New Strains for Tissue-Specific RNAi Studies in Caenorhabditis elegans

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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. 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. By measuring changes in the fatty acid products of the desaturase enzymes that synthesize PUFAs, 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 thus is more appropriate for construction of a robust tissue-specific RNAi strains. Using newly constructed strains in the rde-1(null) background, we found considerable desaturase activity in intestinal, epidermal, and germline tissues, but not in muscle. The RNAi-specific strains reported in this study will be useful tools for C. elegans researchers studying a variety of biological processes.

KEYWORDS fatty acid desaturase RNA interference

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 (Timmons and Fire 1998; Fraser et al. 2000; Ashrafi et al. 2003; 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 system-wide 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 dsRNA binding protein which forms a complex with Dicer to bind long dsRNA and cleave it into ~22 bp interfering RNAs (22G siRNAs) (Tabara et al. 2002; Knight and Bass 2001; Parker et al. 2006; Raman et al. 2017).

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 (Tabara et al. 1999; Parrish and Fire 2001; Steiner et al. 2009). 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 a large family of Argonaute proteins (Smardon et al. 2000; Sijen et al. 2001; Yigit et al. 2006; Xu et al. 2018). 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. 2019).

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; Yigit et al. 2006; Winston et al. 2002). 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.

Gene knockdown by RNAi is useful for elucidating gene function, but it does not provide information about the function of genes in specific tissues. To assess tissue specific gene function, investigators have employed tissue specific transgenic rescue of key RNAi machinery (Sijen et al. 2001; Qadota et al. 2007). 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 and it is also essential that the promoter is expressed only in specific cell types.

Our 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 two 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. We present here a collection of new strains for tissue-specific RNAi and report that intestine and epidermal tissues produce the highest amounts of desaturated fatty acids, while the germline also shows activity of omega-3 and delta-5 fatty acid desaturases.

**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 at 20°C or at 25°C (for *act-5* and *dpy-7* experiments) until worms reached young adult stage.

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(ne219)*; N2; NL2098, *rfe-1(pk1417)*; N2; NL3511, *ppw-1(pk1425)*; WM26, *rde-10(hb20)*; VS27, *rde-11(hb37)*; WM30, *rde-3(ne298)*; WM119, *sago-2(tm894)* *ppw-1(tm914)*; C60A4::*tm887* F58G1.1(*tm1019*); H10D12.2(*tm1144*); *sago-1(tm1195)*; nsl10 X; WM49, *rde-4(ne301)* III, H1C196, sid-1(*gt9*) V; WM118, *rde-1(ne300)* V, nsl9 [*myo-3::HA::RDE-1 + rol-6(*su1006)*]. The WM45, *rde-1(ne300)* V strain was provided by Craig Mello and the DCL569 strain was provided by Di Chen (Zou et al. 2019).

**Construction of new tissue-specific RNAi strains**

The transgenes *frs1I7* and *frs2I1* are single copy insertions on chromosome II (ttTi5605 location) of pNP160 (mtl-2p::RDE-1_3′rde-1 ttTi5605) and pSO21 (col-62p::RDE-1_3′rde-1 ttTi5605) respectively by CRISPR using a self-excisng cassette (SEC) (Dickinson et al. 2015). The strain pNP160 was obtained by insertion of the promoter of mtl-2 which is only expressed in the intestine into the pNP154 vector. pSO21 was obtained by insertion of the promoter of col-62 which is only expressed in the adult in the epidermis into the pNP154 vector. pNP154 was made from a vector containing the SEC for single insertion on Chromosome II at the position of ttTi5605 (pAP087, kindly provided by Ari Pani) (Taffoni et al. 2020). pNP160 and pSO21 were injected in *rde-1(ne300)* at 10 ng/μl together with pDD12 (orf-3p::Cas9) at 40 ng/μl, pCF190 (myo-2p::mCherry) at 2.5 ng/μl, pCF104 (myo-3p::mCherry) at 5 ng/μl, and #46168 (eef-1A.1p::CA39-SV40-NLS)3’Ibb-2 (Friendland et al. 2013) at 30 ng/μl. Non-fluorescent roller worms were selected then heat shocked to remove the SEC by FloxP as described in (Dickinson et al., 2015). The intestine-specific 1GI839 *rde-1(ne300)* V; *frs1I7[pNP160(mtl-2p::RDE-1_3′rde-1 ttTi5605) II; frs8I7[pl-nlp-29p::GFP, col-12p::DsRed] IV]* and the adult-skin-specific 1GI846 *rde-1(ne300)* V; *frs2I1[pSO21(col-62p::RDE-1_3′rde-1 ttTi5605) II; frs7I7[pl-nlp-29p::GFP, col-12p::DsRed] IV]* transgenic strains were subsequently obtained by conventional crosses and all genotypes were confirmed by PCR or sequencing. All constructs were made using Gibson Assembly (NEB Inc., MA) and confirmed by PCR or sequencing.

