bioRxiv preprint doi: https://doi.org/10.1101/283325; this version posted March 15, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Previously characterized *Caenorhabditis elegans* RNA interference-resistant mutants retain substantial silencing capacity

Jason S. Watts, Quentin Guenther, James Dalelio, Jennifer L. Watts

School of Molecular Biosciences and Center for Reproductive Biology

Washington State University, Pullman, WA 99164-7520

bioRxiv preprint doi: https://doi.org/10.1101/283325; this version posted March 15, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Gene silencing in RNAi mutants

Keywords: RNA interference, fatty acid desaturation, tissue-specific RNAi

Corresponding author:

Jennifer L. Watts

School of Molecular Biosciences and Center for Reproductive Biology

Washington State University, Pullman, WA 99164-7520

Telephone: (509)335-8554

Email: jwatts@wsu.edu

bioRxiv preprint doi: https://doi.org/10.1101/283325; this version posted March 15, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Abstract

RNA interference is a powerful tool for dissecting gene function. In *Caenorhabditis elegans*, ingestion of double stranded RNA causes strong, systemic knockdown of target genes. Further insight into gene function can be revealed by tissue-specific RNAi techniques. Currently available tissue-specific *C. elegans* strains rely on rescue of RNAi function in a desired tissue or cell in an otherwise RNAi deficient genetic background. In a classroom setting, we attempted to assess the contribution of specific tissues to polyunsaturated fatty acid (PUFA) synthesis using currently available tissue-specific RNAi strains. We discovered that *rde-1 (ne219),* a commonly used RNAi-resistant mutant strain, retains considerable RNAi capacity against RNAi directed at PUFA synthesis genes. Using GC/MS, we measured changes in the fatty acid products of the desaturase enzymes that synthesize PUFAs in a gene dosage sensitive manner. With this method, we tested a panel of previously described RNAi-deficient mutant strains, and found that almost all of them retained a certain degree of RNAi capacity. Importantly, we found that the before mentioned strain, *rde-1 (ne219)* and the reported germline only RNAi strain, *rrf-1 (pk1417)* are not appropriate genetic backgrounds for tissue-specific RNAi experiments. However, the knockout mutant *rde-1 (ne300)* was strongly resistant to dsRNA induced RNAi, and may be appropriate for construction of robust tissue-specific RNAi strains.

bioRxiv preprint doi: https://doi.org/10.1101/283325; this version posted March 15, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Introduction

RNA interference (RNAi) is an evolutionarily ancient defense mechanism against viruses and transposable elements. *Caenorhabditis elegans* has been a powerful system for discovering the molecular mechanisms underlying the RNAi phenomena and its role in gene regulation (Fire et al., 1998; Grishok, 2005; Hammond, 2005). The discovery that double stranded RNA (dsRNA) expressed by bacteria and ingested by the worm could effectively silence target genes revolutionized the use of RNAi as a tool for high-throughput, large-scale genetic knockdown studies in *C. elegans* (Ashrafi et al., 2003; Fraser et al., 2000; Timmons and Fire, 1998; Yigit et al., 2006).

Genetic screens for mutants that are resistant to RNAi have been essential for elucidating the mechanism of processing exogenously introduced double stranded RNAs and inducing gene silencing (Zhuang and Hunter, 2012). The feeding method of RNAi delivery is successful in *C. elegans* because of two membrane proteins called SID-1 and SID-2 that facilitate the uptake of double stranded RNA into cells (Winston et al., 2002; Winston et al., 2007). Both mutant strains grow and reproduce normally, but are resistant to systematic RNAi delivered by the feeding method. Other screens for viable mutants with RNAi deficiency (RDE mutants) revealed genes coding for many highly-conserved activities required for RNAi, including RDE-4, a double-stranded RNA binding protein which forms a complex with Dicer to bind long double-stranded RNAi and cleave it into ~22 bp interfering RNAs (siRNAs) (Knight and Bass, 2001; Parker et al., 2006; Tabara et al., 2002).

bioRxiv preprint doi: https://doi.org/10.1101/283325; this version posted March 15, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

In *C. elegans*, RDE-1 is the primary Argonaute component of the RNA induced silencing complex (RISC), which degrades the passenger strand of the siRNA and uses the remaining strand to target mRNA for use as a template for synthesis of secondary siRNAs (Parrish and Fire, 2001; Steiner et al., 2009; Tabara et al., 1999). Production of the siRNAs amplifies the signal and are used as the final targeting signal for degradation of newly formed mRNAs. The *C. elegans* genome encodes multiple homologs of certain components of the RNAi machinery, including four RNA-dependent RNA polymerases (RdRPs), including RRF-1, and 27 known Argonaute proteins (Sijen et al., 2001; Smardon et al., 2000; Yigit et al., 2006). The RdRPs amplify silencing by using the primary siRNA/mRNA complex as a template for synthesizing secondary siRNAs. RDE-3, a member of the polymerase beta nucleotidyltransferase superfamily is required for siRNA accumulation (Chen et al., 2005). RDE-10 and RDE-11 form a complex that promotes secondary siRNA amplification (Yang et al., 2012).

Identifying mutants that are resistant to the induction of RNAi by double stranded RNA has typically involved screening with a visible phenotype such as lethality, movement defects, or suppression of a fluorescent transgene (Tabara et al., 1999; Winston et al., 2002; Yigit et al., 2006). Mutant strains in which the treated worms failed to display the knockdown phenotype were considered RNAi resistant. These methods have proven efficient and invaluable for the discovery of RNAi pathway genes. However, the qualitative nature of these visible phenotypes makes them insufficient to determine if a specific mutation in an RNAi pathway gene completely inhibits RNAi capacity, or only enough to prevent the visible phenotype.

bioRxiv preprint doi: https://doi.org/10.1101/283325; this version posted March 15, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Gene knockdown by RNAi is useful for elucidating gene function, but RNAi by feeding does not provide information about the function of genes in specific tissues. To assess tissue specific gene function, investigators have employed tissue specific mutants, and tissue specific transgenic rescue of key RNAi machinery (Qadota et al., 2007; Sijen et al., 2001). Accurate analysis of experiments performed in tissue specific systems depends on RNAi functioning only in the desired cells and tissues. This is achieved by expressing a transgene under the control of a tissue-specific promoter in the background of an RNAi-deficient mutant. Any residual RNAi activity in off-target tissues can lead to erroneous conclusions, thus it is essential that the mutant strain used is completely RNAi-deficient.

