The *Caenorhabditis elegans spe-38* gene encodes a novel four-pass integral membrane protein required for sperm function at fertilization

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Summary

A mutation in the *Caenorhabditis elegans spe-38* gene results in a sperm-specific fertility defect. *spe-38* sperm are indistinguishable from wild-type sperm with regards to their morphology, motility and migratory behavior. *spe-38* sperm make close contact with oocytes but fail to fertilize them. *spe-38* sperm can also stimulate ovulation and engage in sperm competition. The *spe-38* gene is predicted to encode a novel four-pass (tetraspan) integral membrane protein. Structurally similar tetraspan molecules have been implicated in processes such as gamete adhesion/fusion in mammals, membrane adhesion/fusion during yeast mating, and the formation/function of tight-junctions in metazoa. In antibody localization experiments, SPE-38 was found to concentrate on the pseudopod of mature sperm, consistent with it playing a direct role in gamete interactions.

Key words: Fertilization, Sperm, Oocyte, *C. elegans*, Tetraspan, *spe-38*

Introduction

Fertilization is a unique process in which two gametes, one sperm and one egg, come together to generate a diploid zygote. Successful fertilization involves a series of well-coordinated events that include sperm-egg recognition, signaling, adhesion and fusion (for reviews, see Primakoff and Myles, 2002; Singson et al., 2001; Vacquier, 1998; Yanagimachi, 1994). Various biological molecules have been implicated in these events, and defects in the molecules involved can result in sterile or semi-fertile phenotypes.

Studies in marine invertebrates and mammals have strongly influenced our current understanding of fertilization. In many species, sperm must first interact with the extracellular matrix of the egg (Hoodbhoy and Dean, 2004; Kamei and Glabe, 2003; Yanagimachi, 1998). This extracellular egg coat (e.g. the zona pellucida in mammals and the vitelline membrane in sea urchins) not only physically protects the oocyte, but also provides a substrate for species-specific sperm binding and induces essential sperm-specific responses (e.g. the acrosome reaction). After sperm have penetrated the egg coat, direct gamete cell-cell interactions can occur (Foltz and Lennarz, 1993; Kaji and Kudo, 2004). In mammals, sperm proteins that mediate egg binding and fusion are thought to include the surface-associated protein DE (Ellerman et al., 2002), the immunoglobulin-like protein Izumo (Inoue et al., 2005), and the sperm ADAM proteins (fertilin α, fertilin β and cyritestin) (Evans, 2001). On the egg side of the equation, integrins (Evans, 2001), GPI-anchored proteins (Alfieri et al., 2003) and the tetraspanin CD9 (Kaji et al., 2000) are thought to mediate sperm binding and/or fusion. The relationship between these various molecules and their precise biochemical function during fertilization are poorly understood and remain controversial (Cho et al., 2000).

Our current understanding of the molecular machinery required for the steps of fertilization remains fragmentary and would be significantly bolstered by identifying additional core components. The nematode *C. elegans* is an excellent model system for such studies (Singson, 2001). *C. elegans* exists primarily as a self-fertile hermaphrodite that produces both sperm and oocytes, and less frequently as a male that produces only sperm. In fertile hermaphrodites, self and outcross sperm are stored within a spermatheca. *C. elegans* oocytes are produced in an 'assembly line'-like fashion by the hermaphrodite gonad. As oocytes undergo meiotic maturation and ovulation, they enter the spermatheca and come into contact with the crawling sperm, which employ an amoeboid mode of cellular motility. The coordination of cellular events and gamete presentation leads to extremely efficient utilization of sperm; essentially every functional sperm fertilizes an oocyte (Kadandale and Singson, 2004; Ward and Carrel, 1979). The zygote then completes its meiotic divisions, secretes a protective egg shell, passes through the hermaphrodite uterus and is laid prior to hatching.

Powerful forward and reverse genetic approaches have identified several new genes required for fertilization in *C. elegans* (Geldziler et al., 2004; Singson et al., 1998; Xu and Sternberg, 2003). Spermatogenesis-defective (*spe*) hermaphrodites lay unfertilized oocytes and are self-sterile (L'Hernault and Singson, 2000), but they produce viable

progeny when crossed with wild-type males, thus permitting the propagation of these mutations to subsequent generations. A few *spe* genes are required for sperm function specifically during fertilization rather than for the meiotic and morphogenetic events of spermatogenesis (Singson, 2001). The first of these genes to be cloned and phenotypically analyzed was *spe-9* (Putiri et al., 2004; Singson et al., 1998; Zannoni et al., 2003). The *spe-9* gene encodes a sperm transmembrane protein with multiple epidermal growth factor (EGF) repeats. Because its amino acid sequence and structural organization is similar to ligands of the Notch/LIN-12/GLP-1 family of receptor molecules, SPE-9 is a plausible candidate for the sperm ligand for an as yet unidentified oocyte receptor (Singson, 2001). Mutants of a second gene, *trp-3* (also known as *spe-41*), phenocopy *spe-9* mutants (Xu and Sternberg, 2003). *trp-3* encodes a TRPC-type (transient receptor potential canonical) calcium-conducting ion channel, and is proposed to regulate calcium flux during sperm-oocyte interactions at fertilization.

Clearly, many key components in the molecular mechanics of *C. elegans* fertilization have yet to be identified. Here, we report the phenotypic and molecular analysis of *spe-38*. Homozygous *spe-38* mutants exhibit sperm-specific fertility defects that are similar to animals that lack functional copies of *spe-9* or *trp-3*.

Materials and methods

Nematode strains, genetic mapping and phenotypic analysis

C. elegans strains were bred and maintained at 16°C, 20°C or 25°C using standard techniques (Brenner, 1974). For temperature-sensitive (ts) mutations, 25°C was the non-permissive temperature. Bristol (N2) was the wild-type strain. All other strains were Bristol-derived except for the Hawaiian strain (H) CB4856 that was used in the single nucleotide polymorphism (SNP)-mapping studies. The genetic markers and deficiencies used were: *dpy-5(e61)*, *unc-75(e950)*, *spe-38(eb44)*, *stu-10(oj14)*, *sqv-5(n3039)*, *qDf-7*, *hDf-17*, *spe-9(eb19), spe-9(hc52*ts*), fer-1(hc1)*, *trp-3/spe-41(sy693)*, *fem-1(hc17*ts*)*, *fog-2(q71), him-5(e1490)*. The *spe-38(eb44)* mutation was isolated from an ethyl methanesulfonate (EMS) mutagenesis conducted by Mako Saito and Tim Schedl (M. Saito and T. Schedl, personal communication). Descriptions of all other strains can be found in WormBase (Harris et al., 2004). Many strains were constructed in a *him-5(e1490)* genetic background, which increases the frequency of males without causing adverse effects on sperm (Nelson and Ward, 1980).

Phenotypic analysis of *spe-38* mutants was conducted essentially as described for other fertility mutants (Kadandale and Singson, 2004; Putiri et al., 2004; Singson et al., 1999; Singson et al., 1998; Xu and Sternberg, 2003). The number of self progeny for wild type (N2), *spe-38* mutants and the transgenic rescue stocks was determined by placing single worms on separate culture plates and counting entire individual broods. Ovulation rates were assessed by scoring the total number of embryos and unfertilized oocytes laid either during a specific time period or over the entire lifetime of a worm. In male fertility tests, four *spe-38* or wild-type males were crossed with single *dpy-5* hermaphrodites for 24 hours at 20°C. After two days, the total number of Dpy (self) and Non-Dpy (outcross) progeny were scored. Sperm competition (Singson et al., 1999) and mosaic analysis experiments (Herman, 1995; Singson et al., 1998) employed the transgenic rescuing stock *spe-38(eb44), asEx67 [A.1+A.2, myo-3::GFP]*. *spe-38* animals lacking the rescuing transgene were identified as failing to express the *myo-3::GFP* reporter in either their somatic tissue or progeny. Sperm isolation and in vitro activation protocols were performed as described by L'Hernault and Roberts (L'Hernault and Roberts, 1995). In sperm motility and localization experiments, feminized *fem-1(hc17*ts*)* or *fog-2(q71)* females were crossed with *spe-38(eb44)*; *him-5(e1490)* males for one day at 25°C, and male sperm within the hermaphrodite spermatheca were visualized either by light microscopy, and/or by DAPI staining. For light microscopic observation of ovulation and sperm-oocyte contact, animals were anesthetized in a solution of M9 salts containing 0.1% tricaine and 0.01% tetramisole, as described by McCarter et al. (McCarter et al., 1999).

Molecular biology, identification and analysis of the *spe-38* gene

Molecular techniques not described in detail here can be found in Sambrook et al. (Sambrook et al., 1989). The position of the *spe-38* gene was determined using standard linkage mapping (Sulston and Hodgkin, 1988) and single nucleotide polymorphism (SNP)-mapping (Swan et al., 2002) (see Table S1 in the supplementary material). Transgenic worms (Mello et al., 1991) were generated carrying PCR products corresponding to different combinations of the Y52B11A.1 and Y52B11A.2 genes. Through genetic crosses, the transgenes were then transferred into a *spe-38* mutant background where their competence to rescue the *spe-38* defect was assessed by brood size analysis. The structure of the Y52B11A.1 and Y52B11A.2 genes was confirmed by analysis of PCR products from a male-derived cDNA library (Achanzar and Ward, 1997). The nature of the *spe-38(eb44)* mutation was identified by amplifying the Y52B11A.1 gene from a lysate of *spe-38(eb44)* worms and comparing its sequence with that of the N2 strain.

