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Report

Current Biology

Forward Genetics Identifies a Requirement for the Izumo-like Immunoglobulin Superfamily *spe-45* Gene in *Caenorhabditis elegans* Fertilization

Highlights

- A novel genetic screen aids the isolation of sterile mutants in *C. elegans*
- The *spe-45* gene encodes an Izumo-like sperm immunoglobulin superfamily protein
- *spe-45* mutants suggest a conserved sperm function from worms to mammals
- Analysis of other mutants from the screen should identify new fertility factors

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In Brief

Singaravelu et al. screened for sterile mutants in *C. elegans* and identified SPE-45, an Izumo-like immunoglobulin superfamily protein. Despite direct contact of gametes in the reproductive tract, *spe-45* mutant sperm cannot fertilize oocytes. Additional mutants from the screen hold the promise of finding new components of fertility pathways.

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Report

Forward Genetics Identifies a Requirement for the Izumo-like Immunoglobulin Superfamily *spe-45* Gene in *Caenorhabditis elegans* Fertilization

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SUMMARY

Fertilization is a conserved process in all sexually reproducing organisms whereby sperm bind and fuse with oocytes. Despite the importance of sperm-oocyte interactions in fertilization, the molecular underpinnings of this process are still not well understood. The only cognate ligand-receptor pair identified in the context of fertilization is sperm-surface Izumo and egg-surface Juno in the mouse [1]. Here we describe a genetic screening strategy to isolate fertilization mutants in *Caenorhabditis elegans* in order to generate a more complete inventory of molecules required for gamete interactions. From this screening strategy, we identified, cloned, and characterized *spe-45*, a gene that encodes an Izumo-like immunoglobulin superfamily protein. Mammalian Izumo is required for male fertility and has the same basic mutant phenotype as *spe-45*. Worms lacking *spe-45* function produce morphologically normal and motile sperm that cannot fuse with oocytes despite direct contact in the reproductive tract. The power of this screen to identify proteins with ancient sperm functions suggests that characterization of additional mutants from our screen may reveal other deeply conserved components in fertility pathways and complement studies in other organisms.

RESULTS AND DISCUSSION

A Genetic Screen for Sterile Mutants

We designed a forward genetic screen to isolate *C. elegans* mutants defective in fertilization. We used the strain *sem-2(n1343);Is[Pelt-7::gfp;rol-6(su1006)]* for our screening (Figure S1) and isolated 12 temperature-sensitive (ts) mutants and 23 non-conditional sterile mutants. Since this was a pilot screen, we did not attempt to pick every single candidate from our screen, and our screen did not reach saturation. Hence, the same screening strategy can be employed to isolate a wealth of mutants going forward that would help us understand the biology of fertilization. The feasibility of high-throughput screening in *C. elegans* can complement the use of other model organisms in which other strategies are more conducive, such as biochemical analyses of sperm or eggs in the sea urchin and in vitro fertilization in the mouse [2, 3]. To validate the utility of our screening strategy, we decided to characterize one of our new ts mutants, *spe-45(as38)*. Adult *spe-45* mutants are healthy, and all pre-fertilization events [4] are normal in *spe-45(as38)* at all culture conditions (Table S1).

SPE-45 Is Required for Fertility in Both Sexes

Hermaphrodites that are homozygous for *spe-45(as38)* mutation are completely self-sterile at 25°C. However, at 20°C and 16°C, the *spe-45(as38)* mutants produce modest numbers of progeny, suggesting that the *as38* allele that we isolated is a ts mutant (Figures 1A). In contrast, *spe-45(tm3715)* hermaphrodites were profoundly sterile under all culture conditions (Figure 1A) [5]. We conclude that SPE-45 is required for fertility in hermaphrodites. Further, *spe-45* mutant hermaphrodites with either allele proved to be fertile upon crossing with wild-type males, indicating that the fertility defect in *spe-45* mutants is restricted to their sperm and not their oocytes.

To test the fertility of males carrying *spe-45* mutations, we crossed them to *fem-1* mutant hermaphrodites. *fem-1* mutants do not produce any sperm and hence do not produce any self-progeny [6]. When crossed with *him-5* males, the *fem-1* animals produce cross-progeny. However, the *spe-45(as38);him-5* males fail to sire progeny when crossed with *fem-1* at 25°C, indicating that SPE-45 is required for fertility in males, as well. (Figure 1B).

