

A whole-mount in situ hybridization method for microRNA detection in *Caenorhabditis elegans*

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ABSTRACT

Whole-mount in situ hybridization (WISH) is an outstanding method to decipher the spatiotemporal expression patterns of microRNAs (miRNAs) and provides important clues for elucidating their functions. The first WISH method for miRNA detection was developed in zebrafish. Although this method was quickly adapted for other vertebrates and fruit flies, WISH analysis has not been successfully used to detect miRNAs in *Caenorhabditis elegans*. Here, we show a novel WISH method for miRNA detection in *C. elegans*. Using this method, *mir-1* miRNA was detected in the body-wall muscle where the expression and roles of *mir-1* miRNA have been previously elucidated. Application of the method to *let-7* family miRNAs, *let-7*, *mir-48*, *mir-84*, and *mir-241*, revealed their distinct but partially overlapping expression patterns, indicating that miRNAs sharing a short common sequence were distinguishably detected. In *pash-1* mutants that were depleted of mature miRNAs, signals of *mir-48* miRNA were greatly reduced, suggesting that mature miRNAs were detected by the method. These results demonstrate the validity of WISH to detect mature miRNAs in *C. elegans*.

Keywords: *C. elegans*; miRNA; in situ hybridization; *mir-1*; *let-7*

INTRODUCTION

MicroRNAs (miRNAs) are endogenous RNAs of ~22 nucleotides (nt) that play pivotal roles in the regulation of gene expression through binding to target mRNAs. The chief determinant of target recognition is base-pairing between an mRNA and a part of a miRNA: the nucleotides at positions 2–8 from the 5' end of the miRNA, which is referred to as the seed sequence. A group of miRNAs that share a seed sequence is classified into a miRNA family. To date, there have been 434 miRNAs identified in *Caenorhabditis elegans* (Kozomara and Griffiths-Jones 2014), though the functions of most miRNAs remain elusive. In the canonical pathway of miRNA biogenesis, miRNA genes are initially transcribed into long primary-miRNAs (pri-miRNAs), which are subsequently processed into precursor-miRNAs (pre-miRNAs) in the nucleus. After transport to the cytoplasm, the pre-miRNAs are processed into mature miRNAs. As the expressions of miRNAs are transcriptionally controlled, the spatiotemporal expression patterns of miRNAs have been examined using animals transgenic for *gfp* fusion with a miRNA gene promoter (Martinez et al. 2008). However, many lines of evidence indicate that several miRNAs are also regulated post-transcriptionally (Ha and Kim 2014). Therefore, it is

important to detect mature miRNAs in cells, tissues, and bodies.

Whole-mount in situ hybridization (WISH) is a natural choice for this purpose, and was first developed in zebrafish embryos (Wienholds et al. 2005). In this method, oligonucleotide probes including locked nucleic acids (LNAs) are effective for the acquisition of intense hybridization signals and increased specificity. WISH methods for miRNA detection soon became available for embryos of mouse, chicken, medaka, and fruit fly (Sokol and Ambros 2005; Ason et al. 2006; Darnell et al. 2006; Kloosterman et al. 2006). In addition, a significant improvement in the intensity of hybridization signals has been achieved by fixation of sectioned mouse tissues with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), which reacts with phosphates at the 5' end of miRNAs to introduce phosphoramidate linkages, resulting in all of the nucleotides of the miRNAs being accessible to probes for hybridization (Pena et al. 2009). Despite the efficient use of WISH methods, WISH analysis has still not been successfully used to detect miRNAs in *C. elegans*.

In this study, we developed a novel WISH method for miRNA detection in *C. elegans* at both embryonic and

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post-embryonic stages by combining procedures, i.e., the use of LNA probes and EDC fixative, and the WISH methods for mRNA detection that we previously established in *C. elegans* (Tabara et al. 1996; Motohashi et al. 2006, 2015). To assess the new WISH method, we examined the expression patterns of *mir-1* miRNA because the cell-autonomous roles of this miRNA have been elucidated. *mir-1* miRNA is required to regulate synaptic transmission at neuromuscular junctions in body-wall muscle cells (Simon et al. 2008), which lie underneath the hypodermis in four longitudinal stripes over the length of the animal. To show distinguishable detection of miRNAs that share a seed sequence, we compared the expression patterns of *let-7* family miRNAs, *let-7*, *mir-48* (also known as *lin-58*), *mir-84*, and *mir-241*, of which deletion mutants are available and have been well studied for developmental phenotypes (Reinhart et al. 2000; Abbott et al. 2005). To confirm the detection of mature miRNAs, we conducted WISH analysis in *pash-1* mutants. As *pash-1* encodes an RNA-binding protein necessary for the processing of pri-miRNAs, pri-miRNAs accumulate, and mature miRNAs are depleted in *pash-1* mutants (Denli et al. 2004; Lehrbach et al. 2012). The detection of mature miRNAs was also supported by the subcellular localization of WISH signals.