In a classroom setting, we sought to involve undergraduate students in an inquiry-based research project. Our original research objective was to determine various tissue contributions to the overall degree of fatty acid desaturation in the nematode *C. elegans* using tissue-specific RNAi strains and feeding RNAi directed toward fatty acid desaturase genes. We used a biochemical method employing gas chromatography/mass spectrometry (GC/MS) to monitor the flux of fatty acid desaturation reactions from substrates to products as a quantitative approach to measuring the RNAi silencing capacity in previously characterized *C. elegans* mutant strains. Our quantitative method revealed that some *C. elegans* strains deemed "RNAi deficient" retained substantial RNAi silencing capacity when exposed to the feeding method of RNAi delivery, and therefore the available strains used for tissue-specific RNAi are not useful for determining tissue-specificity of gene function.

bioRxiv preprint doi: https://doi.org/10.1101/283325; this version posted March 15, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Materials and Methods

Worm maintenance and RNAi

NGM growth media was supplemented with 100 µg/mL ampicillin, 2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), and seeded with the appropriate HT115 RNAi bacteria. RNAi constructs for *fat-1*, *fat-4*, and empty vector control were obtained from the Ahringer RNAi library and sequence verified (Fraser et al., 2000). Synchronized L1 larvae were plated onto the RNAi plates and allowed to grow for 2-3 days until worms reached young adult stage. Data reported in this study used a combination of biological replicates generated in a genetics teaching lab by Washington State University undergraduate students, while other data was obtained in the research lab. The following strains were provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440): N2, wild type; WM27, *rde-1(ne2019)* V; NL2098, *rrf-1(pk1417)* I; NL3511, *ppw-1(pk1425)* I; VS26, *rde-10(hj20)* I; VS27, *rde-11(hj37)* IV; WM30, *rde-3(ne298)* I; WM119, *sago-2(tm894) ppw-1(tm914)* I; *C06A1.4(tm887) F58G1.1(tm1019)* II; *M03D4.6(tm1144)* IV; *sago-1(tm1195)* V; nels10 X; WM49, *rde-4(ne301)* III, HC196, *sid-1(qt9)* V. The WM49, *rde-1(ne300)* V strain was provided by Craig Mello.

Fatty acid analysis

To measure fatty acid composition, approximately 400 young adult stage worms (containing 0-8 embryos) were washed from feeding plates with water on ice and washed once to remove residual bacteria. After settling on ice again, as much water as possible was removed (~90%). Fatty acids were converted to methyl esters for analysis as previously described (Shi et al., 2013). The worm suspensions were incubated for 1 hour at 70°C in 2 ml of 2.5 % sulfuric acid in

bioRxiv preprint doi: https://doi.org/10.1101/283325; this version posted March 15, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

methanol. Following incubation, the reactions were stopped by adding 1ml of water and then mixed thoroughly with 200 µl of hexane to extract the resulting fatty acid methyl esters. We measured relative amount of fatty acid methyl esters by injecting 2 µl of the hexane layer onto an Agilent 7890 GC/5975C MS in scanning ion mode.

Statistics

At least three biological replicates were used for analysis, include one set that was analyzed by students in an advanced undergraduate genetics lab course. Within each biological experiment, samples were collected in triplicate. Data were analyzed by comparing the control to the RNAi knockdown using t-tests, which were corrected for multiple comparisons using the Holm-Sidak method with an alpha value of 0.05.

Determination of desaturation index and percent RNAi deficiency

Desaturation index for each strain was determined by comparing the relative peak areas of individual fatty acid methyl esters using the following formulas.

FAT-1 desaturation index

DI = (20:4n-3 +20:5n-3)/(20:3n-6 + 20:4n-6)

FAT-4 desaturation index

DI = (20:4n-6+20:5n-3)/(20:3n-6 + 20:4n-3)

Data and Reagent Policy

All *C. elegans* stocks and RNAi feeding clones are available upon request. File S1 contains all of the GC/MS data used to calculate the FAT-1 and FAT-4 desaturation indices.

bioRxiv preprint doi: https://doi.org/10.1101/283325; this version posted March 15, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Results

Using polyunsaturated fatty acid desaturation to assess RNAi efficiency

Fatty acids are the building blocks of the lipids that cells use for energy storage, membrane structure, and signaling. *C. elegans* obtains fatty acids from its bacterial diet, and also has the capacity to generate fatty acids *de novo* from acetyl-CoA (Watts and Ristow, 2017). Saturated fatty acids obtained from the diet or synthesized *de novo* are further modified by chain elongation and the addition of one or more double bonds, (Watts and Browse, 2002) greatly enhancing the structural diversity and utility of fatty acids available to the cell. Based on published RNA sequencing studies (Cao et al., 2017; Chikina et al., 2009) and on reporter strains constructed in our lab, we suspected that fatty acid desaturases are expressed in various tissues in *C. elegans*, with the highest expression in the intestine, the tissue responsible for absorption of dietary fats and modification of synthesized and dietary fats for incorporation into membrane lipids, and as well as for storage lipids contained in lipid droplets and yolk (Watts and Ristow, 2017). We reasoned that using tissue specific RNAi strains, and determination of fatty acid composition by GC/MS, we could establish the relative contributions of various tissues to overall fatty acid composition. Furthermore, this was an appropriate project to undertake in a laboratory setting, because students can perform the growth and RNAi treatment of *C. elegans* in the lab, as well as the fatty acid extractions for the GC/MS analysis.

