widely regarded as a powerful addition to cell-autonomous RNAi. As stated previously, the absence of systemic RNAi is not evidence for the absence of RNAi in the organism. RNAi may simply be more difficult to trigger and therefore detect in the absence of efficient delivery to all cells. In the next section, in which we describe the mechanism of cell autonomous RNAi, we make particular note of how understanding such mechanisms can help researchers enhance cell autonomous RNAi, and therefore increase the potency of experimentally-induced RNAi.

MECHANISM OF AUTONOMOUS RNA INTERFERENCE

Mechanism of exogenous RNAi processing

In *C. elegans*, when exogenously introduced long dsRNA (>100 basepairs) is introduced into a cell, it is bound by a protein complex that contains RDE-4 and DCR-1. RDE-4 contains two copies of a conserved dsRNA-binding motif and binds as a dimer to dsRNA (Knight and Bass, 2001; Tabara *et al*. 2002). DCR-1 is a well-conserved RNase III endoribonuclease that cleaves dsRNA into short (~22 nucleotide) interfering RNAs (siRNAs) (Zamore *et al*. 2000; Knight and Bass, 2001; Pak and Fire, 2007; Habig *et al*. 2008). Biochemically, these double-stranded siRNAs have on each strand a 5′ monophosphate, a free 3′ hydroxyl group and 2 nucleotides of overhang at the 3′ end (Macrae *et al*. 2006).

The RDE-4/DCR-1 complex also includes two Dicer-related helicases of unknown function (DRH-1 and 2) (Duchaine *et al*. 2006) as well as various members of the large Argonaute (AGO) family, defined by signature PAZ and PIWI domains (Song *et al*. 2004). The AGO proteins are thought to be the catalytic machinery of RNAi-based silencing (Czech and Hannon, 2011). The PAZ domain is hypothesized to interface with DCR-1 (Paddison and Vogt, 2008).

In *C. elegans*, the Ago protein RDE-1 binds to double-strand siRNA produced by the DCR-1 complex and cleaves the passenger strand to produce a single-stranded guide siRNA (Parrish and Fire, 2001; Tomari *et al*. 2004; Steiner *et al*. 2009). In most species, the primary Ago protein – like RDE-1 – uses the guide strand to identify cognate mRNAs and, once bound, the slicer activity cleaves the mRNA between the 10th and 11th positions of the siRNA-mRNA complementary region via the activity of the Ago's RNase H catalytic domain (Hall, 2005); this particular event seems to be absent in *C. elegans* (Steiner *et al*. 2009). In *C. elegans*, single-strand siRNA produced by the sequential action of DCR-1 and RDE-1 on the long triggering dsRNA is referred to as a primary siRNA. Through still mysterious processes, an RdRP produces from the siRNA-mRNA complex many copies of so-called secondary siRNAs, that are principally anti-sense to, and distributed towards, the 5′ end of the cognate mRNA (Alder *et al*. 2003; Pak and Fire, 2007). In *C. elegans* somatic cells, the primary RdRP is RRF-1, while in the germline the primary RdRP appears to be EGO-1. These RdRPs are at least partially functionally redundant (Smardon *et al*. 2000). The 5′ end of these RdRP dependent secondary siRNAs contain triphosphate residues, indicating that they represent primary synthesis products; that is, they are not produced by DCR-1 cleavage reactions. The secondary siRNAs are both more abundant than primary siRNAs and target an expanded sequence region on the cognate mRNA. These abundant secondary siRNAs interact with so-called secondary Argonautes (SAGOs) (Yigit *et al*. 2006). These secondary siRNA-SAGO complexes appear to be directly involved in sequence-dependent mRNA degradation. Since many SAGOs lack an active RNase H domain, precisely how they degrade mRNA remains unclear. It has been suggested that in *C. elegans*, mRNA targeted for PTGS are preferentially transported to P bodies or GW bodies (Ding *et al*. 2005; Jakymiw *et al*. 2005; Liu *et al*. 2005). It has been recently shown that these SAGOs, which seem to be responsible for the bulk of the silencing, are poorly conserved compared to the other RNAi components, possibly providing another reason why *C. elegans* RNAi is so efficient compared to that of other species (Dalzell *et al*. 2011).