The *eb44* mutation was mapped to linkage group I between the markers *dpy-5* and *unc-75*. The same two markers were used for two- and three-factor mapping, and this localized *eb44* within a one map unit interval on the right arm of LG I (see Table S1 in the supplementary material, Fig. 6A). Three genes associated with fertility defects (*stu-10*, *sqv-5* and *spe-9*) had been previously mapped to this region, but complementation analysis indicated that the *eb44* was not an allele of any of these genes (see Table S1). *eb44* was thus considered to define the *spe-38* gene. *eb44* also complemented two deficiencies in the region, *qDf7* and *hDf1*. This *qDf7* result was surprising because *qDf7* was thought to span the *spe-38* region, and it suggests that *qDf7*, like many other *C. elegans* deficiencies, is molecularly complex (P. Kadandale and A.S., unpublished). Single nucleotide polymorphisms that generated restriction fragment length polymorphisms (SNIP-SNPs) between N2 and Hawaiian (H) strains of worms were used to further position *spe-38* on the physical map. N2/H hybrids were generated by crossing *spe-38(eb44)*; *dpy-5(e61)* and *spe-38(eb44)*; *unc-75(e950)* homozygous hermaphrodites to wild-type Hawaiian males. Recombinant offspring from the hybrid worms (i.e. Dpy Non-Spe or Unc Non-Spe) were isolated and lines were established. Worm lysates were prepared for 41 such individual lines, and SNP analysis was carried out by PCR amplification using specific primers in the region of the SNP followed by restriction digestion using specific enzymes. Data from five SNIP-SNPs (see Table S1 in the supplementary material) effectively positioned *spe-38* between the two cosmids F49D11 and W02B9 (Fig. 6A). This region of approximately 125 kb contains at least 14 predicted genes. After sequencing PCR products from this sub-region, we identified three new SNPs (Y52B11 SNP1-3) that can only be detected by sequencing. Using these new SNPs, analysis of several Dpy Non-Spe and Unc Non-Spe recombinants localized *spe-38* to a small region on the yeast artificial chromosome (YAC) Y52B11A that contains only three predicted genes, Y52B11A.1, Y52B11A.2 and Y52B11A.3.

For the transgenic studies, PCR products were co-injected with the *myo-3::gfp* selectable marker (pPD118.20 Fire Lab Vector Kit). PCR products corresponding to the Y52B11A.1 and Y52B11A.2 genes were generated using the following primers:

3′A.1, 5′-CGATTATTGCCGTATTGCGTGTTCT-3′;
5′A.1, 5′-ACTTTCTGACTCCACGTGCGACTAC-3′;
5′A.1+, 5′-GCTATTACCATCACATTATCCGCTTTC-3′;
3′A.2, 5′-GCACACGTAGGGAGTTTAAAATTGA-3′; and
5′A.2, 5′-CGCTAGGTGAGGCTCAGAGACTAC-3′;

To sequence the Y52B11A.1 gene from *spe-38(eb44)* the following primers were used to generate appropriate PCR products:

P1, 5′-TGTGTACATTATCACAATCACGATTTGG-3′; and
P2, 5′-GGCGGAAAATTTGAGAAAATCTGA-3′.

Sequencing confirmed that the *eb44* mutation was a deletion of 270 base pairs from nucleotide position 1:10975258 to nucleotide position 1:10975528. In place of the missing sequence were 17 bases with the sequence GCCCTTTCAACCCATTT.

The sequence of the SPE-38 protein was compared with other proteins in the database using BLASTP analysis and HHpred (Altschul et al., 1990; Soding, 2005). A Kyte-Doolittle (Kyte and Doolittle, 1982) plot and topology algorithm (Pasquier et al., 1999) was used to predict transmembrane domains within SPE-38 and PRM1. A search for protein motifs and structural similarities with other tetraspan molecules was carried out using the SMART web-based tool (Schultz et al., 2000).

Immunofluorescence, western analysis and microscopy

To generate SPE-38 antibodies, rabbits were initially pre-screened to identify those whose sera lacked cross-reactivity with *C. elegans* spermatids. Negatives were injected with keyhole-limpet hemocyanin-coupled peptides corresponding to SPE-38 amino acids 101-114 (antibodies generated by Zymed Laboratory).

For the isolation of sperm, animals were dissected in sperm media containing dextrose (SM) (Machaca et al., 1996; Nelson and Ward, 1980). Spermatids were isolated from celibate wild-type or *spe-38* males. In vivo-activated spermatozoa were dissected directly from the spermatheca and uteri of unmated hermaphrodites, wild-type hermaphrodites mated to wild-type males, or females (*fog-2* or *fem-1*) mated to mutant (*spe-38* or *fer-1*) males. For in vitro-activation experiments, celibate males were dissected directly into sperm media containing either Pronase E (200 mg/ml) or 60 mM triethanolamine (Shakes and Ward, 1989).

Western blots were done as described by Sambrook et al. (Sambrook et al., 1989). Preparations from exactly 400 *him-5* or *spe-38, him-5* males were loaded into each lane. Equivalent loading was also assayed by Coomassie blue staining. The *spe-38, him-5* homozygous males were selected individually from our transgene-balanced stock by the lack of transgene GFP marker expression. SPE-38 localization in spermatids or mature spermatozoa was carried out using previously described fixation and staining protocols (Strome and Wood, 1982; Zannoni et al., 2003), or with live cell staining. For live cell staining experiments, worms were dissected directly on Color Frost Plus slides (Fisher Scientific) in 20 μl SM containing polyclonal anti-SPE-38 antisera (1:100 dilution), and subsequently incubated in a humid chamber at room temperature for 30 minutes. Samples were then fixed in –20°C methanol for 30 minutes. After three washes in phosphate-buffered saline (PBS), the slides were blocked for 30 minutes in PBS containing 0.5% BSA and 0.1% Tween-20, before a 1.5-hour room temperature incubation with affinity-purified TRITC-conjugated (Jackson ImmunoResearch Laboratories) or Alexa Fluor 488-conjugated (Molecular Probes) goat anti-rabbit antisera (Ig Fc fragment specific). Slides were dip washed in PBS and mounted with GelMount (Biomedia) containing DAPI. Monolayers of cryomethanol-fixed sperm were prepared as previously described (Golden et al., 2000), with the addition of a 0.2% Triton X-100 permeabilization step following the initial post-fixation wash. In some experiments, dissected sperm were fixed, in the absence of a freeze-crack step, in 4% paraformaldehyde in SM for 20 minutes at room temperature. Similar conditions were used for immunostaining with either anti-SPE-9 (Zannoni et al., 2003) or the mouse monoclonal antibody 1CB4 (Okamoto and Thomson, 1985). 1CB4 staining was detected using Cy3-conjugated (Jackson ImmunoResearch Laboratories) or Alexa Fluor 488-conjugated (Molecular Probes) affinity-purified goat anti-mouse secondary antibody. For the peptide competition experiments, excess lyophilized SPE-38 peptide was incubated on ice with diluted (1:100) anti-SPE-38 antibody for 30 minutes. The peptide-Ab solution was clarified by centrifugation before use, as above.

Samples were prepared for transmission electron microscopy according to Hall (Hall, 1999). DAPI analysis of whole-mount and dissected hermaphrodite gonads was performed according to Miller and Shakes (Miller and Shakes, 1995). Unstained images were obtained using Nomarski differential interference contrast (DIC) microscopy. Epifluorescence images were captured on either an Olympus BX-60 or Zeiss Axioplan compound microscope equipped with either a Cooke or Optronics cooled CCD camera. Images were edited in IP software, Adobe Photoshop, or Deneba Systems Canvas 9. For the co-localization experiments, images were captured using a Nikon Eclipse TE300 microscope equipped with a Biorad Radiance 2100-AGR3Q confocal/multiphoton system and processed using LaserSharp 2000 (v. 6.0) imaging software.

Results

spe-38(eb44) mutants are sterile due to a sperm-specific defect

Unmated hermaphrodites that are homozygous for the *spe-38(eb44)* mutation are sterile and never produce any progeny regardless of culture temperature (Fig. 1A). However, *spe-38* hermaphrodites produce viable progeny when crossed to wild-type (N2) males, indicating that *spe-38* oocytes are functional. Such crosses allowed for the propagation of the *spe-38(eb44)* mutation and provided many generations of backcrossing. The *spe-38(eb44)* mutation is recessive; heterozygous *eb44/+* animals have wild-type fertility. Because *spe-38* hermaphrodites display no additional mutant phenotypes, we conclude that these hermaphrodites have a sperm-specific defect.

To assess male fertility, we compared the ability of *spe-38* and wild-type males to sire outcross progeny from morphologically marked, but otherwise wild-type hermaphrodites. In sharp contrast to wild-type controls, *spe-38* males failed to sire any outcross progeny (Fig. 1B, Fig. 5B). This infertility was not due to an inability to mate, as *spe-38* mutant males were found to transfer wild-type levels of sperm (see below) and exhibited wild-type mating behavior (Liu and Sternberg, 1995). Therefore, the *spe-38* mutant defect affects both male and hermaphrodite sperm, and *spe-38* mutant sperm are incapable of fertilizing either *spe-38* or wild-type oocytes.