We focused on two of the FA desaturase enzymes, FAT-1, an omega-3 desaturase, and FAT-4, a Δ5 desaturase (Watts and Browse, 2002). FAT-1 adds double bonds between the third and

bioRxiv preprint doi: https://doi.org/10.1101/283325; this version posted March 15, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

fourth carbons of a FA from the methyl end (omega-3), whereas the FAT-4 Δ5 desaturase adds double bonds between the fifth and sixth carbon counting from the carboxyl end of the fatty acid. **Figure 1** shows the fatty acid desaturation pathway, highlighting the substrates used by, and products synthesized by FAT-1 (**Figure 1A**) and FAT-4 desaturases (**Figure 1C**). *C. elegans* grown on bacteria expressing double stranded RNA complementary to *fat-1* or *fat-4* grow normally and reproduce, as do *fat-1* and *fat-4* mutants (Watts and Browse, 2002).

Loss of function mutations and RNAi treatment of *fat-1* and *fat-4* lead to reduced ratios of specific fatty acid products to substrates, a relationship that can be quantified as the desaturation index. When grown in lab conditions, on plates with *E. coli* HT115 bacteria, *C. elegans* typically shows a desaturation index of 5-6 corresponding to FAT-1 activity, meaning the abundance of omega-3 fatty acid products is 5-6 fold more than the omega-6 substrates. For FAT-4, the desaturation index is typically 2.5-3, meaning the Δ5 desaturated products are 2.5-3 fold more abundant than their substrates (see Materials and Methods for desaturase index calculations). In *fat-1* or *fat-4* null mutants, the corresponding desaturation indices drop to zero, because there are no omega-3 or delta-5 unsaturated PUFAs formed in the mutant strains. Feeding RNAi is a very efficient means to knock down FAT-1 and FAT-4 activity, and in a wild type background, *fat-1* RNAi causes a drop in the desaturation index from 5-6 to 0.2. Similarly, *fat-4* RNAi causes a drop in the desaturation index of 2.5-3 down to 0.1 (**Figure 1 and Table 1**). An RNAi-resistant strain treated with *fat-1* or *fat-4* RNAi would be expected to show a similar desaturation index as an untreated strain. **Figure 1B and 1D** show typical chromatographs of the wild type control strain, as well as a chromatograph of a strongly

bioRxiv preprint doi: https://doi.org/10.1101/283325; this version posted March 15, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

resistant mutant strain and a chromatograph of a strain that is only partially RNAi resistant when fed *fat-1* or *fat-4* RNAi.

The *rde-1 (ne219)* strain is only partially RNAi resistant. In a classroom setting, we sought to determine the extent of fatty acid desaturation in various tissues by using the RNAi-deficient strain *rde-1(ne219)*, as well as transgenic strains in which the *rde-1* gene was expressed under control of tissue-specific promotors in the *rde-1(ne219)* mutant background (Qadota et al., 2007). Interpretation of these types of tissue specific RNAi experiments relies on the absence of RNAi activity in the *rde-1* mutant strain. The students in the class found that the *rde-1(ne219)* strain, which contains a single glutamate to lysine substitution at a conserved residue, retained considerable RNAi silencing activity. The FAT-1 desaturation index fell from 6.0 to 2.6 during *fat-1* RNAi feeding treatment, while the FAT-4desaturation index fell from 2.5 to 0.8 during *fat-4* RNAi treatment (**Figure 2A, Table 1**). Students therefore could not interpret the data from the tissue-specific rescue strains, because of the remaining RNAi capacity of the *rde-1(ne219)* mutant strain. These results were confirmed with several additional biological replicates in a more highly-controlled setting of the research lab.

We obtained a second *rde-1* mutation, *rde-1(ne300)*, which is predicted to contain a premature stop codon within the protein's PIWI domain (Tabara et al., 1999). In contrast to the results with *rde-1(ne219)*, we found the *rde-1(ne300)* strain to be strongly RNAi deficient. Treatment with *fat-1* or *fat-4* RNAi did not significantly change desaturation index compared to treatment with empty vector control. Previous reports concluded that *rde-1 (ne219)* was highly resistant

bioRxiv preprint doi: https://doi.org/10.1101/283325; this version posted March 15, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

to RNAi against several genes when using lethality and fluorescence as indicators (Qadota et al., 2007; Tabara et al., 1999). Quantification of metabolite products of targeted enzymes, therefore, provides a more sensitive, quantitative method for assessing the RNAi efficiency of reduction-of-function mutations.

Germline and somatic specific RNAi

The apparent tissue-specificity of several different RNA-dependent RNA polymerases and Argonaute proteins enabled the popular method for delineating whether a gene is acting in somatic tissue or in the germ line in *C. elegans*. Knock down of a gene of interest in the background of *rrf-1*, which was reported to be deficient in RNAi in somatic cells (Sijen et al., 2001), has been compared with a knockdown in the *ppw-1* background, which is deficient in RNAi in the germ line (Tijsterman et al., 2002). Similar to the tissue-specific RNAi experiments described above, student's experiments seeking to quantify the relative contributions of somatic or germline activity of FAT-1 and FAT-4 desaturases were uninterpretable, because both strains high levels of RNAi efficiency. Remarkably, the strain carrying the *rrf-1(pk1417)* mutation retained RNAi activity capable of knocking down *fat-1* and *fat-4* nearly to the same extent as wild type **(Figure 2 B and Table 1)**. Several years ago, however, Kumsta and Hansen demonstrated that *rrf-1 (pk1417)* maintained RNAi capacity in the intestine and in the hypodermis (Kumsta and Hansen, 2012). In spite of their careful analysis and clear evidence of RNAi activity in somatic tissues of *rrf-1*, researchers continue to publish studies using *rrf-1* as a mutant lacking RNAi activity in somatic cells (Webster et al., 2017). Our expectation was that most FAT-1 and FAT-4 desaturation activity would be found in the somatic tissues of intestine

bioRxiv preprint doi: https://doi.org/10.1101/283325; this version posted March 15, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

and hypodermis, and we suspect that ample RNAi activity remained in the *rrf-1(pk1417)* mutants because this strain is not truly deficient in RNAi activity in intestinal and hypodermal tissues, supporting the findings of Kumsta and Hansen (Kumsta and Hansen, 2012).

