RNA interference may result in unexpected phenotypes in Caenorhabditis elegans

Evandro A. De-Souza, Henrique Camara, Willian G. Salgueiro, Raíssa P. Moro, Thiago L. Knittel, Guilherme Tonon, Silas Pinto, Ana Paula F. Pinca, Adam Antebi, Amy E. Pasquinelli, Katlin B. Massirer and Marcelo A. Mori

1Program in Molecular Biology, Federal University of São Paulo, São Paulo 04044-020, Brazil, 2Program in Molecular Biology and Biotechnology, Instituto de Bioquímica Médica Leopoldo de Meis, Federal University of Rio de Janeiro, Rio de Janeiro 21941-902, Brazil, 3Department of Biochemistry and Tissue Biology, University of Campinas, Campinas, São Paulo 13083-862, Brazil, 4Program in Genetics and Molecular Biology, University of Campinas, Campinas, São Paulo 13083-970, Brazil, 5Max Planck Institute for Biology of Ageing, Cologne 50931, Germany, 6Cologne Excellence Cluster on Cellular Stress Responses in Ageing-Associated Diseases (CECAD), University of Cologne, Cologne 50931, Germany, 7Division of Biology, University of California, San Diego, La Jolla, California 92093-0349, USA, 8Center for Molecular Biology and Genetic Engineering, University of Campinas, CBMEG-UNICAMP, Campinas, São Paulo 13083-875, Brazil and 9The Structural Genomics Consortium - UNICAMP, University of Campinas, Campinas, São Paulo 13083-875, Brazil

Received October 19, 2018; Revised February 20, 2019; Editorial Decision February 21, 2019; Accepted March 01, 2019

ABSTRACT

RNA interference (RNAi) is a valuable technique to determine gene function. In Caenorhabditis elegans, RNAi can be achieved by feeding worms bacteria carrying a plasmid expressing double-stranded RNA (dsRNA) targeting a gene of interest. The most commonly used plasmid vector for this purpose is L4440. However, it has been noticed that sequences within L4440 may elicit unspecific effects. Here, we provide a comprehensive characterization of these effects and their mechanisms and describe new unexpected phenotypes uncovered by the administration of unspecific exogenous dsRNA. An example involves dsRNA produced by the multiple cloning site (MCS) of L4440, which shares complementary sequences with some widely used reporter vectors and induces partial transgene silencing via the canonical and antiviral RNAi pathway. Going beyond transgene silencing, we found that the reduced embryonic viability of mir-35-41(gk262) mutants is partially reversed by exogenous dsRNA via a mechanism that involves canonical RNAi. These results indicate cross-regulation between different small RNA pathways in C. elegans to regulate embryonic viability. Recognition of the possible unspecific effects elicited by RNAi vectors is important for rigorous interpretation of results from RNAi-based experiments.

INTRODUCTION

RNA interference (RNAi) is an evolutionarily conserved pathway used by multiple species as a mechanism of genome surveillance, antiviral defense and/or gene silencing (1). Given its widespread application, RNAi has been used as a powerful discovery tool, as well as a biotechnological and pharmaceutical asset (2). The identification that exogenous double-stranded RNA (dsRNA) can target and silence RNAs in animals was first made in Caenorhabditis elegans, when it was shown that injection of dsRNA molecules into worms results in silencing of genes that express similar sequences (3). In C. elegans, long dsRNA is recognized by the dsRNA-binding protein RDE-4 and then cleaved by the type III ribonuclease DCR-1 to generate shorter dsRNA fragments of ~22 base pairs (i.e. siRNA) (4–6). Short single-stranded RNA fragments are afterward loaded onto the Argonaute RDE-1 to promote gene silencing (7). Upon matched complementarity, the target RNA is degraded (8).

The first RNAi experiments used microinjection to deliver dsRNA to C. elegans, but later it was shown that RNAi can also be induced by feeding worms with bacteria harboring a plasmid in which the target gene sequence is partially cloned into a multiple cloning site (MCS) flanked by two antiparallel T7 promoters (9). The antiparallel T7 promoters produce dsRNA in the bacteria, which in turn targets the gene of interest in the worm when ingested (9). Using this strategy, RNAi feeding libraries were generated by two different groups using the L4440 plasmid (a modified version of the pBlueScript plasmid) (10,11) and the Escherichia coli strain HT115 (DE3) —a strain carrying a defective RNase

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III and an isopropylthiogalactoside (IPTG)-inducible T7 polymerase gene (12). Given their whole genome coverage and easy access, these libraries are frequently used as the delivery method of choice for RNAi-mediated gene silencing in *C. elegans*.

Another commonly used technique in *C. elegans* is the generation of transgenic lines expressing tagged fluorescent proteins (13). These transgenes are often used as readouts for protein subcellular localization and gene expression, but in some genetic backgrounds, such as in *lin-35* mutants, they can be silenced in somatic cells simply by the activation of the RNAi pathway (14). Curiously, microRNAs as the mir-35-41 cluster regulate RNAi responsiveness through *lin-35* (15), and differently from most of the microRNAs in *C. elegans*, loss of mir-35-41 leads to severe phenotypes, such as a temperature-sensitive reduction in progeny viability and fecundity (16, 17). These results suggest a possible physiological crosstalk between the RNAi pathway, transgene silencing, microRNA expression, embryonic development and germline function.

Years of experiments using RNAi in *C. elegans* have revealed unexpected results in our hands and in the hands of others. It has been previously observed that the backbone of the L4440 vector silences transcription of somatic, LacZ-containing transgenes in a mechanism named RNAi-induced Transcriptional Gene Silencing (RNAi-TGS) (18). This mechanism involves chromatin modifier proteins (e.g. HPL-2) and the canonical RNAi machinery (18). Non-specific dsRNA targeting the bacterial tetA gene was also found to promote GFP silencing in transgenic worms (19).

In this study, we provide a comprehensive analysis of conditions where worms have their phenotypes modified when grown in the presence of exogenous dsRNA, in particular the type produced by the L4440 vector. We describe new mechanisms of multicopy transgene silencing by the L4440 vector and observe that exogenous dsRNA interferes with embryonic development of a *mir-35-41* mutant strain. We therefore conclude that unspecific effects can be much more common than previously anticipated when using standard RNAi techniques. We describe these effects and their mechanisms as a cautionary note for the unrestrictive use of the RNAi technology and introduce new variables for consideration in RNAi studies in *C. elegans* and potentially other species.

**MATERIALS AND METHODS**

**Strains and maintenance of *C. elegans***

Nematodes were maintained under standard conditions as previously described (20, 21). In short, they were kept at 20°C in streptomycin (100 μg/ml) supplemented Nematode Growth Medium (NGM) with a lawn of OP50-1 *E. coli* as the food source, unless stated otherwise. Strains with *gfp-1(e2141)* background were maintained at 15°C to prevent infertility and were grown at 25°C during experiments. Strains used in this study, including transgenes and co-injection markers are described in Supplementary Table S1. Some strains were provided by the Caenorhabditis Genetics Center (Minneapolis, MN).

