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Establishment of a tissue-specific RNAi system in *C. elegans*

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Abstract

In *C. elegans*, mosaic analysis is a powerful genetic tool for determining in which tissue or specific cells a gene of interest is required. For traditional mosaic analysis, a loss-of-function mutant and a genomic fragment that can rescue the mutant phenotype are required. Here we establish an easy and rapid mosaic system using RNAi (RNA mediated interference), using a *rde-1* mutant that is resistant to RNAi. Tissue-specific expression of the wild type *rde-1* cDNA in *rde-1* mutants limits RNAi sensitivity to a specific tissue. We established hypodermal-and muscle-specific RNAi systems by expressing *rde-1* cDNA under the control of the *lin-26* and *hlh-1* promoters, respectively. We confirmed tissue-specific RNAi using two assays: (1) tissue-specific knockdown of GFP expression, and (2) phenocopy of mutations in essential genes that were previously known to function in a tissue-specific manner. We also applied this system to an essential gene, *ajm-1*, expressed in hypodermis and gut, and show that lethality in *ajm-1* mutants is due to loss of expression in hypodermal cells. Although we demonstrate tissue-specific RNAi in hypodermis and muscle, this method could be easily applied to other tissues.

Keywords: Muscle; Hypodermis; Mutant; Mosaic; Phenotype

1. Introduction

The expression pattern of a gene of interest implicates the function of that gene in specific cells or tissues. In the case of a gene that is expressed in a complicated or broad pattern, however, it is difficult to determine the specific requirements for that gene in a particular tissue or subset of cells, due to potential defects arising from neighboring tissues.

In *C. elegans*, mosaic analysis is a powerful genetic tool for determining in which tissues or specific cells a gene of interest is required (Hedgecock and Herman, 1995; Herman, 1995). Originally, before the use of transgenic lines, a chromosomal duplication was used for mosaic analysis. More recently, however, a transgenic approach has been taken to produce mosaics. In this procedure, a genomic fragment rescuing a loss-of-function mutation is injected into mutant worms. The injected DNA forms an extrachromosomal array, which is heritably transmitted to subsequent generations. Occasionally, this extrachromosomal array is not segregated into one of the daughter cells during mitosis, resulting in the production of daughter cells that lack the rescuing array. Using this property of extrachromosomal arrays, mosaic animals can be obtained. Several markers for mosaic analysis exist, such as *ncl-1* (Hedgecock and Herman, 1995) and *sur-5*::NLS::GFP (Yochem

Abbreviations: RNAi; RNA mediated interference; GFP; green fluorescent protein; NLS; nuclear localization signal; dsRNA; double stranded RNA.

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et al., 1998). In the case of the *ncl-1* system, the genotype of each cell can be assessed by the size of nucleoli, since *ncl-1* mutants have enlarged nucleoli. In the case of *sur-5*::NLS:GFP, the presence of the extrachromosomal array in a given cell can be easily assessed by nuclear GFP fluorescence. In both systems, a loss-of-function mutant and a genomic fragment that can rescue the mutant phenotype are required. As an alternative to these methods, rescue of a mutant phenotype can be attempted by driving expression of the wild-type gene using cell/tissue specific promoters. Even in this system, a mutant in the gene of interest and a cloned genomic fragment are required.

Here we describe a mosaic system using RNAi (RNA-mediated interference) and a *rde-1* mutant that is resistant to RNAi (Tabara et al., 1999). In *C. elegans*, RNAi is widely used for rapid and convenient knockdown of most genes of interest (Fire et al., 1998). We show that tissue-specific expression of wild-type *rde-1* in *rde-1* mutant worms confers RNAi sensitivity only in the tissues expressing *rde-1*. Since many tissue-specific promoters are available for *C. elegans*, this system will be applicable to many tissues or selected cells.

2. Materials and methods

2.1. Strains and worm procedure

Nematodes were grown at 20 °C on NGM agar plates with *Escherichia coli* strain OP50 (Brenner, 1974). We used Bristol N2 as wild type and WM27 (*rde-1 (ne219) V*) and KW1309 (*rde-1 (ne219) dpy-11 (e224) V*) as the *rde-1* mutant (Tabara et al., 1999). The genotypes of strains used in this study are:

NR220 (*kzIs7*[pKK1260(*lin-26p::nls::gfp*), pRF4(*rol-6* marker)]);
NR221 (*rde-1(ne219) V; kzIs8*[pKK1260(*lin-26p::nls::gfp*), pRF4(*rol-6* marker)]);
NR222 (*rde-1(ne219) V; kzIs9*[pKK1260(*lin-26p::nls::gfp*), pKK1253(*lin-26p::rde-1*), pRF4(*rol-6* marker)]);
NR230 (*kzIs17*[pPD93.97(*myo-3p::nls::gfp*), pRF4(*rol-6* marker)]);
NR225 (*rde-1(ne219) V; kzIs12*[pPD93.97(*myo-3p::nls::gfp*), pRF4(*rol-6* marker)]);
NR229 (*rde-1(ne219) V; kzIs16*[pPD93.97(*myo-3p::nls::gfp*), pKK1253(*lin-26p::rde-1*), pRF4(*rol-6* marker)]);
NR320 (*rde-1 (ne219) V; kzEx320*[pDM#715(*hlh-1p::rde-1*), pTG96(*sur-5p::nls::gfp*)]);
NR321 (*rde-1(ne219) V; kzEx321*[pKK1253(*lin-26p::rde-1*), pTG96(*sur-5p::nls::gfp)*]);
NR350 (*rde-1(ne219) V; kzIs20*[pKK1253(*lin-26p::rde-1*), pTG96(*sur-5p::nls::gfp)*]).

NR220 and NR221 have an integrated array containing pKK1260 (*lin-26p::nls::gfp*, 10 μg/ml) and pRF4 (*rol-6* dominant marker, 100 μg/ml) (Mello et al., 1991) in the wild-type or *rde-1* mutant background, respectively. NR222 has an integrated array containing pKK1253 (*lin-26p::rde-1*, 10 μg/ml), pKK1260 (*lin-26p::nls::gfp*, 10 μg/ml) and pRF4 (*rol-6* dominant marker, 100 μg/ml) in the *rde-1* mutant background. NR230 and NR225 have an integrated array containing pPD93.97 (*myo-3::nls::gfp*, 10 μg/ml) and pRF4 (*rol-6* dominant marker, 100 μg/ml) in the wild-type or *rde-1* mutant background, respectively. NR229 has an integrated array containing pKK1253 (*lin-26p::rde-1*, 10 μg/ml), pPD93.97 (*myo-3::nls::gfp*, 10 μg/ml) and pRF4 (*rol-6* dominant marker, 100 μg/ml) in the *rde-1* mutant background. NR320 and NR321 have extrachromosomal arrays containing pDM#715 (*hlh-1p::rde-1*, 10 μg/ml) or pKK1253 (*lin-26p::rde-1*, 10 μg/ml) with pTG96 (*sur-5p::nls::gfp*, 100 μg/ml) as a marker in the *rde-1* mutant background. NR350 have integrated arrays containing pDM#715 (*hlh-1p::rde-1*, 10 μg/ml) with pTG96 (*sur-5p::nls::gfp*, 100 μg/ml) as a marker in the *rde-1* mutant background. Injection of plasmids into worms was performed as described (Mello and Fire, 1995). The dominant Rol marker (pRF4) (Mello and Fire, 1995) or GFP marker (pTG96) (Yochem et al., 1998) were used as transformation markers. Integration of extrachromosomal arrays was done by the UV irradiation method (Mitani, 1995).

2.2. Molecular biology

RNAi was performed as described (Fire et al., 1998). The procedure for feeding RNAi was described previously (Mercer et al., 2006). To synthesize double-stranded RNA (dsRNA) for RNAi experiments, we used the Large-scale T7 RNA Transcription Kit (Novagen Inc, Wisconsin). We used PCR products derived from plasmids harboring cDNAs as templates for synthesizing dsRNA. For synthesizing dsRNA for GFP, *hlh-1*, and *ajm-1*, we used PCR products amplified from pKK1351 (GFP cDNA in pBluescript KS+), yk523e12 (cDNA EST clone containing *hlh-1*), and yk28c11 (cDNA EST clone containing *ajm-1*) (yk clones are gifts from Dr. Yuji Kohara) using primers CMo24 (5′-TTG TAA AAC GAC GGC CAG-3′) and CMo422 (5′-GCG TAA TAC GAC TCA CTA TAG GGA ACA AAA GCT GGA GCT-3′). dsRNA for *lin-26* was produced by using PCR product amplified from H13–4 (RB2 cDNA clone containing *lin-26*) (Tsuboi et al., 2002) with primers T7-GAD (5′-GCG TAA TAC GAC TCA CTA TAG GGC AAA CCC AAA AAA AGA GAT C-3′) and GAD-T7 (5′-CGC TAA TAC GAC TCA CTA TAG GGG TTG AAG TGA ACT TGC GCG G-3′).