While RDE-1 and most SAGOs function in the cytoplasm, recent work has shown that one of the SAGOs, NRDE-3, shuttles secondary siRNAs into the nucleus. NRDE-3 has the signature PIWI and PAZ domains of an Ago protein, but also contains a nuclear localization signal required for its function (Guang *et al*. 2008). Once inside the nucleus, NRDE-3 interacts with a complex of nuclear RNAi-silencing factors, including the well conserved novel protein NRDE-2 (Guang *et al*. 2010). The nuclear RNAi complex is guided by the siRNA to nascent transcripts and effects transcriptional silencing by impeding RNA polymerase elongation and recruiting histone methyltransferase activity (Guang *et al*. 2010). This mechanism is likely the basis for heterochromatin modifications and other transcriptional gene-silencing phenomena phenotypically linked to RNAi (Motamedi *et al*. 2004; Grishok *et al*. 2005; Claycomb *et al*. 2009). The synergistic PTGS and TGS mechanisms are summarized in Fig. 4.

Regulators of exogenous RNAi

Mutations that enhance RNAi silencing have been identified by various means. Mutations in genes required for production of endogenous siRNA-silencing pathways were identified in screens for enhanced neuronal RNAi (Eri mutants) and discovered serendipitously when analyzing the phenotype of worms deleted for the RdRP *rrf-3* (Simmer *et al*. 2002; Kennedy *et al*. 2004). Another large class of mutants is in the worm Rb Tumor suppressor pathway, which appears to enhance RNAi by partial soma to germline transformation (Wang *et al*. 2005). It is not clear if this transformation replaces somatic RNAi with germline RNAi, which is particularly robust, or adds additional capacity to the somatic RNAi pathway. Eri mutants were initially sought for their ability to increase the discovery of RNAi phenotypes in large-scale feeding RNAi screens. For example, feeding wild-type worms 447 different RNAi foods resulted in only 307 expected loss-of-function phenotypes, while performing the same screen in the *rrf-3* mutant background resulted in 436 loss-of-function phenotypes (Simmer *et al*. 2003). Because these mutants are enhanced for RNAi, it indicates that the wild-type *eri* genes function directly or indirectly to inhibit RNAi. Mechanistic investigations to date indicate that the enhanced RNAi phenotypes reflect indirect effects rather than the action of direct negative regulators.

The Eri class of enhancers are related by their facultative association with DCR-1 (Duchaine *et al*. 2006; Gent *et al*. 2009; Pavelec *et al*. 2009). To date, nine Eri loci have been described (Table 1), including five in widely conserved genes (Simmer *et al*. 2002; Kennedy *et al*. 2004; Duchaine *et al*. 2006; Fischer *et al*. 2008; Pavelec *et al*. 2009). These genes are required for the production or stability of endogenous siRNAs (Asikainen *et al*. 2007). The current