**RNAi by feeding**

RNAi plates were supplemented with 1 mM of Isopropyl β-D-1-thiogalactopyranoside (IPTG), tetracycline (12.5 μg/ml) and ampicillin (100 μg/ml), unless stated otherwise. HT115(DE3) *E. coli* bacteria transformed with the L4440 vector (empty vector or carrying specific fragments of cDNA) were inoculated in LB medium overnight. When HT115(DE3) was used without a vector, only tetracycline and IPTG were added to NGM. RNAi clones were available from the Ahringer’s RNAi library. Double RNAi assays were performed as previously described (22–24). Briefly, worms were fed a 1:1 mixture of two sorts of RNAi bacteria grown overnight (OD = 1.5) and concentrated (10×). The RNAi clone targeting luciferase was generated previously (25). For *mir-35-41* (gk262) mutant experiments, RNAi plates were prepared using 6 mM of IPTG.

**Cloning**

For MCS removal [L4440 (-MCS)], we digested the L4440 vector with BglII and KpnI, blunted the ends using T4 DNA polymerase and religated the plasmid using the Quick Ligation kit (New England Biolabs). For T7 promoter removal [L4440 (-T7)], we PCR amplified the L4440 vector backbone sequence upstream of the T7 promoters. The PCR fragment was ligated into the L4440 MCS. This specifically removed the T7 promoters maintaining the remaining of the L4440 plasmid including the MCS. Additional digestions were performed with KpnI and XmaI to remove part of the MCS. All these plasmids were confirmed by sequencing (data not shown). L4440 (GFP) was a gift from the Ruvkun lab. Plasmids were transformed into TOP10 E. coli (One Shot® iTOP10 Chemically Competent *E. coli*), clones were selected and DNA was extracted using QIA-GEN Plasmid Mini Kit followed by enzyme digestions or sequencing confirmation. Plasmids were then used to transform HT115(DE3) *E. coli*, which was used to feed worms.

**mir-35-41* (gk262) viability**

Worm viability was assayed as previously described (15). Briefly, *mir-35-41* (gk262) worms were kept at 20°C, synchronized by standard bleaching procedure and the yielding eggs were left to hatch in M9 buffer at 20°C. After hatching, ~100 animals were pipetted onto plates containing RNAi bacteria and transferred to 25°C. Forty-eight hours later, three adult/late L4 worms were transferred to new plates of the same RNAi and were allowed to lay eggs at 25°C for 24 h. After 24 h, parents were removed from each plate and the number of fertilized eggs laid and hatched larvae were counted. Forty hours later, viable progeny (L3 or older) was scored and the percentage of progeny viability was calculated as the ratio of viable progeny/laid eggs. Unfertilized oocytes were not included in the counting. For experiments done in Figure 3A, a single *mir-35-41* (gk262) hermaphrodite grown for 50 h at 25°C in HT115(DE3) was crossed with *mir-35-41* (gk262) II, wwi88 [mir-35-41p::GFP + unc-119(+)] male worms grown for 60 h at permissive temperature (15°C). The worms were kept crossing for 24 h at 25°C, and subsequently, GFP positive eggs and larvae were
counted. After 48 h, GFP positive L3 worms were counted and progeny viability was calculated.

RNA extraction and RT-qPCR
Worms were harvested in TRIzol (Life Technologies). Total RNA was extracted as previously described (26). For GFP and pre-GFP mRNA quantification, worms were kept at 35°C for 1 h before RNA extraction to induce daf-16 expression in the N2 background. cDNA was produced using High-Capacity cDNA Reverse Transcription kit (Life Technologies). Quantitative reverse-transcriptase PCR (RT-qPCR) was performed using Maxima SYBR-Green Master Mix (Fermentas) in an ABI 7000 detection system (Applied Biosystems), and his-10 was used as an endogenous control. Primer sequences used in this work can be found in Supplementary Table S2.

dsRNA synthesis and microinjection
cDNA corresponding to gfp was PCR amplified using the L4440-GFP plasmid as a template and a pair of primers containing T7 and SP6 promoter sequences. The PCR product was gel purified using Qiagen gel purification kit and eluted in DEPC-treated water. For ssRNA synthesis, two PCR fragments flanked by the T7 promoters of L4440, dsRNA synthesis and soaking
PCR fragments flanked by the T7 promoters of L4440, L4440 (gfp) or L4440 (rde-1) plasmids were amplified and used as templates for dsRNA synthesis using the MEGAscript T7 Transcription Kit (ThermoFisher). Soaking was done by adding 1 μl of ddH2O containing ~100 synchronized L1 worms to 10 μl soaking buffer (5.5 mM KH2PO4; 10.5 mM Na2HPO4; 17.2 mM NaCl; 0.05% gelatin; 3 mM spermidine) containing 8 μg dsRNA. Worms were maintained at 20°C for 48 h and then transferred to 60-mm plates until day 0 of adulthood, when phenotypes were assessed.

Identification of L4440-produced dsRNA
RNAi plates were prepared with the addition or not of IPTG. Plates were seeded with bacteria harboring L4440 or gfp RNAi and incubated overnight at room temperature for T7 polymerase induction by IPTG and production of dsRNA. On the next day, plates were washed with LB and centrifuged at 4°C, 8000 × g for 4 min. Total RNA was extracted by addition of 1 ml TRIzol to bacterial pellet. Samples were homogenized by quick vortex, incubated at 68°C for 5 min and RNA was extracted according to manufacturer’s protocol. A 10 μl reaction mix containing 5 μg of total bacterial RNA, 0.32 M NaCl and 0.1 ng/μl RNase A was incubated for 5 min at 37°C. Samples were purified by phenol:chloroform:isoamyl alcohol (29:28:1) extraction, resuspended in 15 μl DEPC-treated water and 10 μl were loaded onto a 1% agarose gel.

For RT-qPCR, L4440 vector plus or minus MCS was grown overnight on LB media. Bacteria were 10× concentrated and split into two vials. One vial was incubated at 20°C overnight with 1 mM IPTG and the other with vehicle. In the following day, cultures were centrifuged at 4°C, 8000 × g for 4 min. The supernatant was discarded, 500 μl of TRIzol was added to bacterial pellet, and total RNA was extracted and subjected to DNase treatment. cDNA RT-qPCR was performed as described before, and E. coli gyrA was used as endogenous control.