**Fatty acid analysis**

To measure fatty acid composition, approximately 1000 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 hr at 70°C in 2 ml of 2.5% sulfuric acid in 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 ontoto an Agilent 7890 GC-5975C MS in scanning ion mode.

**Worm size and GFP fluorescence analyses**

Size was measured as time of flight = TOF as arbitrary units and expression of the nlp-29p::gfp reporter was quantified with the COPAS Biosort (Union Biometrica). The ratio of GFP/size was then
calculated to normalize the fluorescence (Pujol et al. CB 2008). The results shown are representative of at least 3 independent experiments.

Statistics for desaturase index comparisons
At least 3 biological replicates were used for analysis. Within each biological experiment, samples were collected in triplicate. P values shown in Table S1 were determined by comparing the control empty vector 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
\[ DI = \frac{(20:4n-3 + 20:5n-3)}{(20:3n-6 + 20:4n-6)} \]

FAT-4 desaturation index
\[ DI = \frac{(20:4n-6 + 20:5n-3)}{(20:3n-6 + 20:4n-3)} \]

Data availability
Worm strains, data and reagents are available upon request. Accompanying the manuscript is one supplemental table, Table S1. Supplemental material available at figshare: https://doi.org/10.25387/g3.12968096.

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 (Chikina et al. 2009; Cao et al. 2017) we suspected that fatty acid desaturases are expressed in various tissues in C. elegans, especially 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.

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 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

Figure 1 Determination of desaturation index. RNAi efficiency was measured by the ability to knock down the fatty acid desaturase genes fat-1 and fat-4 with RNAi by feeding. We calculated the desaturation index for each treatment, defined as the ratio of fatty acid desaturation products (red) to their substrates (blue) (A and C). Representative chromatographs reveal that in RNAi competent worms (control strain), treatment with RNAi against either fat-1 (B) or fat-4 (D) causes accumulation of substrates (blue) and depletion of products (red) leading to a low desaturation index. Example chromatographs of an RNAi “resistant strain” show a similar desaturation indices as the empty vector control treated worms, while chromatographs of the “partially resistant” strain show intermediate activity.
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 null 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, wild type C. elegans typically shows a desaturation index of 4-6 corresponding to FAT-1 activity, meaning the abundance of omega-3 fatty acid products is 4-6 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 desaturation 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 (Watts and Browse 2002). 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 4-6 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 strongly resistant mutant strain and 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

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 promoters 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. We 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-4 desaturation index fell from 2.5 to 0.8 during fat-4 RNAi treatment (Figure 2A, Table 1). We 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.

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 to RNAi against several genes when using lethality and fluorescence as indicators (Tabara et al. 1999; Qadota et al. 2007). 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

| Table 1 Desaturation indices of strains used in this study |
|---------------------------------|----------------|----------------|---------------|
| Strain | Gene (allele) | Protein/Function | Reported RNAi phenotype | Mutation |
|--------|---------------|-----------------|-------------------------|---------|
| N2     | wild type     | RNA-dependent polymerase | susceptible | none |
| NL298  | rde-1          | Argonaute/Piwi   | RNAi-deficient         | 400 bp deletion |
| NL3511 | rde-1          | Argonaute/Piwi   | RNAi-deficient in soma | 1004 bp deletion |
| VS26   | rde-1          | Argonaute/Piwi   | RNAi-deficient in germline | |
| VS27   | rde-1          | Argonaute/Piwi   | RNAi-deficient | |
| WM20   | MAGO           | dsRNA binding protein | RNAi-deficient | multiple |
| WM49   | rde-1          | Argonaute/Piwi   | RNAi-deficient | |
| WM45   | rde-1          | Argonaute/Piwi   | RNAi-deficient | |
| HC196  | siRNA channel | dsRNA channel | RNAi-deficient | |