To make pKK1351, the *Sac* I/*Eco*R I fragment (GFP cDNA) of pPD95.67 (a plasmid for construction of GFP fusion, a gift from Dr. Andy Fire) was cloned into pBluescript SK+ . pKK1260 contains 3.7 kb upstream sequence of *lin-26* (den Boer et al., 1998) derived from the F18A1 cosmid and NLS-GFP cDNA from pPD95.67. For making pKK1253, *rde-1* cDNA derived from yk296b10 (cDNA EST clone containing *rde-1*, a gift from Dr. Yuji Kohara) was cloned downstream of the 3.7 kb upstream promoter sequence of *lin-26*. For expression of NLS-GFP in muscle cells, we used pPD93.97 (a gift from Dr. Andy Fire). pDM#715 was made by cloning of a fragment of yk296b10 (*rde-1* cDNA) into pPD52.99 (a plasmid for expression under the control of *hlh-1* promoter, a gift from Dr. Andy Fire). A plasmid for RNAi by feeding for the *unc-98* gene was described previously (Mercer et al., 2006).

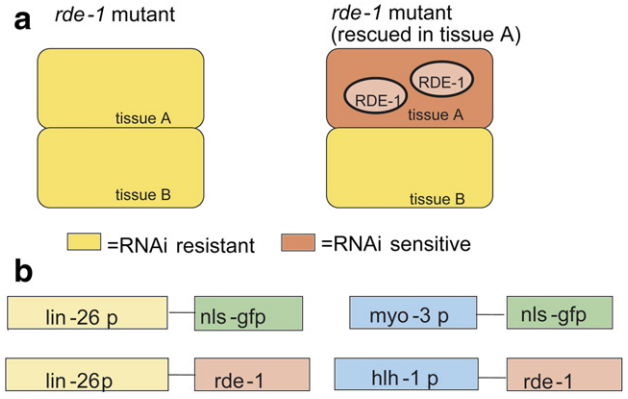

Fig. 1. Strategy of tissue-specific RNAi using the *rde-1* mutant a: Yellow colour shows RNAi resistant cells. Red colour shows RNAi sensitive cells. Expression of *rde-1* in tissue A should result in RNAi sensitivity in tissue A, but not tissue B. b: Plasmids used in this study. The *lin-26* promoter (3.7 kb 5′ sequence) was placed upstream of NLS-GFP or *rde-1*, resulting in hypodermis-specific NLS-GFP or *rde-1* expression. The *myo-3* promoter (2.4 kb 5′ sequence) was fused to NLS-GFP, resulting in a muscle-specific NLS-GFP expressing plasmid (pPD93.97). The *hlh-1* promoter (3.0 kb 5′ sequence) was fused to *rde-1*, resulting in a muscle-specific *rde-1* expressing plasmid.

2.3. Staining and microscopy

Immunostaining was performed as described (Soto et al., 2002) using MH27 monoclonal antibodies, which specifically recognizes the AJM-1 protein (Francis and Waterston, 1985; Koppen et al., 2001). Estimation of GFP fluorescence and phenotype of embryos was performed by microscopy 24 h after injection of each dsRNA. The procedure for polarized light microscopy was described previously (Mercer et al., 2006).

3. Results

3.1. Tissue specific expression of rde-1

rde-1 mutant worms are reported to be resistant to RNAi, and *rde-1* functions in a cell autonomous fashion (Tabara et al., 1999). We reasoned that expression of wild-type *rde-1* in a specific tissue in *rde-1* mutants should rescue susceptibility to RNAi only in that tissue (Fig. 1 a). Initially, we chose hypodermis as a model tissue. For hypodermal expression of *rde-1*, we used the *lin-26* promoter (den Boer et al., 1998). LIN-26 is an essential transcription factor required for the differentiation of hypodermis and is expressed specifically in the hypodermis beginning in mid embryogenesis and continuing to adulthood. We expressed the *rde-1* cDNA under the control of a *lin-26* hypodermal-specific promoter element (Labouesse et al., 1996) in *rde-1* mutant worms (four constructs are shown in Fig. 1 b). Worms harboring an extrachromosomal array containing this construct displayed no defects in growth or movement. To avoid mosaic expression of the *lin-26p::rde-1* construct, we prepared worms containing integrated arrays. To test hypodermis-specific rescue of *rde-1*, we chose muscle as a negative control tissue (see Sections 3.2 and 3.3).

