Report

Current Biology

# The Immunoglobulin-like Gene *spe-45* Acts during Fertilization in *Caenorhabditis elegans* like the Mouse *Izumo1* Gene

## Highlights

- Identification of *C. elegans spe-45* as a homolog of the mouse *Izumo1*-like gene
- Actual involvement of *spe-45* exclusively during fertilization like mouse *Izumo1*
- Evolutionary conservation of a function(s) between SPE-45 and IZUMO1

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## In Brief

Mouse IZUMO1 is needed exclusively in sperm during fertilization and null mutants are sterile. Nishimura et al. discovered *C. elegans spe-45*, which encodes a protein like IZUMO1. The *spe-45* mutant defects are confined to sperm and similar to *Izumo1*, suggesting that a protein involved in fertilization has been conserved for ~1 billion years.

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# The Immunoglobulin-like Gene *spe-45* Acts during Fertilization in *Caenorhabditis elegans* like the Mouse *Izumo1* Gene

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## SUMMARY

**The *Caenorhabditis elegans spe-9* class genes, which show specific or predominant expression in the male germline, are indispensable for fertilization [1, 2]. However, due to the rapid evolution of genes involved in reproduction, we do not currently know if there are *spe-9* class genes in mammals that play similar roles during fertilization to those found in *C. elegans*. In mice, the *Izumo1* gene encodes a sperm-specific transmembrane (TM) protein with a single immunoglobulin (Ig)-like domain that is absolutely required for gamete fusion [3, 4]. In this study, we hypothesized that *C. elegans* has a new member of the *spe-9* class genes coding for an IZUMO1-like protein. We screened *C. elegans* microarray data [5, 6] to identify male germline-enriched genes that encode membrane proteins with Ig-like domains. A deletion (*tm3715*) in one such gene (*F28D1.8*) caused hermaphrodites to show a male germline-dependent self-sterility, so we have named it *spe-45*. Mutant *spe-45* worms seemed to normally undergo spermatogenesis (spermatid production by meiosis) and spermiogenesis (spermatid activation into actively motile spermatozoa). *spe-45* mutant spermatozoa, however, could not complete gamete fusion, which is a characteristic of all *spe-9* class mutants [1, 2]. Moreover, *spe-45* self-sterile worms were rescued by a transgene expressing chimeric SPE-45 protein in which its Ig-like domain was replaced by the Ig-like domain from mouse IZUMO1. Hence, *C. elegans* SPE-45 and mouse IZUMO1 appear to have retained a common function(s) that is required during fertilization.**

## RESULTS AND DISCUSSION

Gamete fusion during fertilization is required to create a zygote. Several studies have revealed that the sperm immunoglobulin (Ig)-like protein IZUMO1 is essential for sperm-oocyte fusion in the mouse [3, 4, 7–9], but it is not yet clear how IZUMO1 is involved in gamete fusion.

*Caenorhabditis elegans* is a useful model to investigate the molecular basis of gamete fusion for two reasons: First, *C. elegans* spermatozoa directly bind to and fuse with the oocyte plasma membrane during fertilization [1, 2]. Second, mutants lacking any of the SPE-9 class proteins (SPE-9 [10–12], SPE-38 [13, 14], SPE-41/TRP-3 [14, 15] and SPE-42 [16, 17]) have been recovered, all of which have defects exclusively during fertilization.

We postulated that *C. elegans* spermatozoa might possess an IZUMO1-like protein(s) that is required for fertilization. This study was undertaken to test this hypothesis.

### *F28D1.8* Was Identified as a Candidate Mouse *Izumo1*-like Gene

As shown in Table S1, we searched for candidate genes in the *C. elegans* genome (release number WBcel235) using the SMART program [18]. Mouse *Izumo1* shows testis-specific gene expression and it encodes a single-pass transmembrane (TM) protein with a single Ig-like domain (Figure S1A). Therefore, among the 62 predicted Ig-like *C. elegans* genes, we first chose *F28D1.8*, *F28E10.2b*, *B0273.4c*, *K04D7.4a*, *C01G6.8a*, *T02C5.3b*, *T04A11.3*, and *Y32G9A.8*, all of which possess one Ig-like and one TM domain.