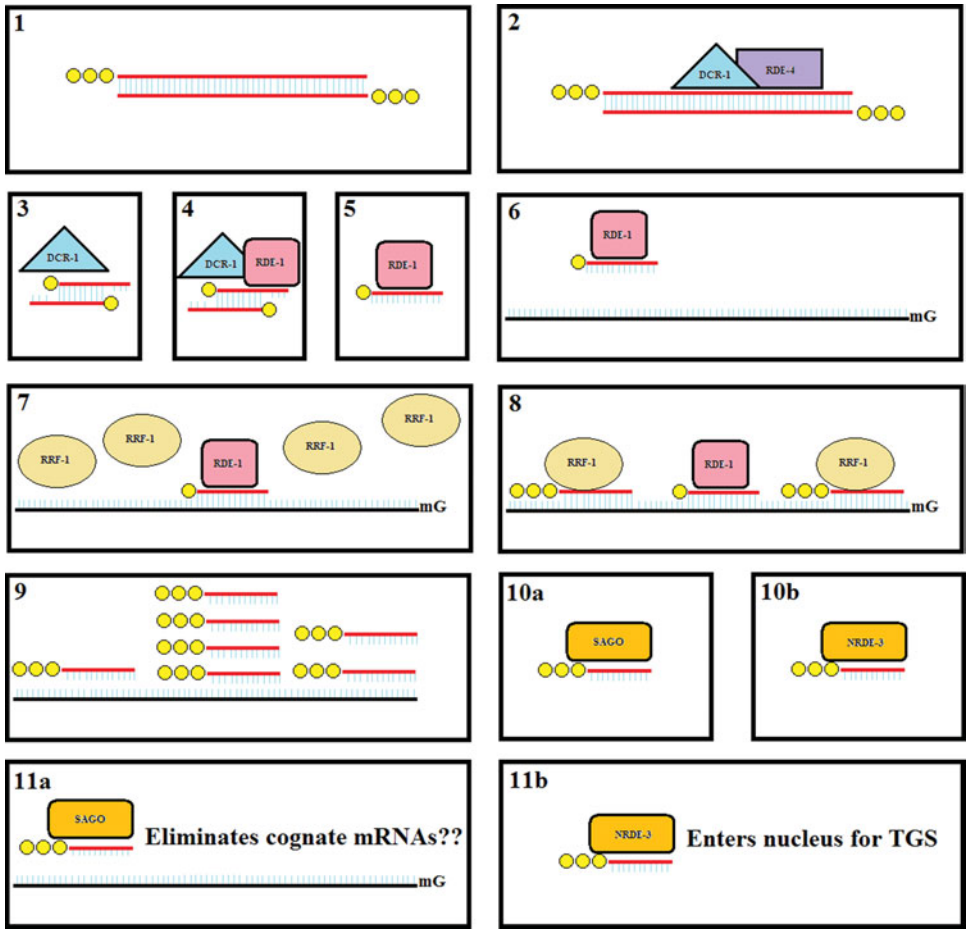

Fig. 4. Summary of the exogenous RNAi pathway in *C. elegans*. (1) Post delivery *in vitro* synthesized long (>100 bp) dsRNA (red) with 5′ triphosphate (yellow) ends is (2) bound by the RDE-4 (purple) and DCR-1 (cyan) complex. (3) The endonuclease DCR-1 dices the long dsRNA into of ~20 bp ds-siRNAs with two nucleotide single stranded 3′ ends. The dicer products have 5′ monophosphate and 3′ hydroxyl ends. (4) Interaction with the Argonaute RDE-1 (pink) leads to slicing of the passenger strange producing (5) a single-stranded ~22 nucleotide guide siRNA bound to RDE-1. (6) This primary ss-siRNA guides RDE-1 to its cognate mRNA (black). (7) In a mechanistically unclear step, the RdRP RRF-1 (coffee) is recruited to the RDE-1-siRNA-mRNA complex (8) leading to the production of many unprimed secondary siRNAs with 5′triphosphate ends. (9) Most of these secondary siRNAs match the originally targeted region, but secondary siRNAs anti-sense to regions both 5′ and 3′ to the originally introduced long dsRNA are also produced. (10a) In a second mechanistically unclear step, these secondary siRNAs become associated with cytoplasmic secondary Argonautes (SAGOs – orange) or (10b) the nuclear localized Argonaute NRDE-3. (11a) The secondary siRNAs then guide the cytoplasmic SAGOs to cognate mRNAs and via yet another mechanistically unclear step lead to the elimination of the mRNAs. (11b) NRDE-3 shuttles the secondary siRNAs into the nucleus where they guide transcriptional gene silencing processes.

model for this *eri*-class is that the relatively abundant endogenous siRNAs compete with siRNAs produced from experimentally introduced dsRNA for limiting effector molecules, for example the SAGO proteins (Lee *et al*. 2006; Yigit *et al*. 2006). Thus mutations in the Eri genes reduce the number of endogenous siRNAs and indirectly increase access to limiting components of silencing pathway(s). These limiting RNAi resources have been proposed to be secondary AGOs (Yigit *et al*. 2006), DICER (Mikuma *et al*. 2004), and even the dsRNA channel SID-1 (Winston *et al*. 2002; Calixto *et al*. 2010); in each case, over-expression increases RNAi efficacy.