Microscopy and quantification of GFP
For DCR-1::GFP quantification, synchronized worms on day 0 of adulthood were anesthetized using 0.1% sodium azide and scanned using InCell Analyzer 2200 (GE Healthcare). GFP fluorescence was quantified in the posterior intestine region located right after the spermatheca, where the DCR-1::GFP is specific (27). For the nuclear localization assays of DAF-16::GFP or GFP::DAF-16, animals were anesthetized using 0.1% sodium azide on day 0 adult stage, and the number of worms with nuclear GFP in intestinal cells was quantified (InCell Analyzer 2200, Cytation 5, or Zeiss Axio Imager Z1 microscopes). We scored a worm as having nuclear DAF-16 localization if at least 20% of its intestinal cells had noticeable GFP signal in the nucleus. For some experiments, in order to facilitate visualization of DAF-16::GFP, worms were exposed to heat shock treatment at 34°C for 1 h. Representative images were shown in some pictures. All images were processed using ImageJ software (http://rsbweb.nih.gov/ij/).

Statistical analysis
Results are presented as mean ± S.E.M. Statistical tests were performed in GraphPad Prism (GraphPad Software)
Figure 1. Transgenes are silenced upon the exposure of worms with HT115 bacteria harboring the L4440 vector. Worms were grown on plates seeded with different bacterial strains. OP50-1 is the standard E. coli feeding bacteria. HT115 is the RNAi feeding E. coli strain, and it may carry the L4440 plasmid for the production of dsRNA (L4440). L4440 targeting gfp was used as a positive control (L4440-gfp). All experiments were performed three times with at least six worms per group each time. Representative images using a 4× objective microscope of worms on day 0 of adulthood. (A) MAM13/ mamIs1 [dcr-1p::DCR-1::GFP + pUN24 (Y66H1B.3p::Y66H1B.3::GFP) + pJK590 (lag-2p::GFP)] strain was grown at 20°C, photographed, and fluorescence was quantified. Arrows indicate the area used for fluorescence quantification (posterior intestine region) (B). CF1935/ daf-16(mu86) I; glp-1(e2141) III; muIs109 [daf-16p::GFP::DAF-16 cDNA + odr-1p::RFP] worms were grown at 25°C to suppress the germline, and DAF-16 nuclear localization was observed in intestinal cells on day 0 of adulthood. (C) MS438/ irIs25 [elt-2::NLS::GFP::lacZ + rol-6(su1006)] strain was grown at 20°C. Dashed lines represent worm area. ***P < 0.001; compared to OP50-1, using one-way ANOVA and Dunnett’s multiple comparison post-hoc test.

using one-way ANOVA (more than two groups), two-tailed student’s t-test (two groups, equal variance) and non-parametric test (two groups, unequal variance).

RESULTS

RNAi vectors silence different transgenes in C. elegans

In a standard RNAi protocol for C. elegans, worms are fed with dsRNA-expressing HT115(DE3) bacteria (henceforth called HT115 for simplicity) transformed with the L4440 plasmid (9). Our experience using this protocol led us to intriguing observations that we considered important to report here. We observed that ingestion of HT115 bacteria carrying the RNAi empty vector L4440 resulted in diminished fluorescence in three independent transgenic strains expressing multicopy GFP reporters: MAM13/ mamIs1 [dcr-1p::DCR-1::GFP + pUN24 (Y66H1B.3p::Y66H1B.3::GFP) + pJK590 (lag-2p::GFP)] (Figure 1A), CF1935/ daf-16(mu86) I; glp-1(e2141) III; muIs109 [daf-16p::GFP::DAF-16 cDNA + odr-1p::RFP] (Figure 1B) and MS438/ irIs25 [elt-2::NLS::GFP::lacZ + rol-6(su1006)] (Figure 1C). The dcr-1p::DCR-1::GFP construct is based on the pPD95.75 vector backbone and was successfully used to rescue the dcr-1(ok247) null allele (27). The daf-16p::GFP::DAF-16 cDNA construct in the muIs109 transgene uses part of the L2911/pPD103.87 vector backbone and is widely applied in the aging field to rescue daf-16 mutations and to report DAF-16 expression and sub-cellular localization (28–33). elt-2::NLS::GFP::lacZ is an elt-2 reporter construct based on the pPD96.04 vector that was found before to be silenced by L4440 (18). In all cases, more than 90% of the worms had at least 50% of transgene fluorescence silenced upon L4440 exposure.

LacZ-containing transgenes like the irIs25 were shown to be silenced by complementary RNA expressed by the backbone of L4440 (18) (for L4440 map and sequence, see Supplementary Figure S1 and Information). Hence, the majority of the clones obtained from L4440-based RNAi libraries are expected to inhibit irIs25 [elt-2::NLS::GFP::lacZ + rol-6(su1006)] expression and potentially other LacZ-harboring transgenes. Likewise, all L4440-based RNAi clones that we tested silenced mamIs1 (Figure 2A) and mamIs2 [ges-1p::dcr-1::gfp + pUN24...
with at least 10 worms per group. 

- **P**one-way ANOVA and Tukey’s multiple comparison post-hoc test. 

**odr-1p::RFP**

0.01 and ***

16(mu86)I

20% of intestinal cells with nuclear GFP localization. The experiment was repeated three times. 

- **P**ing different RNAi clones. OP50-1 was used as a reference. Experi-

seeded with HT115 bacteria without the RNAi cloning vector or car-

fluorescence in at least 10 worms grown on plates (lag-2p::GFP)

- **P**::DCR-1::GFP + pUN24 (Y66H1B.3p::Y66H1B.3::GFP) + pJK590

(lag-2p::GFP) fluorescence in at least 10 worms grown on plates

seeded with HT115 bacteria without the RNAi cloning vector or car-

rying different RNAi clones. OP50-1 was used as a reference. Experiment was repeated three times. 

- **P**roportion of worms with at least 20% of intestinal cells with nuclear GFP localization. The CF1935/ daf-

16(mu86)I; gfp-1(e2141) III; muls109 [daf-16p::GFP::DAF-16 cDNA + odr-1p::RFP] strain was used. Mean of at least three biological replicates with at least 10 worms per group. ***P < 0.001; compared to L4440, using one-way ANOVA and Tukey’s multiple comparison post-hoc test. **P < 0.01 and ***P < 0.001

( Y66H1B.3p::Y66H1B.3::GFP) + pJK590 (lag-2p::GFP)] transgenes, independently of whether these transgenes were integrated into the genome or were extrachromosomal arrays (Supplementary Figure S2 and data not shown). Interestingly, these transgenes do not contain LacZ sequences, but their shuttle vector (i.e. pPD95.75) exhibited significant overlap with the L4440 vector at their backbones (alignments displayed at the Supplementary Material).