To look for genes with elevated expression in the male germline, we compared the DNA microarray data of masculinized *fem-3(q23 gf)* worms and feminized *fem-1(hc17ts)* worms [5, 6] (shown as "male-to-female" [M/F] ratios in Table S1). M/F ratios of the *spe-9* class genes such as *spe-9*, *spe-38*, and *spe-41/trp-3* were 5.54, 2.73, and 6.59, respectively. Hence, if the M/F ratio of a certain gene was more than 2.50, we judged it to be a good candidate. Among eight candidates, *F28D1.8* showed the highest ratio (M/F = 4.72), and *Y32GA9.8* had no available expression data. Thus, sex-dependent expression of these two genes was further examined by RT-PCR (Figure 1A). Similar experiments were also carried out for *spe-42*, which has male germline-specific expression, and for ubiquitously expressed *rpa-1* as controls [16]. Our RT-PCR analysis demonstrated that *F28D1.8*, but not *Y32G9A.8*, has male germline-enriched gene expression.

*F28D1.8* is ~2.4 kb in length and it is composed of eight exons on *C. elegans* chromosome IV (Figure 1B). The *tm3715* allele

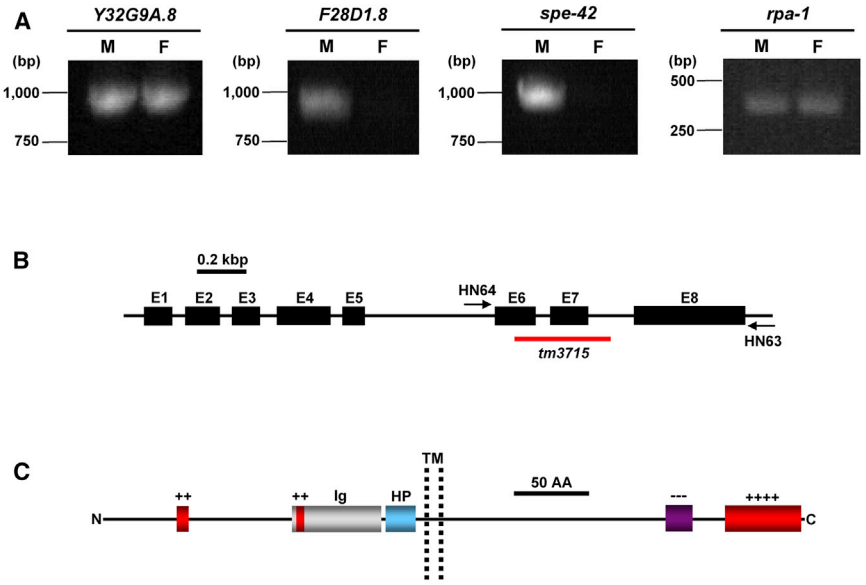



**Figure 1. The *C. elegans F28D1.8* Gene Is a Mouse *Izumo1*-like Gene**

(A) RT-PCR analysis of candidate *C. elegans* genes encoding Ig-like TM proteins. Sex-specific expression of the *C. elegans* genes *F28D1.8* and *Y32G9A.8*, in addition to *spe-42* (specific to the male germline) and *rpa-1* (common to the male and female germlines), were examined. M, male germline (using *fem-3(q23 gf)* worms); F, female germline (using *fem-1(hc17ts)* worms).

(B) The genomic structure of *F28D1.8*. *F28D1.8* is an ~2.4-kb gene consisting of eight exons (E1–E8). The red, thick bar shows the area deleted in the *tm3715* allele. Arrows indicate the annealing sites for the HN64 (forward) and HN63 (reverse) primers that were used for PCR analyses of the *tm3715* deletion. For this study, we used the DNA sequence that was revised by Dr. A. Krauchunas and Dr. A. Singson (for details, see [19]).

(C) The predicted protein structure of F28D1.8. The SOSUI program predicts a hydrophobic region (HP) outside of the transmembrane domain (TM) (http://harrier.nagahama-i-bio.ac.jp/sosui/). The deduced amino acid sequence also contains positively (+) and negatively (−) charged regions, and numbers of the "+" and "−" symbols represent relative numbers of basic and acidic residues, respectively. AA, amino acid; Ig, immunoglobulin-like domain.

