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Molecules and Cells

A Possible Role for FRM-1, a *C. elegans* FERM Family Protein, in Embryonic Development

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FRM-1 is a member of the FERM protein superfamily containing a FERM domain, which is a highly conserved protein-protein interaction module found in most eukaryotes. Although FRM-1 is thought to be involved in linking intracellular proteins to membrane proteins, the specific role for FRM-1 remains to be elucidated. In an effort to explore the biological function of FRM-1, we examined the phenotype of *frm-1(tm4168)* mutant worms. We observed that *frm-1(tm4168)* worms have a delayed hatching phenotype. Twelve hours after being laid, when virtually all wild-type eggs had hatched, only 64% of *frm-1(tm4168)* eggs had hatched. About 3% of *frm-1(tm4168)* eggs failed to hatch, even 3 days after they had been laid. We also found that *frm-1(tm4168)* mutants displayed a temperature-sensitive sterility phenotype. About 13% of *frm-1(tm4168)* worms were unable to produce eggs or produced nonviable eggs at 25°C. In contrast, less than 1% of wild-type animals were sterile at this temperature. At 20°C, neither the mutant nor wild type appeared to be sterile. Western blot analysis indicates that FRM-1 is expressed throughout the developmental stages with the strongest expression at the egg stage. Immunostaining experiments revealed that FRM-1 is mainly localized to the plasma membrane of most if not all cells at an early embryonic stage and to the plasma membrane of P cells during the late embryonic stages. GFP fusion experiments showed that FRM-1 can be expressed in the pharynx and intestine at the larval and adult stages. Our data suggest that FRM-1 may participate in diverse biological processes, including embryonic development.

INTRODUCTION

The FERM protein superfamily is characterized by the presence of a FERM (band 4.1, Ezrin, Radixin, Moesin) domain (Chishti et al., 1998). The FERM domain, a conserved, approximately 300-amino-acid module, is involved in linking intracellular proteins to the plasma membrane (Chishti et al., 1998). The FERM protein superfamily comprises a highly diverse group of proteins that includes band 4.1 proteins, ezrin-radixin-moesin (ERM) proteins, talins, merlin (NF2 tumor suppressor), protein tyrosine phosphatases, and focal adhesion kinases (Diakowski et al., 2006; Tepass, 2009). Thus, members of the FERM protein superfamily perform a wide range of functions from maintenance of cell shape to regulation of cell signaling and development.

Among the members of the FERM protein superfamily, proteins containing a FERM-adjacent (FA) domain, an approximately 60-amino-acid region immediately C-terminal to the FERM domain, are considered to be derived from a common ancestor (Bains, 2006). The best characterized members of the FERM-FA proteins are band 4.1 proteins, which include 4.1R (erythrocyte), 4.1G (general), 4.1N (neuron), and 4.1B (brain). The FA domain of the FERM-FA proteins contains conserved motifs for PKC and PKA (Bains, 2006), suggesting that the FA domain plays a regulatory role. For instance, a previous study showed that phosphorylation of the FA domain of 4.1R by PKC interfered with its ability to form a ternary complex with spectrin and actin, which resulted in reduced membrane stability (Manno et al., 2005). The band 4.1 proteins have also been shown to interact with and regulate the membrane localization of various receptors. For example, 4.1N interacts with D2 and D3 dopamine receptors (Binda et al., 2002) and AMPA receptors (Lin et al., 2009; Shen et al., 2000) and facilitates the cell surface expression of these receptors. Additionally, 4.1G and 4.1B are required for the proper localization and activation of parathyroid hormone receptors (Saito et al., 2005) and NMDA receptors (Hoy et al., 2009), respectively.

A homology search identified 16 proteins containing the FERM domain in *Caenorhabditis elegans* (Van Furden et al., 2004). ERM-1, the sole ERM protein present in *C. elegans*, has been reported to be necessary for intestinal lumen morphogenesis (Gobel et al., 2004; Van Furden et al., 2004). MAX-1/FRM-6, which contains two pleckstrin homology (PH) domains, a myosin tail homology (MyTH4) domain, and a FERM domain, plays a role in motor neuron axon guidance (Huang et al., 2002). UNC-112 interacts with integrin in the muscle cell membrane and may regulate the cell-matrix adhesion (Rogalski et al., 2000). The functions of other FERM-containing proteins, however, have yet to be determined. In the present study, we investigated the function of FRM-1, a *C. elegans* homolog of band 4.1 proteins. Deletion mutant analysis and expression

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studies suggest that FRM-1 may be involved in diverse biological processes, including embryonic development.