3.2. Examination of tissue specificity using GFP markers

To examine the tissue specificity of RNAi in *rde-1* mutant worms expressing the wild type *rde-1* gene in the hypodermis, we conducted two assays. The first assay utilized GFP markers expressed specifically in either hypodermis or muscle using the plasmids shown in Fig. 1 b. These plasmids contain NLS-GFP under the control of the *lin-26* promoter for hypodermal expression (den Boer et al., 1998) or the *myo-3* promoter for muscle expression (Okkema et al., 1993). We confirmed hypodermal or muscle-specific GFP expression in the nucleus in both cases (data not shown). We co-injected *lin-26p::rde-1* DNA together with either *lin-26p::nls::gfp* or *myo-3p::nls::gfp* plasmids, created transgenic lines, and then integrated these extrachromosomal arrays into the genome. Worm strains used in this assay (Fig. 2) have the following characteristics. In the NR220, NR221, and NR222 strains, the GFP marker is expressed in the hypodermis, and in NR230, NR225, and NR229, the GFP marker is expressed in muscle. NR220 and NR230 are wild type strains, in which RNAi is effective in all tissues, including hypodermis and muscle. NR221 and NR225

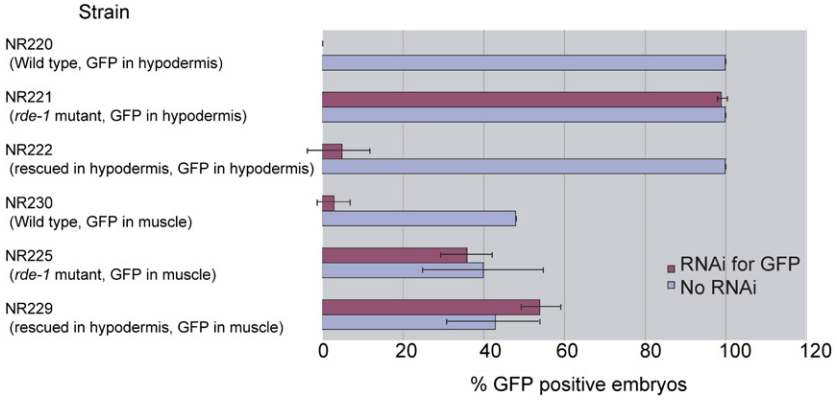

Fig. 2. Estimation of tissue specific RNAi system using GFP markers "Strain" shows the worm strain used for each assay with its properties. Purple bar represents percentage of GFP positive embryos after RNAi for GFP. Blue bar represents percentage of GFP positive embryos after no RNAi. Double sided T-bar shows standard deviation.

are *rde-1* mutant strains, in which RNAi is inactive in all tissues including hypodermis and muscle. NR222 and NR229 are hypodermis-specific RNAi strains, in which the *rde-1* mutation is rescued by the *rde-1* cDNA solely in the hypodermis. As shown in Fig. 2, *gfp(RNAi)* eliminated GFP fluorescence in the hypodermis and muscle in wild type strains (i.e., NR220 and NR230), and these effects were suppressed by the *rde-1* mutation (i.e., NR221 and NR225) in both hypodermis and muscle. In the hypodermis-specific RNAi strain expressing GFP in the hypodermis, *gfp(RNAi)* effectively eliminated GFP fluorescence. However, in the hypodermis-specific RNAi strain expressing GFP in muscle, *gfp(RNAi)* had no effect on GFP fluorescence (Fig. 2). These results show that expression of the *rde-1* cDNA in hypodermis can rescue the *rde-1* mutation only in hypodermis, not in muscle, and that as a result, RNAi is effective only in the hypodermis.

3.3. Examination of tissue specificity using embryonic lethal genes

As a second assay to confirm tissue specificity, we chose to test two genes that are essential for embryonic development. One is *lin-26* (Labouesse et al., 1994; Labouesse et al., 1996), which is expressed only in the hypodermis; *lin-26(RNAi)* causes embryonic lethality. The other is *hlh-1* (Krause et al., 1994), which is expressed only in muscle; *hlh-1(RNAi)* causes embryonic and larval lethality. Both LIN-26 and HLH-1 are essential for tissue differentiation and function in a cell-autonomous manner. Double stranded RNA prepared from *lin-26* and *hlh-1* cDNAs were injected into worms with the following genotypes: wild type, *rde-1* mutant, and *rde-1* mutant rescued in the hypodermis. *lin-26(RNAi)* in wild type showed 100% lethality, in contrast to *lin-26 (RNAi)* in the *rde-1* mutant, in which 0% lethality was observed. In the case of *rde-1* rescued in the hypodermis, *lin-26(RNAi)* resulted in 31% lethality (Fig. 3). RNAi of *hlh-1* caused 81% lethality in wild type, but 0% in the *rde-1* mutant and 0% in the hypodermal rescued *rde-1* (Fig. 3), again demonstrating that the RNAi effect in muscle is not rescued by *rde-1* expressed in the hypodermis. From these results, we conclude that the expression of *rde-1* under the control of the *lin-26* promoter can rescue the *rde-1* mutation solely in the hypodermis and that as a result, RNAi is only effective in the hypodermis in these worms.