RESULTS AND DISCUSSION

Critical parameters to obtain WISH signals in *C. elegans*

During the search for WISH conditions that fit *C. elegans*, we noticed two critical parameters for better hybridization signals: the temperature of incubation with EDC to fix the animals and the concentration of proteinase to partially digest the fixed animals. There is a trade-off between these parameters. The fixation of mouse sectioned tissues with EDC is initially carried out by incubation at 25°C (Pena et al. 2009), which is subsequently increased to 50°C to achieve near-complete phosphoramidate bond formation in a shorter time (Renwick et al. 2013). Cross-linking of miRNAs with EDC for Northern blotting also shows that increasing the reaction temperature up to 60°C improves miRNA detection (Pall et al. 2007). More cross-linking at higher temperatures must lead to more retention of miRNAs, leading to stronger hybridization signals. However, cross-linking with EDC acts not only on miRNAs but also on proteins to form peptide bonds, which make the proteins resistant to proteinase (Sung et al. 2003; Renwick et al. 2013). As the surfaces of *C. elegans* larvae and adults are overlaid with cuticle layers composed of collagens, the layers have to be partially digested by proteinase so that WISH reagents, such as probes, can penetrate inward. More fixation inhibits this process further and necessitates more proteinase for proper digestion. For this reason, we evaluated the incubation temperature with EDC in combination with subsequent digestion by various concentrations of proteinase. As a result, we found that optimized conditions to generate intense signals with animals

were fixation with EDC at 60°C and subsequent digestion by proteinase K at 2 mg/mL: This concentration is so high that unfixed animals are easily dissolved. For embryos, slight modification was made to treat the tough eggshells.

WISH signals of *mir-1* miRNA were detected in muscle tissues

The expression patterns of *mir-1* miRNA were examined by the WISH method throughout all developmental stages. Staining for *mir-1* miRNA was observed in the body-wall muscle at the adult stage (Fig. 1A–C). Additionally, *mir-1* miRNA was detected in the anal depressor muscle (Fig. 1A) and the pharynx (Fig. 1A,B), which is a muscular tube used to suck food. The miRNA was also detected in sex-specific muscles, i.e., the vulval muscle in hermaphrodites (Fig. 1C) and the diagonal muscle in males (Fig. 1B). Analyses of larvae by the method also gave signals in these muscles at each of the four larval stages (Fig. 1D–G). Pharyngeal signals were detected in embryos at the 500-cell stage and later (Fig. 1H–K). The expression patterns of *mir-1* miRNA are consistent with tissues where the *gfp* reporter driven by the promoter of the *mir-1* gene is expressed (Supplemental Fig. S1; Martinez et al. 2008; Simon et al. 2008). Furthermore, we confirmed that the signals of *mir-1* miRNA were lost in *mir-1* deletion mutants (Supplemental Fig. S2). These results are in agreement with the cell-autonomous roles of *mir-1* miRNA in the body-wall muscle, and suggest additional roles of the miRNA in the other muscle tissues.

WISH signals of *let-7* family miRNAs were detected in distinct but partially overlapping tissues

The expression patterns of four *let-7* family miRNAs, *let-7*, *mir-48*, *mir-84*, and *mir-241*, were compared at the fourth larval (L4) stage (Supplemental Fig. S3), as Northern blot analyses show that mature forms of all the miRNAs start to accumulate at the mid-larval stage and reach exceedingly high levels at the L4 stage (Reinhart et al. 2000; Bracht et al. 2004; Abbott et al. 2005). In the head, staining in the pharynx was observed for *let-7* and *mir-84* miRNAs, whereas staining in neurons of the head ganglia was observed for all of the miRNAs (Fig. 2A,D,G,J). WISH analysis of every miRNA showed staining in the vulva (Fig. 2B,E,H,K). Signals of *let-7*, *mir-48*, and *mir-241* miRNAs were detected in the rectum (Fig. 2C,F,I,L). *mir-48* miRNA was also detected in seam cells (Fig. 2M). Consistent with the expressions of all the miRNAs in the vulva, *let-7* mutants and *mir-48 mir-241; mir-84* triple mutants show a phenotype of bursting through the vulva (Reinhart et al. 2000; Abbott et al. 2005), and the activity of *let-7* miRNA is cell-autonomously required to rescue the bursting phenotype in *let-7* mutants (Ecsedi et al. 2015).