Spermatogenesis and Sperm Activation Is Normal in Both Sexes of *spe-45(as38)*

The spermathecae of *spe-45* hermaphrodites contain and retain sperm, just like wild-type control animals (Figure 2A), indicating that the sperm production is uncompromised and that the spermatozoa are likely to be motile in *spe-45(as38)* hermaphrodites. To assess male-derived sperm morphology, we isolated sperm

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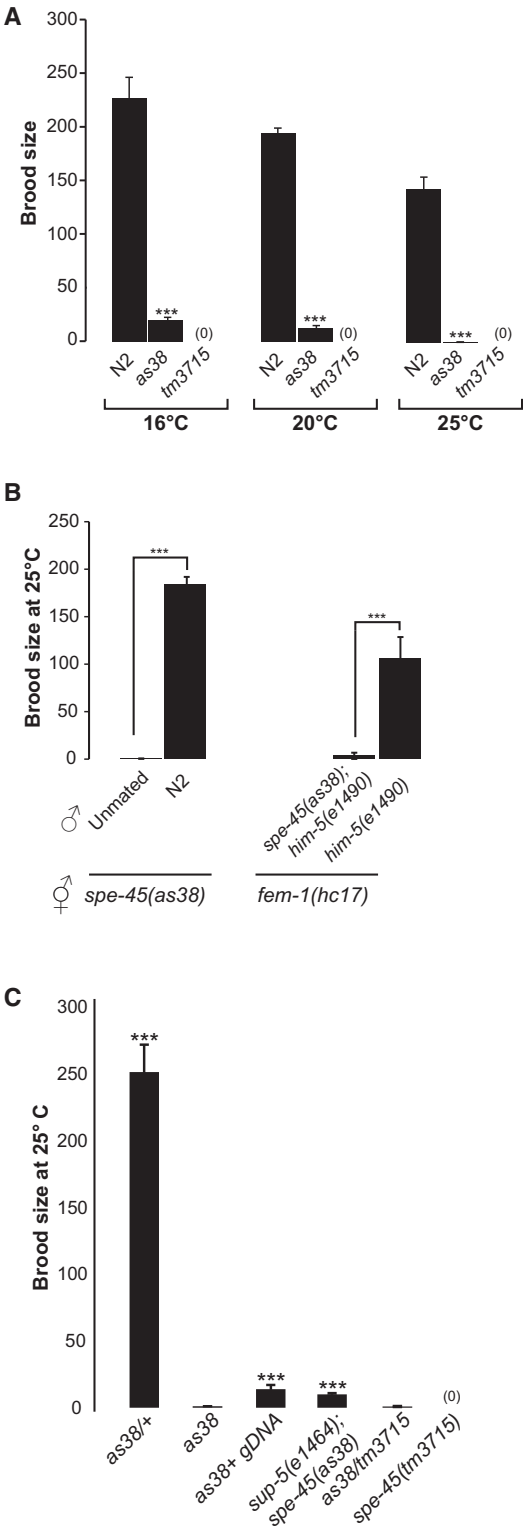

Figure 1. Worms with Mutations in *spe-45* Are Sterile

(A) Brood sizes (self-progeny) of wild-type, *spe-45(as38)*, and *spe-45(tm3715)* mutants at the indicated temperature. ***$p < 0.001$ between the wild-type and *spe-45(as38)*. See also Figure S1 for the screening method that identified *spe-45(as38)* and Table S1 for a summary of pre-fertilization events.

(B) Brood sizes of *spe-45(as38)* hermaphrodites left unmated or mated with N2 males at 25°C and of *fem-1(hc17)* females mated with *spe-45(as35);him-5(e1490)* or *him-5(e1490)* males. ***$p < 0.001$.

(C) Brood sizes of the indicated genotypes at 25°C. *spe-45* gDNA refers to extrachromosomal array comprising the admixture of the fosmids WRM061bG12, WRM0641cH06, WRM066cF06, WRM0631aA01, and WRM0624aC09.

Error bars indicate the SEM.

from *spe-45;him-5* males, either in sperm media alone or in the presence of the in vitro activator pronase (Figure 2B). As viewed under DIC optics, both the spermatids and spermatozoa were indistinguishable in both quality and apparent quantity from that of control *him-5* males. SPE-45 is dispensable for in vitro activation since pseudopods were present in over 90% of sperm from both wild-type and *spe-45* mutant sperm. We also examined hermaphrodite-derived sperm from dissected *spe-45* mutants. These sperm were activated in vivo with fully formed pseudopods and were indistinguishable from wild-type sperm (Figure 2B). Taking these data together, we conclude that spermatogenesis and sperm activation is normal in both sexes of *spe-45* mutants.