Sperm from *spe-38* mutants are indistinguishable from wild-type sperm

To determine whether the fertility defects exhibited by *spe-38* worms were due to abnormal sperm morphology, we closely compared *spe-38* sperm to wild-type sperm. When examined using DIC optics, spermatozoa from *spe-38* mutant males and hermaphrodites were indistinguishable from wild-type spermatozoa (Fig. 2A-C). Notably, spermiogenesis, the maturation of spherical, sessile spermatids into polar, motile spermatozoa (Muhlrad and Ward, 2002; Shakes and Ward, 1989), was unaffected; *spe-38* sperm activated normally both in vitro (Fig. 2B) and in vivo (Fig. 2C). When examined using transmission electron microscopy (TEM), spermatozoa within

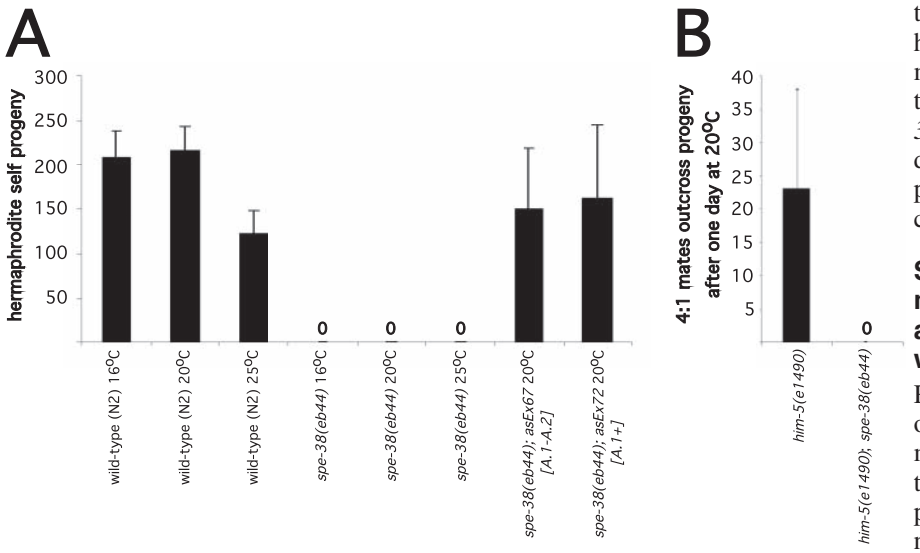

Fig. 1. *spe-38* mutant worms are sterile. (A) Quantification of hermaphrodite self-fertility with (*asEx67 [A.1-A.2]*, *asEx72 [A.1+]*) or without rescuing transgenes. (B) Quantification of male fertility. Four males were crossed to single *dpy-5* hermaphrodites that were then allowed to produce non-Dpy outcross progeny for one day. (A,B) Progeny counts of the indicated genotypes (*x*-axis) were performed on a minimum of eight individuals or crosses. Error bars indicate s.d.

the reproductive tract of adult *spe-38* hermaphrodites also exhibited wild-type morphology (Fig. 2D-F). As in wild type, the membranous organelles (MOs) in *spe-38* sperm fuse with the plasma membrane during spermiogenesis to form permanent pores surrounded by an electron dense collar (Fig. 2F).

Sperm from *spe-38* mutants can migrate to the site of fertilization and make contact with oocytes without sperm entry

Regardless of their source (hermaphrodite or male) all *C. elegans* sperm must be motile in order to maintain their position in the spermatheca against a continual flow of passing oocytes. In *spe-38* hermaphrodites, normal meiotic maturation and ovulation events that deliver the mature oocyte to the sperm within the spermatheca could be observed directly with light microscopy (Fig. 2C, Fig. 3A-D). Although we observed several instances of direct gamete contact within the spermatheca, no fertilization occurred, as indicated by the rows of unfertilized oocytes within the uteri

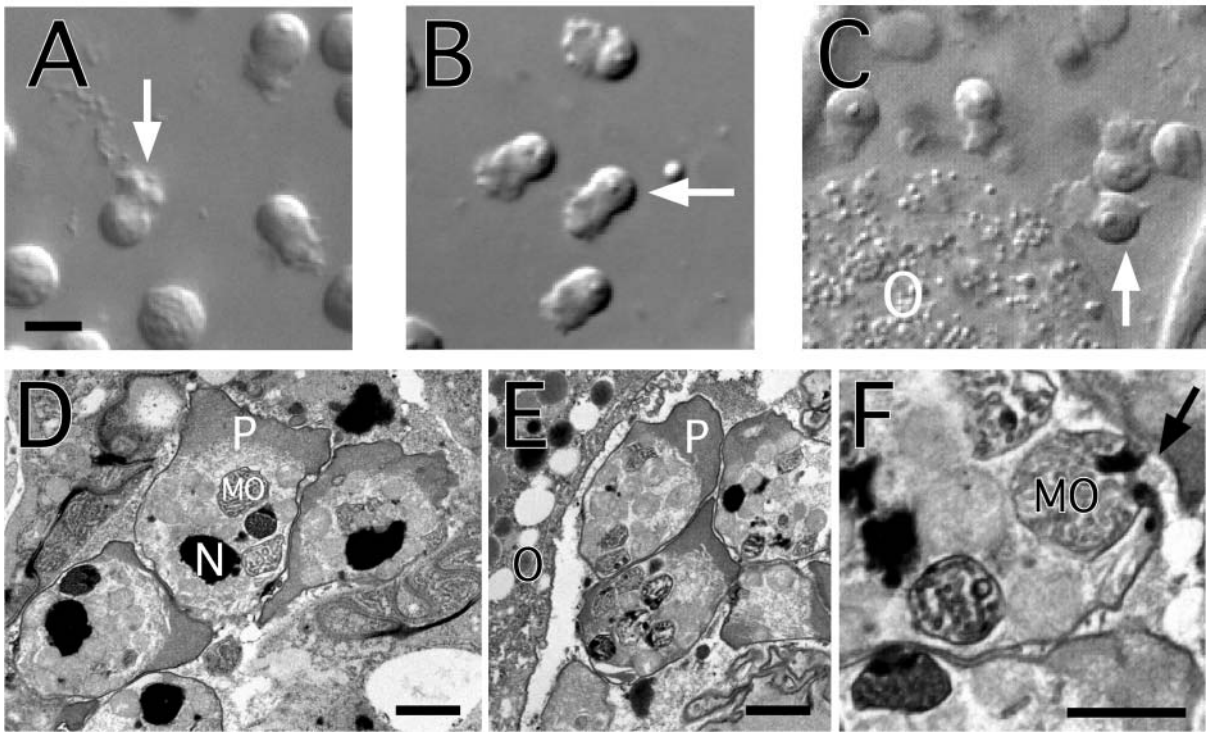

Fig. 2. The morphology of *spe-38* mutant sperm is indistinguishable from that of wild-type sperm. (A-C) White arrows indicate examples of spermatozoa in Nomarski DIC images. In vitro-activated sperm from wild-type (A) and *spe-38(eb44)* (B) male worms appear identical. (C) In vivo-activated hermaphrodite sperm within the spermatheca of an unmated *spe-38(eb44)* hermaphrodite are indistinguishable from wild-type sperm (A). Some of these sperm are in direct contact with the oocyte. (D-F) Transmission electron micrographs of *spe-38(eb44)* sperm within *spe-38(eb44)* hermaphrodites. The ultrastructural details of *spe-38* sperm are indistinguishable from those of wild type, including the sperm chromatin mass (N), the pseudopod (P) and the fused membranous organelles (MOs). (F) Close-up view of MOs in *spe-38(eb44)* sperm highlights the fusion pore (black arrow) surrounded by an electron-dense collar. Scale bars: in A, 5 μm for A-C; 1 μm in D,E; 0.5 μm in F.

of unmated *spe-38* hermaphrodites (Fig. 3A-D). Furthermore, just like wild-type sperm, any *spe-38* sperm that were displaced into the uterus by passing oocytes were able to actively crawl back into the spermatheca.

Sperm that are deposited into the hermaphrodite uterus by males must migrate to the spermatheca. Our sperm competition data (see below) suggests that male-derived *spe-38* sperm can migrate to the spermatheca and displace hermaphrodite sperm. However, in order to directly observe the accumulation of male-derived sperm in the spermatheca, we crossed *spe-38* males to *fem-1* females and tracked sperm accumulation by DAPI staining of whole animals. This assay also gave us a way to roughly compare the number of sperm transferred. Unmated wild-type hermaphrodite controls have many sperm in the spermatheca (Fig. 4A). Unmated *fem-1* mutant females lack endogenous sperm, have an empty spermatheca, and accumulate mature oocytes within their proximal gonad arm. Because of the low rates of ovulation, oocytes with endomitotically replicating (emo) DNA are often present close to the spermatheca (Fig. 4B, Fig. 5A) (Doniach and Hodgkin, 1984; Miller et al., 2003). During mating, wild-type (Fig. 4C) and *spe-38* (Fig. 4D,E) males transfer comparable amounts of sperm to *fem-1* females. In both cases, transferred sperm can be detected in the spermatheca, where they stimulate ovulation and relieve the back up of oocytes within the gonad arm. However, *fem-1* females mated to *spe-38* males fail to produce viable progeny and their uteri fill with what appears to be unfertilized oocytes containing the characteristic emo DNA (Fig. 4E, Fig. 5B). Identical results were obtained when *spe-38* males were crossed to *fog-2* females (see below).