To confirm that these overlapping expressions of the *let-7* family miRNAs are not due to cross-hybridization among them, the expressions of the miRNAs were examined in their

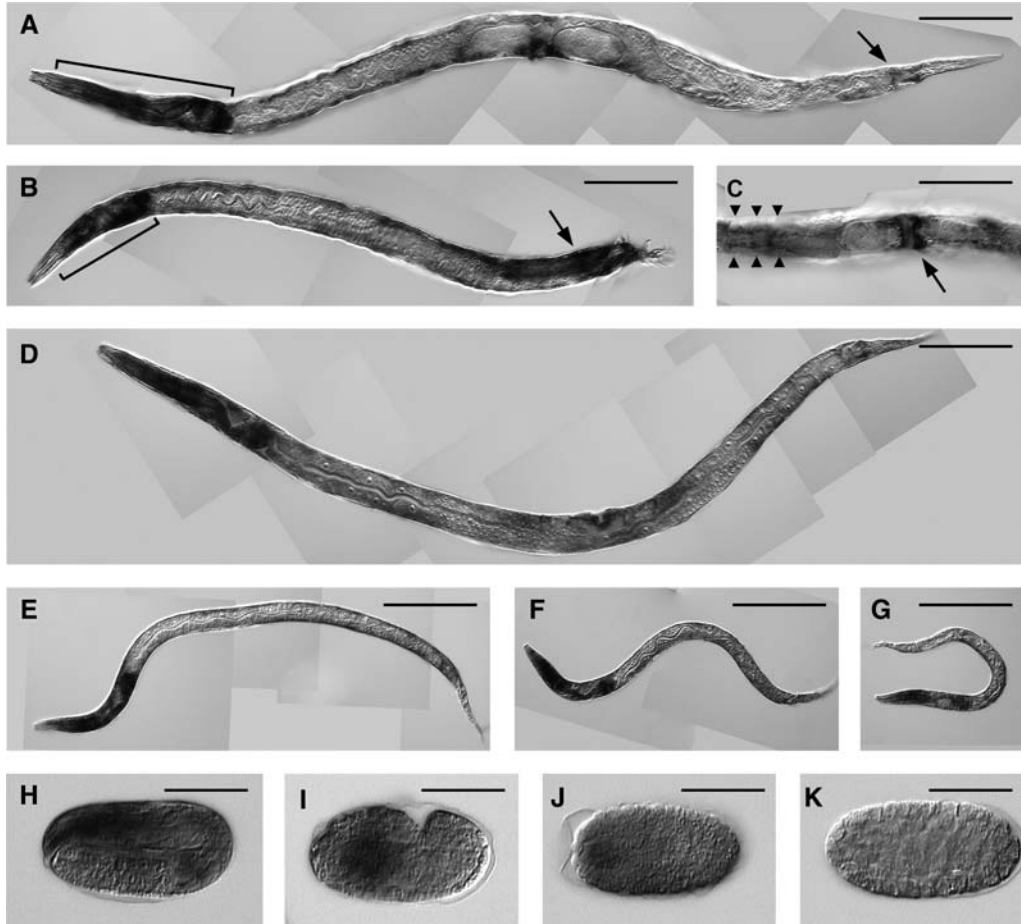


FIGURE 1. WISH analysis for detection of *mir-1* miRNA. Images show an adult hermaphrodite (A), an adult male (B), and a ventral view of the vulva of an adult hermaphrodite (C). The brackets designate the pharynx (A,B). The arrows indicate the anal depressor muscle (A), the diagonal muscle (B), and the vulval muscle (C). The arrowheads show the body-wall muscle (C). Larvae are at the L4 (D), L3 (E), L2 (F), and L1 (G) stages. Embryos are at the fourfold (H), 1.5-fold (I), 500-cell (J), and 200-cell (K) stages. Scale bars, 100 μ m (A–G) and 30 μ m (H–K).

deletion mutants. Using the following three strains of double deletion mutants, *mir-48 mir-241*, *mir-48; mir-84*, and *mir-241; mir-84*, loss of the expressions of *mir-48*, *mir-84*, and *mir-241* miRNAs were found only in the strains that had the deletion of the corresponding miRNA genes (Supplemental Fig. S4A–L). As to *let-7*, lethality associated with the vulval bursting made it difficult to collect a large number of mutant animals for WISH analysis. Therefore, we utilized a deletion mutant strain whose lethality was rescued by the *let-7* transgene. As the transgene was generated as an extra-chromosomal array and the array was unstably inherited, transgenic animals segregated progeny with or without the transgene. The vulval bursting phenotype was an indicator that animals had lost the transgene and had been fixed just after the incidence of bursting because the extrusion of internal organs through the rupture leads to severe damage to the body. While animals with a normal vulva showed staining, animals with the phenotype gave no signal of *let-7* miRNA (Supplemental Fig. S4M,N). These results indicate that the WISH signals of the *let-7* family miRNAs are miRNA specific.