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| VS27   | rde-1          | Argonaute/Piwi   | RNAi-deficient | |
| WM20   | MAGO           | dsRNA binding protein | RNAi-deficient | multiple |
| WM49   | rde-1          | Argonaute/Piwi   | RNAi-deficient | |
| WM45   | rde-1          | Argonaute/Piwi   | RNAi-deficient | |
| HC196  | siRNA channel | dsRNA channel | RNAi-deficient | |

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| VS27   | rde-1          | Argonaute/Piwi   | RNAi-deficient | |
| WM20   | MAGO           | dsRNA binding protein | RNAi-deficient | multiple |
| WM49   | rde-1          | Argonaute/Piwi   | RNAi-deficient | |
| WM45   | rde-1          | Argonaute/Piwi   | RNAi-deficient | |
| HC196  | siRNA channel | dsRNA channel | RNAi-deficient | |
for delineating whether a gene is acting in somatic tissue or in the germ line in *C. elegans*. Knockdown 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, experiments seeking to quantify the relative contributions of somatic or germline activity of FAT-1 and FAT-4 desaturases were uninterpretable, because both strains showed 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 2B 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 and epidermis, 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). Recent studies demonstrated that *EGO-1*, an RNA-dependent RNA polymerase that acts in the germline, also functions in intestinal tissue, and therefore acts in the RNAi pathway in the absence of *RRF-1* (Ravikumar *et al.* 2019).

**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 performed the RNAi and GC-MS analysis of various RNAi-deficient mutants using empty vector, *fat-1*, and *fat-4* RNAi and calculated the desaturation indices. 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).

**Figure 2** Desaturation indices for FAT-1 and FAT-4 of control (N2) and RNAi-deficient strains. (A) *rde-1(ne219)* and *rde-1(ne300)* 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 (MAGO and *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 (P values reported in Table S1), 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.
The RDE-4 strain was partially resistant to RNAi, as was the multiple Argonaute (MAGO) 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). 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.

Construction of new tissue-specific RNAi strains for intestine- and epidermis-specific RNAi

In order to determine the extent of fatty acid desaturation in various tissues, we needed to obtain or construct new strains with the rde-1(null) background. For intestine-specific RNAi, we used the mtl-2 promoter to rescue rde-1 in the rde-1(ne300) strain (Pujol et al. 2008). For epidermis-specific RNAi, we used the col-62 promoter, which is expressed from the L4 stage onward, to rescue rde-1 in the rde-1(ne300) strain (Taffoni et al. 2020). To confirm the intestine-specific RNAi knockdown, we used RNA corresponding to the act-5 gene, which encodes an essential actin known to be expressed in the intestine (MacQueen et al. 2005). When subjected to act-5 RNAi, only the wild-type and the intestine-specific strain arrested their development, while the epidermal-specific strain reached adulthood to the same extent as the rde-1 resistant strain (Figure 3A). To confirm the tissue-specificity of these promoters, we used a transgenic strain containing the nlp-29p::GFP reporter, which is activated in the epidermis upon cuticle disruption (Pujol et al. 2008), including dpy-7 inactivation (Dodd et al. 2018). Only the epidermal-specific and the wild-type strains present a reduced size and an activation of nlp-29p::GFP, while the intestinal-specific strain was similar to the rde-1 resistant strain (Figure 3B). These results confirm the specificity of the intestinal- and epidermal-specific RNAi strains.