MATERIALS AND METHODS

C. elegans strains

The *C. elegans* Bristol N2 strain was used in this study. The *frm-1(tm4168)* mutant strain FX04168 was obtained from the National Bioresource Project for the Nematode (Japan). Nematodes were grown and maintained as described previously (Brenner, 1974).

Phenotype analysis

To analyze the delayed hatching phenotype, ten to fifteen gravid adults were placed onto one NGM plate seeded with OP50 and allowed to lay eggs for 2 h. After the adult worms were removed, the plate was incubated at 20°C, and the number of unhatched eggs was counted at various time points. For the temperature-sensitive sterility test, worms that failed to yield viable progeny were classified as sterile. For the egg-retention assay, 1-day-old adults were individually placed in a drop of 1 M NaOH and 2% sodium hypochlorite solution and the number of eggs retained in the uterus was counted. To measure the egg-laying rate, L4-stage worms were incubated on a growth plate for one day. Each adult worm was transferred to a fresh plate, and the number of eggs laid in 1 h was counted.

RNAi assay

RNAi against *frm-1* was performed by feeding worms with *E coli* expressing *frm-1* dsRNA as described by Timmons and Fire (1998) and Shin et al. (2008).

Monoclonal antibody production and Western blot analysis

To generate the monoclonal antibody specific for FRM-1, an *frm-1* cDNA fragment encoding the middle region of FRM-1 (365-557 aa) was inserted into the *E. coli* expression vector pET21a. The FRM-1 fragment was purified by Ni^{2+}-column chromatography. Monoclonal antibodies were produced by standard procedures. To analyze the development-specific expression of FRM-1, synchronized worms were collected in M9 buffer and washed twice with M9 buffer. SDS-PAGE sample buffer (60 mM Tris-Cl, 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol, 0.1% bromophenol blue) was added, and the samples were boiled for 5 min. In the case of eggs, cells were lysed by sonication in M9 buffer for 5 min before the addition of SDS-PAGE sample buffer. After centrifugation, the supernatants were subjected to electrophoresis on a 10% polyacrylamide gel. After blotting to PVDF membrane (Millipore), an anti-FRM-1 monoclonal antibody was applied to detect FRM-1 expression.

Immunostaining

Antibody staining of embryos was performed using the freeze-cracking method as previously described (Kang et al., 2009). Primary (anti-FRM-1 mouse IgG) and secondary antibodies were added at the dilution of 1:1,000. Immuno-stained images were acquired using a confocal microscope (ZEISS LSM 700, Carl Zeiss), a 100X 1.30 NA oil lens (Carl Zeiss), and the ZEN2009 software (Carl Zeiss). Images were taken as four sections along the z-axis at 0.5 μm intervals and were merged into a single projection image using the ZEN2009 software.

FRM-1::GFP expression

A plasmid construct for FRM-1::GFP expression was generated by polymerase chain reaction (PCR) and standard cloning procedures. The PCR reaction was performed using Takara PrimeSTAR polymerase. The PCR product containing an approximately 6.3 kb 5′ upstream sequence and the first 19-bp coding region of the *frm-1b* gene (the cosmid ZK270 was used as the template with FUF2 and FR2 as primers) was ligated into the *Bam*HI site of the promoterless GFP expression vector pPD95.79. Germ-line transformation of the *gfp*-fusion construct into the *C. elegans* N2 strain was performed as described by Mello et al. (1991). The GFP expression was observed using a confocal microscope (Leica TCS SP2 AOBS).

Primer sequences were as follows (restriction enzyme sites are underlined): FUF2, 5′-ATAGGATCCAGACGGTGCGACCTTGCA-3′; FR2, 5′-ATAGGATCCGATCTCTATCGGTACCCA-3′.