miRNAs in the cytoplasm, but not pri-miRNAs, were detected by the WISH method

Precursors of miRNAs also contain complementary sequences to probes for the miRNAs. To inspect miRNA forms detected by our method, WISH analysis was conducted in temperature-sensitive *pash-1(mj100)* mutants, which were depleted of mature miRNAs after cultivation at the restrictive temperature (Lehrbach et al. 2012). WISH signals of *mir-48* miRNA were considerably weaker in *pash-1* mutants when grown under the restrictive conditions than in control animals, which were *pash-1* mutants under permissive conditions and wild-type animals under restrictive conditions (Fig. 3A–C). Faint staining was predominantly observed in the cytoplasm of the *pash-1* mutants (Fig. 3A), suggesting that *mir-48* pri-miRNAs, which accumulated in the nucleus, hardly contributed to WISH signals. One explanation for this result is that the pri-miRNAs were not retained in the fixed animals, because pri-miRNAs contain a 5'-cap structure (Bracht et al. 2004), and the structure prevented the

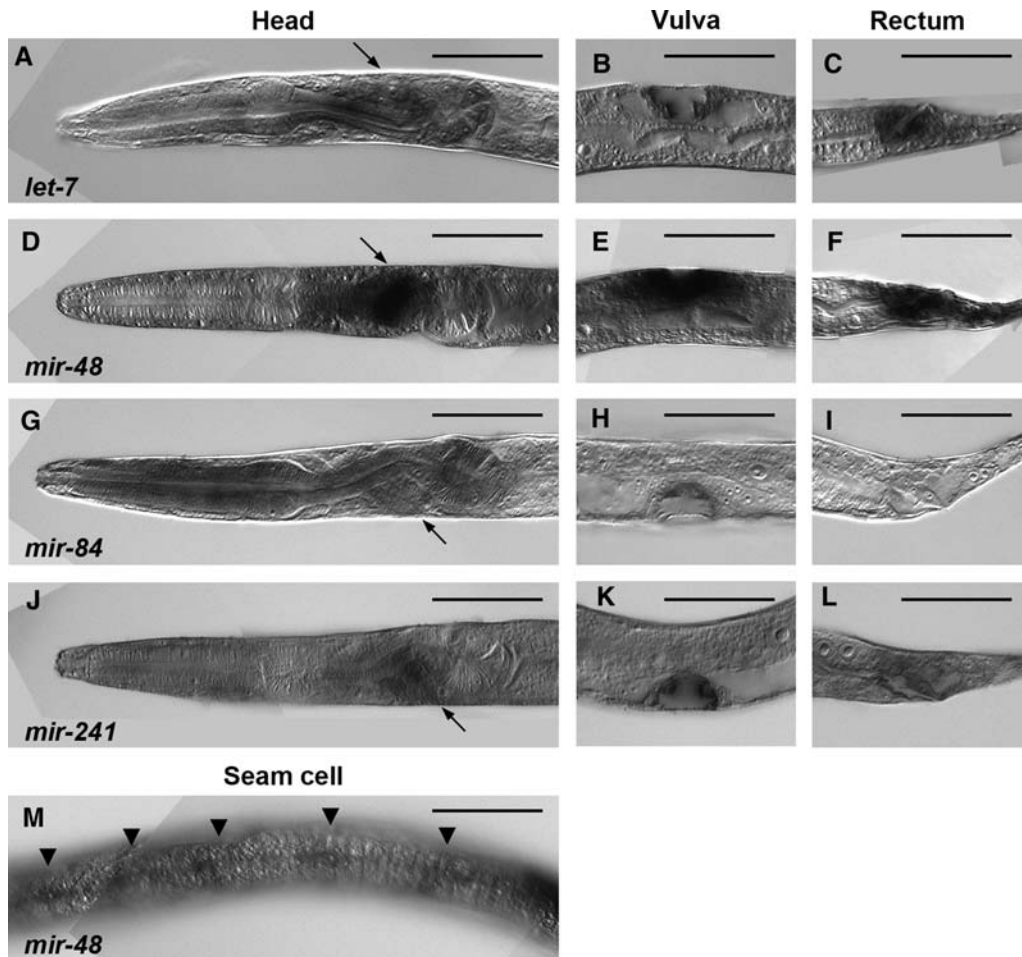


FIGURE 2. WISH analysis for detection of *let-7* family miRNAs at the L4 stage. The family members are *let-7* (A–C), *mir-48* (D–F,M), *mir-84* (G–I), and *mir-241* (J–L). Images show the head region (A,D,G,J), the vulva (B,E,H,K), the rectum (C,F,I,L), and seam cells (M). The arrows indicate neurons of the head ganglia (A,D,G,J). The arrowheads point to seam cells (M). Whole-body images corresponding to these partial images are shown in Supplemental Figure S4. Scale bars, 50 μ m.

formation of phosphoramidite linkages with EDC, which reacts with free phosphates at the 5'-end of miRNAs.