See also Figure S1 and Table S1.

deletes 418-bp nucleotides from the *F28D1.8* sequence (Figure 1B). The predicted F28D1.8 protein (492 amino acids) contains a hydrophobic region and acidic and basic amino acid clusters, as well as one Ig-like and one TM domain (Figure 1C). *tm3715* deletes a part of *F28D1.8* that encodes the TM and cytoplasmic tail domains, likely resulting in a non-functional or absent protein.

## *F28D1.8* Is a *spe* Gene

We examined the self-fertility of wild-type (N2) and *tm3715* hermaphrodites (Figures 2A and 2B). N2 hermaphrodites produced ~290, ~290, and ~140 self-progeny at 16°C, 20°C, and 25°C, respectively, whereas *tm3715* hermaphrodites produced no progeny at any tested temperature by self-fertilization (Figure 2A). The same *tm3715* mutants, however, laid ~181, ~340, and ~190 unfertilized oocytes at 16°C, 20°C, and 25°C, respectively (Figure 2B). Numbers of unfertilized oocytes that had been laid by N2 worms were, as expected (Figure S2), fewer than those by *tm3715* worms (~40, ~140, and ~5 unfertilized oocytes at 16°C, 20°C, and 25°C, respectively) (Figure 2B). When *tm3715*, *spe-9(eb19); him-5(e1490)*, and *fem-1(hc17ts)* hermaphrodites were outcrossed to *him-5(e1490)* males, which are proficient in mating and produce fertilization-competent sperm, ~210, ~240, and ~110 F1 progeny, respectively, were produced at 20°C (Figure 2C). The *spe-9(eb19)* [10–12] and *fem-1(hc17ts)* [13, 16] hermaphrodites produce fertilization-competent oocytes, but self-fertilization does not occur due to defective or no self-sperm. Therefore, the data shown in Figure 2C suggest that oocytes of *spe-45* mutants are at least equally competent to be fertilized, as compared with those of *spe-9* and *fem-1* mutants.

If *tm3715* affects a *spe-9* class gene, males would produce sperm that could outcompete hermaphrodite-derived sperm after copulation [13, 16, 20] (see also Figure S2). Since *dpy-5(e61)* is a recessive mutant that causes a Dpy phenotype (smaller and fatter worm shape than wild-type) [21], we used *dpy-5* mutant hermaphrodites to distinguish self- and outcross progeny. As shown in Figure 2D, unmated *dpy-5* mutants produced only self-progeny (~60 worms) [13, 16, 20]. After mating to non-Dpy *him-5* males, *dpy* hermaphrodites produced ~80 non-Dpy outcross progeny, whereas Dpy progeny were reduced (~40 worms). Outcrossing of *dpy-5* mutants to *tm3715; him-5* males again resulted in reduced numbers of Dpy progeny (~30 worms), but this time non-Dpy progeny were not observed. Thus, the fertilization-incompetent sperm of *tm3715* males are capable of outcompeting *dpy-5* self-sperm.

These data suggest that the *tm3715*-induced self-sterility was restricted to male germline functions, exhibiting typical Spe (spermatogenesis-defective) phenotypes [1, 2]. *F28D1.8* was originally named *oig-7* (one Ig domain-7), but this name is based on just its protein structure [22]. Therefore, we renamed this gene *spe-45*, hereafter, based on its loss-of-function phenotype.

## Production and Activation of *spe-45(tm3715)* Spermatids Occurs Normally

*C. elegans* male germline functions are divided into three pivotal steps: spermatid production during meiosis (spermatogenesis), spermatid activation into spermatozoa (spermiogenesis), and fertilization. Dissected *him-5(e1490)* and *spe-45(tm3715); him-5(e1490)* males each released spermatids that are indistinguishable in number (Figures 2E and 2H) and cytology (Figures 2F and 2I), suggesting that spermatogenesis occurs normally in *spe-45* mutants.