To distinguish whether the *spe-38* defect blocks sperm entry or an early post-fertilization step, these presumably unfertilized oocytes were isolated from the uteri of animals stained with DAPI. In the newly fertilized oocytes of wild-type hermaphrodites, the highly condensed sperm chromatin mass can be easily distinguished from the meiotically dividing oocyte chromatin (Fig. 4F). In the youngest in utero oocytes of unmated *spe-38* hermaphrodites (Fig. 4G) or *fog-2* females that were crossed to *spe-38* males (Fig. 4I), no sperm DNA could be detected. As these older unfertilized oocytes aged, they became emo and accumulated high levels of DNA (*fem-1* female×*spe-38* males, Fig. 4E; unmated *spe-38* hermaphrodites, Fig. 4H; *fog-2* female×*spe-38* males, Fig. 4J). By contrast, wild-type sperm successfully enter *spe-38* oocytes, and the resulting embryos develop normally within the uterus (Fig. 4K). Taken together, these data indicate that, although *spe-38* mutant sperm are fully motile and can migrate to the correct location in the reproductive tract, they fail to enter oocytes.

spe-38 sperm are competent to stimulate ovulation and can participate in sperm competition

In *C. elegans*, oocytes must undergo meiotic maturation and ovulation in order to be fertilized (McCarter et al., 1999). The major sperm protein (MSP) functions as a signaling molecule to induce the meiotic maturation and ovulation of oocytes above basal levels (Miller et al., 2003). This signal helps the worms to avoid wasting metabolically costly oocytes when no sperm are present in the reproductive tract. To examine whether *spe-38* mutant sperm are signaling competent, we

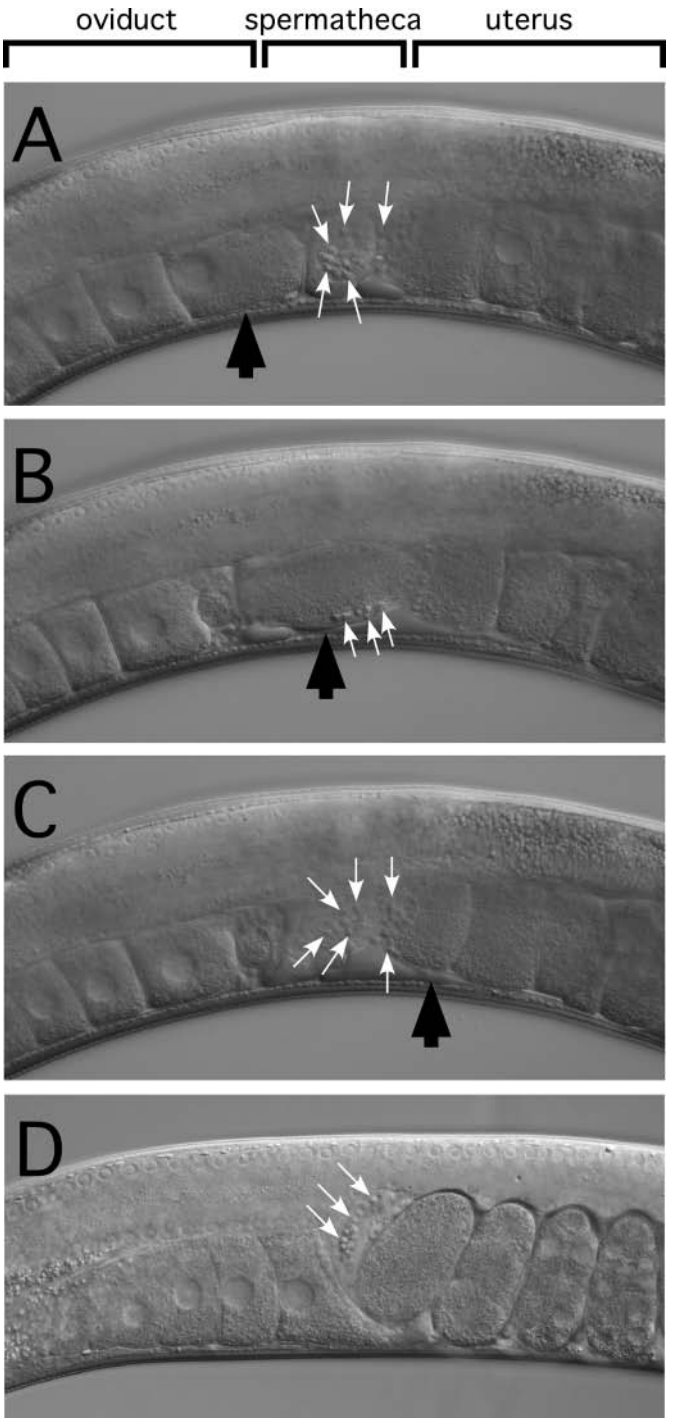

Fig. 3. Meiotic maturation and ovulation. (A-C) Three sequential Nomarski DIC micrographs showing normal meiotic maturation, ovulation and sperm-oocyte contact in a *spe-38(eb44)* hermaphrodite. The black arrows indicate the position of the same oocyte as it moves from the oviduct (A) to the spermatheca (site of sperm storage; B) and finally to the uterus (C). The oocyte spent ~7 minutes in contact with sperm in the spermatheca without being fertilized. The uterus is filled with previously unfertilized oocytes. (D) A wild-type hermaphrodite has a uterus that is filled with developing embryos. Unlike unfertilized oocytes, developing embryos are oval and are enclosed within a clearly defined eggshell. In all panels, the white arrows indicate examples of sperm and their position.

quantified the total lifetime number of ovulations of mutant and wild-type worms. Mutant *spe-38* hermaphrodites ovulated at levels slightly but not statistically lower than the wild-type controls (Fig. 5A). By contrast, hermaphrodites that lack either sperm (Fig. 5A, see *fem-1*) or the ability to respond to the MSP signal ovulate at only basal rates (McCarter et al., 1999; Miller et al., 2003; Singson et al., 1998). Because the pattern and rates of ovulation may be affected by a multitude of variables, such as sperm numbers, sperm aging, hermaphrodite aging and the influence of successful fertilization events, we also examined the ability of *spe-38* mutant sperm to modulate ovulation rates in age matched *fem-1* females. As assessed by the number of eggs and/or oocytes laid on the culture plate (Kadandale and Singson, 2004), *spe-38* mutant sperm induced ovulation rates that were again comparable to wild-type controls (Fig. 5B).

In *C. elegans*, male sperm engage in sperm competition and outcompete hermaphrodite-derived sperm. The larger male-derived sperm displace the smaller hermaphrodite sperm from the reproductive tract such that male-derived sperm fertilize almost all of the hermaphrodite's oocytes (LaMunyon and Ward, 1998; Singson et al., 1999). The ability to compete successfully depends on numerous factors, including sperm size, motility and the ability to locate the spermatheca. When tested for their capacity to engage in sperm competition, *spe-38* male-derived sperm were found to significantly suppress hermaphrodite self-fertility (Fig. 5C). This result suggests that male-derived *spe-38* mutant sperm can engage in effective sperm competition (size, motility, etc.) despite their inability to fertilize oocytes.

The *spe-38* gene encodes a novel four-pass (tetraspan) integral membrane protein

To address the molecular role of SPE-38 in wild-type sperm-oocyte interactions, we cloned the *spe-38* gene. We mapped *spe-38* to a small region of chromosome I (Fig. 6A) corresponding to the yeast artificial chromosome (YAC) clone Y52B11A. This region contains the predicted genes Y52B11A.1, Y52B11A.2 and Y52B11A.3, but only Y52B11A.1 has been reported to have a male-enriched transcript (Reinke et al., 2004). An approximately 7 kb PCR product from genomic DNA that contained both the Y52B11A.1 and Y52B11A.2 (A.1+A.2) genes was produced and injected to generate transgenic worms. This transgene significantly rescued *spe-38* mutant sterility (Fig. 1A, Fig. 6A), as many viable progeny were produced. In fact, this transgene allowed us to genetically balance homozygous *spe-38* mutants. We did note that our transgenic stocks produced broods that were on average lower than wild-type ones. Incomplete transgene rescue for germline-expressed genes is common because such genes are often expressed poorly in simple transgenic arrays (Putiri et al., 2004; Seydoux and Schedl,