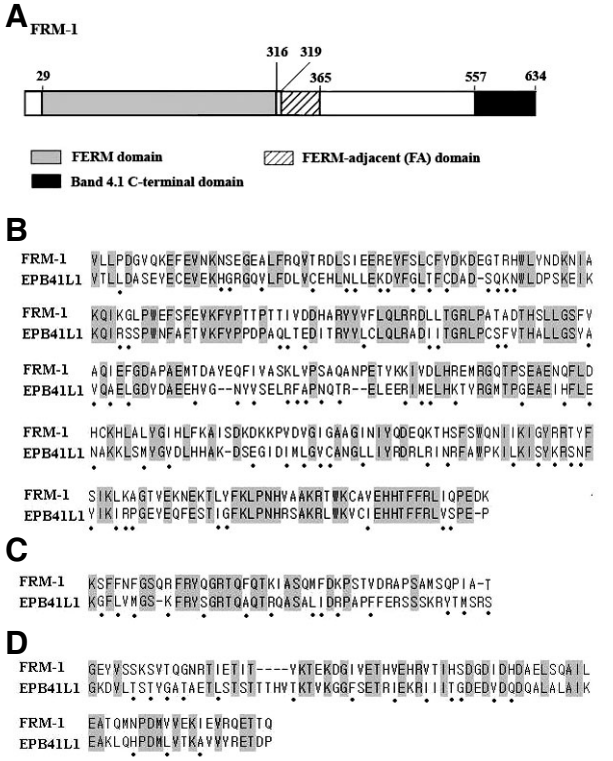

Fig. 1. Structural features of FRM-1. (A) A schematic representation of the FRM-1 domains. FRM-1 is made up of 634 amino acids containing a FERM domain (29-316 aa), a FERM-adjacent (FA) domain (319-365 aa), and a band 4.1 C-terminal domain (557-634 aa). (B-D) An amino acid sequence alignment of the FRM-1 domains (B, FERM domain; C, FA domain; D, band 4.1 C-terminal domain) with domains of EPB41L1, the human 4.1N protein, is shown. Identical and similar amino acids are indicated by grey boxes and dots, respectively.

RESULTS AND DISCUSSION

C. elegans FRM-1 is closely related to human 4.1N protein

FRM-1, a 634-amino-acid protein encoded by the *frm-1b* (ZK270.2b) gene (www.wormbase.org), contains the following three conserved domains: a FERM domain, an FA domain, and a band 4.1 C-terminal domain (Fig. 1A). A homology search indicated that FRM-1 is highly homologous to EPB41L1, the human 4.1N protein (Fig. 1B). The FERM domain exhibits 43.1% amino acid sequence identity (124/288) and 67.4% similarity