3.4. Application to other tissues

To verify our "tissue-specific RNAi" system, we used the *hlh-1* promoter for muscle-specific expression (Krause, 1995). We established two lines in which *rde-1* mutants harbor the *rde-1* cDNA under control of either the *lin-26* or *hlh-1* promoter (Table 1). For these two lines, we carried out *lin-26(RNAi)* and *hlh-1(RNAi)*. We scored worms showing lethal phenotypes from GFP positive worms containing extrachromosomal arrays in each case (Table 1). *hlh-1(RNAi)* resulted in lethality only in the strain in which *rde-1* was rescued in muscle. Similarly, *lin-26 (RNAi)* only resulted in lethality in the strain rescued for *rde-1* function in the hypodermis (Table 1). These results demonstrate that as with the *lin-26* promoter in the hypodermis, the *hlh-1* promoter can be used to induce muscle-specific RNAi.

3.5. Tissue-specific analysis of ajm-1 function

We applied the hypodermis-specific RNAi system to a gene expressed in multiple tissues. We chose *ajm-1*, which is expressed in hypodermis, pharynx, and gut (Koppen et al., 2001). The *ajm-1* gene encodes a protein that localizes to apical junctions in epithelial cells, and is required for junctional integrity. *ajm-1(RNAi)* causes embryonic lethality (Koppen et al., 2001). To determine whether loss of function of *ajm-1* in hypodermis but not other tissues causes lethality, we carried out *ajm-1(RNAi)* in embryos rescued for RNAi only in the hypodermis. *ajm-1(RNAi)* in wild type showed 100% lethality; in contrast, *ajm-1(RNAi)* in the *rde-1* mutant yielded 0% lethality (Table 2), confirming previous results. *ajm-1(RNAi)* in the hypodermis-rescued *rde-1* strain showed a high percentage of lethality (Table 2). RNAi of *ajm-1* in a hypodermis-rescued *rde-1*strain also eliminated AJM-1 immunostaining using MH27, a monoclonal antibody specific for AJM-1 (Francis and Waterston, 1985; Koppen et al., 2001), in the hypodermis, but not in gut (Fig. 4), confirming that RNAi is effective only in the hypodermis. These results suggest that *ajm-1* expression in hypodermal cells is essential for its function, and further

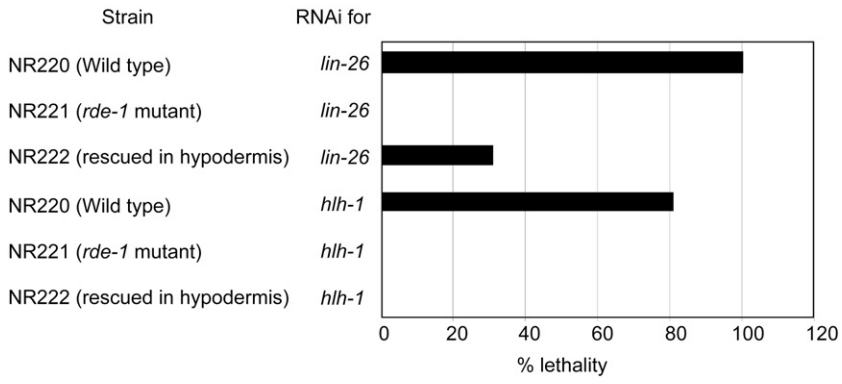

Fig. 3. Estimation of the efficacy of the tissue specific RNAi system using embryonic lethal genes "Strain" shows the worm strain used for each assay with its properties. "RNAi for" shows which gene was used in the assay. Black bar represents percentage of embryos that showed lethality.

Table 1
Muscle-and hypodermis-specific RNAi system

Strain	Genotype	RNAi	Lethal phenotype (%)
NR320	*rde-1 (ne219) V; kzEx320*[pDM#715 (*hlh-1p::rde-1*), pTG96(*sur-5p::nls::gfp*)]	*lin-26*	0
NR320	*rde-1 (ne219) V; kzEx320*[pDM#715 (*hlh-1p::rde-1*), pTG96(*sur-5p::nls::gfp*)]	*hlh-1*	23.5
NR321	*rde-1(ne219) V; kzEx321*[pKK1253 (*lin-26p::rde-1*), pTG96(*sur-5p::nls::gfp*)]	*lin-26*	84.5
NR321	*rde-1(ne219) V; kzEx321*[pKK1253 (*lin-26p::rde-1*), pTG96(*sur-5p::nls::gfp*)]	*hlh-1*	0

