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The sperm surface localization of the TRP-3/SPE-41 Ca^{2+}-permeable channel depends on SPE-38 function in *Caenorhabditis elegans*

Gunasekaran Singaravelu [a,*], Indrani Chatterjee [a], Sina Rahimi [a], Marina K. Druzhinina [a], Lijun Kang [b], X.Z. Shawn Xu [b], Andrew Singson [a]

[a] *Waksman Institute of Microbiology, Piscataway, NJ, USA*
[b] *Life Sciences Institute and Department of Molecular & Integrative Physiology, University of Michigan, Ann Arbor, MI 48109, USA*

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ABSTRACT

Despite undergoing normal development and acquiring normal morphology and motility, mutations in *spe-38* or *trp-3*/*spe-41* cause identical phenotypes in *Caenorhabditis elegans*—mutant sperm fail to fertilize oocytes despite direct contact. SPE-38 is a novel, four-pass transmembrane protein and TRP-3/SPE-41 is a Ca^{2+}-permeable channel. Localization of both of these proteins is confined to the membranous organelles (MOs) in undifferentiated spermatids. In mature spermatozoa, SPE-38 is localized to the pseudopod and TRP-3/SPE-41 is localized to the whole plasma membrane. Here we show that the dynamic redistribution of TRP-3/SPE-41 from MOs to the plasma membrane is dependent on SPE-38. In *spe-38* mutant spermatozoa, TRP-3/SPE-41 is trapped within the MOs and fails to reach the cell surface despite MO fusion with the plasma membrane. Split-ubiquitin yeast-two-hybrid analyses revealed that the cell surface localization of TRP-3/SPE-41 is likely regulated by SPE-38 through a direct protein–protein interaction mechanism. We have identified sequences that influence the physical interaction between SPE-38 and TRP-3/SPE-41, and show that these sequences in SPE-38 are required for fertility in transgenic animals. Despite the mislocalization of TRP-3/SPE-41 in *spe-38* mutant spermatozoa, ionomycin or thapsigargin induced influx of Ca^{2+} remains unperturbed. This work reveals a new paradigm for the regulated surface localization of a Ca^{2+}-permeable channel.

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Introduction

A thorough understanding of fertilization will enable us to design a better strategy to either facilitate the process, as in fertility clinics, or to prevent the unintended conception—depending on the need. As a step towards understanding fertilization, genetically tractable model organisms facilitate the identification of the gene products required for fertility. The nematode *Caenorhabditis elegans* is an excellent model to understand the physiology of reproduction. *C. elegans* comes in two sexes: self-fertile hermaphrodites and males. The hermaphrodites produce sperm and oocytes, whereas males produce exclusively sperm.

In both sexes, diploid germ cells undergo mitotic proliferation and then enter into meiotic division to give rise to spherical, non-motile spermatids. The spermatids are rapidly converted into active, motile amoeboid spermatozoa through a post-meiotic differentiation process known as spermiogenesis or sperm activation (see Fig. 1A). The mature spermatozoon extends a pseudopod which is responsible for the motility of the sperm.

Spermatids contain specialized secretory vesicles called membranous organelles (MOs). During spermiogenesis, the MOs fuse with the plasma membrane forming continuous fusion pores and release their contents outside the sperm. The fusion of MOs with the plasma membrane is essential for fertility and mutants defective in MO-plasma membrane fusion are sterile (Achanzar and Ward, 1997). *C. elegans* lack an acrosome but, instead, show fusion of MOs. Although there are a number of differences between MOs and acrosome, the fusion of both organelles are Ca^{2+} mediated events and there are other significant similarities, indicating that both processes place proteins essential for fertilization on the sperm cell surface (Washington and Ward, 2006).

Mature, motile sperm are stored in a structure called the spermatheca. Oocytes are ovulated into spermatheca where they meet sperm and get fertilized. Genetic analysis of *C. elegans* has identified several genes that are essential for fertility in sperm or oocytes (Singson et al., 2008). The genes that function in sperm are classified as the *spe* group (spermatogenesis defective) and the genes that function in oocytes are classified as the *egg* group. The *spe* genes are further divided into many subgroups based on the developmental stage during which their activities are required (Nishimura and L'Hernault, 2010).

We are focusing our attention on the *spe-9* class of mutants (Singson, 2001) that are defective in sperm-function, namely the inability of the mutant sperm to execute fertilization, despite the

* Corresponding author at: 190, Frelinghuysen Road, Piscataway, NJ 08854, USA. Fax: +1 732 445 5735.
E-mail address: guna@waksman.rutgers.edu (G. Singaravelu).

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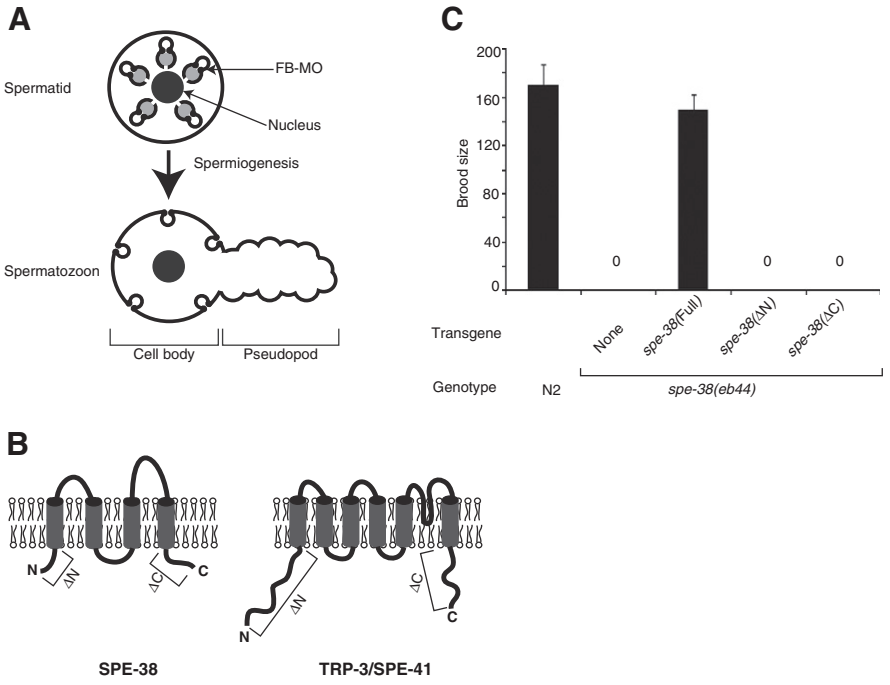

Fig. 1. (A) The cartoon showing the morphological changes associated with the spermiogenesis. (B) The schematic diagram depicting the putative topology of SPE-38 and TRP-3/SPE-38 proteins. Regions of amino acids deleted in constructs used in split-ubiquitin yeast two hybrid assay and/or transgenic worms are indicated. (C) Brood size of wild type or *spe-38(eb44)* mutants with or without the full length or truncated forms of *spe-38* transgenes. A minimum 15 worms from each genotype were analyzed.

successful production and differentiation into motile spermatozoa. The currently published members of *spe-9* class include *spe-9* (Singson et al., 1998), *spe-38* (Fig. 1A) (Chatterjee et al., 2005), *spe-41/trp-3* (Fig. 1B) (Xu and Sternberg, 2003), and *spe-42* (Kroft et al., 2005).