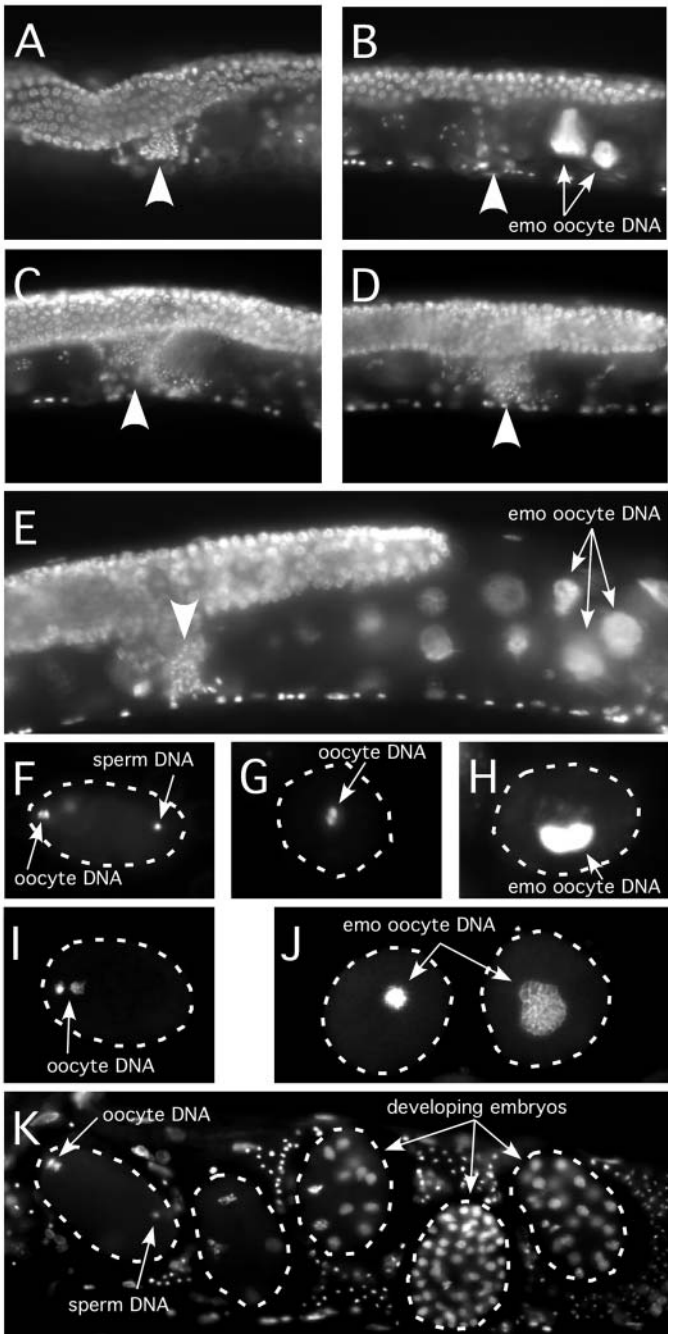

Fig. 4. *spe-38* sperm exhibit normal transfer and migratory behavior, but fail to penetrate oocytes. (A-E) Partial images of whole-mount DAPI-stained hermaphrodites or genetic females, in which the oviduct (left), spermatheca (large arrowheads) and uterus (right) are shown. In such DAPI-stained preparations, the small dense chromatin mass of mature spermatozoa distinguishes them from other somatic- and germ-cell types. (A) In an unmated wild-type hermaphrodite, numerous sperm (small bright spots) are present within the spermatheca. (B) An unmated *fem-1* female lacks sperm within her spermatheca, and her uterus is filled with unfertilized, endomitotic (emo) oocytes (arrows). (C-E) Upon mating, sperm from either wild-type (C) or *spe-38(eb44)* males (D,E) populate the spermatheca of *fem-1* females. (F) In a newly fertilized wild-type oocyte, the sperm chromatin remains as a single, highly condensed DNA mass, while the oocyte chromosomes are undergoing their meiotic divisions. (G,H) Sperm chromatin masses were never observed in either young (G) or older (H) emo oocytes from the uteri of unmated *spe-38* hermaphrodites. (I,J) Young (I) and older (J) emo oocytes from the uteri of *fog-2* females crossed with *spe-38; him-5* males. (K) A series of developing embryos within the uterus of a *spe-38* hermaphrodite mated to wild-type males. A condensed sperm chromatin mass is visible in the meiotic-stage embryo (far left). The broken lines outline the oocytes and embryos.

2001). Individually, neither the Y52B11A.1 nor the Y52B11A.2 gene alone could rescue fertility (Fig. 6A). However, a 3.7 kb genomic fragment that included the Y52B11A.1 gene plus additional upstream sequences, including all of the last intron of the Y52B11A.2 gene (A.1+), rescued fertility to levels comparable to rescue by the the A.1+A.2 fragment (Fig. 1A, Fig. 6A). The structure of the Y52B11A.1 gene (Fig. 6B) was confirmed by sequencing of PCR products generated from a male-derived cDNA library (Achanzar and Ward, 1997). We confirmed that Y52B11A.1 and Y52B11A.2 are separate genes, neither gene is transpliced, and neither gene is listed in the operon database (Blumenthal and Gleason, 2003).

To further confirm our identification of the *spe-38* coding region and to determine the nature of the *eb44* mutation, DNA from *spe-38* mutant worms was amplified and sequenced. The PCR product amplified from *eb44* mutant worms was significantly smaller than the corresponding PCR product amplified from wild-type worms (Fig. 6C). Sequencing confirmed that *eb44* contained a deletion of 270 base pairs, in which all of exon 4 and parts of flanking introns 3 and 4 are missing (Fig. 6B). In place of the missing sequence were 17 bases with the sequence GCCCTTTCAACCCATTT. This deletion not only disrupts proper mRNA splicing but is also predicted to generate a truncated SPE-38 protein with three or four frame shift-encoded residues after amino acid 74 (Fig. 7A, and see below).

If the altered transcript produced by the *eb44* mutant was stable and spliced from intron 3 to intron 5, a frame shift would occur after amino acid 74, yielding the short sequence QMG followed by an opal stop codon. If intron 3 was not spliced to intron 5, the resulting transcript would also have a frame shift after amino acid 74, and code for the short amino acid sequence NLNA followed by an opal stop codon. We cannot formally rule out the possibility that the *eb44* mutation is not a simple loss-of-function mutation, and/or could produce a protein with an altered function that would interfere with fertility. However, we believe this is unlikely as *eb44* is completely recessive and homozygous mutants are rescued by the wild-type transgene (see above).

The *spe-38* gene is predicted to encode a small protein of 179 amino acids (Fig. 7A). BLASTP and HHpred analysis using *C. elegans* SPE-38 as a query did not identify strong homologies, apart from *C. briggsae* SPE-38 (CBG24396; Fig. 7). The two amino acid sequences are 55% identical and 64% similar. The identical and similar amino acids are evenly distributed across the length of the molecules, except for an extended loop between transmembrane domains 1 and 2 in Cb-SPE-38. When this loop is not included in the comparison, the two molecules have 61% identity and 71% similarity. Hydropathy and topology algorithms (see Materials and methods) predicted that SPE-38 has four transmembrane domains (Fig. 7A,B). Using the SMART web-based tool and visual comparison, we identified other four-pass integral membrane proteins that have similar domain arrangements

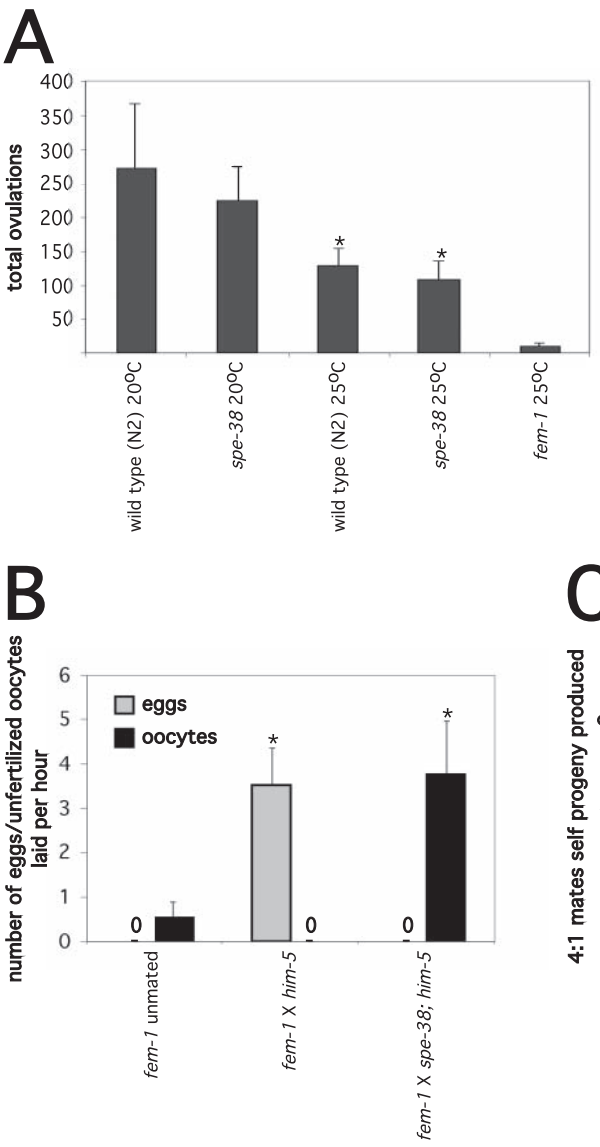

Fig. 5. *spe-38* sperm accelerate ovulation rates and participate in sperm competition. (A) The level of ovulation in unmated *spe-38* and wild-type (N2) hermaphrodites is significantly higher than in unmated *fem-1* controls (*P<0.001, when compared *fem-1* at 25°C, Student's *t*-test). (B) Quantitative analysis of fertilized and unfertilized oocytes laid by unmated *fem-1* females, as well as *fem-1* females crossed to either wild-type *him-5* males or mutant *spe-38; him-5* males. Sperm from wild-type and *spe-38* males induce comparable ovulation rates, which are significantly higher than those of the unmated *fem-1* controls (*P<0.001, Student's *t*-test). (C) Hermaphrodite sperm from morphologically marked (*dpy-5*), but otherwise wild-type, hermaphrodites are out-competed by male sperm from either wild-type *him-5* or mutant *spe-38(eb44); him-5* males. In both cases, the self-fertility of mated *dpy-5* hermaphrodites was significantly depressed when compared with unmated controls (*P<0.001, Student's *t*-test). All counts for the indicated genotypes were performed on a minimum of eight individuals or crosses. Error bars indicate s.d.

(relatively small, lacking channel features, and possessing two loops of varying sizes) (Schultz et al., 2000). The available database contains many such predicted small tetraspan integral membrane proteins, and several have been implicated in important cell-cell interactions (Fig. 7B).