Table 2
Application of hypodermis-specific RNAi system to the *ajm-1* gene

Strain	Genotype	Lethal phenotype (%)	No phenotype (%)	*N*
NR220	*kzIs7*[pKK1260(*lin-26p::nls::gfp*), pRF4(*rol-6* marker)]	100	0	23
NR221	*rde-1(ne219) V; kzIs8*[pKK1260(*lin-26p::nls::gfp*), pRF4(*rol-6* marker)]	0	100	64
NR222	*rde-1(ne219) V; kzIs9*[pKK1260(*lin-26p::nls::gfp*), pKK1253(*lin-26p::rde-1*), pRF6(*rol-6* marker)]	56	42	115

confirm the tissue specificity of RNAi using tissue-specific promoters to rescue *rde-1* function in *rde-1* mutants.

3.6. Postembryonic muscle-specific RNAi by the feeding method

To determine whether our tissue-specific RNAi system might also work postembryonically, we performed RNAi by feeding for *unc-98*. The *unc-98* gene is expressed primarily in adults and *unc-98* mutants show a characteristic birefringent needle phenotype by polarized light microscopy (Mercer et al., 2003). Bacteria expressing dsRNA of the *unc-98* gene were fed to either wild type, the *rde-1* mutant, or the *rde-1* mutant carrying an integrated array containing wild type *rde-1* cDNA fused to the *hlh-1* promoter. The progeny from each strain were subjected to polarized light microscopy. Progeny from wild type showed the needle phenotype as previously reported (Mercer et al., 2003), whereas those from the *rde-1* mutant strain did not show the needle phenotype (Fig. 5). Although the percentage of adult worms showing the needle phenotype is not high (20%), the progeny from the muscle-specific RNAi strain showed the needle phenotype (Fig. 5), suggesting that the tissue-specific RNAi system can be applied to a gene which functions at postembryonic stages by the feeding procedure.

4. Discussion

4.1. A tissue-specific RNAi system

In this report, we demonstrate a tissue-specific RNAi method in *C. elegans*, using the hypodermis and muscle as test cases. Based on two lines of evidence, we showed the tissue specificity