In this study, we focused our attention on two of the *spe-9* class genes, *spe-38* and *trp-3/spe-41*. SPE-38 is a novel membrane protein and its biochemical function is unknown. TRP-3 homologs are found in higher animals, including humans and have been implicated in a variety of diseases (Kiselyov et al., 2007), such as cancer (Bodding, 2007), heart disease (Eder and Molkentin, 2011), kidney disease (Dietrich et al., 2010) and pain (Chung et al., 2011). TRP channels are also present in the sperm of many animals including, *C. elegans* (Xu and Sternberg, 2003), mouse (Jungnickel et al., 2001) and human (Castellano et al., 2003) where they are required for fertility.

The mammalian sperm undergoes a specialized exocytosis process called the acrosome reaction upon binding with the outermost layer of the oocyte, zona pellucida (ZP) (Ikawa et al., 2010). The acrosome reaction is prerequisite for successful fertilization. The ZP induced acrosome reaction relies on TRP channels in the mouse, suggesting the importance of TRP channels in mammalian fertility (Jungnickel et al., 2001). Sperm motility is an important determinant of male fertility and the motility of human sperm is inhibited by a TRP channel inhibitor (Castellano et al., 2003). The presence and functional importance of TRP channels in the sperm of worms and mammals suggest that TRP channels are broadly utilized across the phyla and might help constitute an evolutionarily conserved biological pathway.

Here we show that SPE-38 and TRP-3/SPE-41 can physically interact with each other and the proper localization of TRP-3/SPE-41 channels to the cell surface requires SPE-38 function. Furthermore, we show that the mislocalized TRP-3/SPE-41 channel can function while still within the fused MO. The establishment of the link between TRP-3/SPE-41 and SPE-38 would enable us to better understand the pathophysiology of various diseases caused by the aberrant TRP channel localization or function. Given the paucity of available knowledge on SPE-38 function, our current results provide more clues on its cellular function. Perhaps SPE-38 directly binds to and facilitates translocation of TRP-3/SPE-41 and, possibly, other target proteins that must translocate from MO vesicles to the plasma membrane.

Materials and methods

Strains

The following strains were used in this study: N2, *dpy-5(e61)I*, *dpy-5(e61)I;him-5(e1490)V*, *dpy-5(e61) spe-38(eb44)I*, *dpy-5(e61) spe-38(eb44)I;him-5(e1490)V*, *trp-3(sy693)III*, *trp-3(sy693)III;him-5(e1490)V*, *spe-38(eb44)I;him-5(e1490)V;asEx78[Pspe-38::spe-38genomicDNA::spe-38 3′UTR + Pmyo-3::gfp]*, *dpy-5(e61) spe-38(eb44)I;him-5(e1490)V;asEx79[Pspe-38::spe-38cDNA::spe-38 3′UTR + Pmyo-2::gfp]*, *dpy-5(e61) spe-38(eb44)I;him-5(e1490)V;asEx80[Pspe-38::spe-38cDNA Full::spe-38 3′UTR + Pmyo-3::gfp]*, *N2;asEx81[Pspe-38::spe-38 cDNA ΔN::spe-38 3′UTR + Pmyo-3::gfp]*, *N2;asEx82[Pspe-38::spe-38 cDNA ΔC::spe-38 3′UTR + Pmyo-3::gfp]*, *dpy-5(e61) spe-38(eb44)I;him-5(e1490)V;asEx79[Pspe-38::spe-38cDNA Full::spe-38 3′UTR + Pmyo-2::gfp]; asEx81[Pspe-38::spe-38 cDNA ΔN::spe-38 3′UTR + Pmyo-3::gfp].* All strains were maintained on *Escherichia coli* seeded plates and manipulated as previously described (Brenner, 1974).

Constructs utilized for generating transgenic worms

We generated three constructs for our current study, all of which drives the expression of either full length or truncated forms of *spe-38* under the control of *spe-38* regulatory elements (Chatterjee et al., 2005). The construct *Pspe-38::spe-38 cDNA(full)::spe-38 3′UTR*, drives the expression of full length *spe-38* coding sequence. The constructs, *Pspe-38::spe-38 cDNA(ΔN)::spe-38 3′UTR* and *Pspe-38::spe-38 cDNA(ΔC)::spe-38 3′UTR* drive the expression of SPE-38 lacking the N and C termini, respectively.

N2 lysate was used as a template to amplify *spe-38* promoter and *spe-38 3′UTR*. A 1851 bp fragment upstream of the *spe-38* start codon was amplified by PCR to obtain the *spe-38* promoter. A 500 bp

fragment downstream of the *spe-38* stop codon was amplified by PCR to obtain the *spe-38 3′UTR*. The *spe-38* cDNA was used as a template to amplify either the full length *spe-38* cDNA or the truncated *spe-38* cDNA. The *spe-38* cDNA was amplified in such a way that the resultant amplicon would have "extra" 20 bp on either end. The "extra" sequence on the 5′ and 3′ end corresponds to 20 bp preceding and succeeding *spe-38* ORF, respectively. The full length or truncated *spe-38 cDNA* was ligated to the *spe-38 3′UTR* by adapting overlap extension PCR. Finally, the *spe-38* promoter was ligated to either the full length or truncated *spe-38 cDNA* fused with the *spe-38 3′UTR* by overlap extension PCR. The sequences of the primers and the primer pairs utilized in this study are summarized in Supplementary materials. All the PCR was carried out using Expand long template PCR system (Roche, Indianapolis, USA), in accordance to the manufacturer's recommendation. All PCR products were cloned into PCR Blunt using Zero Blunt PCR cloning kit (Invitrogen, USA) and the inserts were sequenced in their entirety.