Previously-described RNAi-resistant strains show varied susceptibility to RNAi feeding

Our findings that the *rde-1(ne219)* strain and the *rrf-1* strain were not RNAi-deficient as described led us to use our biochemical GC/MS method to quantitatively assess the degree of RNAi deficiency in other strains reported to be RNAi deficient. We again engaged undergraduate students in the genetics lab course to perform a screen, and findings were confirmed in the research lab. Teams of undergraduates performed the RNAi and GC/MS analysis of various RNAi-deficient mutants and then presented the background literature on their mutant, as well as their findings regarding the fatty acid composition after *fat-1* and *fat-4* RNAi treatment, and their calculations of the desaturation indices.

Students tested several known components of the exogenous RNAi pathway for resistance to RNAI against *fat-1* and *fat-4*. We found that *sid-1* mutants were resistant to RNAi of *fat-1* and *fat-4* induced by the feeding method, as expected (**Figure 2C and Table 1**). The RDE-4 strain was partially resistant to RNAi, as was the WM119 strain, which carries mutations in several Argonaute proteins, including SAGO-1 and SAGO-2 that are known to interact with secondary siRNA (Yigit et al., 2006). The strain carrying a mutation in the gene encoding the nucleotidyltransferase RDE-3 was also partially resistant to *fat-1* and *fat-4* RNAi. Neither *rde-10 (hj20),* nor *rde-11 (hj37)* were significantly RNAi resistant in our study (**Figure 2C and Table 1**).

bioRxiv preprint doi: https://doi.org/10.1101/283325; this version posted March 15, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

The range of desaturase activity measured in various strains demonstrated that our method is a precise way to measure the degree of RNAi-deficiency in *C. elegans* mutant strains.

bioRxiv preprint doi: https://doi.org/10.1101/283325; this version posted March 15, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Discussion

We have demonstrated that several previously isolated RNAi resistant mutants retain significant RNAi capacity against genes involved in fatty acid desaturation. Fatty acid desaturation activity is highly dependent on gene dosage, strains heterozygous for fatty acid desaturase mutations show desaturation indices that are intermediate between wild type and mutant (Watts and Browse, 2002). This allows a quantitative analysis of remaining desaturase activity after RNAi induction by feeding of the *fat-1* omega-3 desaturase gene or the *fat-4* Δ5 desaturase gene. Some mutant strains tested in this study carry point mutations in the gene of interest, and thus the resulting proteins may have some remaining enzymatic function. This demonstrates that our method of assessing RNAi deficiency could be useful for studying the impact of changing specific residues of proteins involved in exogenous RNAi. Of the strains we tested, only *rde-1 (ne219), rde-1 (ne300), rde-3 (298), rde-4 (ne301), sid-1 (qt9),* and the multiple secondary Argonaute mutant WM119 were resistant to RNA. And of those, only the null mutant *rde-1 (ne300)* and *sid-1(qt9)* were completely resistant to *fat-1* and *fat-4* RNAi.

We analyzed two mutant alleles of the Argonaute encoding gene, *rde-1*. The *rde-1* has been reported to be essential to the success of exogenous RNAi. The primary evidence being that the *rde-1 (ne219)* allele was completely resistant to *pos-1* RNAi, which normally causes lethality (Tabara et al., 1999). However, we provide evidence that the *ne219* allele retains significant RNAi processing capacity. The *ne219* allele contains a single base pair mutation causing a change from glutamate to lysine in the predicted PAZ RNA binding domain. The *ne219* strain must either retain function via residual RDE-1 function, or by bypassing RDE-1 through an

bioRxiv preprint doi: https://doi.org/10.1101/283325; this version posted March 15, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

unknown mechanism. However, the deletion allele, *rde-1 (ne300)* is completely resistant to RNAi against fatty acid desaturase genes, which suggests that RDE-1 function is indispensable. The *ne219* allele is was used as the RNAi deficient genetic background for tissue specific rescue of RNAi function, in which the *rde-1* gene was expressed under tissue specific backgrounds in the *rde-1(ne219)* mutant background (Qadota et al., 2007). This technique of tissue-specific RNAi has been cited in over 70 publications, including these recent studies (Chun et al., 2017; Han et al., 2017; Jeong et al., 2017; Liu et al., 2017; Shamalnasab et al., 2017). Our findings indicate that the results of these studies must be carefully interpreted, and future studies of gene knockdowns in specific tissues should not use the *rde-1(ne219*) background, because this strain is not completely RNAi deficient.

Evidence from RNAi sequencing and reporter gene constructs made in our lab indicate that *fat-1* and *fat-4* genes are expressed in multiple tissues, with highest expression in the intestine (Cao et al., 2017; Chikina et al., 2009). The RNA-dependent RNA polymerase RRF-1 was originally thought to be required for somatic RNAi in *C. elegans* (Sijen et al., 2001; Smardon et al., 2000). We found that *fat-1* and *fat-4* were efficiently knocked down in *rrf-1 (pk1417)*, to nearly wild-type levels. The *rrf-1 (pk1417)* contains a large deletion that eliminates a large conserved region of the protein (Sijen et al., 2001). The mutation is likely a null, which suggests that RRF-1 activity is not required for efficient RNAi in somatic tissues, including the intestine.