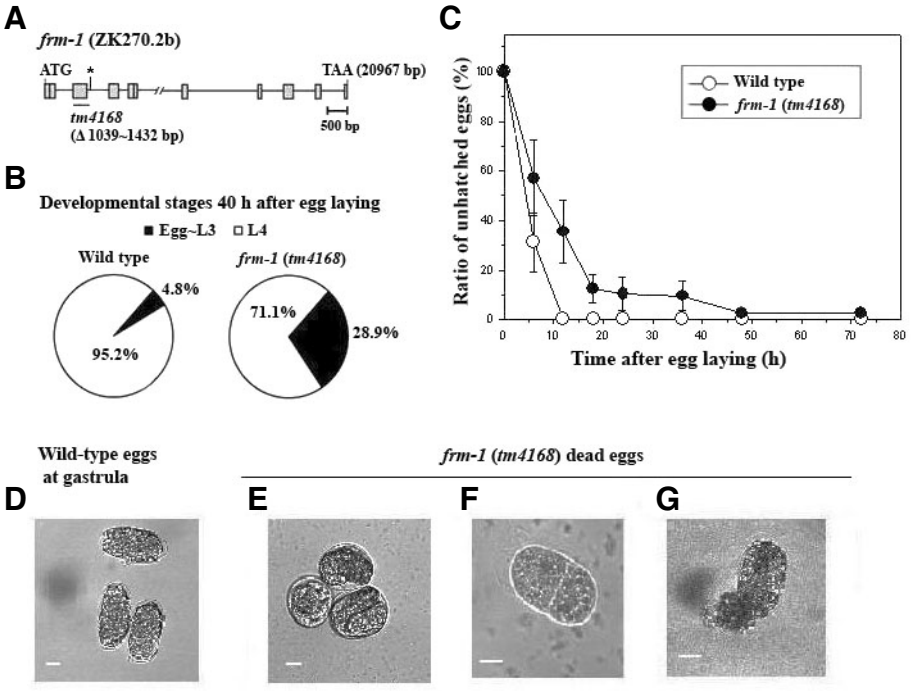

Fig. 2. *frm-1(tm4168)* worms exhibit a delayed hatching phenotype. (A) The genomic organization of the *frm-1* gene, which consists of 11 exons (boxes) and 10 introns (lines), is shown. The sixth intron is extremely large (~14 kb) and the full length is not shown here. ATG and TAA represent the translation initiation and termination codons, respectively. The *frm-1(tm4168)* mutant allele has a 394 bp deletion. An asterisk indicates the putative site of the premature stop codon. The mutant animals are predicted to synthesize an FRM-1 fragment of 118 amino acids. (B) A comparison of growth rates between mutant and wild-type animals is shown. The developmental stages of worms 40 h after egg laying were determined to measure the growth rate. The growth rate of *frm-1(tm4168)* mutants was slower when compared to the wild type. (C) A time course of hatching after egg laying is shown. A delay in hatching of *frm-1(tm4168)* mutants was observed. Wild-type gastrula-stage eggs (D) and *frm-1(tm4168)* dead eggs (E-G) were observed under a DIC microscope. Scale bars indicate 10 μm.

(194/288) to EPB41L1. The identity and similarity of the FRM-1 FA domain to EPB41L1 are 38.3% (18/47) and 68.1% (32/47), respectively. The identity and similarity of the FRM-1 band 4.1 C-terminal domain to EPB41L1 are 39.7% (31/78) and 60.3% (47/78), respectively. Although FRM-1 is expected to perform functions similar to the human 4.1N protein, the precise role of FRM-1 in *C. elegans* is not known. To explore the biological function of FRM-1, we analyzed the phenotype of an *frm-1* deletion mutant and examined the expression pattern of the *frm-1* gene.

frm-1(tm4168) mutation results in a delayed hatching phenotype

In this study we used the *frm-1(tm4168)* mutant strain, which has a 394-bp deletion including a large portion of the third exon (Fig. 2A), for phenotype analysis. This deletion generates a premature stop codon and yields a truncated FRM-1 with only 118 N-terminal amino acids. The overall morphological characteristics of *frm-1(tm4168)* worms were indistinguishable from those of wild-type worms; however, we did notice retarded growth in *frm-1(tm4168)* animals during development. Therefore, we compared the growth rate of *frm-1(tm4168)* to that of the wild type by counting the number of L4-stage worms after 40 h from the eggs being laid. Close to 30% of the mutant eggs (28.9 ± 2.4%, n = 171) had not reached the L4 stage, whereas less than 5% of the wild-type eggs (4.8 ± 1.8%, n = 190) had not reached this stage (Fig. 2B). To determine the critical period for this retarded growth, we compared the mutants and wild-type worms during a hatching time course (Fig. 2C). About 57% of mutant eggs (57.3 ± 14.5%, n = 388) had not hatched after 6 h, whereas about 31% of wild-type eggs (30.9 ± 9.8%, n = 388) were unhatched. After 12 h, about 36% of mutant eggs (36.2 ± 12.8%) had not hatched, whereas nearly all the wild-type eggs had hatched. About 3% of the *frm-1* mutant eggs (2.9 ± 0.7%) had not hatched after 72 h (which is considered embryonically lethal). A role for FRM-1 in egg hatching is also supported by the observation that inactivation of FRM-1 by RNAi results in the delayed hatching phenotype. After 12 h, about 23% of the worms treated with *frm-1* dsRNA (23.2 ± 4.6%, n = 156) had not hatched, but only 1% of the worms treated with control dsRNA (1.4 ± 0.6%, n = 172) had not hatched.

To test the possibility that the *frm-1(tm4168)* mutation delays growth during larval stages, in addition to delaying hatching, we compared the growth rates between wild-type and mutant worms from the L1 to L4 stages. We could not detect any significant difference. Most of the wild-type (98.8 ± 1.0%, n = 163) and *frm-1(tm4168)* worms (98.4 ± 0.9%, n = 199) grew from L1 to L4 larvae within 32 h. Our data indicate that the *frm-1(tm4168)* mutation delays hatching and does not affect the growth rate after hatching.

frm-1(tm4168) animals exhibit a temperature-sensitive sterility phenotype

When *frm-1(tm4168)* worms were raised at 20°C, only a tiny fraction of the mutants (less than 1%) showed a sterile phenotype (Table 1). When mutant worms were cultured at 25°C, however, the sterile phenotype increased to 12.7%. In contrast, the number of sterile wild-type worms did not significantly increase at 25°C (still less than 1%). Moreover, knockdown of the *frm-1* gene by RNAi in worms cultured at 25°C resulted in an increase in the number of sterile worms (4.2 ± 2.4%, n = 180). On the other hand, knockdown of the *frm-1* gene did not cause an increase in sterility in worms cultured at 20°C. This temperature-sensitive sterility phenotype exhibited by *frm-1(tm4168)* worms suggests that FRM-1 is responsible for viable egg production in a temperature-dependent manner.