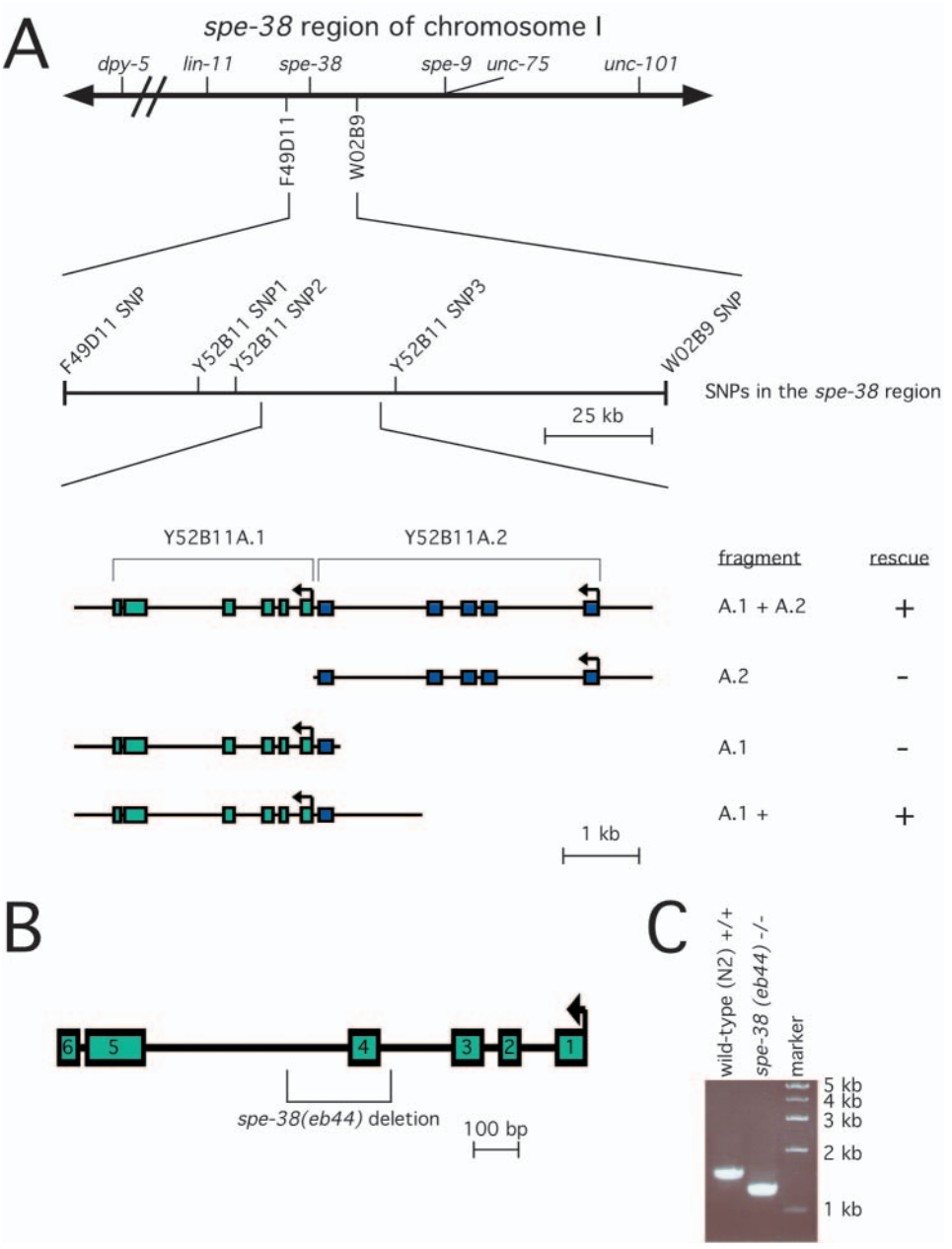

Fig. 6. Molecular cloning of the *spe-38* gene. (A) Genetic and physical maps of the *spe-38* region of chromosome I. Expanded views below the genetic map show the relative position of informative single nucleotide polymorphisms (SNPs), and the structure/orientation of the Y52B11A.1 (*spe-38*) and Y52B11A.2 genes. Exons are indicated by the blue and green boxes. The transgenic rescue profile of DNA fragments containing these genes is shown on the right. (B) Expanded view showing the intron and exon structure of the *spe-38* gene. Exons are indicated by the green boxes and are numbered from right to left to keep orientation consistent with the genetic map. The 270 bp deleted in the *spe-38(eb44)* mutant strain are indicated by the bracket under the structural representation of the gene. (C) PCR characterization of the *spe-38(eb44)* mutation. Primers designed to amplify an ~1.5 kb DNA fragment from wild-type worms amplify a smaller DNA fragment from *spe-38(eb44)* homozygous animals. Marker sizes are indicated to the right of the gel.

spe-38 function is required in sperm

In addition to the fact that *spe-38* mutants display sperm-specific fertility defects, several independent lines of evidence suggest that SPE-38 is required only in sperm. (1) *spe-38* mutant hermaphrodites and males have no detectable somatic defects. (2) Published microarray experiments identified Y52B11A.1 as a sperm-enriched gene (Reinke et al., 2004), and we were able to amplify *spe-38* from a male-derived library (Achanzar and Ward, 1997). (3) In mosaic studies, self-fertile hermaphrodites from a line of *spe-38* hermaphrodites containing the A.1+A.2 transgene always transmitted the transgene through their germ line to the next generation. By contrast, when self-sterile hermaphrodites from this same line were crossed with wild-type males, the resulting progeny never transmitted the transgene. (4) SPE-38 localizes to sperm in immunolocalization studies (see below).

SPE-38 protein localization in spermatids and spermatozoa

To analyze the cellular distribution of SPE-38, we obtained polyclonal anti-peptide antisera to the large putative extracellular loop (amino acids 101-114) of SPE-38 (underlined sequence in Fig. 7A). Relative to other tetraspan proteins, this loop is unique to SPE-38 and could be essential to its function. In cryomethanol-fixed and permeabilized spermatids, SPE-38 appears to be enriched in large structures near the cell cortex (Fig. 8A). Identical results were obtained with paraformaldehyde-fixed and permeabilized spermatids (data not shown). The specificity of our antisera was confirmed in three independent ways. (1) Fixed and permeabilized wild-type spermatids failed to stain when the antisera was pre-incubated with competing peptide (Fig. 8B). (2) Fixed and permeabilized *spe-38(eb44)* sperm failed to stain (Fig. 8C). (3) A western blot of wild-type and *spe-38(eb44)* males revealed a protein of the expected molecular weight in the wild type but not in the mutant sample (Fig. 8D).

Ultrastructural studies indicate that *C. elegans* spermatids possess only four major organelles: a central

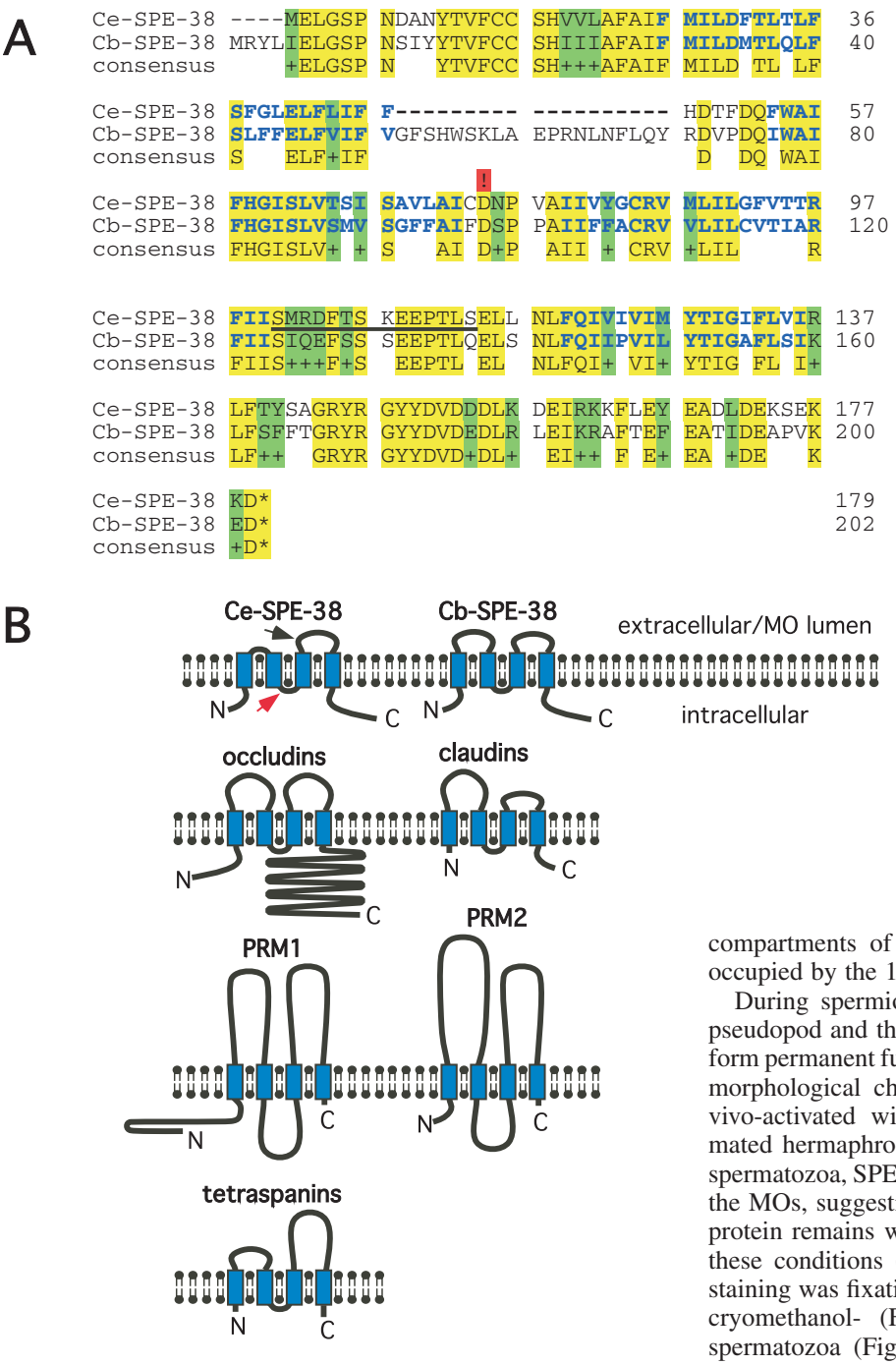

Fig. 7. The amino acid sequence of SPE-38 and a visual comparison of the predicted structure of SPE-38 with that of several tetraspan integral membrane proteins that are thought to function in cell-cell interactions. (A) The predicted and aligned amino acid sequences (single letter code) of *C. elegans* (Ce-SPE-38) and *C. briggsae* (Cb-SPE-38) SPE-38 proteins. Yellow shading indicates identical amino acids; green shading indicates similar amino acids; predicted transmembrane domains are shown in bold blue letters. The sequence used to generate the anti-SPE-38 sera is underlined, and the first amino acid missing in the *spe-38(eb44)* mutation (aspartic acid) is indicated by red shading. (B) A schematic representation of Ce-SPE-38 and Cb-SPE-38, and comparisons with other tetraspan integral membrane proteins. Transmembrane domains are indicated with blue boxes; the black arrow indicates the loop domain used for peptide synthesis and the red arrow indicates the aspartic acid residue position in *eb44* noted in A. The mutant gene is not predicted to code for any wild-type protein sequence beyond this residue. The schematics for the various proteins are derived from previous reports (Boucheix and Rubinstein, 2001; Heiman and Walter, 2000; Hemler, 2003; Tsukita and Furuse, 1999), and/or from our hydropathy plot and domain structure analysis (see Materials and methods).