The question of how silencing is occurring in the intestine without RRF-1 has at least two possible answers. First, another RdRP could be functioning in the intestine. The *C. elegans*

bioRxiv preprint doi: https://doi.org/10.1101/283325; this version posted March 15, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

genome encodes four RdRPs including *rrf-1*, although only *rrf-1* and *ego-1* are known to act on exogenous dsRNA (Sijen et al., 2001; Smardon et al., 2000). A second explanation suggested by Kumsta and Hansen was that in the intestine the concentration of dsRNA, and thus primary siRNA may be high enough that amplification by RdRPs wouldn't be necessary for silencing. Interestingly, we found that mutants lacking either RDE-10 or RDE-11 were not significantly resistant to *fat-1* or *fat-4* RNAi. RDE-10 and RDE-11 form a complex that promotes siRNA amplification, which is further evidence that siRNA amplification is not required for efficient silencing in the intestine.

We found that the WM119 strain was partially resistant to *fat-1* and *fat-4* RNAi. WM119 contains mutations in several Argonaute proteins, including SAGO-1 and SAGO-2 that are known to interact with secondary siRNA (Yigit et al., 2006). If RdRP activity is not required for intestinal RNAi, then we would not expect that the SAGOs would be required either. However, it is possible that secondary siRNAs overwhelm the RNAi machinery, preventing silencing by primary siRNAs, but without the SAGOs, the secondary siRNAs produced by RdRP cannot be used for silencing.

Overall, this study proved to be a valuable exercise for undergraduate students. In addition to learning about the discoveries regarding the mechanisms of exogenous RNAi, students were exposed to inquiry-based primary research, and learned the value of appropriate controls and of quantitative methods for evaluating gene function. For their presentations, students read and interpreted primary literature, and integrated their findings with previous reports of RNAi-

bioRxiv preprint doi: https://doi.org/10.1101/283325; this version posted March 15, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

deficient mutant strains. While we were unable to answer our original research question regarding tissue contributions to overall fatty acid composition in *C. elegans*, our findings are valuable for researchers contemplating tissue-specific RNAi approaches in *C. elegans*.

Acknowledgements

We thank Washington State University students enrolled in MBioS402, Genetics Lab, during the years of 2014, 2015, and 2016 for generating some of the data presented in this study. We thank Craig Mello for the *rde-1(ne300)* strain. Some *C. elegans* strains were provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). Funding was provided by grants from the National Institutes of Health (T32GM083864 to JSW and R01DK074114 to JLW).

bioRxiv preprint doi: https://doi.org/10.1101/283325; this version posted March 15, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