Generation of transgenic worms

The *spe-38* constructs (100 μg/ml) were mixed with a transformation marker (100 μg/ml) and injected into the gonads of N2 hermaphrodites (Kadandale et al., 2009; Mello and Fire, 1995). The F1 self-progeny were screened for the transformants and successive three to four generations were followed to establish a stable transgenic line. At least three independent lines were generated for each transgene construct. The transgenes were introduced into *spe-38(eb44)I; him-5(e1490)V* by genetic crosses.

Generation of transgenic worms harboring double extra-chromosomal arrays

In order to obtain large number of *spe-38(eb44)* mutants expressing truncated SPE-38 for the immunostaining studies, we generated a transgenic worm that expresses both full length coding sequence of *spe-38* (so the strain could be propagated) and truncated *spe-38* (to determine the effects of the transgene on sperm function). First, we crossed *spe-38(eb44)I;him-5(e1490)V;asEx79[Pspe-38::spe-38 cDNA Full::spe-38 3′UTR + Pmyo-2::gfp]* hermaphrodites with *him-5(e1490)V; asEx81[Pspe-38::spe-38 cDNA ΔN::spe-38 3′UTR + Pmyo-3::gfp]* males. The F1 generation was screened for a very rare hermaphrodite that carried both transgenes, as judged by the expression of GFP in body wall muscle and pharyngeal muscle. We set up 64 crosses to obtain a rare hermaphrodite that possessed both transgenes. The F2 generation of the worms carrying both transgenes were singly cloned into twenty four well plates and were screened for the segregation of worms exhibiting a Spe phenotype. From the subsequent generation, we singly cloned several worms exhibiting the expression of GFP either in the pharyngeal muscle or in the body wall muscles or both pharyngeal and body wall muscle or none. We selected a clone in which all worms expressing GFP in pharyngeal muscles are completely fertile and all the worms that do not express GFP are completely sterile. The genotype of the resultant strain is *spe-38(eb44)I;him-5(e1490)V;asEx79[Pspe-38::spe-38cDNA Full::spe-38 3′UTR + Pmyo-2::gfp];asEx81[Pspe-38::spe-38 cDNA ΔN::spe-38 3′UTR + Pmyo-3::gfp].*

The males and hermaphrodites segregating from the aforementioned strain that expresses GFP in body wall muscle alone were checked for fertility. The *spe-38(eb44)I;him-5(e1490)V;asEx81[Pspe-38::spe-38 cDNA ΔN::spe-38 3′UTR + Pmyo-3::gfp]* males were crossed with *fem-3(e1996 ts)* hermaphrodites at 25 °C for 3–4 days to check the male fertility. The L4 hermaphrodites of the genotype *spe-38(eb44)I;him-5(e1490)V;asEx81[Pspe-38::spe-38 cDNA ΔN::spe-38 3′ UTR + Pmyo-3::gfp]* were transferred to a fresh plate and their self-fertility was analyzed. A similar strategy was utilized for the analysis of the fertility of *spe-38(eb44)I;him-5(e1490)V;asEx82[Pspe-38::spe-38 cDNA ΔC::spe-38 3′UTR + Pmyo-3::gfp]* worms.

Immunostaining of spermatids and spermatozoa

Immunostaining of *C. elegans* sperm was conducted as described previously (Chatterjee et al., 2005; Strome and Wood, 1982; Zannoni et al., 2003). The L4 males segregating from *spe-38(eb44)I;him-5(e1490)V;asEx79[Pspe-38::spe-38cDNA Full::spe-38 3′UTR + Pmyo-2::gfp];asEx81[Pspe-38::spe-38 cDNA ΔN::spe-38 3′UTR + Pmyo-3::gfp]* worms were examined for the expression of GFP in their body wall muscle alone and were transferred onto a fresh plate 1 day before the dissection of worms. The males were dissected on a drop of 1× sperm medium (50 mM HEPES, 25 mM KCl, 45 mM NaCl, 1 mM $MgSO_4$, 5 mM $CaCl_2$, 10 mM Dextrose; pH 7.8) to release the spermatids. The dissection of the worms was carried out as described previously (Singaravelu et al., 2011). Approximately twenty worms were dissected to obtain a sufficient number of sperm for immunostaining. The sperm were fixed using 4% paraformaldehyde in sperm medium for 30 min. The slides were washed in PBS and treated with sodium borohydrate (0.5 mg/ml in PBS) for 15 min. Following the wash, the spermatids were permeabilized by the treatment of 0.5% Triton-X 100 in PBS for 5 min and then the slides were washed with PBS. The samples were blocked in 20% normal goat serum for 15 min and washed with PBS. The rabbit α-TRP-3/SPE-41 polyclonal antibody and mouse 1CB4 monoclonal antibody (1:500) were added on the slide and incubated overnight at 4 °C. The unbound primary antibodies were washed and 50 μl each of AlexaFluor 488 conjugated goat anti-rabbit antibody (1:200) and Cy-3 conjugated goat anti-mouse antibody (1:200) were added and incubated for 2 h at room temperature. The unbound secondary antibodies were washed with PBS. Finally 7 μl of DAPI in GelMount (1 μg/ml) was applied over the sample and a cover slip was placed. The images were captured using Zeiss axioplan compound microscope equipped with X-cite series 120 as a source of illumination. A similar strategy was employed for staining the spermatids with α-SPE-38 antibody and for staining the spermatids from *spe-38(eb44)I;him-5(e1490)V;asEx82[Pspe-38::spe-38 cDNA ΔC::spe-38 3′UTR + Pmyo-3::gfp]* males. For staining spermatozoa, the males were dissected onto a drop of sperm medium containing 20 μg/ml of Pronase E.