condensed chromatin mass, its associated centriole, multiple mitochondria, and numerous membranous organelles (MOs). MOs are unique nematode sperm organelles. In spermatids they are unfused, fully internal structures, which abut the cortex of spermatids (Ward et al., 1981; Wolf et al., 1978). To test whether the SPE-38 localization pattern reflected its presence within MOs, permeabilized spermatids were co-stained with anti-SPE-38 antibody and the monoclonal antibody 1CB4, which specifically binds to MOs (Okamoto and Thomson, 1985). Analysis using confocal/multiphoton microscopy revealed a significant but incomplete overlap of the two staining patterns (Fig. 9A). This result indicates that, within spermatids, SPE-38 predominately localizes to sub-compartments of the MO that only partially overlap those occupied by the 1CB4 antigen.

During spermiogenesis, nematode sperm extend a motile pseudopod and their MOs fuse with the plasma membrane to form permanent fusion pore structures. To assess whether these morphological changes alter the distribution of SPE-38, in vivo-activated wild-type spermatozoa were dissected from mated hermaphrodites. In fixed and permeabilized wild-type spermatozoa, SPE-38 antisera stained both the pseudopods and the MOs, suggesting that a significant fraction of the SPE-38 protein remains within the fused MOs of spermatozoa under these conditions (Fig. 8A). The ratio of MO to pseudopod staining was fixation dependent, with stronger MO staining in cryomethanol- (Fig. 8A) than in paraformaldehyde-fixed spermatozoa (Fig. 8C). Under live-cell staining conditions, where our antisera did not have access to internal epitopes, SPE-38 localized predominantly to the pseudopods of wild-type spermatozoa (Fig. 8A). Importantly, pseudopod binding was not observed when the antibody was preincubated with peptide (Fig. 8B). This live-cell staining result supports our prediction of SPE-38$^{101–114}$ as an extracellular loop domain.

These localization patterns suggest a model in which SPE-38 moves from the unfused MOs of spermatids to the plasma membrane of the pseudopods of spermatozoa. In a crucial test of this model, SPE-38 remained inaccessible to antibody binding under live-cell staining conditions of either wild-type spermatids (Fig. 8A) or *fer-1* spermatozoa (Fig. 9B), which were previously characterized as having abnormally short pseudopods and unfused MOs (Ward et al., 1981). When these

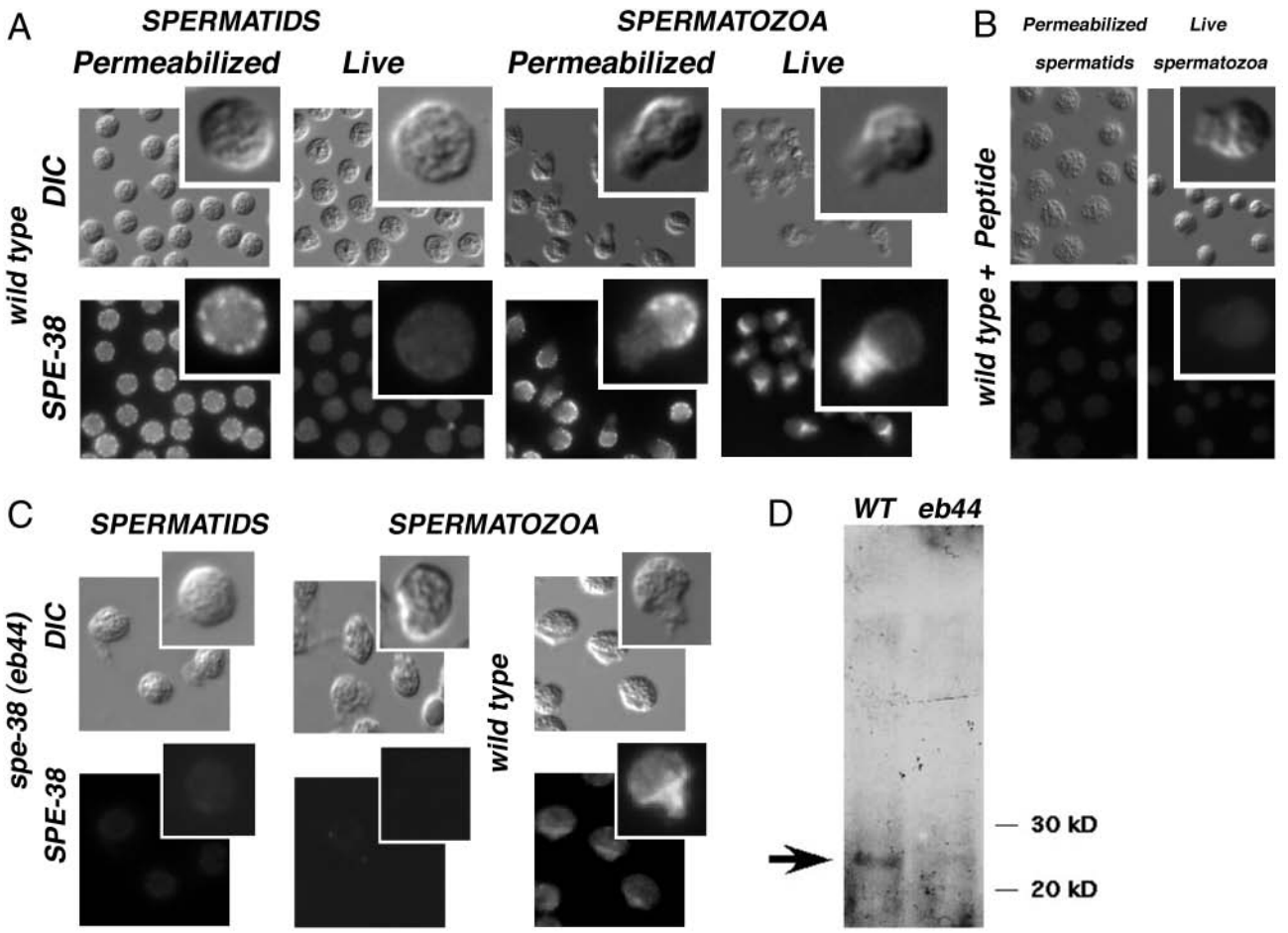

Fig. 8. Localization of SPE-38 in spermatids and spermatozoa. SPE-38 was detected using a peptide polyclonal antibody against a putative extracellular loop domain of SPE-38 (SPE-38$^{101–114}$). (A) DIC (top) and epifluorescence (bottom) images of in vivo-activated wild-type spermatids and spermatozoa. Permeabilized cells were cryomethanol fixed and permeabilized with Triton X-100. Live cells were incubated with antibody prior to fixation and were not permeabilized. Insets are enlargements of cells from the same or equivalent fields. (B) Sperm prepared as described in A, but incubated with anti-SPE-38 antibody that was pre-incubated with competing peptide. (C) DIC (top) and epifluorescence (bottom) images of in vitro-activated *spe-38(eb44)* and wild-type sperm. Cells were paraformaldehyde fixed and premeabilized with Triton X-100. (D) Western blot of wild-type and *spe-38(eb44)* adult males. Each lane contains exactly 400 male worms. Equivalent loading was confirmed by Coomassie Blue staining. The sequence coding the antigenic peptide (SPE-38$^{101–114}$) is deleted in *eb44* mutants.

fer-1 spermatids and spermatozoa were fixed and permeabilized, SPE-38 antisera bound to SPE-38 in the unfused MOs (Fig. 9B).

SPE-38 is not required to localize SPE-9 or to maintain membrane domains

As an integral membrane protein, SPE-38 could function to regulate the localization of other proteins. To test whether SPE-38 is required to either specifically localize SPE-9, another sperm-specific fertility protein, or more generally establish and/or maintain the integrity of membrane domains within crawling spermatozoa, the localization patterns of both SPE-9 and the MO-marker (1CB4) were examined in *spe-38* mutant spermatids and spermatozoa. Neither marker was mislocalized in *spe-38(eb44)* spermatozoa (Fig. 9C), suggesting that SPE-38 does not function in this manner. Conversely, in reciprocal experiments, neither *spe-9* nor *trp-3/spe-41* proved to be essential for the proper localization of SPE-38 (I.C., unpublished).