Other phenotypes found in *frm-1(tm4168)* mutant animals

In addition to the delayed hatching and temperature-sensitive sterility phenotypes, a small fraction of *frm-1(tm4168)* worms

Table 1. *frm-1(tm4168)* mutation causes a temperature-sensitive sterility phenotype

	Sterile worms[a] (%) at 20°C	Sterile worms[a] (%) at 25°C
Wild type	0.0 ± 0.0 (n = 261)	0.6 ± 0.1 (n = 179)
frm-1(tm4168)	0.2 ± 0.1 (n = 304)	12.7 ± 2.5 (n = 154)

Sterility of 1-day-old adults was measured.
[a]Sterile worms did not produce eggs or produced eggs that failed to hatch.

(less than 1%) had larval lethality, small size, or abnormal vulva phenotype. In addition, about 6% of mutant worms showed an abnormal egg-retention phenotype. With this phenotype, the mutants had more or fewer eggs retained in the uterus than did the wild type (Supplementary Fig. S1A). Furthermore, some mutants (less than 3%) exhibited a slower egg-laying rate than the wild type (Supplementary Fig. S1B).

frm-1(tm4168) mutant embryos that fail to hatch display diverse morphologies

The dead *frm-1* mutant eggs, which did not hatch after 72 h, displayed aberrant morphologies when compared to the wild type (Figs. 2D-2G). The dead eggs were arrested at various stages (e.g., two-cell stage, gastrula, or comma stage). A significant number of the dead eggs were circular, unlike the oval shape of the wild-type eggs (Figs. 2D and 2E). Some dead eggs showed abnormal division (Fig. 2F), and a small fraction of eggs had ruptured (Fig. 2G). These observations indicate that FRM-1 may be required for various processes during embryonic development.

Expression pattern of the *frm-1* gene

To gain further insight into the function of FRM-1, we set out to determine the expression pattern of FRM-1 in *C. elegans*. First, we examined the level of FRM-1 expression at various developmental stages by Western blot analysis using an anti-FRM-1 monoclonal antibody. This monoclonal antibody detected a protein band of approximately 72 kDa in the wild-type sample but no band in the *frm-1* mutant sample (Fig. 3A, right), indicating that this antibody is specific for FRM-1. FRM-1 expression was most prominent in the embryonic stage compared to the larval and adult stages (Fig. 3A, left). The FRM-1 band of the L1 stage appeared to be somewhat denser than those bands of the L2 to adult stages. The strong expression of FRM-1 during embryogenesis, combined with the delayed hatching phenotype associated with the *frm-1(tm4168)* mutation, implies that FRM-1 plays an important role in embryonic development.

Next, we investigated the cellular localization of FRM-1 by immunostaining (Fig. 3B). At an early embryonic stage, FRM-1 was mainly localized to the plasma membrane of most, if not all, cells. This expression pattern indicates that FRM-1 may participate in membrane functions, like other members of the FERM protein superfamily. As the developmental process continued, the membrane expression of FRM-1 was diminished in all cells except for the P cells. It has been well established that eggs are derived from the P cells, and this robust P-cell-specific expression of FRM-1 raises the possibility that FRM-1 may be involved in normal egg production. At the larval and adult stages, we were unable to detect any specific FRM-1 expression in our immunostaining experiments because of false-positive signals (data not shown). Distributive cellular localization, along with a relatively weak expression of FRM-1 during theses stages, could account for this result.