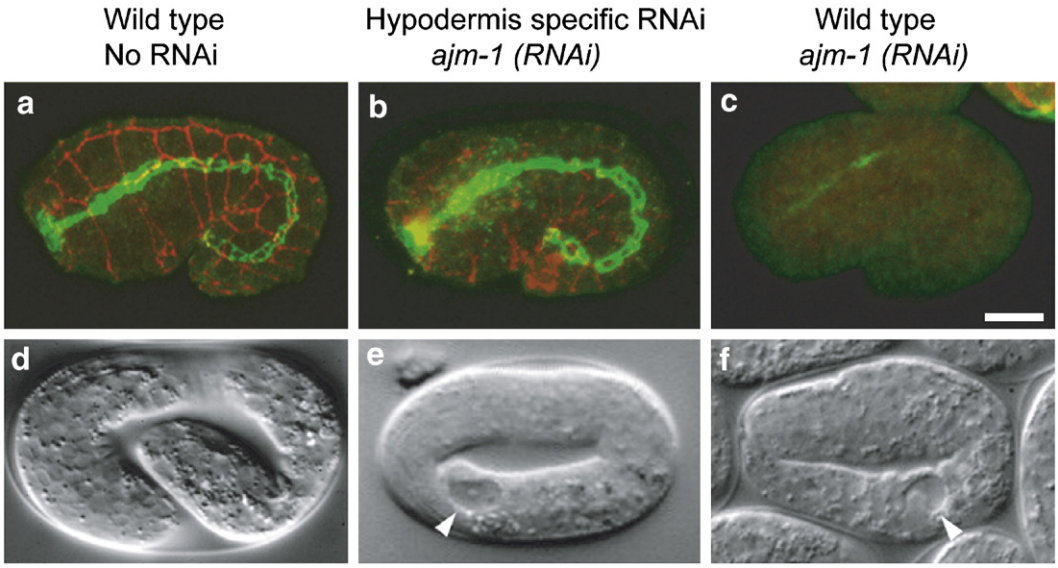

Fig. 4. Tissue-specific depletion of AJM-1 via *ajm-1*(RNAi) in wild type and hypodermis-rescued *rde-1* embryos. a–c, MH27 (a monoclonal antibody specific to AJM-1) staining of wild type, *ajm-1(RNAi); rde-1 [plin-26::rde-1]* and *ajm-1(RNAi)* embryos. Hypodermal MH27 signal is shown in red (a projection of three confocal sections of the surface of the embryo), and pharyngeal and intestinal signal is shown in green (a projection of two central confocal sections). In the wild type, MH27 staining is bright and even in all epithelia (a). *ajm-1(RNAi)* in the strain rescued for *rde-1* in the hypodermis (b) results in marked reduction of MH27 staining in the hypodermis, but no effect in the pharynx and intestine. *ajm-1(RNAi)* in a wild-type embryo causes almost complete loss of MH27 signal from all embryonic epithelia (c). d–f, Nomarski phenotypes of wild type, *ajm-1(RNAi); rde-1 [plin-26::rde-1]* and *ajm-1(RNAi)* embryos. A wild type embryo is shown at the 3-fold stage (d). *ajm-1(RNAi)* causes a nearly identical 2-fold arrest phenotype in both the strain rescued for *rde-1* in hypodermis (e) and in the wild type (f). However, in the case of the strain rescued for *rde-1* in hypodermis, embryonic lethality is only observed in 50% of the embryos, while 100% lethality is observed in a wild-type background. Scale bar represents 10 μm. Arrowheads indicate vacuoles characteristic of the *ajm-1* loss of function phenotype.

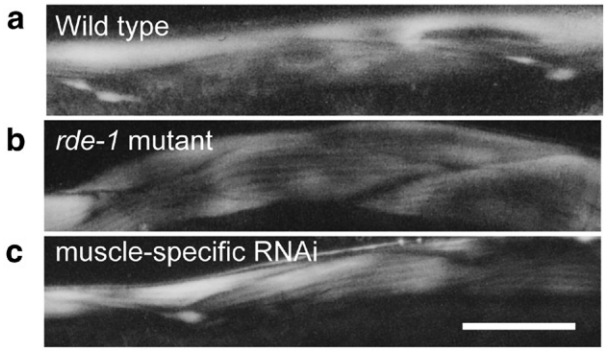

Fig. 5. Muscle-specific feeding RNAi for the *unc-98* gene in adult worms All three panels show polarized light microscope images of progeny from worms fed with bacteria expressing dsRNA for *unc-98*. (a) Wild type strain; (b) the *rde-1* mutant strain; (c) muscle-specific RNAi strain. Birefringent needles, which are characteristic of *unc-98* mutants, are observed in (a) and (c). Scale bar represents 20 μm.

of this system. First, by using GFP markers expressed in either the hypodermis or muscle, we showed that expression of wild-type *rde-1* in the hypodermis can confer RNAi sensitivity only in the hypodermis. GFP is not affected by other cellular functions and is a sensitive reporter of gene activity (Chalfie et al., 1994), and thus confirms the tissue specificity of our technique. Second, we confirmed tissue specificity by using genes essential for embryonic development that are expressed either in the hypodermis (*lin-26*) (Labouesse et al., 1996) or muscle (*hlh-1*) (Krause, 1995). Results from functional assays indicate that hypodermis-or muscle-specific RNAi has the desired effect, and can be applied for practical "mosaic" analysis. Furthermore, using this hypodermis-specific RNAi system, we revealed that the expression of *ajm-1* in the hypodermis is important for its function during development, thus confirming the usefulness of this tissue-specific RNAi system. A previous report suggested that RDE-1-dependent tissue-specific RNAi can function postembryonically in hypodermis and muscle using promoters different from the ones used in our study (*dpy-7* promoter for hypodermis and *myo-3* for muscle) to perturb *nas-37* function (Suzuki et al., 2004). Although this work did not verify tissue-specific knockdown using protein assays as we have done here, this previous work supports our idea that the RDE-1-dependent tissue-specific RNAi system is useful for the characterization of tissue-specific function of a given gene.

4.2. Efficiency of RNAi in the tissue-specific RNAi system

When we injected *lin-26* dsRNA into worms rescued for RNAi only in the hypodermis, only 31% of progeny from injected worms showed a lethal phenotype (Fig. 3). Injection of *ajm-1* dsRNA resulted in only 56% lethality (Table 2). In the case of muscle-specific RNAi (Table 1), only 23% of progeny injected with *hlh-1* dsRNA showed a lethal phenotype. All three dsRNA injections caused lethality in 81–100% of progeny from wild type worms (Fig. 3, Tables 1 and 2), suggesting that rescue of *rde-1* in the hypodermis or muscle was not complete. As described previously, maternal and zygotic expression of *rde-1* are required for complete rescue of the *rde-1* mutant (Tabara et al., 1999). In our tissue-specific RNAi system, *rde-1* is expressed from extrachromosomal or integrated arrays. Expression of those arrays is silent in the germline, resulting in a lack of maternal expression. Furthermore, in our tissue-specific RNAi system, the *rde-1* gene is regulated by the *lin-26* or *hlh-1* promoters. These promoters are reported to drive expression beginning during mid-embryogenesis (Krause et al., 1994; Labouesse et al., 1996). Due to the exclusively zygotic expression of *rde-1* by the *lin-26* or *hlh-1* promoters, it is therefore likely that complete rescue of *rde-1* in the hypodermis or muscle is not achieved because the hypodermis or muscle lacks maternal *rde-1* expression. To overcome this reduced efficiency of RNAi, we attempted to utilize the double mutant, *rde-1; rrf-3*, since it has been reported that *rrf-3* increases RNAi sensitivity (Simmer et al., 2002). However, *rrf-3* is also known to cause genome instability and the double mutant genotype was very unstable (soon after we constructed the *rde-1; rrf-3* strain, the *rde-1* mutation was lost).