We next examined spermatid activation into spermatozoa in sperm medium (SM) [23, 24] containing the bacterial protease mixture Pronase [25]. Spermatozoa from *spe-45; him-5* males

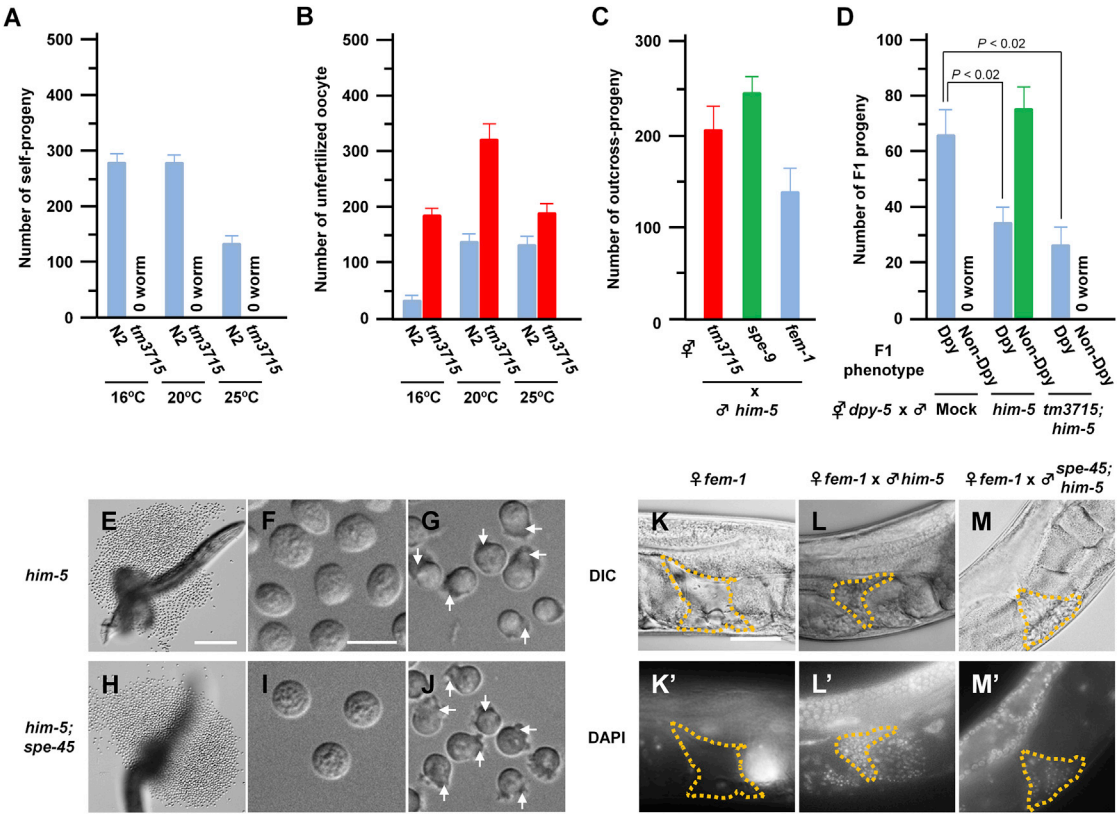



**Figure 2. Phenotypic Analysis of *F28D1.8* (*spe-45*) Mutant Worms**

(A and B) The deletion allele *tm3715* of *F28D1.8* (*spe-45*) causes hermaphroditic self-sterility. N2 (n = 26) and *spe-45(tm3715)* (n = 24–30) worms were grown at 16°C, 20°C, and 25°C, and the numbers of self-progeny (A) and unfertilized oocytes (B) produced by those hermaphrodites were determined. Data for N2 wild-type (light-blue bars) and *spe-45(tm3715)* (red bars) hermaphrodites are shown as the mean ± SEM.

(C) The self-sterility of *spe-45(tm3715)* worms is due to a male germline defect. After *tm3715* (n = 15; red bar), *spe-9(eb19); him-5(e1490)* (n = 13; green bar) and *fem-1(hc17ts)* (n = 10; light-blue bar) hermaphrodites were mated with *him-5(e1490)* males; numbers of outcross progeny were determined and are shown as the mean ± SEM.

(D) *spe-45* male spermatozoa can outcompete hermaphrodite-derived spermatozoa for oocytes. *dpy-5(e61)* worms (n = 10) were sired by mock (self-progeny only; a no mating negative control), *him-5(e1490)*, or *tm3715; him-5(e1490)* males, and numbers of self-progeny (Dpy; light-blue bars) and outcross progeny (Non-Dpy; green bar) were counted (mean ± SEM).