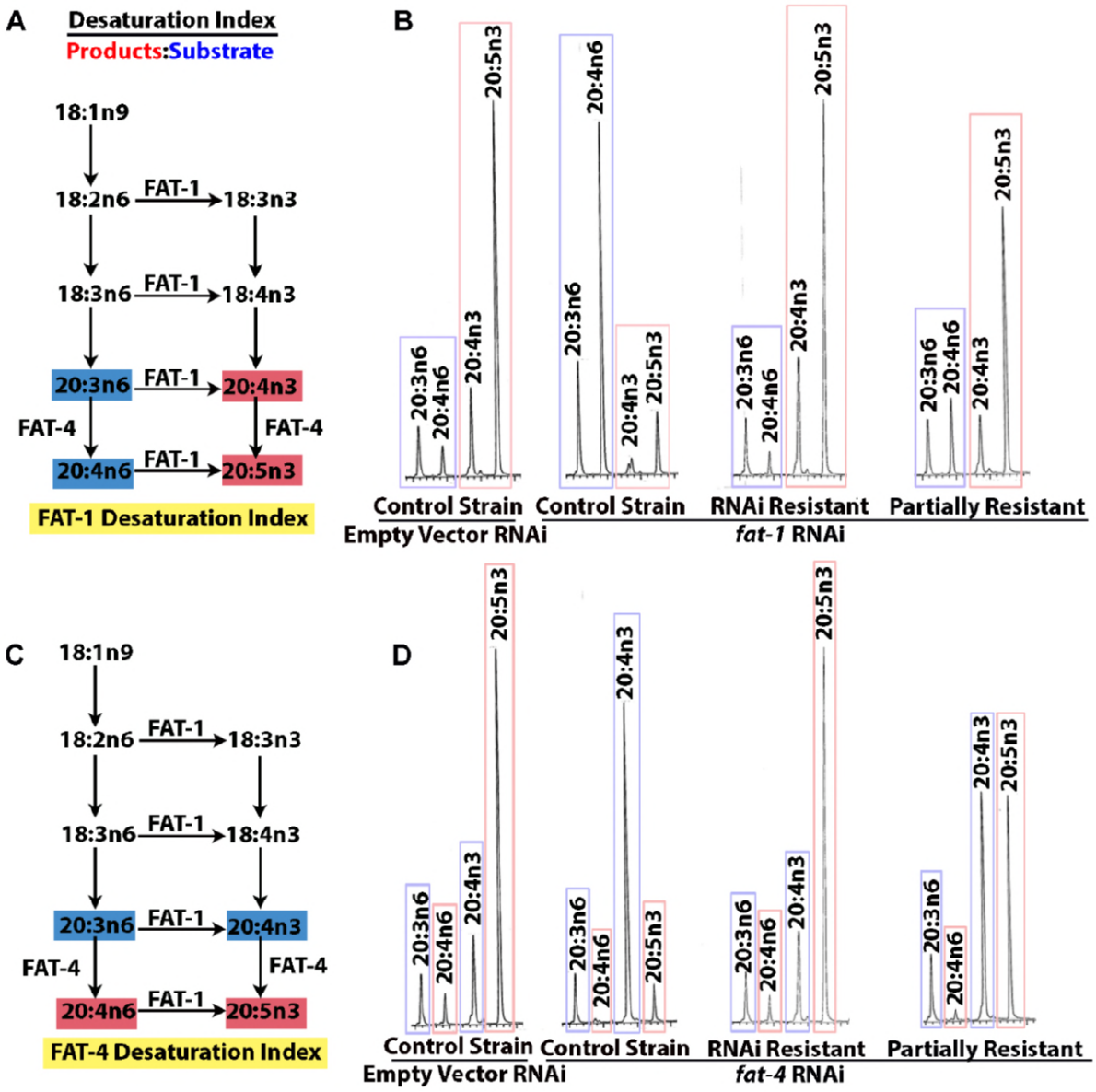

Figure 1. Determination of desaturation index. To measure RNAi deficiency in mutant worm strains, we knocked down the fatty acid desaturase genes *fat-1* and *fat-4* with RNAi by feeding and determined the ratio of fatty acid desaturation products (red) to their substrates (blue) and termed this the desaturation index for each desaturation reaction. The chromatographs demonstrate that in RNAi competent worms (control strain), treatment with RNAi against either *fat-1* (A) or *fat-4* (D) causes accumulation of substrates (blue) and depletion of products (red). Example chromatographs of and RNAi "resistant strain" show a high ratio of products to substrates (Desaturation index), similar to empty vector control treated worms, and chromatographs of the "partially resistant" strain have an intermediate desaturation index.

bioRxiv preprint doi: https://doi.org/10.1101/283325; this version posted March 15, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

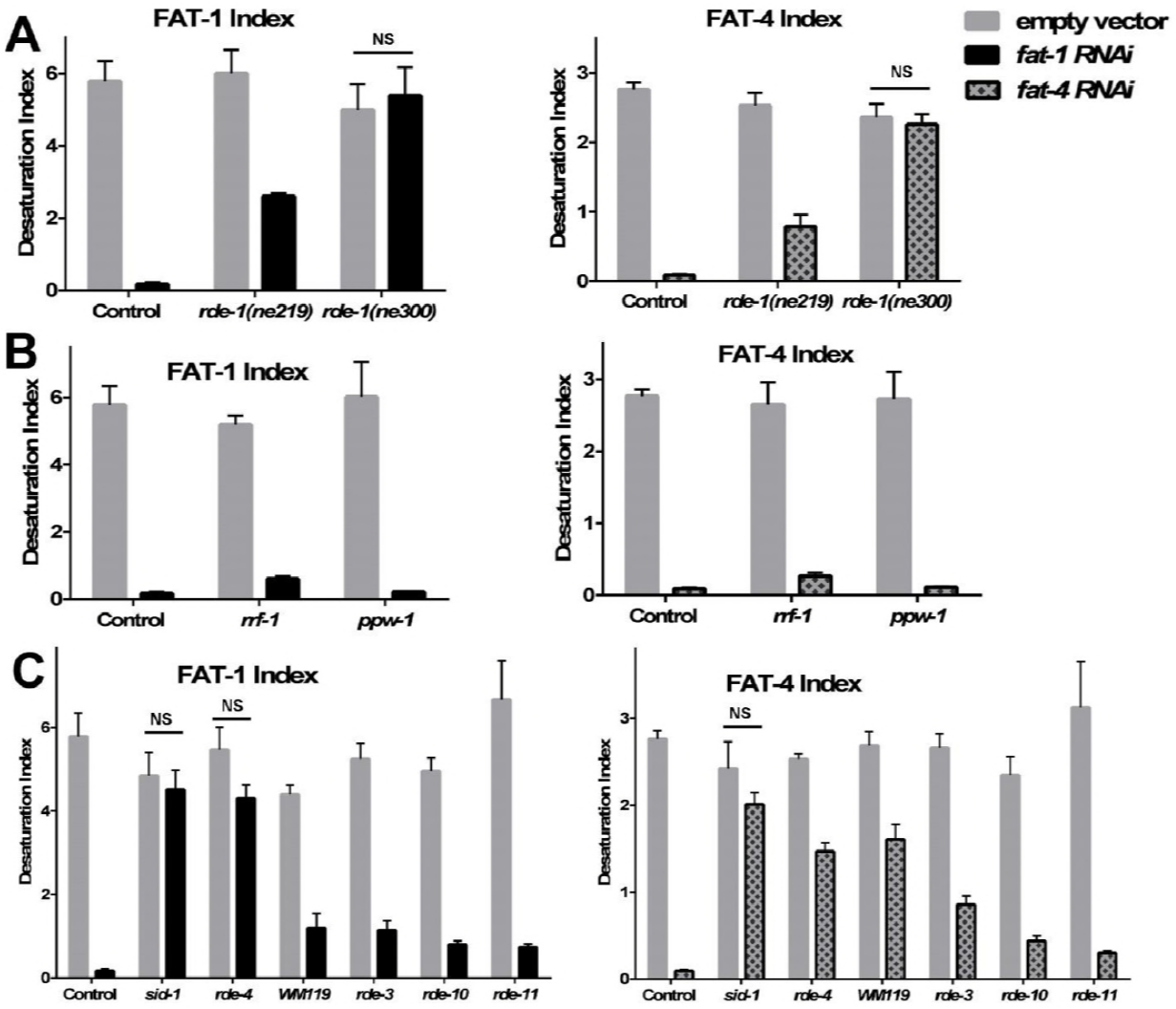

Figure 2. Desaturation indices for FAT -1 and FAT-4 of control (N2) and RNAi-deficient strains