Interestingly, muls109 [daf-16p::GFP::DAF-16 cDNA + odr-1p::RFP] also does not contain LacZ sequences, but in contrast to the dcr-1-expressing transgenes, it was silenced by some, but not all tested L4440-based RNAi clones, arguing that the backbone of the L4440 plasmid alone cannot explain this phenotype (Figure 2B). Moreover, we observed marginally significant, 5- to 10-fold decreases in daf-16 (P value = 0.381) or DAF-16 target sod-3 (P value = 0.0476) mRNA expression, respectively, when CF1935 worms were fed L4440-harboring bacteria (Supplementary Figure S3A and B). Despite the variation in the control group, L4440 exposure resulted in a robust 74% reduction in gfp mRNA expression, while there were no significant changes at the pre-mRNA level, suggesting that cytoplasmic silencing is the main responsible for the L4440 effect (Supplementary Figure S3C). To exclude the possibility that L4440 was targeting the daf-16 pathway itself, we measured dauer entry (a daf-16-dependent phenotype) in daf-2 (e1370) mutants and found that the vector did not interfere with the penetrance of the phenotype (Supplemental Figure S4A). Additionally, we did not observe L4440-induced transgene silencing using an alternative DAF-16 reporter construct where GFP was fused to the C-terminus of DAF-16 [i.e. zIs336 (daf-16p::DAF-16a/b::GFP; rol-6)] (Supplementary Figure S4B and C). Together, these data demonstrate that the L4440 vector targets specific transgenes and silences them through distinct mechanisms.

dsRNA expressed by the L4440 vector acts through the RNAi pathway to silence the muls109 transgene

Importantly, we observed that L4440 produces a non-specific dsRNA from its MCS (Supplementary Figure S5A–D), which does not match any genomic or transcriptomic sequence in C. elegans (Supplementary Figure S5E), or the irIs25 and zIs356 transgenes (data not shown), but does target two complementary sequences in the muls109 transgene, which were carried with the gfp sequence from the L2911/pPD103.87 vector and fused with the daf-16 gene (34) (Supplemental Figure S6A) (sequences for transgenes and L4440 can be accessed in Supplementary Material).

The two matching sequences had a 47-bp long size with identity of 94% and E value of 3 × 10⁻¹² and a 38-bp long size with identity of 100% and E value of 9 × 10⁻¹⁶, respectively. We hypothesized that these matches determine how the L4440 vector silences the muls109 transgene.

Indeed, we removed the MCS region or the T7 promoters of the L4440 plasmid [L4440 (-MCS) and L4440 (-T7), respectively] and found that the vector could no longer silence the muls109 transgene (Figure 3A). In contrast, the irIs25 transgene was silenced by the L4440 (-MCS) vector but not by the L4440 (-T7) construct (Figure 3B), in agreement with a role of the backbone in this particular modality of transgene silencing. Furthermore, when we used an RNAi construct in which the MCS of L4440 was totally replaced by the luciferase gene or when we removed part of the L4440 MCS region that is complementary to the muls109 transgene, we blunted L4440-mediated silencing (Supplementary Figure S6B). To further support our hypothesis, when we tested other GFP-expressing transgenes constructed using GFP derived from vectors similar to the one used to construct muls109 (i.e. pBluescript-based vectors), we found that they were also silenced by L4440 (Table 1) (27, 28, 35–40). Importantly, all of these vectors had sequences that significantly overlapped with L4440, while the muls109 and ccIs4810 transgenes specifically overlapped with the MCS sequence (sequences in the Supplementary Material). This

Figure 2. L4440 silences different transgenes using distinct pathways. 

(A) Representative experiment showing quantification of mams1 [dcr-1p::DCR-1::GFP + pUN24 (Y66H1B.3p::Y66H1B.3::GFP) + pJK590 (lag-2p::GFP)] fluorescence in at least 10 worms grown on plates seeded with HT115 bacteria without the RNAi cloning vector or carrying different RNAi clones. OP50-1 was used as a reference. Experiment was repeated three times. 

(B) Percentage of worms with at least 20% of intestinal cells with nuclear GFP localization. The two matching sequences had a 47-bp long size with identity of 94% and E value of 3 × 10⁻¹² and a 38-bp long size with identity of 100% and E value of 9 × 10⁻¹⁶, respectively. We hypothesized that these matches determine how the L4440 vector silences the muls109 transgene.

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Figure 3. L4440 inhibits muIs109 expression in an RNAi machinery-dependent manner. (A) Quantification of worms exhibiting GFP::DAF-16 nuclear localization in the CF1935/daf-16(mu86) I; muIs109 [daf-16p::GFP::DAF-16 cDNA + odr-1p::RFP] strain grown on HT115 harboring L4440 with or without MCS (-MCS) or T7 (-T7) promoter elements. ***P < 0.001 compared to L4440, using one-way ANOVA and Tukey’s multiple comparison post-hoc test. (B) MS438/irIs25 [elt-2::NLS::GFP::lacZ+rol-6(su1006)]/worms grown on HT115 harboring L4440 with or without MCS or T7 promoters. Representative images using an 10× objective microscope (GFP, lower panels and phase contrast, upper panels). (C) CF1935/daf-16(mu86) I; gfp-1(e2141) III; muIs109 [daf-16p::GFP::DAF-16 cDNA + odr-1p::RFP] worms were grown on OP50-1 or HT115 bacteria harboring L4440 or a double RNAi 1:1 mix of HT115 (L4440) and HT115 (L4440 + dcr-1 RNAi) or HT115 (L4440 + pos-1 RNAi). Single RNAi against dcr-1 or pos-1 did not compromise muIs109 [daf-16p::GFP::DAF-16 cDNA + odr-1p::RFP] nuclear localization (data not shown and Figure 2B). MAM56/rde-1(ne219); daf-16(mu86) I; gfp-1(e2141) III; muIs109 [daf-16p::GFP::DAF-16 cDNA + odr-1p::RFP] worms were grown on OP50-1, HT115 harboring L4440 or daf-16 RNAI. + or - represents when worms exhibited GFP expression comparable to control or when GFP expression was silenced, respectively. Silencing was defined when more than half of a group of worms had <20% of intestinal cells with GFP localization. All experiments were performed three times with at least eight worms per group each.

Table 1. List of transgenes silenced or not by L4440

| Transgene | Platform | Silenced by L4440 | Strain name |
|-----------|----------|------------------|-------------|
| muIs109   | Bluescript II KS+ | Yes              | CF1935      |
| namIs1    | Bluescript-like | Yes              | MAM13       |
| ecIs4810  | Bluescript II KS+ | Yes              | YG1021      |
| judIs76   | pUC19-derived   | No               | CZ3464      |
| muIs25    | pUC19-derived   | No               | SP2533      |
| qceXc24   | Generated by MosSCI | No          | CS119       |
| oxS1259   | Generated by MosSCI | No          | EG6173      |
| bql2      | Generated by CRISP | No             | BN580       |

Worms were grown on plates with HT115 harboring or not L4440 in the presence or not of IPTG for dsRNA induction. Worms were observed at day 0 of adulthood to check if GFP fluorescence was inhibited by the presence of L4440 + IPTG. The experiment was repeated twice and 10 images with at least 20 worms total were analyzed and total fluorescence was quantified using ImageJ (data not shown). The strains tested were previously described: CF1935 (28), MAM13 (27), YG1021 (35), CZ3464 (36), SP2533 (37), CS119 (38), EG6173 (39), BN580 (40). Shaded rows represent transgenes silenced by L4440 as scored by a blind observer based on the respective controls. CF1935 and YG1021 were not silenced by L4440 (-MCS) (Figure 2 and data now shown).

was not the case for transgenes where GFP was derived from pUC19 plasmids or inserted in the genome by precise techniques such as MosSCI or CRISPR (Table 1 and Supplementary Material). These results demonstrate that the L4440 MCS targets specific complementary sequences in some transgenes, in particular the ones where sequences of pBluescript-based plasmids remained in the construct.