Calcium imaging

Calcium imaging was performed as described previously (Li et al., 2011; Xu and Sternberg, 2003). Axiovert 200 microscope (Carl Zeiss) with Metaflour software (Molecular Devices) and CoolSnap ES2 CCD camera (Photometrics) was used. L4 males were picked and aged for 4 days before being used. After dissection, spermatids were activated for 15 min in Sperm Medium 1 (50 mM HEPES, 25 mM KCl, 45 mM NaCl, 1 mM $MgCl_2$, 5 mM $CaCl_2$, PH 7.8) containing 70 mM tetraethylammonium (TEA), then incubated for 30 min in Sperm Medium 1 with 5 μM Fura-2 AM at 20 °C. For CAC test, mature sperm were incubated for 10 min in Calcium-free Sperm Medium 2 (10 mM HEPES, 4 mM KCl, 110 mM NaCl, 1 mM EGTA, 1 mM $MgCl_2$, PH 7.8), then treated with Sperm Medium 2 (EGTA was replaced with 5 mM $CaC1_2$). For SOC test, mature sperm were incubated for 10 min in Calcium-free Sperm Medium 2 with 0.5 μM ionomycin or 10 μM thapsigargin, and washed twice with Calcium-free Sperm Medium 2, then switched to Sperm Medium 2.

Split-ubiquitin yeast two hybrid analysis

The split-ubiquitin two hybrid analyses were performed using a DUALmembrane kit (Dualsystems Biotech, USA) as per the manufacturer's instruction. Please see Supplementary materials for the summary of primers and plasmids used for constructing various bait and

prey plasmids. The yeast strain NMY51 was first transformed with bait plasmid and were selected on synthetic medium lacking leucine. The yeasts transformed with bait plasmid were then transformed with prey plasmid and were selected on synthetic medium lacking leucine and tryptophan. Expression was also confirmed by western blot analysis. The resultant strain harboring both bait and prey plasmids were examined for their ability or inability to turn on the reporter genes by plating them on synthetic medium lacking Trp, Leu, His and Ade. Yeast cells transformed with both pCCW-Alg5 and pAI-Alg5 express wild type C-terminal ubiquitin (Cub) and wild type N-terminal ubiquitin (Nub) and serve as a positive control. Yeast cells transformed with pCCW-Alg5 and pDL2-Alg5 express wild type Cub and mutant Nub (Ile to Gly at position 3) and serve as negative control. When SPE-38(ΔC) was used as a bait, it failed to interact with SPE-17. So, we believe SPE-38(ΔC) may not be "sticky".

Results

The SPE-38 and TRP-3/SPE-41 proteins play an important role in *C. elegans* sperm. The putative topologies of SPE-38 and TRP-3/SPE-41 proteins were previously described (Chatterjee et al., 2005; Xu and Sternberg, 2003) and are depicted in Fig. 1B. Both SPE-38 and TRP-3/SPE-41 are membrane proteins and mutations in genes encoding either of the proteins cause a similar sterile phenotype. SPE-38 is a nematode-specific novel protein that has four predicted transmembrane domains and short cytoplasmic tails at its N- and C-termini. Our previous genetic analysis conclusively established that *spe-38* is essential for sperm-function. However the mechanistic details of the function of SPE-38 were still elusive. Here we examine the role of SPE-38 in regulating the localization of subset of sperm proteins in *C. elegans*.

A screen for epistatic relationships between sperm function molecules

Based on the SPE-38 topology and mutant phenotype, we hypothesized that SPE-38 might (1) mediate the adhesion of sperm with oocytes during fertilization, (2) be involved in transducing signal(s) during fertilization, or (3) regulate the transport or subcellular localization of other protein(s) that are essential for fertility. Previously we reported that SPE-38 does not influence the localization of SPE-9 and vice versa (Chatterjee et al., 2005). In this study, we systematically analyzed the possible dependence of SPE-38 on the hitherto identified SPE-9 class proteins for its localization (see Table 1, Supplementary Fig. 1). The localization of SPE-38 is comparable to wild type sperm in all *spe-9* class mutants, suggesting that the localization of SPE-38 does not depend on any of these proteins. As a control, we examined the localization of SPE-38 in *fer-1(hc1)* mutant sperm (Achanzar and Ward, 1997). The FER-1 is required for the fusion of MO with plasma membrane during spermiogenesis and hence the MO fails to fuse with plasma membrane in the *fer-1(hc1)* mutant spermatozoa (Washington and Ward, 2006). As expected and previously reported (Chatterjee et al., 2005), the SPE-38 remains localized to MO in the *fer-1(hc1)* spermatozoa (Table 1).

Next, we asked whether SPE-38 might be required for the localization of other SPE-9 class proteins. As shown in Table 2, the localization of TRP-3/SPE-41 was found to be aberrant in *spe-38* mutant spermatozoa. Fig. 2 shows the wild type spermatids doubly stained with a monoclonal antibody 1CB4 that marks MOs (red) and an α-TRP-3/SPE-41 antibody (green). As evidenced from the merged image, the localization of TRP-3/SPE-41 is initially confined to the membranous organelles in wild type spermatids. As the spermatids differentiate into spermatozoa, TRP-3/SPE-41 localizes to the cell surface (Fig. 2). The localization of TRP-3/SPE-41 in *spe-38* mutant spermatids is indistinguishable from that of wild type spermatids. However, as the mutant spermatids differentiate into spermatozoa, TRP-3/SPE-41 fails to reach the cell surface (Fig. 2). It was previously shown by electron microscopy that MOs fuse with the plasma membrane in *spe-38* mutants (Chatterjee et al., 2005). Therefore, the lack of TRP-3/SPE-41 on the plasma membrane is not due to a lack of MO fusion. Our result indicates that SPE-38 is required for the cell surface localization of TRP-3/SPE-41 during or after spermiogenesis in *C. elegans* (Fig. 5). In contrast, the localization of SPE-38 is unperturbed in *trp-3* spermatids as well as *trp-3* spermatozoa (Table 1, Supplementary Fig. 1), indicating that TRP-3/SPE-41 localization is dependent on SPE-38, but not vice versa (Fig. 5).

Table 1
Localization of SPE-38 in the sperm of indicated genetic background relative to that of wild type.

Genotype	Spermatid	Spermatozoa	References
Wild type (N2)	+	+	(Chatterjee et al., 2005)
spe-9(eb19)	+	+	
spe-41(sy693)	+	+	
spe-42(tn1231)	+	+	(Kroft et al., 2005)
fer-1(hc1)	+	Mislocalized	(Chatterjee et al., 2005)
spe-38(eb44)	ND	ND	

Table 2
Localization of other molecules in the sperm of *spe-38(eb44)* mutants relative to wild type sperm.