Tissue-specific differences in RNAi sensitivity among the Eri mutants provides additional support for the competition model, and further suggest that the extent of competition differs among tissues (Zhuang and Hunter, 2011). The tissue-specific differences can be explained by tissue-specific components of a competing small RNA pathway, by relative tissue-specific activities of multiple competing pathways, and even by multiple limiting resources, which may show tissue-specific biases. Interestingly, all nine Eri mutants showed robust maternal rescue and enhanced RNAi in the germline. These observations indicate that not only are these Eri genes expressed and active in the germline, but that maternally synthesized product or the product(s) of their activity is apparently well distributed to somatic tissues in the progeny. This also suggests that

Table 1. Negative regulators of RNA interference in *C. elegans*

Gene name	Conservation	Homologous domains	Notes
eri-1	Wide	siRNase; RNA binding domains	Temperature sensitive sterile at 25 °C
rrf-3/eri-2	Wide	RdRP	Temperature sensitive sterile at 25 °C
eri-3	*Caenorhabditis*	Hydrolase	Temperature sensitive sterile at 25 °C
dcr-1/eri-4	Wide	Helicase domain of DCR-1	Temperature sensitive sterile at 25 °C; weak Eri phenotype
eri-5	Nematodes	Tudor domain	Germline-specific Eri phenotype
eri-6/7	Wide	Helicase	Retrotransposon homologue
ergo-1/eri-8	Wide	Argonaute	
eri-9	*Caenorhabditis*	RNA transferase	
eri-11	*Caenorhabditis*	Oligosaccharyl transferase	

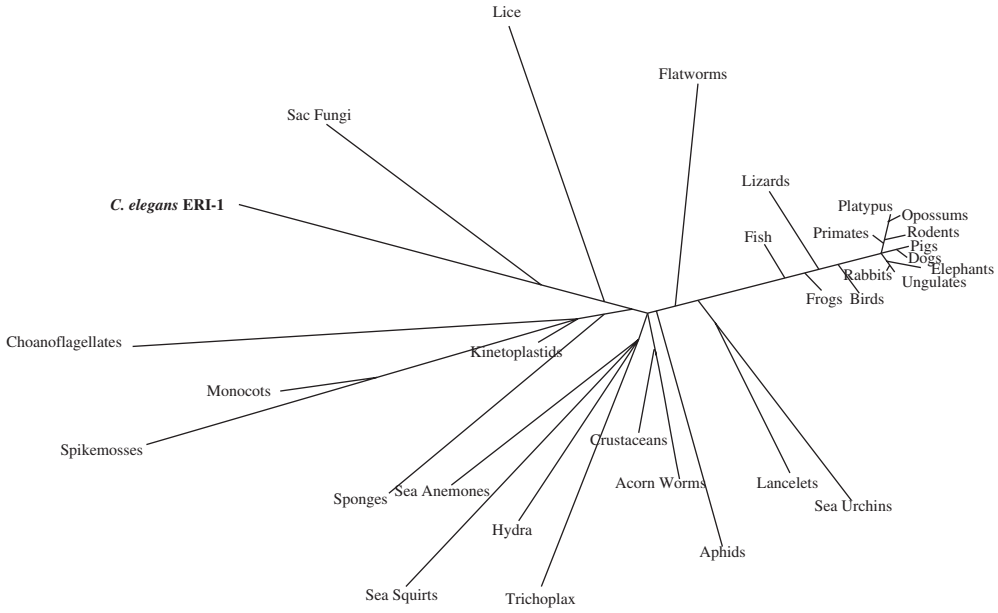

Fig. 5. *C. elegans* ERI-1 is widely conserved. ERI-1, which is important for the production or stability of endogenous siRNA in *C. elegans*, has homologues in many taxonomic groups. Since it is likely that endogenous RNAi processes will compete with exogenous RNAi processes in these species, researchers should not only consider the possibility of enhancing RNAi, but the possibility that exogenous RNAi will interfere with essential endogenous processes (e.g. *eri-1* and *rrf-3* are required for sperm function). The taxonomic tree of *C. elegans* ERI-1 was created using Grishin (protein) distance, with a max sequence difference of 0·85, a fast minimum evolution parameter, and with a radial display representing inferred evolutionary distance.

the maternal contribution to the embryo directly or indirectly includes small RNAs (Zhuang and Hunter, 2011).

These data indicate that exogenous RNAi capacity is regulated by or is responsive to endogenous small RNA-silencing activity levels. Thus the sensitivity of the animal to exogenous dsRNA, whether experimentally introduced, the outcome of a viral infection, or other environmental or genomic stresses, may be tuned by intrinsic or extrinsic events (e.g. pathogens, DNA damage); for instance, systemic RNAi appears to be enhanced by starvation (Winston *et al*. 2002). This could reflect increased dsRNA transport or enhanced RNAi responsiveness mediated by changes in the level of endogenous siRNA levels. Analysis of the Eri class of genes indicates that the endogenous siRNA pathways are important for maturation of sperm (Gent *et al*. 2009; Pavelec *et al*. 2009), and proper chromosomal segregation cannot take place without the secondary Ago *csr-1* (Claycomb *et al*. 2009). In contrast, *rde-4* and *rde-1* mutants, which appear to be specific to exogenous RNAi, do not seem to have any non-RNAi phenotypes (Tabara *et al*. 1999).