Sperm Migration Is Normal in *spe-45(as38)* Mutants

Upon mating, the sperm ejaculated from males should get activated and directionally migrate from the site of ejaculation (vulva) to the site of fertilization (spermatheca). Furthermore, sperm are dislodged from the spermatheca into the uterus during the passage of fertilized oocytes into the hermaphrodite's uterus. These sperm then crawl back into the spermatheca. Several factors orchestrate the directional migration of sperm into the spermatheca [7]. As a defect in sperm migration into the spermatheca can compromise fertility, we asked whether this is the case in *spe-45(as38)* mutants. *fem-1(lf)* worms do not produce any sperm, and hence the spermathecae of *fem-1* animals are devoid of sperm (Figure 2C). Just like wild-type sperm, *spe-45*-male-derived sperm migrate from just inside the vulva to the spermatheca (Figure 2C). This also indicated that male-derived *spe-45* sperm activated normally in vivo. Further, *spe-45* sperm were able to maintain their position in the spermatheca despite passing oocytes. We conclude that *spe-45* mutant sperm are fully motile and display normal migratory behavior in the reproductive tract.

The *spe-45* Gene Belongs to the *spe-9* Class of Genes

Spermatogenesis-abnormal (*spe*) genes are classified based on the phase of the sperm development or sperm differentiation in which their roles are critically important [8]. The "*spe* classes" are named after the founding member of the class. For example, the "*spe-9* class" refers to genes whose loss of function phenocopies *spe-9* mutants where sperm development is normal but sperm are unable to fertilize oocytes despite direct contact with passing oocytes in the spermatheca [9].

Unlike in wild-type worms, whose uteri are filled with developing embryos, the *spe-45* uteri are filled with unfertilized oocytes (Figure 2A), suggesting that despite being motile and in the correct position in the reproductive tract, *spe-45* spermatozoa are incapable of fertilizing oocytes. Staining of wild-type worms with DAPI showed the entry of sperm into the oocytes

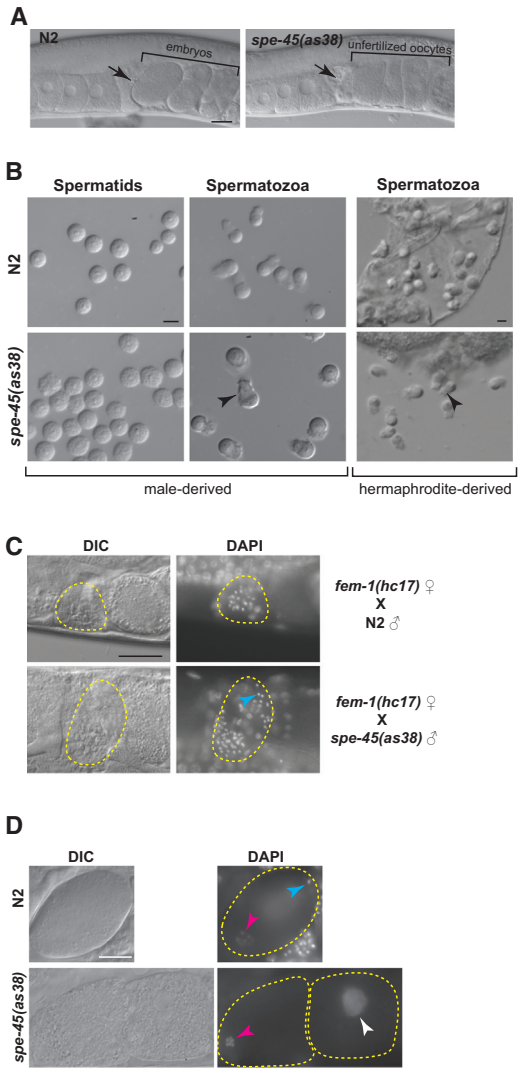

Figure 2. Despite Normal Sperm Morphology and Migratory Behavior, *spe-45* Mutant Sperm Cannot Fertilize Oocytes

(A) Differential interference contrast (DIC) images of wild-type and *spe-45(as38)* reproductive tracts. Black arrows indicate spermathecae containing sperm. The uterus is located at the right side of the spermatheca; the ovary is located at the left side of the spermatheca. Scale bar, 20 μm.

(B) Spermatids and pronase-activated spermatozoa of N2 and *spe-45(as38)* males (left) and spermatozoa from N2 and *spe-45(as38)* hermaphrodites (right). Arrowheads indicate pseudopods of sperm. Scale bar, 5 μm.