Consistent with a role of dsRNA produced by the MCS, L4440-mediated muIs109 transgene silencing was dependent on components of the RNAi pathway such as DRE-1 and DCR-1 (Figure 3C and Supplemental Figure S7). These results provide evidence that the pathways for transgene suppression by the L4440 vector involve RNA-mediated silencing but differ in their mechanism depending on the RNA produced and the target (i.e. muIs109 versus irIs25 transgene). To directly test the role of L4440-produced dsRNA to silence the muIs109 transgene, we delivered by soaking the dsRNA synthesized in vitro using the MCS region of the L4440 plasmid as a template. As predicted, this led to reduced fluorescence in muIs109-
expressing worms, but not in mamIs1 or with a lesser magnitude in irIs25 transgenic (Figure 4), reinforcing the necessity and the specificity of L4440-produced dsRNA to silence the transgenes.

Furthermore, to better comprehend the mechanism underlying the L4440 effect over the mutIs109 transgene, we performed a double RNAi screen directed against genes involved in small RNA pathways. As expected, we confirmed that components of the canonical RNAi pathway (i.e. dcr-1, rde-1, rde-4, and mut-16) were necessary for mutIs109 silencing (Table 2). We also identified drh-1 and rsd-6 as genes required for L4440-mediated silencing (Table 2). drh-1 and rsd-6 are both involved in the antiviral RNAi pathway (41,42). Interestingly, we observed that RNAi targeting these genes did not block the silencing effect mediated by daf-16 RNAi (data not shown), hinting that L4440 acts through a non-canonical RNAi pathway that involves drh-1 and rsd-6. However, L4440 (or daf-16 RNAi) could still silence the mutIs109 transgene in drh-1(ok3495) or rsd-6(pk2011) mutants (Supplementary Figure S8A and B). Given that in the mutant strains drh-1 or rsd-6 are lost during the worm’s entire life cycle and in the double RNAi experiments genes are silenced concomitantly starting at L1, we hypothesized that differences when these genes are lost could explain such discrepancies. Consistent with this notion, when we started applying rsd-6 RNAi in utero, L4440 was able to silence the mutIs109 transgene (Supplementary Figure S8C).

Exogenous dsRNA increases progeny viability of a miRNA mutant strain

mir-35-41 mutants exhibit reduced progeny viability when grown at restrictive temperature (25°C), and this has been linked to embryonic and larval lethality (15,16,43,44). We found that reduced progeny viability of mir-35-41(gk262) hermaphrodites grown at the restrictive temperature (25°C) was paternally rescued when these worms were crossed with mir-35-41(gk262) males grown at permissive temperature (15°C) (Figure 5A). Remarkably, we also found that the mir-35-41(gk262) mutant had its progeny viability of 5% increased to over 30% when worms were fed with HT115 carrying L4440 (Figure 5B). L4440 also promoted fecundity of mir-35-41(gk262) mutants at 25°C, as evidenced by increased brood size (Supplementary Figure S9A). The partial rescue of progeny viability by L4440 was only observed when worms were grown on plates containing IPTG (inducer of T7 RNA polymerase). These findings led to the important and novel conclusion that the effects of L4440 go beyond transgene silencing. However, in this case, a L4440-based gfp RNAi clone also suppressed the decrease of progeny viability in the mir-35-41(gk262) mutant (Figure 5B). Since the mir-35-41(gk262) mutant does not carry transgenes that could be affected by complementarity with the L4440 backbone and we could not find significant overlap between the L4440 backbone and the genome of C. elegans, we hypothesized that dsRNA generated by L4440 could result in general activation of the RNAi pathway and consequently rescue progeny viability. To test whether the phenotype was caused by the L4440 plasmid or simply by the presence of exogenous dsRNA, we

Figure 4. MCS dsRNA from L4440 silences mutIs109 expression. Quantification of fluorescence of (A) MAM13/mamIs1 [dcr-1p::DCR-1::GFP + pUN24 (Y66H1B3p:Y66H1B3::GFP) + pJK590 (lag-2p::GFP)], (B) MAM71/N2; mutIs109 [daf-1p::GFP::DAF-16 cDNA + odr-1p::RFP] and (C) MS438/irIs25 [elt-2p::NLS::GFP::lacZ + rol-6(su1006)] treated with soaking buffer (buffer), MCS dsRNA of L4440 (dsMCS), GFP dsRNA (dsGFP) or rde-1 dsRNA (dsRDE-1) at day 0 of adulthood for 48 h before analysis. All experiments were performed three times with at least 10 worms per group each time. *P < 0.05, ***P < 0.001 compared to ‘buffer’ using one-way ANOVA and Tukey’s multiple comparison post-hoc test.
injected gfp-targeting dsRNA into L3 worms and it recovered the progeny viability of mir-35-41(gk262) mutants to 30% (Figure 5C). This confirms that the mir-35-41(gk262) phenotype suppression occurs independently of the L4440 plasmid and is associated with the presence of exogenous dsRNA.

To better understand the mechanisms, we performed double RNAi screen targeting components of small RNA pathways (the same genes are listed in Table 2) and asked whether any of the clones interfered with the L4440 effect (Table 3). We observed that the majority of the RNAi clones partially recovered progeny viability in mir-35-41(gk262) mutants, once again confirming that dsRNA production is sufficient to attenuate the phenotype. Consistent with this observation, the effect of exogenous dsRNA on mir-35-41(gk262) progeny viability depended on canonical components of the RNAi machinery (i.e. dcr-1 and pash-1) (45) (Table 3). Importantly, we confirmed these findings by crossing mir-35-41(gk262) mutants with rde-4(ne301) mutants, where loss of an important gene involved in dsRNA processing blocked the effects of L4440 (Figure 5D). This was not due to a potential outcross of mir-35-41(gk262) mutants since the effect of L4440 was preserved even when these worms were outcrossed to a wild-type background for one further generation (Supplementary Figure S9B). We also found that knockdown of genes involved in miRNA biogenesis (i.e. dcr-1 and pash-1) counteracted the effects of L4440 on mir-35-41(gk262) mutants (Table 3), although silencing of dcr-1 and pash-1 also reduced progeny viability in wild-type worms (Supplementary Figure S9C).