(E–J) Spermatids of *spe-45* males can be activated into spermatozoa in vitro. Spermatids were released from *him-5(e1490)* (n = 10; E–G) or *spe-45(tm3715); him-5(e1490)* (n = 10; H–J) males in the absence or presence of Pronase, an in vitro spermatid activator [20]. Round, sessile spermatids (E, F, H, and I) could be transformed into amoeboid, motile spermatozoa by Pronase treatment (G and J). We counted more than 100 cells after Pronase-induced activation and found that 84.0% ± 1.6% and 85.6% ± 2.2% (mean ± SEM) of total cells were activated spermatozoa in *him-5* (n = 11) and *spe-45; him-5* (n = 11) males, respectively. Arrows point at the pseudopods of spermatozoa. Scale bars represent 100 μm for (E) and (H) and 10 μm for (F), (G), (I), and (J).

(K–M′) *spe-45* male-derived spermatids activate into spermatozoa in vivo. Mock (no male mating; K and K′), *him-5(e1490)* (L and L′), or *spe-45(tm3715); him-5(e1490)* (M and M′) males were mated to feminized *fem-1(hc17ts)* worms, which contain functionally normal oocytes but no self-sperm. After being stained with DAPI to visualize sperm nuclei (K′–M′), the mated females were observed under a fluorescent microscope to examine the presence of male spermatozoa in the spermatheca. Orange broken lines outline the spermatheca. DIC, differential interference contrast microscopy. The scale bar represents 50 μm.

See also Figure S2.

(Figure 2J) showed similar cytology and activation rate to those observed for *him-5* male-derived spermatozoa (Figure 2G).

We also evaluated whether *spe-45(tm3715)* male-derived spermatids can be activated into spermatozoa in vivo (see Figures 2K–2M′). This experiment used *fem-1(hc17ts)* worms, because they have no self-sperm at 25°C [26]. Consistent with this phenotype, 4′,6-diamidino-2-phenylindole (DAPI)-stained, unmated *fem-1* hermaphrodites lacked detectable sperm within the spermatheca (Figures 2K and 2K′). In contrast, *fem-1* worms were crossed to either *him-5* (Figures 2L and 2L′) or *spe-45; him-5* (Figures 2M and 2M′) males, and both had many spermatozoa within their spermathecae after mating. Therefore, *spe-45(tm3715)* male-derived spermatids were able to activate into spermatozoa in the uterus of *fem-1* hermaphrodites and subsequently to crawl into the spermatheca, suggesting normal in vivo spermiogenesis of *spe-45* male spermatids.

## *spe-45(tm3715)* Spermatozoa Cannot Fertilize Oocytes in the Spermatheca

As shown in Figure S2, ~300 wild-type self-sperm are all consumed by fertilization that occurs in the spermatheca [27]. For this study, fourth larval stage (L4) hermaphrodites were