Antibody	Spermatid	Spermatozoa	Reference
α-SPE-9	+	+	(Chatterjee et al., 2005)
α-SPE-41/TRP-3	+	Mislocalized	
1CB4	+	+	

Functional domains of SPE-38

The absence of SPE-38 homologs from other non-nematode species precludes the prediction of the region(s) of SPE-38 that might be necessary for its function. In order to obtain further clues about the functional determinants of SPE-38, we decided to examine the ability of truncated forms of SPE-38 to rescue *spe-38* mutants. We have previously shown that introducing *spe-38* genomic DNA can rescue *spe-38(eb44)* mutants (Chatterjee et al., 2005). Here we asked whether expressing the *spe-38* cDNA is sufficient enough to rescue the mutant. As shown in Fig. 1C, *spe-38(eb44)* mutants are completely sterile, which can be rescued by introducing a transgene that drives the expression of a full length SPE-38 cDNA from its endogenous promoter. This result suggests that the *spe-38* introns may be dispensable for its expression. SPE-38 truncated at either the N-terminal or C-terminal cytoplasmic tail does not rescue the sterility observed in *spe-38(eb44)* mutants, suggesting that intact cytoplasmic tails are essential for SPE-38 function.

We analyzed at least three independently generated transgenic lines for the genetic rescue of *spe-38* mutants and all our observed results are consistent with each other, suggesting that complexity of the extrachromosomal array might not account for the inability of the truncated forms of *spe-38* to rescue the *spe-38(eb44)*. Furthermore, we could successfully amplify the transgene-specific *spe-38* DNA from all the transgenic worms in a PCR that can distinguish the unspliced form of *spe-38*, which is found in wild type worm, from the *spe-38* cDNA that is incorporated into extrachromosomal array (data not shown). Hence, the failure of some of the transgenes to rescue *spe-38(eb44)* mutants is not due to the failure of their incorporation into extrachromosomal array. Next, we examined the presence of the SPE-38 protein in all transgenic worms by staining their sperm with α-SPE-38 antibody that is raised against a short peptide deleted in the *spe-38 (eb44)* mutant. As reported previously (Chatterjee et al., 2005), and shown in Supplementary Fig. 3, spermatids isolated from wild type worms, and not *spe-38(eb44)* mutants, could be specifically stained with this antibody. Upon introduction of either full length or

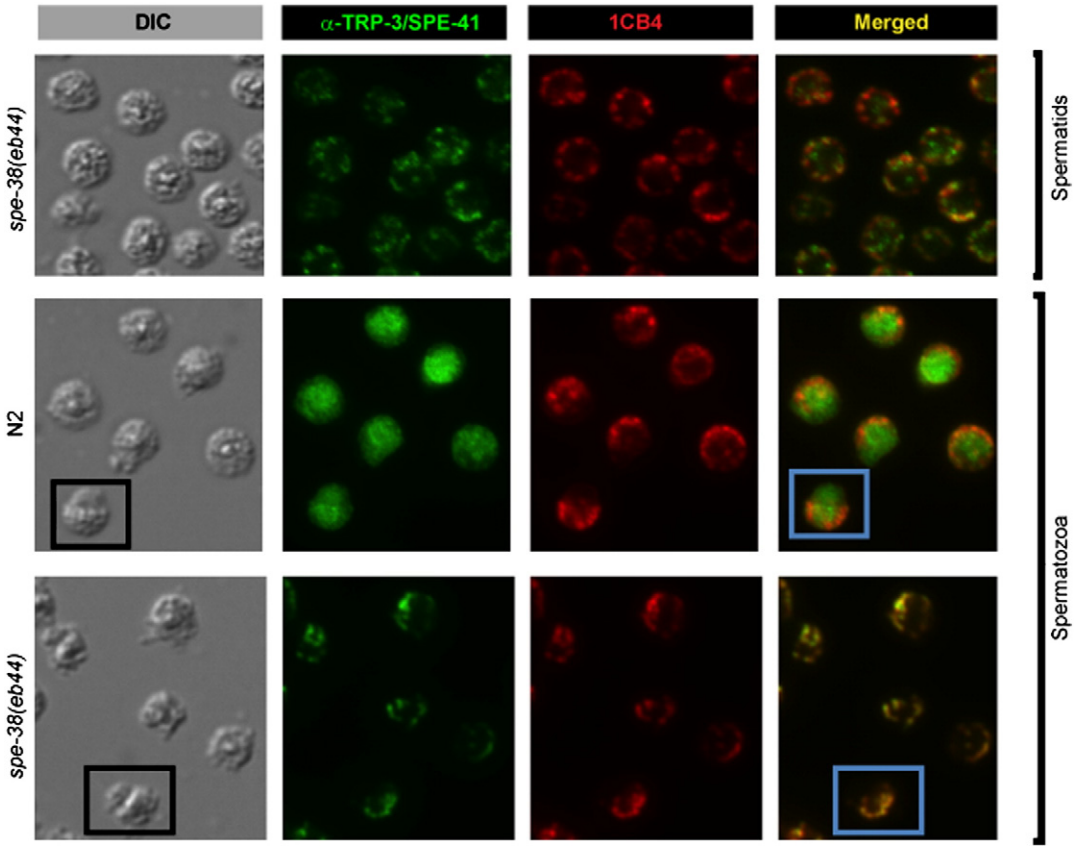

Fig. 2. TRP-3/SPE-41 is mislocalized in *spe-38* mutant. Spermatids or spermatozoa isolated from the males of indicated genotypes were doubly stained with α-SPE-41 antibody (green) and 1CB4 antibody (red).

truncated forms of *spe-38* into *spe-38(eb44)*, the spermatids can be stained with α-SPE-38 antibody (Supplementary Fig. 3), indicating the successful expression of various forms of SPE-38 from the context of extrachromosomal arrays. Despite the successful expression of all examined forms of SPE-38, only those that express full length of SPE-38 could rescue *spe-38(eb44)* mutants, overruling the possibility that failure to rescue *spe-38* mutants might be due to the inability of the expression of truncated proteins. Taken together, our results indicate that the cytoplasmic tails of SPE-38 are necessary for executing its function. Considering the absence of any recognizable domain in SPE-38 and the confinement of SPE-38 homologs within the phylum Nematoda, our results shed light on the functionally important regions of SPE-38.