(C) 4′,6-diamidino-2-phenylindole (DAPI) staining of the *fem-1(hc17)* females mated with either N2 males or *spe-45(as38)* males. The yellow dotted lines indicate the position of each spermatheca. The blue arrowhead indicates an example of sperm DNA. Scale bar, 20 μm.

(D) DAPI staining of N2 and *spe-45(as38)*. The blue arrowhead indicates the compacted sperm chromatin mass in a newly fertilized oocyte; pink arrowheads indicate the meiotic oocyte chromosomes in a newly fertilized oocyte. The white arrowhead indicates the chromosomes of an unfertilized oocyte that have undergone endomitotic replication. The yellow dotted lines indicate the borders of embryos or oocytes. See also Figure S2. Scale bar, 10 μm.

as evidenced from the localization of a small, compact nuclear material, characteristic of the sperm DNA, inside the oocyte (Figure 2D, blue arrow). In contrast, the *spe-45* mutant worms showed no sign of sperm entry into the oocytes, indicating that SPE-45 is required for fertilization in *C. elegans*. The phenotypes of *spe-45* mutants are consistent with *spe-45* belonging to the *spe-9* class of *spe* genes—all phases of sperm development and differentiation remain unaffected; however, since the mutant sperm are incapable of fertilizing oocytes, the unfertilized oocytes undergo endomitotic replication (EMO phenotype) (Figure 2D, white arrow) [10].

A further indictor of lack of sperm entry can be found in the nature of the EMO phenotype seen in oocytes from unmated *spe-45* hermaphrodites. In addition to paternal DNA, sperm also deliver centrosomes to the zygote. When sperm do not enter the oocyte, they do not deliver centrosomes and the EMO DNA forms a single mass as seen in *spe-45* mutants (Figure 2D, white arrow; Figure S2) [10].

Oocytes Function Normally in *spe-45(as38)*

The observed sterility in *spe-45(as38)* could be due to the defect in the sperm, oocyte, or both. At least two observations suggest that SPE-45 is not required in oocytes. First, the brood size of *spe-45(as38)* hermaphrodites could be restored to that of N2 upon crossing with N2 males (Figure 1B), indicating that SPE-45 is dispensable in oocytes. Second, the *spe-45(as38)* hermaphrodites lay large numbers of oocytes (mean = 91 ± 9 SE), suggesting that oogenesis and ovulation are not compromised in *spe-45(as38)* [10].

The Genetic Nature of *spe-45(as38)* Allele

Compared to *spe-45(as38)* homozygous mutants, which do not produce any progeny at 25°C, the *spe-45(as38)* heterozygous mutant produce a significant number of progeny (mean = 252 ± 22 SE, n = 25, $p < 0.001$), indicating that *spe-45(as38)* is recessive (Figure 1C). The phenotype of *spe-45(as38)/sDf27* is Spe, which suggests that the null mutant phenotype is sperm sterile (Spe). The *tm3715* mutation from the Japanese Knockout Consortium harbors a large deletion within the protein-coding region of F28D1.8 (see below). As expected, the *tm3715* allele exhibited a Spe phenotype (Figure 1C). Next, we expected that the *as38* allele should fail to complement the *tm3715* allele, if both are alleles of the same gene. As shown in Figure 1C, there is no significant difference between the brood sizes of *as38/as38* homozygotes and *as38/tm3715* trans-heterozygotes ($p = 0.36$), indicating that same gene is affected in both the *as38* and *tm3715* alleles. The molecular lesions for both alleles also map to the same transcript (Figure 3).

Molecular Identification of *spe-45*

We did standard two-factor, three-factor, and SNP mapping (Figure 3; Table S2) and found that the *spe-45* locus maps to the fourth chromosome between the genomic intervals defined by the cosmids C42C1 and K08E7 (Figure 3A).

We used strains deficient in a defined region of the genome as a tool to further map the locus of *spe-45(as38)*. In complementation tests, the deficiency *sDf22* complemented *spe-45(as38)*; the deficiencies *sDf27* and *sDf21* failed to complement *spe-45(as38)* (Figure 3A). These results suggest that *spe-45* lies within the region that is deleted by *sDf27* and *sDf21* and lies outside the region of *sDf22*. Further, *spe-45* over a non-complementing