The subcellular localization of WISH signals can be clearly visualized by staining with fluorescent dye. Fluorescent signals of *mir-48* miRNA were detected mostly in the cytoplasm of the same tissues as chromogenic signals (Fig. 4A–C; Supplemental Fig. S5A–C). Fluorescent signals of *let-7*, *mir-84*, and *mir-241* miRNAs, however, were obscure due to the low signal-background ratio (Fig. 4D–F; Supplemental Fig. S5D–F, data not shown). As *mir-48* miRNA is the most abundant among the *let-7* family miRNAs, as revealed by high-throughput sequencing analysis of *C. elegans* miRNAs (Kato et al. 2009), it is likely that the amount of the three miRNAs was not enough to obtain intense fluorescent signals. Although *mir-1* miRNA is one of the most abundant miRNAs revealed by sequencing analysis (Kato et al. 2009), fluorescent signals of *mir-1* miRNA were also obscure in muscle tissues except for the pharynx (data not shown). To address the issue that the amount of miRNAs is pivotal to obtain intense fluorescent signals, WISH analysis was conduct-

ed in animals containing a *let-7* transgene, which can provide a large amount of *let-7* miRNAs. Intense fluorescent signals of *let-7* miRNA were detected in the transgenic animals and were almost all localized in the cytoplasm (Fig. 4G–I; Supplemental Fig. S5G–I). These results suggest that increasing the amount of miRNAs by a transgenic strategy is a powerful way to obtain fluorescent high-resolution signals of the miRNAs.

Although we cannot rule out the possibility that pre-miRNAs in the cytoplasm partially contributed to WISH signals, these results suggest that miRNAs in the cytoplasm, which were possibly mature miRNAs, and not pri-miRNAs in the nucleus, were detected by the method.

In this report, we presented a novel WISH method to detect mature miRNAs in *C. elegans*. The validity of the method was evaluated by the detection of *mir-1* miRNA and *let-7* family miRNAs in the tissues where the roles of these miRNAs have been elucidated, and by a lack of detection of the miRNAs in deletion mutants. The detection of mature miRNAs by the method was strongly suggested by the reduction of the WISH signals of *mir-48* miRNA in



FIGURE 3. WISH analysis for detection of *mir-48* miRNA in temperature-sensitive *pash-1* mutants at the L4 stage. Genotypes are *pash-1* (A,B) and wild-type (C). Animals were grown under restrictive conditions (A,C) and under permissive conditions (B). Images on the right in A show magnified views of the boxed regions in the image on the left, and the arrows indicate nuclei of the vulva (top) and the rectum (bottom). Scale bars, 100 μm (A–C) and 30 μm (magnified images in A).

pash-1 mutants, and by localization of the fluorescent signals of the miRNA exclusively in the cytoplasm. The WISH method will be not only useful for miRNA research in *C. elegans*, but the strategy can be adapted for WISH methods to detect miRNAs in other organisms.

MATERIALS AND METHODS

Caenorhabditis elegans strains and culture

The following strains and mutant alleles were used in this study: wild-type Bristol N2, *mir-1*(n4102) I, *pash-1*(mj100) I, *lin-58 mir-241*(nDf51) V, *lin-58*(n4097) V; *mir-84*(n4037) X, *mir-241*(n4315) V; *mir-84*(n4037) X, *let-7*(mm112) *unc-3*(e151) X, and *maIs251* [*mir-1p::gfp*]. Animals were grown at 20°C as previously described (Andachi 2008). To prepare animals under the restrictive conditions, *pash-1* mutants and wild-type controls were grown from eggs at 16°C and subsequently at 25°C for 20 h, which spanned the period from mid to late larval stages. The developmental stage of animals was assessed by gonad morphology.