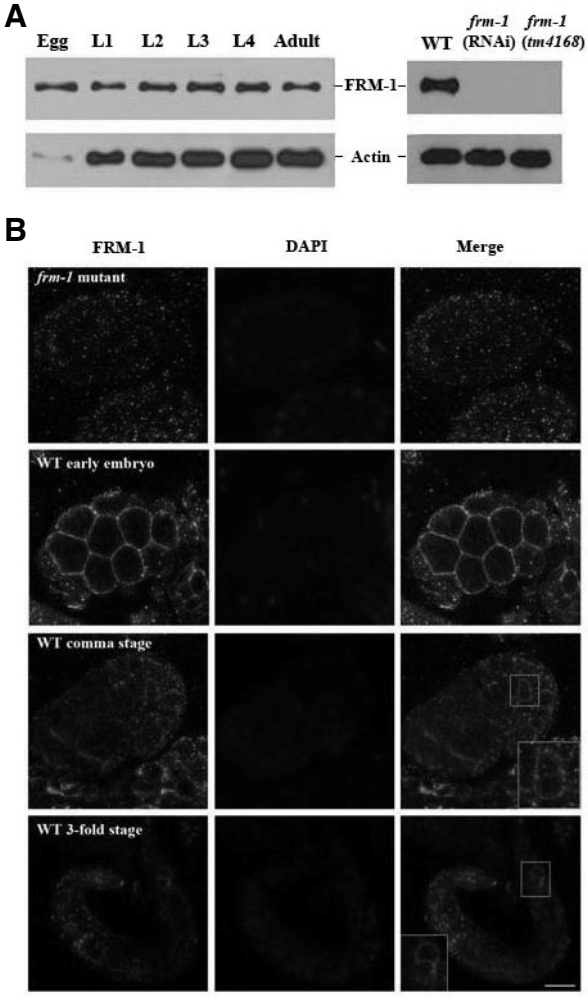

Fig. 3. Analysis of FRM-1 expression. (A) Developmental expression pattern of FRM-1 detected by Western blotting. The actin loading control is shown below. We deliberately loaded smaller amount of egg sample, because the FRM-1 band of the egg sample was very dense. (B) Localization of endogenous FRM-1 is shown. FRM-1 was not observed in *frm-1(tm4168)* embryos. In wild-type (WT) embryos, FRM-1 was mainly localized to the plasma membrane. Membrane localization of FRM-1 was weakened after the comma stage in all cells except for the P cells, the germ cell precursors. Germ cell precursors at higher magnification are shown in the boxes. FRM-1 immunostaining (left panels), DAPI staining (middle panels) and the merged images (right panels) are shown. The scale bar indicates 10 μm.

We then exploited the GFP technique to examine the cell-specific expression patterns of FRM-1 at the larval and adult stages. We made a *gfp*-fusion construct, which contains an approximately 6.3 kb upstream region from the *frm-1b* gene translation initiation site and the first 19-bp coding sequence of the *frm-1b* gene (Fig. 4A). We obtained the worms that incorporated the *gfp*-fusion construct by using the germ-line transformation technique and found that GFP was expressed in the pharynx and intestine from the larval to the adult stages (Fig. 4B).

In summary, *frm-1(tm4168)* mutant animals exhibited diverse phenotypes such as delayed hatching and temperature-sen-

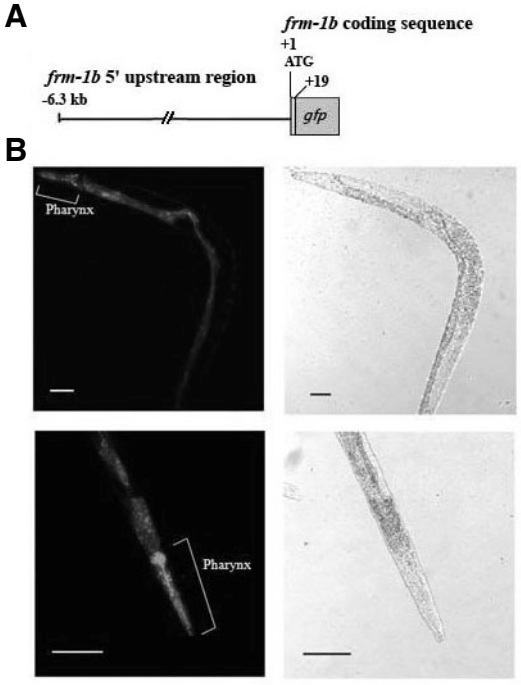

Fig. 4. Expression of FRM-1::GFP in the pharynx and intestine of larvae and adults. (A) A *gfp*-fusion construct containing an approximately 6.3 kb upstream region from the translation initiation site (ATG) and the first 19-bp coding sequence of the *frm-1b* gene is shown. (B) Expression of the *gfp*-fusion construct in the pharynx and intestine at larval and adult stages. The GFP expression patterns in an adult (top) and an L3 larva (bottom) are shown. Confocal fluorescent images (left) and corresponding optical images (right) are shown. Scale bars indicate 100 μm.

sitive sterility. The expression of FRM-1 was most prominent at the egg stage but could be detected throughout developmental stages. FRM-1 was localized to the plasma membrane of most cells at an early embryonic stage and to the plasma membrane of P cells during the late embryonic stages. FRM-1::GFP expression was observed in the pharynx and intestine from the larval to the adult stages. We propose that FRM-1 is involved in many biological functions, including embryonic development.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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