The nature of conservation among the *eri* genes should also be of interest in studying RNAi in other organisms (Altschul *et al*. 1997) (Fig. 5, Table 1). ERI-1 is a well conserved nuclease with siRNase activity (Kennedy *et al*. 2004); RRF-3 is a well conserved RdRP (Sijen *et al*. 2001; Crombach and

Hogeweg, 2011); the *dcr-1/eri-4(mg375)* mutant is a point mutation in the helicase domain of the well conserved DICER protein (Macrae *et al*. 2006; Pavelec *et al*. 2009); ERI-6/7 is a conserved helicase domain (Fischer *et al*. 2008); and ERGO-1/ERI-8 (Pavelec *et al*. 2009) is a well conserved Ago protein. Mutations to these conserved genes in other organisms have been shown to have some similar endogenous defects, such as general RNA processing defects (Ansel *et al*. 2008), but assays in RNAi efficacy have not been thoroughly performed. This area of research holds vast potential for dramatically increasing RNAi applicability and technology. Even more interesting are the potential roles played by the non-conserved *eri* genes specific to *C. elegans* or *Caenorhabditis*; their predicted molecular identities (Kelley and Sternberg, 2009) suggest that hydrolyases and transferases play a large role in small RNA production in *C. elegans* (Table 1). Perhaps such class-specific genes in other organisms hold the key to decreasing the competitive regulation of RNAi. Moreover, mutations to novel or non-conserved genes are less likely to have wide-ranging impacts, while maintaining similar degrees of RNAi hypersensitivity. Therefore, studying organism-specific *eri* genes through genetic screens, if possible, holds tremendous promise for understanding (and pragmatically overcoming) RNAi regulation.

Finally, given that the products of *eri* pathway are effective competitors of the *rde* pathway, it is worthwhile to examine the chemical structures of small RNAs produced by the *eri* pathway. The *eri* gene products produce endogenous siRNAs of 22 or 26 nucleotides that usually begin with a G (22 G or 26 G siRNAs) and contain a 5′ triphosphates (Conine *et al*. 2010; Gent *et al*. 2010; Vasale *et al*. 2010; Welker *et al*. 2010). Perhaps unknown chemical properties of these siRNAs are important for their relative enhanced activity. Attempting to introduce experimental siRNAs which share such properties may thus enhance RNAi efficacy as well (Kim *et al*. 2005*a*).

In *C. elegans*, the core of the RNAi machinery that interacts with experimentally introduced RNAi signals (whether long dsRNAs or siRNAs) is a relatively well-understood framework. Recent advances in deep sequencing revealed more and more of the intricacy and potency of the endogenous small RNA network, as well as its competitive regulation of the exogenous RNAi pathway. Researchers frustrated by the limited utility of RNAi in other species should examine the RNAi regulation perspective to perhaps overcome this seeming impasse. Once the RNAi silencing signal is inside the cell, most organisms from protists to fungi, and from plants to animals, all have some part of the conserved RNAi processing machinery, whether cytoplasmic PTGS or nuclear TGS (Shabalina and Koonin, 2008). It is the relative effectiveness of RNAi that vastly differs (Maida and Masutomi, 2011) and is possibly thwarting broader use of RNAi as a technological resource.

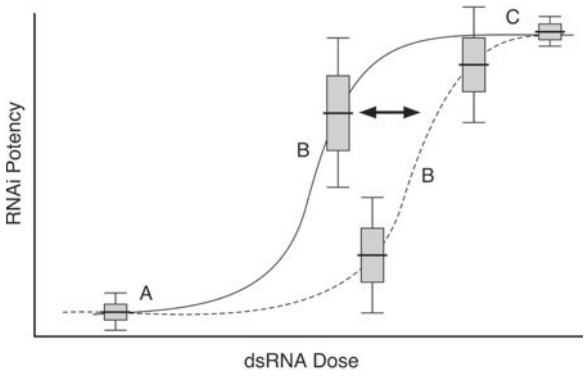

Fig. 6. RNAi phenotypic penetrance is sensitive to dsRNA dose. Measurements of RNAi potency (penetrance) versus dsRNA dose show a sigmoidal relationship with high variability surround the inflection point (B). At low dsRNA dose (A) most worms do not respond, while at sufficiently high dsRNA dose (C) most or all worms do respond. However, slight variations in delivered dsRNA dose at intermediate concentrations can have dramatic effects on perceived phenotypes. Mutations that enhance RNAi tend to shift such dose-response curves toward lower dsRNA dose (solid line) without noticeably affecting the shape of the curve.