Whole-mount *in situ* hybridization analysis of larvae and adults

Synchronized populations of larvae or adults were prepared by cultivation of eggs that were collected by hypochlorite treatment of

adult hermaphrodites. The animals were usually reared in liquid S medium containing *Escherichia coli* strain OP50 or sometimes on NGM plates: Both cultivations gave essentially the same results. The animals were cleaned by the sucrose flotation procedure and chilled on ice. Approximately 300 μL of the packed animals was transferred to a new 2-mL siliconized tube. In the following procedures, incubation was carried out in 1 mL of a specified solution at room temperature for 2 min with continuous rotation of the tube, and was terminated by centrifugation at 3000 rpm for 1 min followed by the removal of supernatant fluid by pipetting, unless otherwise indicated. The animals were incubated in DENT (80% methanol + 20% dimethyl sulfoxide) at -20°C for 10 min. The animals were rehydrated at 4°C through a graded series of 100% methanol, 90% methanol + 10% Fixative-phosphate (80 mM HEPES [pH 6.9], 1.6 mM MgSO_4 , 0.8 mM EGTA, and 100 mM NaCl, 3.7% formaldehyde), 70% methanol + 30% Fixative-phosphate, 50% methanol + 50% Fixative-phosphate, 30% methanol + 70% Fixative-phosphate, and 100% Fixative-phosphate, and then fixed in Fixative-phosphate at 4°C for 20 min. The animals were washed three times in EDC buffer (130 mM 1-methylimidazole [pH 8], 300 mM NaCl), and then incubated in freshly prepared EDC Fixative (160 mM EDC, 130 mM 1-methylimidazole [pH 8], 300 mM NaCl) at 60°C for 1 h. The animals were washed three times in $1\times\text{BO}_3$ (25 mM sodium borate [pH 9]), and then incubated in $1\times\text{BO}_3$ with 10 mM dithiothreitol (DTT) for 20 min. The animals were washed three times in $1\times\text{BO}_3$, and then incubated in $1\times\text{BO}_3$ with 0.6% H_2O_2 for 20 min. The animals were washed three times in PBT (PBS [137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 , 1.5 mM

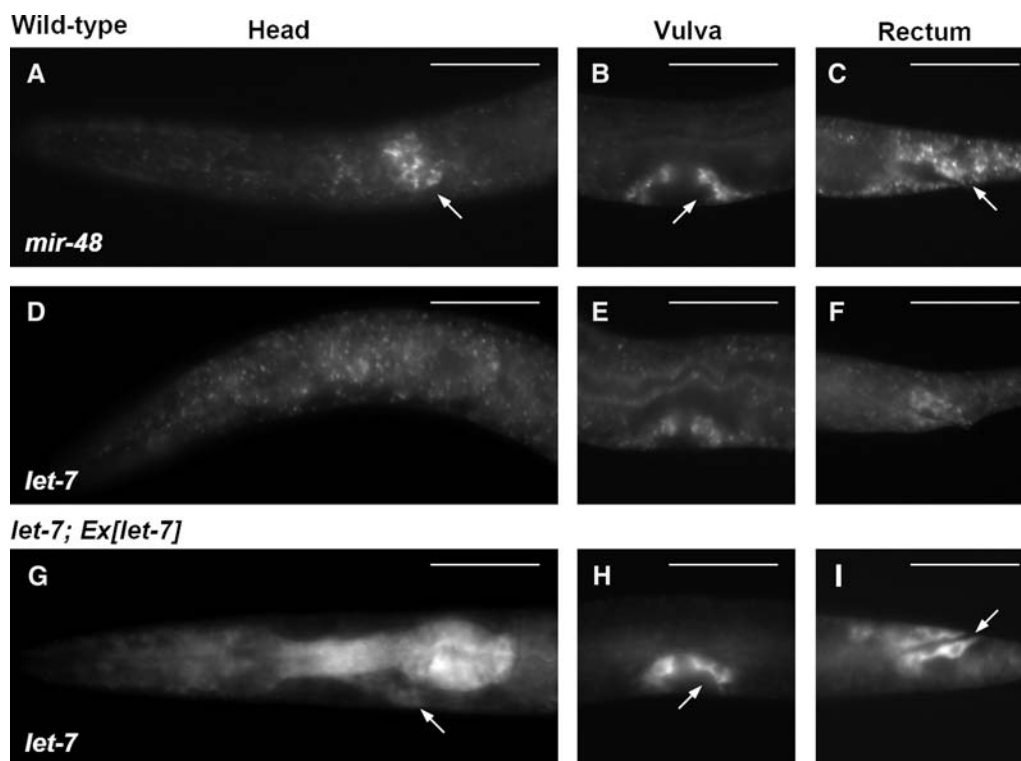


FIGURE 4. Fluorescent WISH analysis for detection of *let-7* family miRNAs at the L4 stage. The family members are *mir-48* (A–C) and *let-7* (D–I). Genotypes are wild type (A–F) and *let-7; Ex[let-7]* (G–I). Images show the head region (A,D,G), the vulva (B,E,H), and the rectum (C,F,I). The white arrows in (A–C,G–I) indicate fluorescent signals in the cytoplasm that surround nuclei, which are observed as circles without staining. Color images corresponding to this figure are shown in Supplemental Figure S5. Scale bars, 50 μ m.