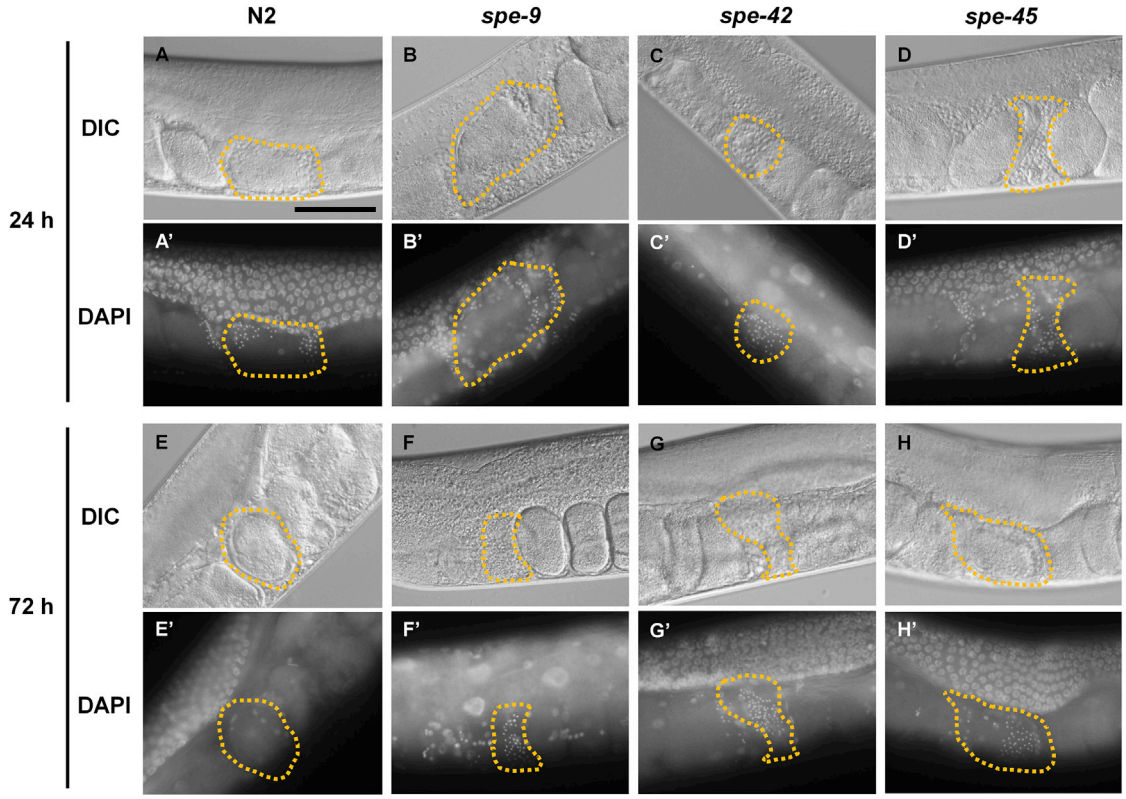



**Figure 3. Self-fertilization Is Not Observed in *spe-45* Mutant Worms**

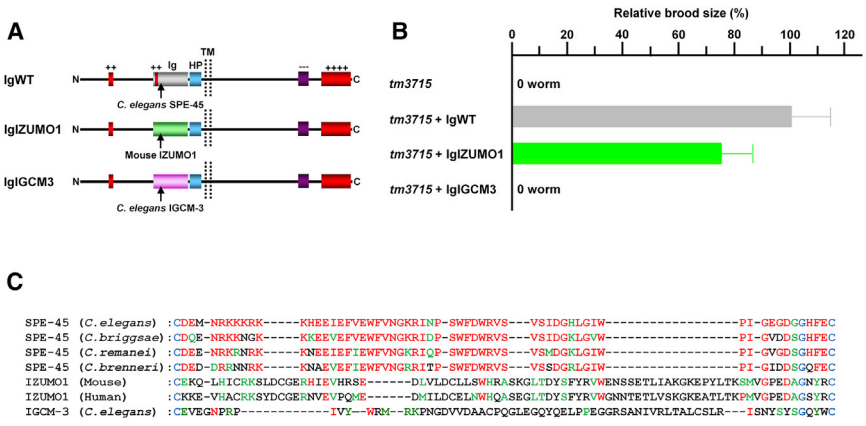
At 24 and 72 hr after the L4 stage, N2 (A, A′, E, and E′), *spe-9(eb19); him-5(e1490)* (B, B′, F, and F′), *spe-42(tm2421)* (C, C′, G, and G′), and *spe-45(tm3751)* (D, D′, H, and H′) hermaphrodites were fixed, treated with DAPI to stain self-sperm nuclei, and then observed to check whether self-sperm numbers are reduced due to participation in fertilization. Orange broken lines highlight the position of the spermatheca. DIC, differential interference contrast microscopy. The scale bar represents 50 μm. See also Figure S2 and Table S2, which summarizes the number of self-sperm in each spermatheca of tested worm strains.

incubated at 20°C for either 24 or 72 hr. Then, those worms were fixed and DAPI-stained to visualize countable self-sperm (Figure 3 and Table S2). In each spermatheca of wild-type N2 hermaphrodites, there were ~140 self-sperm at 24 hr after the L4 stage, but their numbers were reduced to almost zero at 72 hr. Similar data were also obtained in *him-5(e1490)* hermaphrodites (unpublished data). In the known "*spe-9* class" mutants *spe-9(eb19)* [10] and *spe-42(tm2421)* [16] and also in *spe-45(tm3715)* worms, as in the wild-type, there were numerous self-sperm in each spermatheca at 24 hr (~90 *spe-9* sperm, Figures 3B and 3B′; ~220 *spe-42* sperm, Figures 3C and 3C′; and ~180 *spe-45* sperm, Figures 3D and 3D′). However, unlike the wild-type, many self-sperm still resided in the spermathecae of *spe-9* (~50 sperm, Figures 3F and 3F′), *spe-42* (~220 sperm, Figures 3G and 3G′), and *spe-45* (~110 sperm, Figures 3H and 3H′) hermaphrodites even at 72 hr. Intriguingly, numbers of *spe-9* and *spe-45* self-sperm at 72 hr were, respectively, reduced to 52% and 63% of those at 24 hr, whereas numbers of *spe-42* self-sperm at 24 hr were similar to those at 72 hr. Such reduction of *spe-9* self-sperm was also observed previously in hermaphrodites bearing different mutant alleles [28].