FAT-1 and FAT-4 desaturation indices. (A) *rde-1 (ne219)* and *rde-1 (ne30*0) compared to control (N2) reveal that *rde-1 (ne219)* is partially resistant to feeding RNAi and *rde-1 (ne300)* is strongly RNAi resistant. (B) Germline-specific RNAi deficient (*ppw-1*) and somatic-specific RNAi deficient (*rrf-1*) strains both show nearly wild-type levels of RNAi efficiency. (C) Screen of RNAi-deficient strains showing RNAi deficiency (*sid-1* and *rde-4*), partial deficiency (WM119- MAGO, *rde-3*) and nearly normal RNAi efficiency (*rde-10* and *rde-11*) when treated with *fat-1* and *fat-4* RNAi by feeding. Comparisons of RNAi treatment to empty vector treatment showed statistically significant differences, except for comparisons of the strains depicted on the graphs with NS, not significant. The strains with no significant difference in desaturation index are considered to be completely RNAi resistant.

bioRxiv preprint doi: https://doi.org/10.1101/283325; this version posted March 15, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Table.1 Strains used in this study. Rows shaded in blue indicate strains with high RNAi susceptibility, as indicated by a large change in the desaturation indices. Rows shaded in green indicate strains with partial RNAi susceptibility, while strains shown in orange are RNAi deficient.

Strain	Gene (allele)	Protein/Function	Reported RNAi phenotype	Mutation	*fat-1* desat. index	*fat-4* desat. index
N2	wild type		susceptible	none	5.8->0.2	2.9->0.1
NL2098	*rrf-1(pk1417)*	RNA dependent polymerase	RNAi deficient in soma (Sijen et al., 2001)	400 bp deletion	5.2->0.6	2.7->0.3
NL3511	*ppw-1(pk1425)*	Argonaute/piwi	RNAi deficient in germline (Tijsterman et al., 2002)	1504 bp deletion	6.0->0.2	2.7->0.1
VS26	*rde-10(hj20)*	siRNA amplification	RNAi deficient (Yang et al., 2012)	premature stop codon	5.0->0.8	2.3->0.4
VS27	*rde-11(hj37)*	siRNA amplification	RNAi deficient (Yang et al., 2012)	premature stop codon	5.0->0.8	2.6->0.3
WM30	*rde-3(ne298)*	nucleotidyl transferase	RNAi deficient (Chen et al., 2005)	G366R	5.2->1.1	2.7->0.9
WM119	MAGO	multiple Argonautes	RNAi deficient (Yigit et al., 2006)	multiple	4.4->2.0	2.9->1.4
WM49	*rde-4(ne301)*	dsRNA binding protein	RNAi deficient (Tabara et al., 2002)	unknown	5.5->4.3	2.5->1.5
WM27	*rde-1(ne219)*	Argonaute/piwi	RNAi deficient (Tabara et al., 1999)	E414K	6.0->2.6	2.5->0.8
WM45	*rde-1(ne300)*	Argonaute/piwi	RNAi deficient (Tabara et al., 1999)	premature stop codon	5.6->6.0	2.4->2.3
HC196	*sid-1(qt9)*	dsRNA channel	RNAi deficient (Winston et al., 2002)	S536I	5.2->5.0	2.4->2.0

bioRxiv preprint doi: https://doi.org/10.1101/283325; this version posted March 15, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

References

Ashrafi, K., Chang, F.Y., Watts, J.L., Fraser, A.G., Kamath, R.S., Ahringer, J., and Ruvkun, G. (2003). Genome-wide RNAi analysis of Caenorhabditis elegans fat regulatory genes. Nature *421*, 268-272.

Cao, J., Packer, J.S., Ramani, V., Cusanovich, D.A., Huynh, C., Daza, R., Qiu, X., Lee, C., Furlan, S.N., Steemers, F.J.*, et al.* (2017). Comprehensive single-cell transcriptional profiling of a multicellular organism. Science *357*, 661-667.

Chen, C.C., Simard, M.J., Tabara, H., Brownell, D.R., McCollough, J.A., and Mello, C.C. (2005). A member of the polymerase beta nucleotidyltransferase superfamily is required for RNA interference in C. elegans. Curr Biol *15*, 378-383.

Chikina, M.D., Huttenhower, C., Murphy, C.T., and Troyanskaya, O.G. (2009). Global prediction of tissue-specific gene expression and context-dependent gene networks in Caenorhabditis elegans. PLoS Comput Biol *5*, e1000417.

Chun, H., Sharma, A.K., Lee, J., Chan, J., Jia, S., and Kim, B.E. (2017). The Intestinal Copper Exporter CUA-1 Is Required for Systemic Copper Homeostasis in Caenorhabditis elegans. J Biol Chem *292*, 1-14.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., and Mello, C.C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature *391*, 806-811.

Fraser, A.G., Kamath, R.S., Zipperlen, P., Martinez-Campos, M., Sohrmann, M., and Ahringer, J. (2000). Functional genomic analysis of C. elegans chromosome I by systematic RNA interference. Nature *408*, 325-330.

Grishok, A. (2005). RNAi mechanisms in Caenorhabditis elegans. FEBS Lett *579*, 5932-5939.

Hammond, S.M. (2005). Dicing and slicing: the core machinery of the RNA interference pathway. FEBS Lett *579*, 5822-5829.

Han, S., Schroeder, E.A., Silva-Garcia, C.G., Hebestreit, K., Mair, W.B., and Brunet, A. (2017). Mono-unsaturated fatty acids link H3K4me3 modifiers to C. elegans lifespan. Nature *544*, 185-190.

bioRxiv preprint doi: https://doi.org/10.1101/283325; this version posted March 15, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Jeong, D.E., Lee, D., Hwang, S.Y., Lee, Y., Lee, J.E., Seo, M., Hwang, W., Seo, K., Hwang, A.B., Artan, M.*, et al.* (2017). Mitochondrial chaperone HSP-60 regulates anti-bacterial immunity via p38 MAP kinase signaling. EMBO J *36*, 1046-1065.