KH_2PO_4], 0.1% Tween-20), and then digested with proteinase K at a concentration of 2 mg/mL in PBT with 1% β -mercaptoethanol (β -ME) at 37°C for 20 min. The animals were washed three times in PBS, and then fixed in Fixative (80 mM HEPES [pH 6.9], 1.6 mM MgSO_4 , 0.8 mM EGTA, PBS, 3.7% formaldehyde) for 20 min. The animals were finally washed three times in PBS. Aliquots of the animals suspended in PBS were allowed to stick to wells on Teflon printed diagnostic slides that had been coated with poly-L-lysine. The animals on slides were dehydrated through a graded series of 30% ethanol + 70% PBS, 50% ethanol + 50% PBS, 70% ethanol + 30% PBS, 90% ethanol + 10% distilled water, and 100% ethanol for 5 min each. The samples could be stored in ethanol at -20°C for at least 1 mo.

In the following procedures, samples on slides were immersed in a specified solution at room temperature for 5 min, unless otherwise indicated. The animals were rehydrated through a graded series of ethanol with 24 mM HCl, 90% ethanol + 10% distilled water, 70% ethanol + 30% PBS, 50% ethanol + 50% PBS, 30% ethanol + 70% PBS, and 100% PBS. After excess fluid on the slides was wiped off, the animals in each well were covered with 100 μ L of proteinase K at a concentration of 0.5 mg/mL in PBT with 1% β -ME, and incubated at 37°C for 20 min in a moist chamber. The animals were washed in PBS twice, and then fixed in Fixative for 20 min. The animals were washed in PBS twice, and then incubated in PBS containing glycine at a concentration of 2 mg/mL. The animals were incubated in a series of PBS for 5 min, 50% PBS + 50% hybridization buffer (50% deionized formamide, 750 mM NaCl, 75 mM sodium citrate [pH 7.0], 100 μ g/mL heparin, and 2% Blocking Reagent

[Roche]) for 10 min, and hybridization buffer for 10 min. For pre-hybridization, the animals in each well were covered with 100 μ L of hybridization buffer and incubated for 1 h in a moist chamber at a hybridization temperature suitable for each probe. After the buffer was exchanged with 100 μ L of hybridization buffer containing 10 nM heat-denatured probes, the animals were further incubated for 2 h in a moist chamber at the same hybridization temperature. Probes were 5' digoxigenin-labeled oligodeoxynucleotides containing LNAs (Nippon Gene Material). The hybridization temperature for each probe was experimentally determined as follows. A hybridization temperature was tentatively set at 20°C – 25°C below the melting temperature of the probe that was calculated by the manufacturer. WISH experiments were carried out at the hybridization temperature and at slightly different (both higher and lower) hybridization temperatures. Among them, we chose a hybridization temperature at which tissue-specific signals were detected in wild-type animals and the signals were lost in their deletion mutants. See Table 1 for the nucleotide sequences and the hybridization temperatures of probes.

The animals on the slides were immersed in hybridization buffer at the same hybridization temperature with continuous shaking for 10 min twice, and in $0.2\times$ SSC (150 mM NaCl, 15 mM sodium citrate [pH 7.0]) in the same way twice. For blocking, after washing in TNT (100 mM Tris [pH 7.5], 150 mM NaCl, 0.5% Tween-20) twice, the animals in each well were covered with 100 μ L of TNB (100 mM Tris [pH 7.5], 150 mM NaCl, 0.5% Blocking Reagent [TSA Plus Cy3 Kit, PerkinElmer]) and incubated for 30 min in a moist chamber. For staining with fluorescent dye, after the buffer was exchanged

TABLE 1. The nucleotide sequences and the hybridization temperatures of probes

microRNA	Sequence	Hybridization temperature
<i>mir-1</i>	5'-(DIG) <u>TACATAC</u> TTCTTTACATTCCA-3'	50°C
<i>let-7</i>	5'-(DIG)AACTATAACA <u>ACCTACTACCT</u> CA-3'	58°C
<i>mir-48</i>	5'-(DIG)TCGCATCTACTGAGCCTACCTCA-3'	58°C
<i>mir-84</i>	5'-(DIG)TACAATATTACATACTACCTCA-3'	46°C
<i>mir-241</i>	5'-(DIG)TCATTTCTCGCACCTACCTCA-3'	54°C

(DIG) shows 5'-labeled digoxigenin. Locked nucleic acids (LNAs) are indicated by underlines.

with 100 μ L of TNB containing Anti-Digoxigenin-POD Fab fragments (Roche), the animals were further incubated for 1 h in a moist chamber, and then washed in TNT with continuous shaking for 10 min four times. Fluorescent staining was carried out using a TSA Plus Cy3 Kit following the manufacturer's instructions. For staining with chromogenic substrate, the blocked animals in each well were covered with 100 μ L of TNB containing Anti-Digoxigenin-AP Conjugate (Roche), incubated for 2 h in a moist chamber, and then washed in TNT with continuous shaking for 10 min four times. Chromogenic staining was carried out using a DIG Nucleic Acid Detection Kit (Roche) following the manufacturer's instructions, except that incubation in color substrate solution was carried out until staining appeared in the samples: The range of incubation time was from a few hours to a few days.