These data suggest that *spe-9*, *spe-42*, and *spe-45* self-sperm cannot complete fertilization. *spe-42* is possibly involved in the sperm binding to the oocyte plasma membrane, whereas *spe-9* and *spe-45* self-sperm might be able to contact and bind to oocytes but are not capable of undergoing gamete fusion. It is worth noting that mouse sperm lacking IZUMO1 can bind to, but not fuse with, the oocyte plasma membrane [3, 4].

## A Chimeric SPE-45/IZUMO1 Retains In Vivo Function in *C. elegans*

Since Ig-like domains have considerable sequence diversity but similar three-dimensional structure [29, 30], we tested whether the IZUMO1 and SPE-45 Ig-like domains are functionally similar. Constructs encoding wild-type SPE-45 (IgWT) or chimeric SPE-45, in which the SPE-45 Ig-like domain was replaced with the mouse IZUMO1 Ig-like domain (IgIZUMO1), were created (Figure 4A). As a control, we created a chimeric construct that encoded SPE-45 where the natural Ig-like domain was replaced with that of *C. elegans* IGCM-3, a somatic protein with no obvious role during fertilization [22] (IgIGCM3; Figure 4A). These three constructs were used to create transgenes that were evaluated for rescue of *spe-45* self-sterility (Figure 4B). Intriguingly, *spe-45* hermaphrodites bearing the IgIZUMO1 or IgIGCM3 (control) transgene had self-broods that were, respectively, 76.7% or 0% of those with the IgWT transgene. These data suggest that the Ig-like domains of SPE-45 and IZUMO1 have a common function(s) during sperm-oocyte fusion.




C

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SPE-45 (C.elegans) :CDEM-NRKKKRK-----KHEEIEFVEWFVNGKRINP-SWFDWRVS---VSIDGHLGIW----------------PI-GEGDGGHFEC
SPE-45 (C.briggsae):CDQE-NRKKNGK-----KKEEVEFVEWFVNGKRIDP-SWFDWRVS---VSIDGKLGVW----------------PI-GVDDSGHFEC
SPE-45 (C.remanei) :CDEE-NRKRNRK-----KNEEIEFIEWFVNGKRIQP-SWFDWRVS---VSMDGKLGIW----------------PI-GVGDSGHFEC
SPE-45 (C.brenneri):CDED-DRRNNRK-----KNAEVEFIEWFVNGRRITP-SWFDWRVS---VSSDGRLGIW----------------PI-GIDDSGQFEC
IZUMO1 (Mouse)     :CEKQ-LHICRKSLDCGERHIEVHRSE------DLVLDCLLSWHRASKGLTDYSFYRVWENSSETLIAKGKEPYLTKSMVGPEDAGNYRC
IZUMO1 (Human)     :CKKE-VHACRKSYDCGERNVEVPQME------DMILDCELNWHQASEGLTDYSFYRVWGNNTETLVSKGKEATLTKPMVGPEDAGSYRC
IGCM-3 (C.elegans) :CEVEGNPRP-----------IVY--WRM--RKPNGDVVDAACPQGLEGQYQELPPEGGRSANIVRLTALCSLR---ISNYSYSGQYWC
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**Figure 4. Ig-like Domains Can Be Interchangeable between SPE-45 and IZUMO1**

(A) Transgenes used for the rescue assay in this study. We constructed three transgenes encoding SPE-45 protein in which the Ig-like domain was the one naturally found in SPE-45 (IgWT) or it was replaced by those of mouse IZUMO1 (IgIZUMO1) or *C. elegans* IGCM-3 (IgIGCM3).