Discussion

The *spe-38* gene is required for gamete interactions during fertilization

Despite the obvious importance of fertilization, our understanding of its molecular details awaits a more complete inventory of its component molecules. In this study, we have identified the *spe-38* gene as a new component essential for *C. elegans* fertilization. *spe-38* mutants phenocopy other known ‘sperm-sterile’ fertility mutants (Singson, 2001; Singson et al., 1998; Xu and Sternberg, 2003); mutant sperm are incapable of fertilizing wild-type oocytes regardless of whether they are hermaphrodite or male derived. This infertility defect is specific to the fertilization process. Mutant *spe-38* sperm develop normally and ultimately mature into sperm that are morphologically indistinguishable from wild type at the level of both light and electron microscopy. *spe-38* mutant sperm are fully motile and can both migrate to and maintain their position within the spermatheca. Despite having frequent contact with mature oocytes in the spermatheca, *spe-38* mutant sperm never

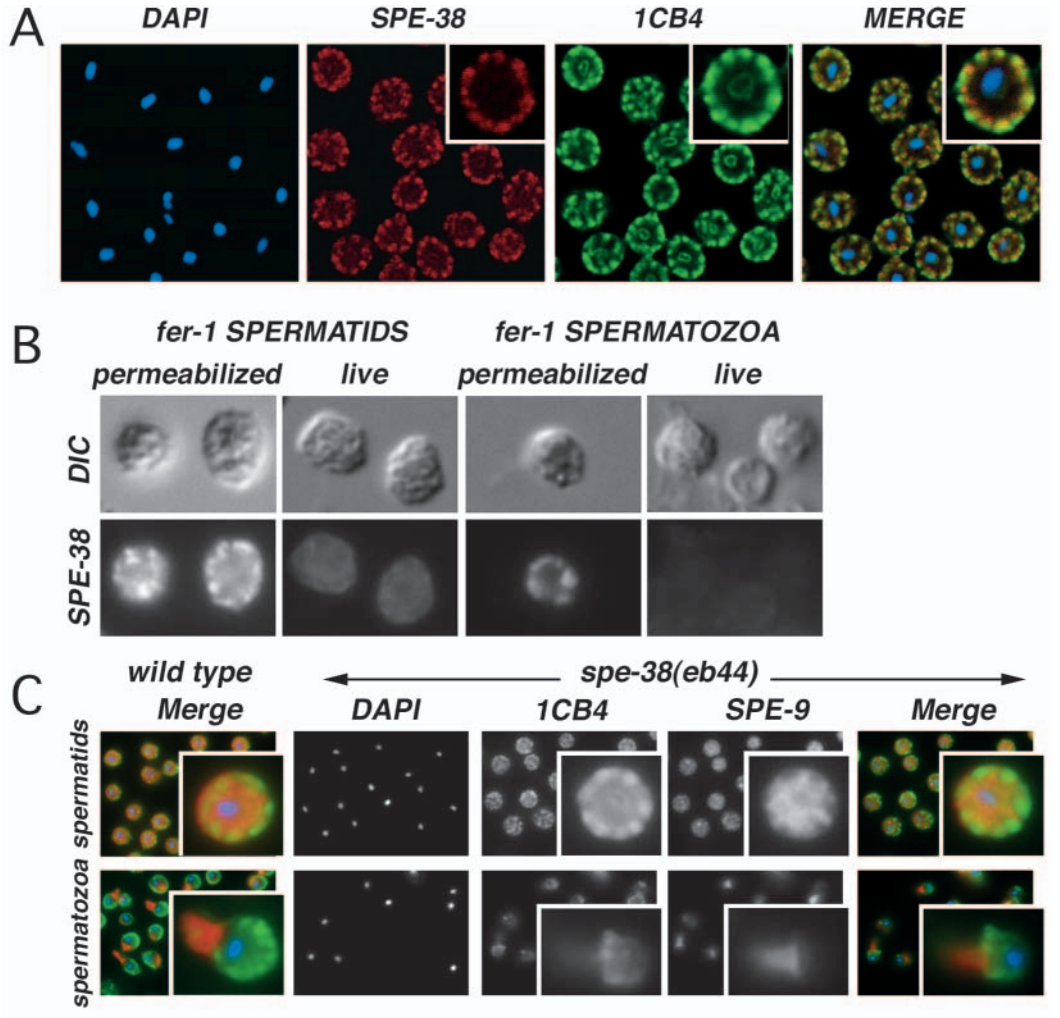

Fig. 9. The dynamic localization of SPE-38 requires MO fusion, but SPE-38 is not required for the dynamic localization of other sperm membrane proteins. (A) Confocal/multiphoton images of wild-type spermatids stained with DAPI (blue), anti-SPE-38 antibody (red) and the monoclonal antibody 1CB4 (Okamoto et al., 1985), which binds to an MO-associated antigen (green). Partial co-localization (yellow) of SPE-38 and 1CB4 staining is shown in the merged image. (B) Anti-SPE-38 immunolocalization in *fer-1(hc17ts)* mutant sperm, which fail to undergo MO fusion during spermiogenesis. Staining is detectable in fixed, permeabilized spermatids and spermatozoa, but not in live, non-permeabilized cells. (C) Localization of the 1CB4 antigen (green), SPE-9 (red) and DNA (blue) in wild-type and *spe-38* sperm.

successfully enter an oocyte. Nevertheless, like other fertilization-specific *spe* mutants (Singson et al., 1999), *spe-38* sperm induce high rates of oocyte maturation/ovulation, and male *spe-38* sperm are capable of successfully out-competing smaller wild-type hermaphrodite sperm.

The role of SPE-38 in wild-type fertilization

The *spe-38* gene is predicted to encode a novel four-pass (tetraspan) integral membrane protein that has no strong sequence homologs apart from its *C. briggsae* counterpart (Fig. 7). SPE-38 has weak homology to a variety of membrane proteins, including several channel protein subunits (see below). However, the loop domains of SPE-38 specifically lack pore-forming consensus sequences. Furthermore, SPE-38 failed to display channel activity when expressed in frog oocytes (I.C., A. Smolyanskaya and L. Bianchi, unpublished).

In addition to channel proteins, there are hundreds of predicted tetraspan integral membrane proteins in the current databases. Many of these molecules have a domain organization that is similar to SPE-38 (see Fig. 7B for examples). Several tetraspan membrane proteins have been implicated in cell-cell interactions, vesicle trafficking and membrane morphogenesis (Hemler, 2003; Hubner et al., 2002; Tsukita and Furuse, 1999). One important sub-group of tetraspans include the mammalian membrane-spanning 4A (*MS4A*) gene family. As a group, these MS4A-related proteins are expressed in many tissues (Ishibashi et al., 2001; Liang and Tedder, 2001) and function within cell-surface oligomeric complexes as signal transducers. One, TETM4, is specifically expressed in human testis (Hulett et al., 2001). Another subgroup of tetraspan proteins, the tetraspanins, have been implicated in both mammalian sperm-oocyte adhesion/fusion and other cell-cell interactions (CD9 and CD81) during both the immune response and nervous system development/function (Bronstein, 2000; Hemler, 2001; Hemler, 2003; Kaji et al., 2002; Kaji et al., 2000; Naour et al., 2000). Twenty *C. elegans* genes fit the specific criteria for tetraspanins (Todres et al., 2000); *spe-38* is not one of those twenty. A third group of tetraspan molecules includes occludins and claudins, which function within polarized epithelia to form cell-cell junctions and barriers to membrane diffusion (Tsukita and Furuse, 1999; Turksen and Troy, 2004). SPE-38 also shares structural similarities with the fungal tetraspan proteins PRM1 and PRM2, which function in membrane adhesion/fusion during yeast mating (Heiman and Walter, 2000). In the absence of definitive structure/function studies, the exact molecular activities of these molecules remain poorly understood. However, in the context of this study, it is notable that various tetraspan molecules are proposed to function as receptors, signal transducers, fusion proteins, and ‘scaffolding or

membrane web' components that function to group other specific cell-surface proteins, thus altering their activity, stability or presentation (Ellerman et al., 2003; Hemler, 2003; Kaji et al., 2000; Maecker et al., 1997; Naour et al., 2000; Yunta and Lazo, 2003). The mutant phenotype of *spe-38* is consistent with it having one or more of these activities during sperm-egg interactions during *C. elegans* fertilization.

The localization of SPE-38, and models for its function during fertilization

We find that SPE-38 concentrates within the membranous organelles (MOs) of spermatids, and that a significant fraction of the protein relocalizes to the pseudopod of the mature, motile and translationally inactive spermatozoa (Figs 8, 9). The MOs appear to be the source of the SPE-38 protein that ends up on the pseudopods of motile spermatozoa, as this relocalization fails to occur in mutants that are defective in MO fusion. The observed localization pattern suggests a number of possibilities concerning how and where SPE-38 could function to promote successful fertilization. The localization of SPE-38 in the MOs of both spermatids and spermatozoa suggests that it could carry out a crucial function within MOs. However, if SPE-38 does play an essential function within MOs, it is not required for the proper ultrastructure, MO-plasma membrane fusion, or spermatozoan motility. As an integral membrane protein, SPE-38 might also plausibly function to regulate the localization of other proteins. However, if so, SPE-38 is neither functioning like an occludin to maintain large-scale membrane domains, nor to specifically localize the sperm-specific fertility protein SPE-9. Additional studies will be required to assess whether SPE-38 is essential either for the localization of other proteins or for the functioning of SPE-9. What our live-cell staining experiments do show is that SPE-38 is present on the pseudopod membrane of mature sperm, where it is positioned to function directly in gamete interactions.

Given the functional diversity of tetraspan proteins, the best clues regarding the biochemical function of SPE-38 may ultimately come from the analysis of its binding partners. For instance, identifying an oocyte binding partner(s) would suggest an egg receptor/ligand function analogous to a model proposed by Ellerman et al. (Ellerman et al., 2003) for the immunoglobulin superfamily (IgSF)/CEA subfamily protein PSG17 binding to the tetraspanin CD9. Alternately, identifying sperm-protein binding partner(s) might support a role for SPE-38 as either a single subunit within a key, but larger, protein complex, or as an essential modulator of key sperm receptor proteins. Additional clues will come from a detailed structure function analysis of SPE-38 in transgenic worm strains. In any case, the current study demonstrates that SPE-38 is a new component of the sperm fertilization machinery, whose function is absolutely required for *C. elegans* sperm-egg interactions. Continued analysis of SPE-38 activity in *C. elegans* promises to give us new insights regarding, not only the molecular mechanisms of fertilization, but also the molecular functions of tetraspan proteins.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/12/2795/DC1

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