IMPLEMENTATION

The hallmarks of RNAi are specificity and potency. In *C. elegans*, dsRNA is not toxic and studies indicate that increasing dsRNA concentration can increase RNAi potency (Rea *et al*. 2007; Zhuang and Hunter, 2011). Similarly, mutations in the Eri genes that reduce competition for limiting small RNA resources (Lee *et al*. 2006) and overexpression of these limiting resources can also increase RNAi potency (Mikuma *et al*. 2004; Yigit *et al*. 2006; Calixto *et al*. 2010). However, there is some possibility that these measures reduce specificity (Pavelec *et al*. 2009). Furthermore, tissues differ in their relative sensitivity: for example, neurons are fairly refractory of RNAi (Kamath and Ahringer, 2003; Kamath *et al*. 2003) whereas the germline is hypersensitive to RNAi (Sijen and Plasterk, 2003). Consequently, mutations that transform somatic cells towards germline can increase RNAi potency (Wang *et al*. 2005). Similarly, developmental stages or environmental conditions can also influence RNAi sensitivity: starved worms are slightly more sensitive to RNAi (Jose and Hunter, 2007). Therefore, in assaying for RNAi efficacy, the gene target expression profile, both temporal and spatial, as well as environmental conditions need to be considered for optimal phenotypic output (Visser *et al*. 2006). These *C. elegans* tissue-specific and developmental sensitivities may be paralleled in other species and should be optimized when implementing RNAi. In summary, to determine whether RNAi is effective in a

species one should first not assume that RNAi is systemic. Consequently gene-specific dsRNA must to be delivered directly to the target cells either by microinjection into syncytial tissues, for example into the germline, or transgenically expressed using either general or tissue specific promoters. Second, the expression or function of the targeted gene must be unambiguously assayed either by directly examining RNA levels (e.g. RNA *in situ* hybridization) or gene function. For example, for *Caenorhabditis* species, we injected a high concentration of species-specific RNA polymerase II subunit dsRNA directly into the germ line to phenocopy application of the RNA polymerase inhibiting toxin alpha-amanitin to embryos (Winston *et al*. 2007).

Although RNAi potency increases with dsRNA dose, it is a common misperception that this relationship is linear. When RNAi potency (phenotypic penetrance) is plotted versus dsRNA dose, one clearly observes a sigmoidal curve (Fig. 6). Curiously, for most phenotypes, the expressivity (the severity of the phenotype) is nearly constant, thus the great variability at the empirically determined intermediate dose range reflects a mixture of strongly affected and non-affected individuals. This dose sensitivity likely underlies much of variation in reported RNAi effects, which in some cases are even contradictory (Rea *et al*. 2007). It is obviously best to use the maximum possible dose, but for potent foods, a simple dose response curve can determine an effective range with minimal variability.

Finally, the majority of the studies on *C. elegans* RNAi, including those referenced in this text, specifically refer to the N2 Bristol strain – the commonly used 'wild type' strain for *C. elegans* research. However, there are variations in RNAi efficacy among wild isolates of *C. elegans* (Felix *et al*. 2011). While the genetic basis for some of these variations is known, such as a polymorphism in a specific RNAi gene (Tijsterman *et al*. 2002), other sources for such differences remain to be identified. Future research into these population-specific RNAi efficacy differences for *C. elegans* and other species will be extremely relevant because it provides a clue as to the evolutionary scale at which changes in RNAi pathways may occur.

It seems reasonable to apply the lessons of the deep mechanistic and phenomenological observation made in *C. elegans*, as a first step towards enabling the highest probability of optimizing RNAi in other species. There will inevitably be species in which RNAi does not work, but the conservation of basal RNAi machinery suggests that more often than not, RNAi will function in most species.

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