TRP-3/SPE-41 can mobilize Ca^{2+} from extracellular milieu in the fused MOs

Two types of calcium channels were detected in *C. elegans* sperm, namely CAC (constitutively active calcium channel) and SOC (store-operated calcium channel) (Xu and Sternberg, 2003). The CAC is constitutively active and permeates Ca^{2+} soon after the introduction of Ca^{2+} in the extracellular milieu. The SOC is activated by the depletion of intracellular Ca^{2+} store, which can be done by the application of ionomycin or thapsigargin. *C. elegans* spermatids exhibit relatively low activity of both CAC and SOC. In contrast, mature spermatozoa exhibit levels of CAC and SOC activity that are higher than those seen in spermatids. Since much TRP-3/SPE-41 fails to exit from MOs in *spe-38(eb44)* spermatozoa, we asked whether TRP-3/SPE-41 can function when all of it is retained within MOs. We examined the activity of CAC and SOC in wild type and *spe-38(eb44)* spermatozoa. As shown in Fig. 3, channel activities of the *spe-38(eb44)* spermatozoa are indistinguishable from the wild type spermatozoa. Specifically, both ionomycin- and thapsigargin-induced Ca^{2+} influx is unperturbed in *spe-38(eb44)* spermatozoa, suggesting that despite being mislocalized, TRP-3/SPE-41 can function in *spe-38(eb44)* spermatozoa. We have previously shown that MOs do fuse with plasma membrane in *spe-38(eb44)* spermatozoa (Chatterjee et al., 2005). Thus, the TRP-3/SPE-41 channel appears to function from fused MOs.

SPE-38 and TRP-3/SPE-41 might physically interact

The phenotypic similarity observed in *spe-38* and *trp-3/spe-41* mutants combined with the overlapping localization in MOs and the epistatic relationship between SPE-38 and TRP-3/SPE-41 prompted us to investigate the potential physical interaction between SPE-38 and TRP-3/SPE-41. Since SPE-38 and TRP-3/SPE-41 are both membrane proteins, we wanted to investigate the potential physical interaction between them in their native environment and hence we performed split-ubiquitin yeast two hybrid analysis. When full length SPE-38 was used as a bait and full length TRP-3/SPE-41 was used as a prey, none of the reporter genes were turned on, as evidenced by the lack of growth of yeast on selective media (Table 3). An identical result was observed when the bait and prey were swapped. We verified the successful expression of SPE-38 and TRP-3/SPE-41 bait or prey proteins in yeast by western blot analysis. Therefore, failure to observe the interaction may not be due to lack of protein production or stability in yeast cells. Our result indicates that full length SPE-38 does not interact with full length TRP-3/SPE-41.

Our initial observation that the cytoplasmic termini of SPE-38 play an important role *in vivo* prompted us to check the physical interaction between truncated forms of SPE-38 with full length TRP-3/SPE-41. We observed a robust growth of yeast colonies on selective plates when full length TRP-3/SPE-41 and SPE-38 lacking 43 amino acids from its C-terminal tail (SPE-38 ΔC) were used as bait and prey,

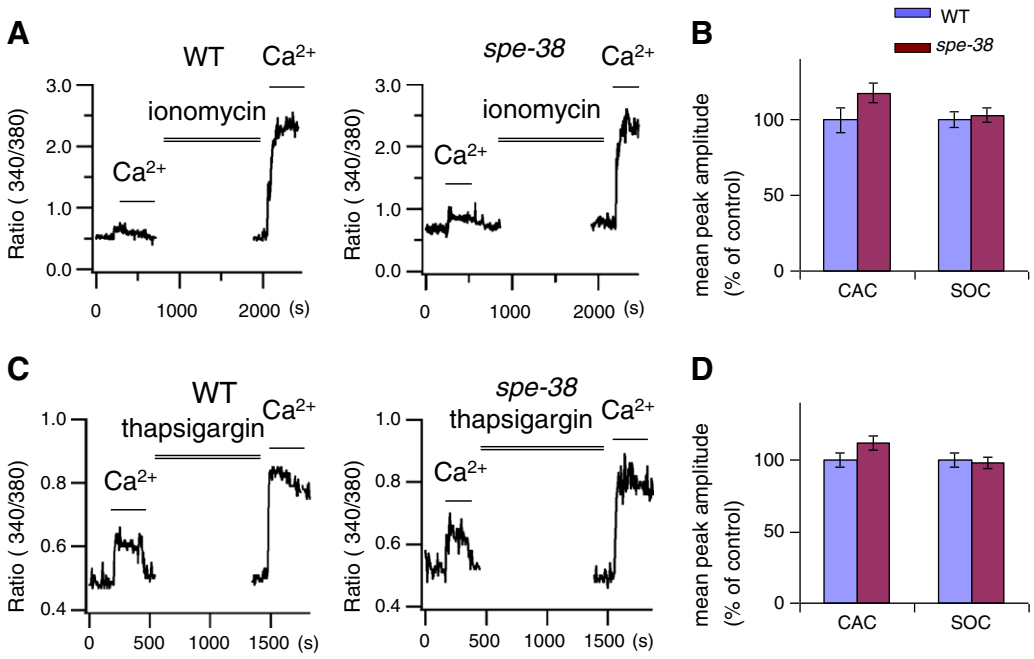

Fig. 3. SOCE remains unperturbed in *spe-38* mutant spermatozoa. (A) Calcium induced Ca^{2+} release and ionomycin induced Ca^{2+} release in wild type and *spe-38(eb44)* spermatozoa. (B) Histogram of mean peak amplitude of CAC and ionomycin-induced SOCE in wild type and *spe-38(eb44)* spermatozoa. (C) Calcium induced Ca^{2+} release and thapsigargin induced Ca^{2+} release in wild type and *spe-38(eb44)* spermatozoa. (D) Histogram of mean peak amplitude of CAC and thapsigargin-induced SOCE in wild-type and *spe-38(eb44)* spermatozoa. Traces are broken during ionomycin or thapsigargin treatment due to the pause of data acquisition to conserve disk space. No change in calcium level was observed during this period.

respectively (Fig. 4). Swapping the bait and prey gave an identical result, lending more credence to our interaction assay. Our results suggest that the C-terminal of SPE-38 might negatively regulate the physical interaction between SPE-38 and TRP-3/SPE-41. SPE-38 was not found to interact with other sperm membrane molecules SPE-9, SPE-19 and SPE-29 (Fig. 4A). This further suggests the specificity of binding between SPE-38 and TRP-3/SPE-41. Depending on the choice of bait and prey, weak or no interaction was observed when the full length of TRP-3/SPE-41 and SPE-38 lacking N-terminal tail (SPE-38 ΔN) were utilized in our split-ubiquitin assay (Table 3). This result suggests that, although both N and C termini are essential for fertility (see structure function analysis above), they interact differentially with TRP-3/SPE-41.