(B) The self-sterility of *spe-45* mutant worms is rescued by the IgIZUMO1 transgene. Non-transgenic *spe-45(tm3715)* hermaphrodites (*tm3715*) produced no F1 progeny (n = 15). Normalizing for *spe-45* worms expressing the IgWT transgene (*tm3715* + IgWT) as having a relative brood size of self-progeny at 100.0% ± 15.0% levels (mean ± SEM; n = 12), the relative brood sizes of the same hermaphrodites expressing the IgIZUMO1 transgene (*tm3715* + IgIZUMO1) and the IgIGCM3 transgene (*tm3715* + IgIGCM3) were 76.8% ± 9.7% (n = 14) and 0% (n = 15), respectively.

(C) Alignment of Ig-like domains. The amino acid sequences of the Ig-like loop regions in *Caenorhabditis* SPE-45 orthologs, human (NCBI: NP_872381.2) and mouse (NCBI: NP_001018013.1) IZUMO1s, and *C. elegans* IGCM-3 (WormBase: WBGene00020160) were aligned. Red and green letters indicates residues that are, respectively, identical or chemically similar to those of *C. elegans* SPE-45. Blue letters indicate residues that are conserved in all of those proteins.
See also Figure S3 and Table S3.

As shown in Figure 4C, although the SPE-45 Ig-like domain is well conserved among four *Canorhabditis* species (~75%–83% identities), there are only limited primary sequence identities of the Ig-like domains between *C. elegans* SPE-45 and mouse, human IZUMO1, or *C. elegans* IGCM-3 (~12%–17% identities). When the entire sequences were compared (Figure S3 and Table S3), *C. elegans* SPE-45 and mouse IZUMO1 showed only 8.7% sequence identity, whereas *Caenorhabditis* SPE-45 orthologs showed only modest identities (~36%–61%). Our data suggest that *C. elegans* SPE-45 is orthologous to mouse IZUMO1, but this is not detectable by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) because of low sequence identity. Prior work showed that the sequences of many fertilization-related proteins are poorly conserved, even among closely related species [31–33], so orthologous proteins that participate in reproduction might be best defined based on their functions, rather than sequence identity.

## Diverse Species Use Proteins with Ig-like Domains during Gamete Interactions

Like SPE-45 and IZUMO1, a diverse group of membrane proteins required for fertilization contain Ig-like domains. *Chlamydomonas* FUS1 [34, 35] and *Arabidopsis* GEX2 [36, 37] are single-pass TM proteins containing Ig-like filamin repeat domains, and they participate in gamete attachment and fusion, respectively. In mice, disruption of the *Bsg* (Basigin) gene, which encodes a single-pass TM protein with dual Ig-like domains, results in sterility of both males and females and in arrest of spermatogenesis [38]. Pre-treatment of sperm with antibodies to the BSG protein blocks sperm interactions with the cumulus cells and the zona pellucida [39]. These data all indicate that proteins with Ig-like domains play important roles during fertilization.

## What Function Does SPE-45 Play during *C. elegans* Fertilization?

Besides the Ig-like domain, SPE-45 exhibits several structural features with unknown roles. First, an N-terminal region of ~150 amino acids might be involved in association with an oocyte partner(s). The Izumo domain [7], which is also an N-terminal region of mouse IZUMO1, has been recently demonstrated to function in gamete fusion through the binding to JUNO, an oocyte IZUMO1 receptor [4, 9]. Second, SPE-45 has a α-helical hydrophobic region between the Ig-like and TM domains (Figure 1C). This region is possibly an interface to form a multimer, as is the case for mouse IZUMO1 [7]. Third, the extracellular region contains two, whereas the intracellular region has one, basic amino acid cluster(s) (Figure 1C). The positively charged regions in SPE-45 might associate with negatively charged substances such as sulfated proteoglycans and phospholipids. An acidic sequence in the SPE-45 intracellular domain possibly folds and binds to the cytoplasmic, basic region within a single SPE-45 protein molecule. At any rate, these structural features are probably prerequisites to regulate the function(s) and localization of SPE-45.

In summary, we identified *C. elegans spe-45*, which shows male germline-enriched gene expression and encodes an Ig-like TM protein that is essential for fertilization, like mouse *Izumo1*. Moreover, our domain-swapping experiments suggested that the Ig-like domains of SPE-45 and IZUMO1 might share a common function(s) during fertilization.

## EXPERIMENTAL PROCEDURES

The experimental procedures are described in the Supplemental Experimental Procedures.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2015.10.056.

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