4.3. Muscle specific promoters

We used the *hlh-1* promoter for muscle-specific expression. Commonly, the *myo-3* promoter is used for muscle-specific expression (Okkema et al., 1993). Based on GFP and lacZ expression results (Okkema et al., 1993), the *myo-3* promoter does drive gene expression largely in muscle cells, a finding we confirmed using *myo-3p::nls::gfp*. We therefore tried to establish a muscle-specific RNAi system using the *myo-3* promoter, but results of both GFP and functional assays showed that RNAi is effective both in hypodermis and muscle cells in such worms (data not shown), suggesting that the *myo-3* promoter may not be sufficiently specific to muscle cells. It is possible that excessive gene expression driven by the *myo-3* promoter results in "leaky" expression of *rde-1* transcripts in other tissues besides muscle. If the amount of *rde-1* function required for rescue of *rde-1* loss of function is less than that required for robust GFP or lacZ expression, such a scenario is feasible. One additional possibility is that RDE-1 protein expressed in muscle cells has non-cell autonomous effects on other tissues. However, the result that hypodermis-specific RNAi, using the *lin-26* promoter, and muscle-specific RNAi, using the *hlh-1* promoter, each yielded tissue-specific effects does not support this possibility. Furthermore, RDE-1 has been reported to be cell-autonomous by others (Tabara et al., 1999). A previous report shows that the *myo-3* promoter does function in muscle-specific RNAi (Suzuki et al., 2004). This discrepancy may be due to the difference between integrated arrays (this study) and extrachromosomal arrays (previous study). In addition, the previous study did not verify tissue-specific knockdown via immunostaining. It may be that partial knockdown of hypodermal expression of *nas*-37 due to "leaky" rescue of *rde-1* function driven by the *myo-3* promoter is insufficient to yield a noticeable phenotype, whereas our functional assays using *lin-26* and *ajm*-1 are more sensitive to "leaky" RNAi effects.

4.4. Further application of the tissue specific RNAi system

Considerable effort has been expended to determine the pattern of expression of many genes and the *cis*-acting elements responsible for their expression. In the mouse and *Drosophila*, a technique for tissue-specific inactivation or knockdown of a specific gene has been established using tissue-specific promoters (Golic, 1991; Herault et al., 1998; Zheng et al., 2000). Our method of tissue-specific RNAi in *C. elegans* also requires tissue-specific promoters. Presently, data is available for several promoters that drive expression in specific tissues, such as hypodermis (Gilleard et al., 1997; den Boer et al., 1998; Sieburth et al., 1999), muscle (Okkema et al., 1993; Krause et al., 1994; Terami et al., 1999), pharynx (Okkema et al., 1993; Kalb et al., 1998; Anyanful et al., 2001), or gut (Fukushige et al., 1998), and some specific cells, such as distal tip cells (Henderson et al., 1994), sex myoblasts (Harfe et al., 1998), and germline cells (Praitis et al., 2001). Many of these promoters should be useful for extending our tissue-specific RNAi system to other tissues.

By using various promoters to drive the expression of the *rde-1* cDNA and a GFP marker, it will be possible to establish additional tissue-or cell-specific RNAi systems in *C. elegans*. These tissue-specific RNAi systems can be used to rapidly identify tissues or cells in which the function of a gene of interest is essential. In particular, for genes functioning in multiple cells or tissues, such as those encoding cytoskeletal components or other essential proteins, this system should be a powerful tool for examining the function of a given gene in a particular tissue or cell type. We have also found that the feeding RNAi procedure is effective in the tissue-specific RNAi system (Fig. 5) and that this system could apply to the genes functioning both at embryonic (Fig. 4) and postembryonic (Fig. 5) stage. Combined with genome-wide RNAi methods (Fraser et al., 2000; Gonczy et al., 2000), the procedures described here will enable genome-wide estimates of tissue specific functions of genes of interest without the use of the more technically difficult traditional mosaic analysis.

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