Finally, by timing RNAi exposure in mir-35-41(gk262) mutants, we found that the decrease in progeny viability could only be reversed if a source of dsRNA was given to parents starting at the L1 stage and remained during in utero development of the progeny (Supplementary Figure S9D). If L4440 was provided late during parent development or to the progeny, no effect was observed. Moreover, this phenotype depended on the temperature in which the parents were raised, as it occurred only when parents were transferred to 25°C at the L1 stage and not at the L4 stage, most likely because in this case progeny viability was already higher (~25%) (Supplementary Figure S9E). Taken together, these results suggest that mir-35-41 deficiency leads to defects in embryogenesis at 25°C and providing an exogenous source of dsRNA or keeping parents at permissive temperatures (particularly the parental line) partially reverse this phenotype. Consistent with a direct effect of dsRNA over the germline, a null mutation in rrf-1—a gene involved in somatic RNAi, but not in germline RNAi (46,47)—did not inhibit L4440 action and rather tended to make mir-35-41(gk262) mutants more sensitive to L4440 (Figure 5D).

DISCUSSION

This study introduces new variables to be considered in RNAi experiments in C. elegans and potentially in other model organisms. By treating worms with bacteria harboring widely used RNAi constructs, we report at least three distinct ways that worms can be affected by the presence of foreign dsRNA. First, we confirmed previous observations that LacZ-containing transgenes, like irIs25, are silenced by the L4440 RNAi vector (18). Importantly, this previous study suggested that the effect of L4440 was limited to a specific class of LacZ-containing transgenes. Here, we provide data showing that L4440 is capable of producing dsRNA to silence a wide range of multicopy transgenes (including GFP-expressing transgenes) and demonstrate new mechanisms of L4440-mediated silencing. For example, we found that the muIs109 transgene is silenced mostly at the mature mRNA level by dsRNA produced by the MCS of the L4440 vector. Consistently, silencing occurs to a much lesser magnitude or does not occur at all when the MCS is partially or totally removed from the L4440 plasmid. We also show that administration of exogenous dsRNA partially reverses embryonic viability in an mir-35-41(gk262) deficient background via a mechanism dependent on the canonical RNAi pathway. These findings not only imply that current RNAi technology in C. elegans may lead to unexpected outcomes that need to be controlled and accounted for in most applications, but also bring insights into a new level of cross-regulation between different small RNA pathways to control embryonic development.

Given the broad implication of these findings, we decided to explore how the MCS of L4440 silences transgenes. We found that carryover sequences from pBluescript-based vectors used to generate GFP-tagged transgenes share sequence similarity with the MCS of the L4440 vector. This is somewhat expected since the L4440 vector is also a modified version of the pBluescript plasmid. In contrast, GFP sequences from pUC19-based transgenes, which lack relevant sequence complementarity with the L4440 vector, are not silenced by L4440. Furthermore, transgenes inserted with techniques such as MosSCI or CRISPR are not affected by L4440. These results suggest that GFP derived from pBluescript-based plasmids should be avoided for transgene construction in strains where L4440-mediated RNAi will be used. In this case, the dsRNA produced by the L4440 MCS can silence transgenes when corresponding pBluescript vector sequences remain in the construct. Based on double RNAi screening and further validation using mutants, we found that the L4440 MCS requires both canonical (i.e. dcr-1, rde-1, rde-4 and mut-16) and non-canonical (i.e. drh-1 and rsd-6) members of the RNAi pathway to silence transgenes. DRH-1 and RSD-6 have been shown to play a role in the antiviral RNAi pathway in C. elegans (41,42), although RSD-6 was first described as a protein necessary for systemic RNAi, and therefore can also participate in the canonical RNAi pathway (48). We found that while daf-16 RNAi silences the muIs109 transgene even in the absence of DRH-1 or RSD-6, L4440 cannot do so under some circumstances. Interestingly, this epistatic interaction is only observed when L4440 is offered concomitantly with either drh-1 or rsd-6 RNAi and not when L4440 is given to drh-1 or rsd-6 mutants. Such discrepancies are unlikely a consequence of unspecific effects of the drh-1 or rsd-6 RNAi clones, which differ largely in sequence. Dilution of L4440 in the double RNAi experiments is also unlikely, since several other RNAi clones did not influence the effects of L4440 in our double RNAI screen and even conditions with low or no IPTG (i.e. very low dsRNA expression) elicited L4440-mediated transgene silencing (Supplemental Figure S8C).
Table 2. Double RNAi screen to assess genes necessary for L4440-mediated muIs109 (daf-16p::GFP::DAF-16 cDNA + odr-1p::RFP) transgene silencing.

| Gene | Single RNAi | Double RNAi | Function |
|------|-------------|-------------|----------|
| cdi-1 | Not silenced | Silenced | Nucleotidyltransferase |
| csw-1 | Not silenced | Silenced | 22G-RNA Argonaute |
| dcr-1 | Not silenced | Not silenced | microRNA and siRNA processing |
| drh-1 | Not silenced | Not silenced | Helicase of the RNAi antiviral pathway |
| ekl-1 | Not silenced | Silenced | RNAi pathway |
| ergo-1 | Not silenced | Silenced | 26G-RNA Argonaute |
| eri-9 | Not silenced | Silenced | DICER-interacting protein |
| hda-4 | Not silenced | Silenced | Histone deacetylase 4 |
| hrde-1 | Not silenced | Silenced | Nuclear Argonaute |
| iri-1 | Not silenced | Silenced | Kinase/Endoribonuclease; UPR activator |
| mut-14 | Not silenced | Silenced | Putative DEAD box RNA helicase |
| mut-16 | Not silenced | Not silenced | Activity required for RNAi |
| nrde-2 | Not silenced | Silenced | Activity required for RNAi |
| nrde-3 | Not silenced | Silenced | Nuclear RNAi factor |
| mut-7 | Not silenced | Silenced | Regulation of clustered 21U-RNA loci |
| mut-14 | Not silenced | Silenced | Nucleotidyltransferase |
| mut-16 | Not silenced | Silenced | Activity required for RNAi |

In the ‘Single RNAi’ column, we describe the effect of the RNAi clone (‘Gene’) alone on transgene expression. In the ‘Double RNAi’ column, we describe the effect of the RNAi clone (‘Gene’) mixed 1:1 with HT115 harboring L4440 on transgene expression. Not silenced, when the majority of the worms had at least 20% of the intestinal cells with nuclear GFP localization. Silenced, when the majority of the worms had less than 20% of the intestinal cells with nuclear GFP localization. Bold rows represent RNAi clones that blocked the L4440 effect. This experiment was performed twice with at least eight worms per group each time.