Strains for germline- and muscle-specific RNAi

We obtained muscle-specific and germline specific RNAi strains, both of which were made in the rde-1(null) background. The germline-specific strain DCL569 uses a single-copy insertion of a transgene expressing rde-1 under control of the sun-1 promoter (Zou et al. 2019). The muscle-specific strain WM118 expresses rde-1 under control of the myo-3 promoter. We used RNAi constructs corresponding to egg-5, dpy-10, fat-7, and pat-4 to test the phenotypic specificity of the tissue-specific RNAi strains. As expected, we observed sterility after egg-3 knockdown primarily in the wild type and germline-specific RNAi strain. We observed paralyzed worms in wild type and in the muscle-specific RNAi strain after treatment with pat-4 and unc-112 RNAi (Table 2).

Desaturase activity occurs in intestine, epidermal, and germline tissues

We performed fat-1 and fat-4 RNAi on wild type, rde-1(ne300), and the tissue-specific RNAi strains and determined the desaturase indices using GC-MS. We found that RNAi activity in intestinal, epidermal and germline tissues partially reduces the desaturation index compared to RNAi performed in rde-1(null) animals, while muscle-specific RNAi showed no detectable change in the desaturation index compared to the rde-1(null) control (Figure 4A-B).

These experiments reveal the highest desaturase activity in intestinal tissues, with detectable desaturase activity in the germline and in the epidermis, but not in muscle.

DISCUSSION

This study used tissue-specific RNAi paired with a highly quantitative method to determine the tissues in C. elegans that are most active in fatty acid desaturation. Importantly, we 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

Figure 3 New strains for intestine- and epidermis-specific RNAi knockdown. (A) The intestine-specific RNAi strain shows developmental arrest on act-5(RNAi) like the wild-type strain, while rde-1 null (rde-1(ne300)) and the adult skin-specific RNAi strain reach adult 48 h after transferring synchronized L1 larvae onto RNAi plates at 25°C. (B) and (C) Similar to the wild-type, the adult skin-specific RNAi strain becomes shorter (B) and presents a high expression of nlp-29 in the epidermis (C) on dpy-7(RNAi), compared to rde-1 null and intestine-specific RNAi strain; the phenotype is observed as in (A) 48 h after transferring synchronized L1 larvae onto RNAi plates at 25°C. Individual data points are shown, with bars depicting the mean and SEM.
Table 2  Efficacy of tissue-specific RNAi strains

| Tissue | Gene | Phenotype | # worms | Stadium, embryo death | Short and stout | Intestine fat-7 | Unc-112 paralyzed | Pat-4 paralyzed |
|--------|------|-----------|---------|----------------------|----------------|----------------|------------------|----------------|
| Germline | rde-1 | Wild type | 100 | 90% | 97.4 | 95.2 |
| | rde-1 | RNAi deficient | 92 | 92 | 97.4 | 95.2 |
| | rde-1 (ne300) | DCL569; rde-1(-) | 99 | 99 | 97.4 | 95.2 |
| | rde-1 (ne300) | IG1839; rde-1(ne300) | 99 | 99 | 97.4 | 95.2 |
| | rde-1 (ne300) | IG1846; rde-1(ne300) | 99 | 99 | 97.4 | 95.2 |
| | rde-1 (ne300) | (ne300) | 99 | 99 | 97.4 | 95.2 |
| | rde-1 (ne300) | :: rde-1(+) | 99 | 99 | 97.4 | 95.2 |
| | rde-1 (ne300) | sun-1p :: rde-1(+) | 99 | 99 | 97.4 | 95.2 |
| | rde-1 (ne300) | mtl-2p :: rde-1(+) | 99 | 99 | 97.4 | 95.2 |
| | rde-1 (ne300) | col-62p :: rde-1(+) | 99 | 99 | 97.4 | 95.2 |

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 unknown mechanism. The deletion allele, rde-1 (ne300) is, in contrast, completely resistant to RNAi against fatty acid desaturase genes, which suggests that RDE-1 function is indispensable. The ne219 allele was used as the RNAi deficient genetic background for tissue specific rescue of RNAi function (Qadota et al. 2007). This technique of tissue-specific RNAi has been cited in over 70 publications, including these recent studies (Shamalnasab et al. 2017; Liu et al. 2017; Jeong et al. 2017; Chun et al. 2017; Han 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 studies indicate that fat-1 and fat-4 genes are expressed in multiple somatic tissues, including epidermis and intestine (Chikina et al. 2009; Cao et al. 2017). The RNA-dependent RNA polymerase RRF-1 was originally thought to be required for somatic RNAi in C. elegans (Smardon et al. 2000; Sijen et al. 2001). We found that fat-1 and fat-4 were efficiently knockdown 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.