Whole-mount *in situ* hybridization analysis of embryos

Caenorhabditis elegans eggs were collected by hypochlorite treatment of adult hermaphrodites. Approximately 100 μ L of the packed eggs was transferred to a new 1.5-mL siliconized tube. The following procedures were carried out at room temperature, unless otherwise indicated. To remove eggshells, the eggs were mixed with 100 μ L of yatalase (Takara Bio) at a concentration of 15 mg/mL in Embryo Handling buffer (300 mM mannitol, 50 mM HEPES [pH 7.2], 10 mM NaCl, 10 mM MgCl₂, 1 mM EGTA, 2 mM NH₄NO₃, 0.1% gelatin) and vortexed for 75 sec. The eggs were washed three times in 1 mL of Embryo Handling buffer, which was removed by pipetting after centrifugation at 3000 rpm for 1 min, and washed once in 1 mL of basal Embryo Handling buffer (300 mM mannitol, 50 mM HEPES [pH 7.2], 10 mM NaCl, 10 mM MgCl₂). Aliquots of the egg suspension in basal Embryo Handling buffer were allowed to stick to wells on Teflon printed diagnostic slides that had been coated with poly-L-lysine. The eggs on slides were immersed in methanol chilled at -20° C for 10 min, and rehydrated at room temperature through a graded series of 100% methanol, 90% methanol + 10% Fixative-phosphate, 70% methanol + 30% Fixative-phosphate, 50% methanol + 50% Fixative-phosphate, 30% methanol + 70% Fixative-phosphate, and 100% Fixative-phosphate for 5 min each, and then fixed in Fixative-phosphate for 20 min. The eggs were dehydrated through a graded series of 30% ethanol + 70% TBS (10 mM Tris [pH 7.5], 100 mM NaCl), 50% ethanol + 50% TBS, 70% ethanol + 30% TBS, 90% ethanol + 10% TBS, and 100% ethanol for 5 min each. The samples could be stored in ethanol at -20° C for at least 1 mo.

In the following procedures, samples on slides were immersed in a specified solution at room temperature for 5 min unless otherwise

indicated. The eggs were rehydrated through a graded series of ethanol with 0.6% H₂O₂, 90% ethanol + 10% TN (100 mM Tris [pH 7.5], 150 mM NaCl), 70% ethanol + 30% TN, 50% ethanol + 50% TN, 30% ethanol + 70% TN, and 100% TN. After washing in EDC buffer twice, the eggs on slides were immersed in freshly prepared EDC Fixative preheated at 60°C, and then incubated at 60°C for 20 min. After the eggs were washed in PBT twice and excess fluid on the slides was

wiped off, the eggs in each well were covered with 100 μ L of proteinase K at a concentration of 2 mg/mL in PBT with 1% β -ME, and incubated at 37°C for 20 min in a moist chamber. After washing in PBS twice, the eggs were fixed in Fixative for 20 min. After washing in PBS twice, the eggs were incubated in PBS containing glycine at a concentration of 2 mg/mL. Hybridization was carried out by the same procedure for larvae and adults.

Transgenics

Caenorhabditis elegans genomic DNA corresponding to nucleotides 11,422–15,339 of cosmid C05G5 (accession number: Z70203) was PCR-amplified to produce a plasmid clone of the *let-7* gene, which was verified by DNA sequencing. *let-7* plasmid DNA at 5 μ g/mL was mixed with pBluescript II KS+ DNA at 75 μ g/mL and DNA of a transformation marker plasmid, *K10C3.4^{promoter}::rfp::unc-54^{3'UTR}* (Andachi 2008), at 20 μ g/mL, and the mixture was injected into adult gonads of the wild-type strain to generate an extrachromosomal array. The array was crossed into the *let-7* background.

Microscopy

Images of chromogenically stained samples were captured using an Axioplan2 microscope (Carl Zeiss) equipped with Nomarski differential interference contrast and a DXC-970MD CCD color camera (Sony), and processed using Photoshop (Adobe Systems) software. Images of fluorescently stained samples and anesthetized animals with the *gfp* reporter were captured using an Axioplan2 microscope equipped with epifluorescence optics and an ORCA-ER CCD camera (Hamamatsu Photonics), and processed using Photoshop software.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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