Table 3. Double RNAi screen to assess genes necessary for L4440-mediated recovery of reduced progeny viability of mir-35-41 (gk262) mutant

| Gene | HT115 (% viability) | L4440 (% viability) | Single RNAi (% viability) | Double RNAi (% viability) | HT115 versus single RNAi | L4440 versus double RNAi |
|------|---------------------|---------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| cdi-1 | 13.01 ± 1.67 | 27.13 ± 1.63 | 29.73 ± 2.01 | 30.34 ± 1.05 | P < 0.0001 | P = 0.5137 |
| csw-1 | 11.68 ± 1.67 | 28.56 ± 1.63 | 28.45 ± 2.01 | 29.79 ± 1.05 | P < 0.0001 | P = 0.9498 |
| dcr-1 | 11.43 ± 2.99 | 26.98 ± 1.59 | 26.06 ± 2.53 | 15.93 ± 2.65 | P = 0.0028 | P = 0.0298 |
| drh-1 | 18.32 ± 3.49 | 34.74 ± 2.50 | 39.05 ± 2.56 | 41.70 ± 5.77 | P = 0.0071 | P = 0.5835 |
| ekl-1 | 10.92 ± 1.34 | 23.67 ± 2.22 | 28.98 ± 2.29 | 27.15 ± 1.75 | P < 0.0001 | P = 0.5955 |
| ergo-1 | 9.74 ± 1.86 | 37.76 ± 12.30 | 49.88 ± 4.26 | 40.34 ± 1.12 | P < 0.0001 | P = 0.9021 |
| eri-9 | 12.30 ± 1.18 | 27.34 ± 2.95 | 27.70 ± 2.17 | 25.87 ± 2.48 | P = 0.0007 | P = 0.9680 |
| hda-4 | 16.48 ± 2.89 | 34.40 ± 2.71 | 34.83 ± 2.29 | 36.18 ± 5.57 | P = 0.0090 | P = 0.9850 |
| hrde-1 | 11.32 ± 1.06 | 23.61 ± 2.37 | 23.18 ± 1.95 | 22.42 ± 1.59 | P = 0.0009 | P = 0.9659 |
| iri-1 | 13.82 ± 1.45 | 38.58 ± 2.09 | 43.19 ± 5.35 | 44.62 ± 3.93 | P < 0.0001 | P = 0.6338 |
| mut-14 | 8.19 ± 1.88 | 36.36 ± 2.42 | 41.68 ± 3.35 | 30.89 ± 3.78 | P < 0.0001 | P = 0.5673 |
| mut-16 | 15.79 ± 1.37 | 30.84 ± 2.98 | 40.67 ± 5.04 | 42.55 ± 4.09 | P = 0.0005 | P = 0.1381 |
| mut-7 | 12.97 ± 1.62 | 22.58 ± 0.93 | 26.27 ± 1.36 | 15.00 ± 1.50 | P < 0.0001 | P = 0.0048 |
| nrde-2 | 12.66 ± 1.27 | 39.93 ± 3.50 | 52.07 ± 3.96 | 54.30 ± 4.11 | P = 0.0002 | P = 0.0647 |
| nrde-3 | 13.83 ± 3.45 | 31.19 ± 3.04 | 37.85 ± 5.04 | 46.17 ± 4.10 | P = 0.0068 | P = 0.0993 |
| pash-1 | 14.60 ± 3.83 | 30.76 ± 3.12 | 11.52 ± 1.42 | 10.11 ± 2.72 | P = 0.8765 | P = 0.0004 |
| prg-1 | 13.10 ± 2.79 | 31.07 ± 7.50 | 21.22 ± 1.66 | 19.37 ± 2.62 | P = 0.5511 | P = 0.2492 |
| rde-1 | 12.54 ± 1.36 | 28.66 ± 3.79 | 29.47 ± 1.27 | 18.02 ± 3.18 | P = 0.0004 | P = 0.0370 |
| rde-4 | 13.81 ± 1.91 | 26.53 ± 1.50 | 17.75 ± 1.42 | 17.12 ± 1.32 | P = 0.3091 | P = 0.0020 |
| rsd-6 | 4.97 ± 5.06 | 11.91 ± 2.04 | 26.06 ± 3.96 | 15.33 ± 1.26 | P = 0.7620 | P = 0.2768 |
| unc-130 | 13.37 ± 0.96 | 26.68 ± 1.42 | 23.69 ± 1.36 | 24.46 ± 1.83 | P = 0.0003 | P = 0.6967 |

In the ‘Single RNAi’ column, we describe the effect of the RNAi clone (‘Gene’) alone. In the ‘Double RNAi’ column, we describe the effect of the RNAi clone (‘Gene’) mixed 1:1 with HT115 harboring L4440. Bold rows represent RNAi clones in which the viability of the ‘double RNAi’ group was statistically different from the ‘L4440’ group. One-way ANOVA and Tukey’s multiple comparison post-hoc tests were performed. Experiments were repeated four to five times with at least nine worms per group each time.