Next, we focused on the domains of TRP-3/SPE-41 that are essential for its interaction with SPE-38. The full length of SPE-38 does not interact with a truncated form of TRP-3/SPE-41 lacking the N-terminus (TRP-3 ΔN), regardless of which of the two proteins were used as a bait or prey. Interestingly, truncated TRP-3/SPE-41 lacking the C terminus (TRP-3 ΔC) can robustly bind with SPE-38 ΔC. This result suggests that the C-terminus of TRP-3/SPE-41 is dispensable for the interaction between SPE-38 and TRP-3/SPE-41 in this two-hybrid assay. Taken together, our results suggest a model where in the C-terminal of SPE-38 plays an inhibitory role and SPE-38 probably binds away from the C-terminus of TRP-3/SPE-41 during the interaction between these proteins.

Table 3
Summary of the split-ubiquitin yeast two-hybrid analysis of the interaction between SPE-38 and TRP-3.

Bait/prey	SPE-38 full	SPE-38 ΔN	SPE-38 ΔC
TRP-3 full	−	−	+++
TRP-3 ΔN	−	−	+++
TRP-3 ΔC	−	−	+++
Bait/prey	**TRP-3 full**	**TRP-3 ΔN**	**TRP-3 ΔC**
SPE-38 full	−	−	+++
SPE-38 ΔN	+	+	+++
SPE-38 ΔC	+++	+	+++

The top rows and left columns indicate the preys and baits utilized in the interaction assays, respectively. Absence of the interaction between the protein pair is indicated as (−). Weakly interacting protein pairs and strongly interacting protein pairs are indicated (+) and (+++), respectively.

Discussions

In this study, we show that the redistribution of the TRP-3 channel in *C. elegans*, from MOs to the cell membrane, as the spermatids differentiate into spermatozoa, depends on SPE-38. Dynamic localization of membrane proteins is a fundamental processes utilized under broader biological context. For example, localization of several membrane proteins such as, chemoattractant receptors, adhesive molecules and integrins are altered during the polarization of leukocytes (Sanchez-Madrid and del Pozo, 1999). Redistribution of proteins during various developmental stages may be very common in other animals as well. For example, several proteins exhibit altered localization after the process of capacitation in mice (Miranda et al., 2009). During capacitation, a transmembrane protein TMEM 190 partly relocates and forms protein complexes on the region of the sperm that participates in sperm–oocyte interaction in mouse (Nishimura et al., 2011). Similarly, dynamic localization of Ran-binding protein 17 is observed during spermatogenesis and spermiogenesis in mice (Bao et al., 2011).

The trafficking of TRP channels in general, is intrinsically interesting, even outside the realm of reproductive biology, since malfunctioning TRP channels are implicated in several disease states. Molecular identities of several proteins that modulate the trafficking of TRP channels have been unraveled in mammals (for review, please refer to Cayouette and Boulay, 2007). Of particular note, RNF24 is a mammalian protein that affects the trafficking of TRPC (Lussier et al., 2008). RNF24 physically interacts with TRPCs and overexpression of RNF24 causes the retention of TRPCs in the Golgi apparatus. Our result shows that SPE-38 physically interacts with TRP-3/SPE-41. However, unlike RNF24, mutation in—and not the overexpression of *spe-*

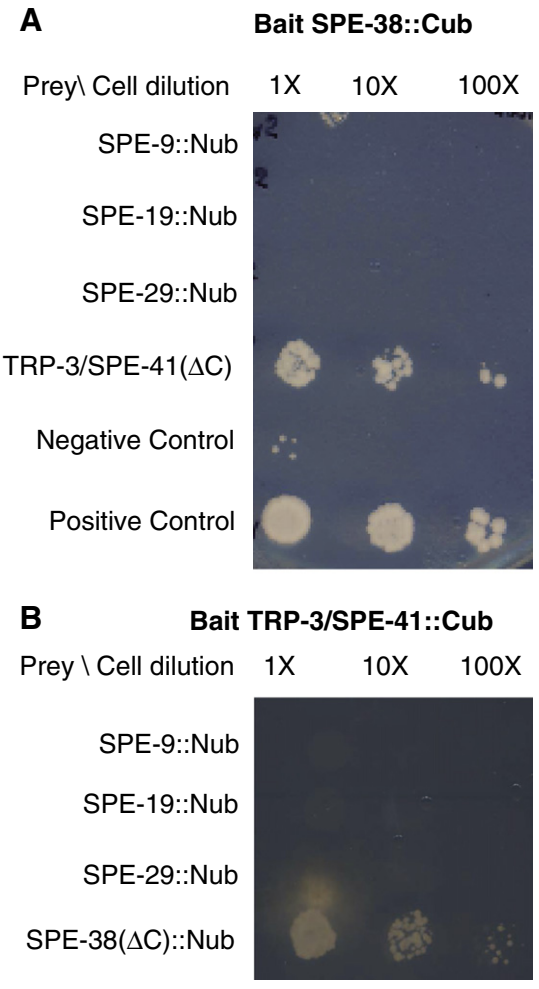

Fig. 4. (A) Split-ubiquitin yeast two hybrid analysis of the interaction between SPE-38 and indicated SPE proteins. Growth of yeast colonies on selective media indicates the physical interaction between the examined protein pairs. Negative control shows the result of the interaction between wild-type Cub and mutant Nub that are known not to interact with each other. Positive control shows the result of the interaction between wild type Cub and wild type Nub that are known to interact with each other (see Materials and methods).

38—causes the retention of TRP-3/SPE-41 in the Golgi derived MO. Taken together, it seems that SPE-38 and RNF24 are examples of positive and negative regulators of the trafficking of TRP channels, respectively. Our results indicate that SPE-38 is a novel regulator of the trafficking of TRP-3/SPE-41 channels in nematodes. Identification of other regulators will shed light on the plasticity of TRP channel activity.

The *spe-38(eb44)* mutants are completely sterile (Chatterjee et al., 2005). The sterility observed in *spe-38* mutants might be owing to the failure of the TRP-3/SPE-41 channel to reach the cell surface following sperm activation. However, *trp-3* null mutants are weakly fertile (Xu and Sternberg, 2003), suggesting that the failure of TRP-3/SPE-41 localization alone might not account for the sterility observed in *spe-38* mutants. Perhaps, in addition to TRP-3/SPE-41, SPE-38 might regulate other unidentified protein(s) that are essential for fertility. Analyzing proteins that interact with SPE-38, in terms of their roles in fertility and their potential dependence on SPE-38 for their localization, might reveal the identities of such protein(s). Alternatively, SPE-38 might play a direct role in fertility, in addition to its role in trafficking TRP-3/SPE-41 channels to the cell surface.