There are at least two possible explanations for how silencing in the intestine without RRF-1 is occurring. First, the EGO-1 RdRP has been shown to function in the intestine, substituting for RRF-1 (Ravikumar et al. 2019). 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 of desaturase genes.

We found that the MAGO strain was partially resistant to fat-1 and fat-4 RNAi. The MAGO strain contains mutations in six 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 overwhelm the RNAi machinery, preventing SAGOs would be required either. However, it is possible that is not required for intestinal RNAi, then we would not expect that the SAGOs, the secondary siRNAs produced by RdRP cannot be used for silencing. By primary siRNAs, but without the SAGOs, the secondary siRNAs overwhelm the RNAi machinery, preventing SAGOs would be required either. However, it is possible that is not required for intestinal RNAi, then we would not expect that the SAGOs, the secondary siRNAs produced by RdRP cannot be used for silencing.

Several tissue-specific RNAi strains have been constructed by others that do not rely on the tissue-specific rescue of rde-1 in the rde-1 mutant background. For example, strains have been made in the sid-1(p3321) mutant background that rescue sid-1 using neuronal, muscle, and intestine-specific promoters (Miles and van Oosten-Hawle 2020; Calixto et al. 2010). We used existing muscle- and germline- specific RNAi strains and constructed new intestine and epidermal-specific strains all made in a rde-1(null) background. With these strains we were able to determine the extent of omega-3 and Δ5 desaturation in various tissues in C. elegans. The lack of RNAi activity in the rde-1(ne300) treated with fat-1RNAi and fat-4RNAi indicates that the rde-1(null) background is appropriate for tissue-specific RNAi experiments.

For both desaturases, we found high desaturase activity in intestinal tissues, as well as significant desaturase activity in the epidermis and the germ line, but not in muscle tissues. Single-cell RNA seq studies reveal fat-1 and fat-4 transcripts in epidermal and germ cells (Chikina et al. 2009; Cao et al. 2017). In agreement with this, the quantitative tissue-specific method reveals significant expression of desaturase activity in epidermal and germline. Lipid metabolism in the epidermis has recently been recognized as influencing system-wide physiological responses (Kruse et al. 2017). Thus C. elegans fatty acid desaturation efficiency in skin cells could influence barrier formation and cuticle development. Similarly, PUFA synthesis in the germline is important for the proper development of the egg shell permeability barrier (Watts et al. 2018). A limitation of our study was that we did not test neuronal-specific RNAi. Neuronal desaturation could contribute to the overall pool of PUFAs in the worm, since promoter GFP fusion experiments demonstrated expression of fat-1 and fat-4 in several types of neurons (Vásquez et al. 2014).

Our findings emphasize that caution must be used in interpreting previous tissue-specific RNAi studies using the rde-1(ne219) and rrf-1 strains. Similarly, researchers should also be aware that the tissue-specific promoters may also exhibit a low level of expression in other tissues, which may explain the slight leakiness in the DCL569 strain. Expression in unexpected tissues was observed with several neuronal promoters expressed from multicopy arrays (Radetskaya et al. 2019). Transgenic approaches manipulating CRISPR-Cas9 activity in a tissue-specific manner (Shen et al. 2014) or using conditional protein degradation methods may be more precise approaches for tissue-specific knockdown experiments in C. elegans (Nance and Frokjaer-Jensen 2019).

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Figure 4 Desaturation indices for wild type, RNAi-deficient (rde-1(ne300)) and tissue-specific RNAi strains. For both fat-1 RNAi (A) and fat-4 RNAi (B), the greatest reduction of RNAi activity occurred in the intestine-specific RNAi strain, revealing intestine as the major tissue of fatty acid desaturation. Comparisons of RNAi treatment to empty vector treatment showed statistically significant differences (P values reported in Table S1), except for comparisons of the strains depicted on the graphs with NS, not significant.
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