Nonetheless, differently from when rsd-6 is silenced in larvae, silencing in utero does not significantly affect the ability of L4440 to suppress the muIs109 transgene (Supplementary Figure S8C). This suggests that the difference between the double RNAi experiments and the experiments where we applied L4440 to mutant worms is timing. While in the mutants drh-1 and rde-6 are constitutively absent, in our double RNAi screen genes were silenced concomitantly with the administration of L4440 starting at the L1 stage. The former could induce a compensatory, redundant pathway to deal with foreign dsRNA during early embryonic development even in the absence of drh-1 and rde-6. Indeed, it is expected that during embryogenesis cells use redundant pathways to preserve genomic integrity and reduce transcriptional noise, and previous work has provided an exam-
Figure 5. Unspecific RNAi reverses the phenotype of decreased progeny viability of mir-35-41(gk262)/VC514 mutants. (A) Percentage of viable progeny of N2 and mir-35-41(gk262)/VC514 hermaphrodite mutants when grown at 25°C in HT115 and crossed with males of their respective background grown at permissive temperature (15°C). For the experimental group (third bar), MAM130/mir-35-41(gk262) II, wvIs8 [mir-35-41p::GFP + unc-119(+)] males were grown for 60 h at 15°C and then crossed with a single mir-35-41(gk262) hermaphrodite that was grown for 50 h at 25°C. The worms were left mating for 24 h at 25°C. After that, GFP positive eggs were counted. Later, L3 worms were counted and viable progeny was calculated. As controls, N2 males were crossed with N2 hermaphrodite (first bar) or mir-35-41(gk262)/VC514 strain was grown at 25°C (second bar) or 15°C (fourth bar) and viable progeny was evaluated. a, b, c and d represent \( P < 0.05 \) in comparison to all the other groups according to one-way ANOVA with Tukey’s multiple comparison post-hoc test. The experiments were repeated four times with at least nine worms scored per group in each experiment. (B) The mir-35-41(gk262)/VC514 mutants show progeny viability of 5% at 25°C. Percent of viable progeny represents the average number of eggs laid that reached the L3 stage. L4440* is a L4440 plasmid from another laboratory (Dr Andrew Chisholm). ** \( P < 0.01 \) L4440 plus IPTG versus minus IPTG. The experiment was repeated two times with at least 276 worms per group. (C) Percent of viable progeny when worms were injected with buffer or dsRNA targeting GFP (dsGFP RNA). About 43 worms per parent were measured. mir-35-41(gk262);ccIs4251[myo-3p::GFP(NLS)::LacZ+myo-3p::GFP] strain were used in this experiment, so we could monitor whether injection with gfp RNAi worked. * \( P < 0.05 \) dsGFP versus buffer. (D) Worms were grown in NGM plates with HT115 harboring L4440 in the presence or not of IPTG for dsRNA induction. Percent viability represents percent progeny that grew up to L3. Results are from at least two independent experiments. At least 85 worms per parent were measured. \( P \) values for L4440 (-) IPTG versus L4440 (+) IPTG using unpaired \( t \)-test are: mir-35(gk262): 0.0678; mir-35(gk262); rde-1(ne300): 0.0282; mir-35(gk262); rrf-1(pk1417): 0.1815; mir-35(gk262); rde-4(ne301): 0.0007. We also found that RNAi may result in undesirable effects over the animal’s physiology. We show that reduced embryonic viability in mir-35-41(gk262) mutants is partially reversed in the presence of exogenous dsRNA including the one produced by the empty vector. In this case, a plasmid where redundant genes can compensate for mutations but not RNAi-mediated silencing (49). Like the muls109 transgene, the mamIs1 and mamIs2 transgenes are also silenced by L4440. The dcr-1 overexpressing constructs were made using the pPD95.75 plasmid (27)—a pBluescript-based vector. However, unlike the muls109 transgene, the dcr-1 transgenes are silenced by multiple RNAi clones lacking part or the entire MCS. These results suggest that this silencing mechanism is independent of the MCS and may be similar to the one observed by Grishok et al., even though we could not find sequences complementary to the LacZ gene in the backbone of pPD95.75. We could nonetheless identify significant sequence overlap between the backbones of pPD95.75 and L4440, indicating that transgene silencing may be mediated by such overlap (Supplementary Material). Moreover, RNA produced by the L4440 backbone could exacerbate an already existing RNAi-dependent silencing process normally occurring in the soma for repetitive transgenes (18, 50). Accordingly, it was recently demonstrated that the L4440 vector does not present T7 terminator sequences and therefore T7-mediated transcription leads to backbone sequence expression (51). These results hint that L4440 could interfere with a wide variety of transgenes in C. elegans, particularly the ones where sequences of pBluescript vectors remain in the construct.
the effect of L4440 was dependent on an upstream component of the canonical siRNA processing machinery (i.e. RDE-4) as assessed first by the double RNAi screen and confirmed by crossing \textit{mir-35-41 (gk262)} with \textit{rde-4 (ne301)} mutants. An intriguing possibility to explain this phenotype is a role for the \textit{mir-35-41} cluster as a sponge for RNA-binding proteins and components of the RNAi pathway. Indeed, \textit{mir-35-41} are among the most highly expressed miRNAs in oocytes and during early embryonic development \cite{17,52,53}, and their absence could alter the stoichiometric balance between the miRNA biogenesis and RNAi pathways \cite{15}, possibly affecting the production of endogenous small RNAs that impair progeny viability (Figure 6). The presence of abundant exogenous dsRNA would in turn rebalance the stoichiometry and restore viability.

Our model is consistent with the fact that \textit{mir-35-41} deficiency leads to RNAi hypersensitivity \cite{15} and that almost all RNAi clones that we tested partially recover the viability of \textit{mir-35-41 (gk262)} mutant progeny, while deficiency in the miRNA pathway (as demonstrated by \textit{pash-1} RNAi) sensitizes \textit{mir-35-41 (gk262)} mutants to reduced embryonic viability, probably by further promoting the flux of small RNA biogenesis toward other branches of the pathway. Alternatively, exogenous dsRNA may potentially suppress targets of \textit{mir-35-41} such as germline RNA-binding proteins like GLD-1 and SUP-26, which genetically interact with \textit{mir-35-41} to control progeny development \cite{17,43,54}.

A search in Pubmed for ‘elegans AND (RNAi OR RNA interference)’ retrieved 3353 published papers as of February 2019. It is safe to speculate that the majority of these papers or upcoming publications have used or will use the L4440 vector at some point, given that the two \textit{C. elegans} RNAi libraries available were constructed using this vector. Furthermore, pBluescript-based plasmids such as pPD103.87 and pPD95.75 are a common way to shuttle and express transgenes in \textit{C. elegans} \cite{55}. Reverse genetics using L4440 as a vector for RNAi feeding has been applied to other organisms namely \textit{Daphnia melanica}, \textit{Ephydatia muelleri}, \textit{Euplotes eurystomus}, \textit{Helicoverpa armigera}, \textit{Mythimna separate}, \textit{Paramecium octaurelia}, \textit{Spodoptera exigua} and \textit{Tethya wilhelma} \cite{56–62}. In light of our results, these observations raise a concern regarding how one should control RNAi experiments going forward, how transgenes should be used to genetically modify animals and how RNAi vectors should be designed to avoid unnecessary sequences. We propose some aspects that need to be considered such as: (i) always testing for the effect of the control vector, (ii) removing the MCS of L4440 or replacing it for an unspecific DNA fragment like the luciferase gene, (iii) using alternative RNAi vectors that share less sequence complementarity with transgenes or vice versa, (iv) using RNAi vectors that
present T7 terminator sequences such as the one recently proposed by Sturm et al. (51), (v) mixing bacteria harboring the RNAi clone targeting the gene of interest with bacteria harboring the control vector (empty or otherwise) in all conditions to account for unspecific effects of the control vector, (vi) dosing the dsRNA levels to achieve efficient silencing without overloading the RNAi machinery, (vii) avoiding pBluescript vector sequences within transgenes and/or (viii) using CRISPR or MosSCI techniques to create reporter strains for RNAi screens. In conclusion, our work has cautionary implications for RNAi studies in *C. elegans* and provides new insights into the complex interplay between foreign dsRNA and animal physiology.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

**ACKNOWLEDGEMENTS**

We thank Dr Elzira Saviani for technical support and members of Antebi, Mori, Massirer and Pasquinielli lab for helpful discussions. We thank the *C. elegans* Genetics Center (CGC) at the University of Minnesota, which is funded by the NIH Office of Research Infrastructure Programs (P40 OD010440), for providing *C. elegans* strains and Dr Hugo Agulaniu, Dr Andrew Chisholm and Dr Gary Ruvkun for providing RNAi clones.

**FUNDING**

Fundação de Amparo à Pesquisa do Estado de São Paulo [2017/01184-9, 2014/10814-8, 2016/02207-0, 2015/01316-7, 2010/52557-0, 2017/08829-5, 2017/22057-5, 2017/03432-0, 2017/04377-2, 2017/01339-2, 2016/15958-3 and 2014/25068-0 to M.A.M.; 2012/00195-3 to K.B.M.; Conselho Nacional de Desenvolvimento Científico e Tecnológico [444424/2014-8 to M.A.M.]; Financiadora de Estudos e Projetos [UNIFESP-RPMI PROINFR A 01/2011]; National Institutes of Health [R35 GM127012 to A.E.P.]. Funding for open access charge: Fundação de Amparo à Pesquisa do Estado de São Paulo.

Conflict of interest statement. None declared.

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