Knight, S.W., and Bass, B.L. (2001). A role for the RNase III enzyme DCR-1 in RNA interference and germ line development in Caenorhabditis elegans. Science *293*, 2269-2271.

Kumsta, C., and Hansen, M. (2012). C. elegans rrf-1 mutations maintain RNAi efficiency in the soma in addition to the germline. PLoS One *7*, e35428.

Liu, F., Xiao, Y., Ji, X.L., Zhang, K.Q., and Zou, C.G. (2017). The cAMP-PKA pathway-mediated fat mobilization is required for cold tolerance in C. elegans. Sci Rep *7*, 638.

Parker, G.S., Eckert, D.M., and Bass, B.L. (2006). RDE-4 preferentially binds long dsRNA and its dimerization is necessary for cleavage of dsRNA to siRNA. RNA *12*, 807-818.

Parrish, S., and Fire, A. (2001). Distinct roles for RDE-1 and RDE-4 during RNA interference in Caenorhabditis elegans. RNA *7*, 1397-1402.

Qadota, H., Inoue, M., Hikita, T., Koppen, M., Hardin, J.D., Amano, M., Moerman, D.G., and Kaibuchi, K. (2007). Establishment of a tissue-specific RNAi system in C. elegans. Gene *400*, 166-173.

Shamalnasab, M., Dhaoui, M., Thondamal, M., Harvald, E.B., Faergeman, N.J., Aguilaniu, H., and Fabrizio, P. (2017). HIF-1-dependent regulation of lifespan in Caenorhabditis elegans by the acyl-CoA-binding protein MAA-1. Aging (Albany NY) *9*, 1745-1769.

Shi, X., Li, J., Zou, X., Greggain, J., Rodkaer, S.V., Faergeman, N.J., Liang, B., and Watts, J.L. (2013). Regulation of lipid droplet size and phospholipid composition by stearoyl-CoA desaturase. J Lipid Res *54*, 2504-2514.

Sijen, T., Fleenor, J., Simmer, F., Thijssen, K.L., Parrish, S., Timmons, L., Plasterk, R.H., and Fire, A. (2001). On the role of RNA amplification in dsRNA-triggered gene silencing. Cell *107*, 465-476.

bioRxiv preprint doi: https://doi.org/10.1101/283325; this version posted March 15, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Smardon, A., Spoerke, J.M., Stacey, S.C., Klein, M.E., Mackin, N., and Maine, E.M. (2000). EGO-1 is related to RNA-directed RNA polymerase and functions in germ-line development and RNA interference in C. elegans. Curr Biol *10*, 169-178.

Steiner, F.A., Okihara, K.L., Hoogstrate, S.W., Sijen, T., and Ketting, R.F. (2009). RDE-1 slicer activity is required only for passenger-strand cleavage during RNAi in Caenorhabditis elegans. Nat Struct Mol Biol *16*, 207-211.

Tabara, H., Sarkissian, M., Kelly, W.G., Fleenor, J., Grishok, A., Timmons, L., Fire, A., and Mello, C.C. (1999). The rde-1 gene, RNA interference, and transposon silencing in C. elegans. Cell *99*, 123-132.

Tabara, H., Yigit, E., Siomi, H., and Mello, C.C. (2002). The dsRNA binding protein RDE-4 interacts with RDE-1, DCR-1, and a DExH-box helicase to direct RNAi in C. elegans. Cell *109*, 861-871.

Tijsterman, M., Okihara, K.L., Thijssen, K., and Plasterk, R.H. (2002). PPW-1, a PAZ/PIWI protein required for efficient germline RNAi, is defective in a natural isolate of C. elegans. Curr Biol *12*, 1535-1540.

Timmons, L., and Fire, A. (1998). Specific Interference by ingested dsRNA. Nature *395*, 854.

Watts, J.L., and Browse, J. (2002). Genetic dissection of polyunsaturated fatty acid synthesis in Caenorhabditis elegans. Proc Natl Acad Sci U S A *99*, 5854-5859.

Watts, J.L., and Ristow, M. (2017). Lipid and Carbohydrate Metabolism in Caenorhabditis elegans. Genetics *207*, 413-446.

Webster, C.M., Pino, E.C., Carr, C.E., Wu, L., Zhou, B., Cedillo, L., Kacergis, M.C., Curran, S.P., and Soukas, A.A. (2017). Genome-wide RNAi Screen for Fat Regulatory Genes in C. elegans Identifies a Proteostasis-AMPK Axis Critical for Starvation Survival. Cell reports *20*, 627-640.

Winston, W.M., Molodowitch, C., and Hunter, C.P. (2002). Systemic RNAi in C. elegans requires the putative transmembrane protein SID-1. Science *295*, 2456-2459.

bioRxiv preprint doi: https://doi.org/10.1101/283325; this version posted March 15, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Winston, W.M., Sutherlin, M., Wright, A.J., Feinberg, E.H., and Hunter, C.P. (2007). Caenorhabditis elegans SID-2 is required for environmental RNA interference. Proc Natl Acad Sci U S A *104*, 10565-10570.

Yang, H., Zhang, Y., Vallandingham, J., Li, H., Florens, L., and Mak, H.Y. (2012). The RDE-10/RDE-11 complex triggers RNAi-induced mRNA degradation by association with target mRNA in C. elegans. Genes Dev *26*, 846-856.

Yigit, E., Batista, P.J., Bei, Y., Pang, K.M., Chen, C.C., Tolia, N.H., Joshua-Tor, L., Mitani, S., Simard, M.J., and Mello, C.C. (2006). Analysis of the C. elegans Argonaute family reveals that distinct Argonautes act sequentially during RNAi. Cell *127*, 747-757.

Zhuang, J.J., and Hunter, C.P. (2012). RNA interference in Caenorhabditis elegans: uptake, mechanism, and regulation. Parasitology *139*, 560-573.