The split-ubiquitin yeast two-hybrid analysis suggests that SPE-38 physically interacts with TRP-3/SPE-41, providing a mechanistic explanation for the *modus operandi* of TRP channel trafficking by SPE-38. Interestingly, truncated SPE-38, and not, the full length of SPE-38 interacted with the full length of TRP-3/SPE-41 in our analysis. This could be due to the steric hindrance of the full length of proteins utilized in split-ubiquitin yeast two-hybrid analysis, which could lead to a false-negative result. It is not uncommon to observe a pair of proteins known to interact with each other failing to interact with each other in yeast-based interaction assays (Huang et al., 2007). Alternatively, the C-terminus of SPE-38 might negatively regulate the physical interaction with TRP-3/SPE-41 and masking the C-terminus might promote the interaction with TRP-3/SPE-41. The SPE-38 and TRP-3/SPE-41 proteins need to be confined to the same subcellular compartment (MOs) at one stage and be separated at a later stage. SPE-38 and TRP-3/SPE-41 are both localized to MOs in spermatids. In mature spermatozoa, the TRP-3/SPE-41 localizes to the whole plasma membrane whereas SPE-38 localizes to the pseudopod. Such dynamic changes in the localization of TRP-3/SPE-41 and SPE-38 would be impossible, if both proteins were to bind with each other constitutively. We hypothesize that the C-terminus of SPE-38

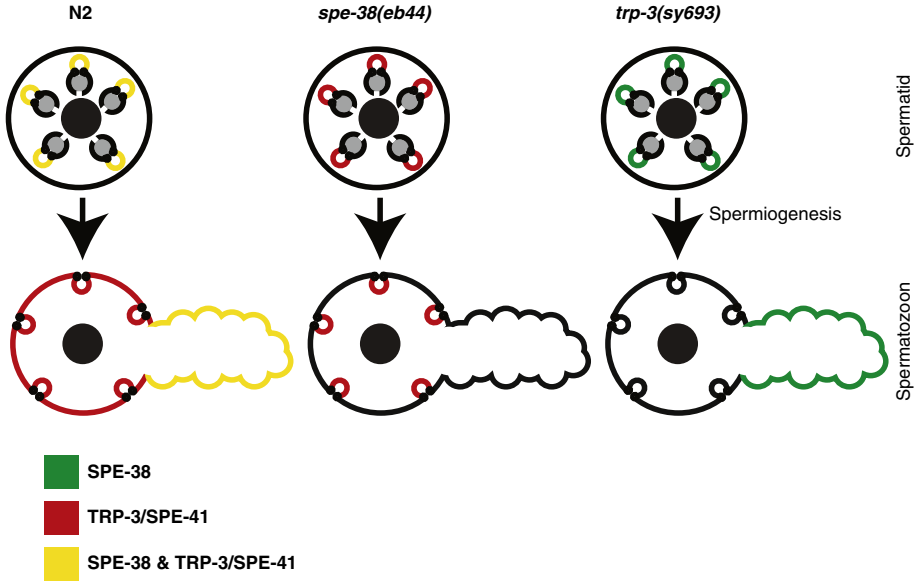

Fig. 5. The cartoon showing the localization of SPE-38 and TRP-3/SPE-41 in the spermatids and spermatozoa of indicated genotype. The localization of both SPE-38 and TRP-3/SPE-41 are confined to membranous organelles (MOs) in wild type, as well as *spe-38(eb44)* mutant spermatids. In wild-type spermatozoa, SPE-38 is localized to pseudopods and TRP-3/SPE-41 is localized to the plasma membrane. In *spe-38(eb44)* mutant spermatozoa, TRP-3/SPE-41 fail to reach cell surface and are trapped in MOs.

might function as a "switch" for its physical interaction with TRP-3/SPE-41, such that it can interact with TRP-3/SPE-41 when masked by other proteins.

Despite being mislocalized, the activity of TRP-3/SPE-41 remains unperturbed in *spe-38(eb44)* spermatozoa, which suggest several possibilities. First, the channel activity of TRP-3 might be separable from its role in fertility. Consistent with this idea, the TRP channel has been shown to exhibit dual roles in *Drosophila*, where it functions as a cation channel as well as a molecular anchor (Wang et al., 2005). Second, although the magnitude of calcium influx remains unperturbed in *spe-38(eb44)* mutants, it could be the spatial distribution of calcium influx that dictates fertility in *C. elegans*. As the localization of TRP-3/SPE-41 is altered in *spe-38(eb44)* mutant, the resulting spatial differences in Ca^{2+} influx might produce a qualitatively different signal. It is known that calcium influx can produce a variety of biological functions, where the specificity of a given function is determined by its spatial and temporal profile (Parekh, 2009). Third, a subtle difference in the quantity of Ca^{2+} influx left undetected in our experiment might be a decisive factor in *C. elegans* fertility.

Spermiogenesis is a rapid process (5 min) and does not require new protein synthesis. One way to accomplish this rapid cellular differentiation is through the redistribution of proteins to different subcellular compartments. Therefore, when sperm undergo several developmental and physiological changes, it is accompanied by changes in the dynamics of a myriad of proteins. Successful fertilization depends on controlled localization of many such proteins present in sperm and oocytes. The sperm proteins required for binding with the oocyte, for example, need to be sequestered into the cytoplasmic vesicles (MOs) in immature spermatids to prevent the precocious, abortive binding with oocyte. Equally important, these proteins should be quickly brought to the cell surface during or after the maturation process, so as to allow the fertilization to proceed successfully.

In conclusion, we unraveled a novel genetic and physical interaction between two critical sperm function molecules, SPE-38 and TRP-3/SPE-41 in *C. elegans*. Mutations in either of the genes cause a similar sterile phenotype. Both proteins co-localize in spermatids and then get differentially distributed in mature sperm (See Fig. 4). The final localization and/or activity of these molecules likely depend on the regulated interaction between these proteins. The SPE-38 is likely to regulate other molecules critical for sperm function. Our study reveals a new mechanism of regulated interaction between two membrane molecules influencing distribution and/or activity critical to cellular function.

Supplementary materials related to this article can be found online at doi:10.1016/j.ydbio.2012.02.037.

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