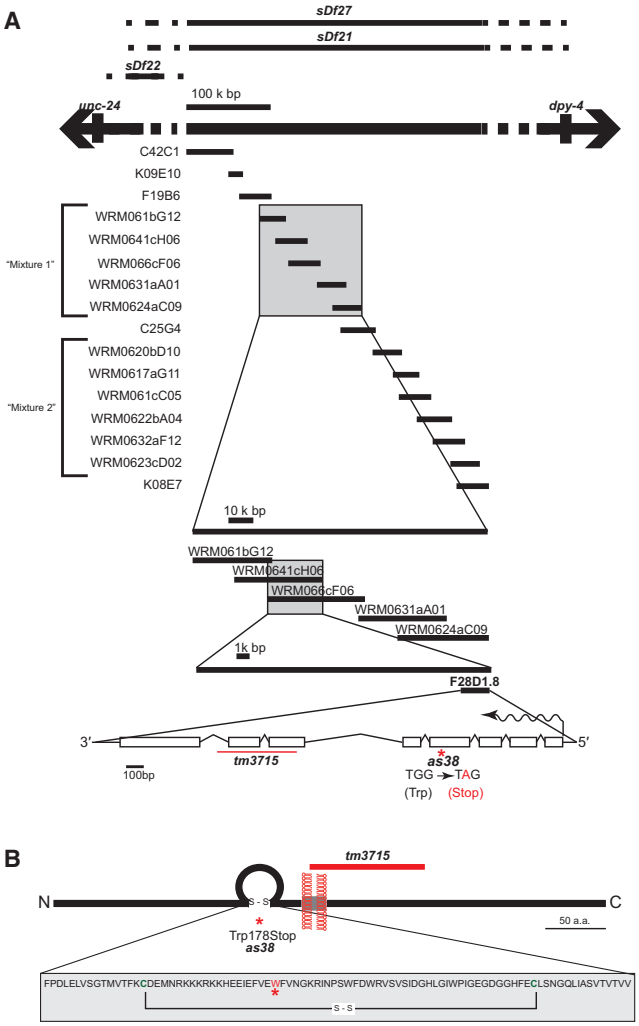

Figure 3. Molecular Analysis Reveals that SPE-45 Is an Immunoglobulin Superfamily Protein

(A) Genomic locus of *spe-45(as38)*. See also Table S2. Complementing and non-complementing deficiencies are indicated at the top of the figure. The list of cosmids and fosmids tested for the genetic rescue of *spe-45(as38)* is indicated. A single extrachromosomal array carrying a mixture of all fosmids listed in mixture 1—but not mixture 2—rescued the *spe-45(as38)* phenotype. Introns and exons of the *spe-45* gene are indicated as lines and boxes, respectively; the direction of the transcription is shown as wavy arrow.

(B) Schematic diagram of the SPE-45 protein. Complete sequence of the immunoglobulin domain of the SPE-45 is indicated in the box. See also Figure S3 for full amino acid sequence of SPE-45.

deficiency has no new phenotypes and still has only a Spe mutant phenotype.

Results from three-factor mapping, deficiency mapping, and complementation testing suggested that the *as38* mutation resides within the interval of an approximately 400 kb region. We introduced the extrachromosomal arrays representing this genomic interval into the *as38* mutant to test which, if any, could rescue the *as38* Spe phenotype. Among the tested arrays, the fosmids WRM0641cH06 and WRM066cF06 rescued the Spe phenotype of *as38* (Figures 1C and 3A), indicating that the molecular lesion responsible for *as38* phenotype should lie somewhere in the overlapping region of these two fosmids.

We performed whole-genome sequencing of the *spe-45(as38)* and our laboratory N2 strains to identify variants unique to *spe-45(as38)*. We discovered 23 candidate mutations on chromosome IV, defined as homozygous, non-synonymous variants. Of those, only one mapped to the interval delimited by the rescuing fosmids RM0641cH06 and WRM066cF06. The presence of that variant in *spe-45(as38)* was confirmed independently by PCR amplification and Sanger sequencing. The variant is a G→A transition in the gene F28D1.8 and encodes a nonsense mutation predicted to truncate the protein at Trp185. The gene name *oig-7* (for one immunoglobulin domain) has been proposed on the basis of structural prediction [11], but no functional studies had been reported. Additional analyses (described below) confirm the identity of F28D1.8 as *spe-45*, and we use that designation hereafter.

Suppression of *spe-45(as38)*

We observed a point mutation altering the tryptophan codon (TGG) to a stop codon (TAG) in the *as38* allele of the *spe-45* gene (Figure 3B). If this mutation is indeed responsible for the observed Spe phenotype, we hypothesized that *sup-5(e1464)* should be able to at least partially rescue the *spe-45(as38)* mutant phenotype [12]. The *sup-5(e1464)* allele harbors a mutation in a gene encoding tRNA, such that the anti-codon of tRNA carrying tryptophan is TAG. Therefore, *sup-5(e1464)* is expected to insert Trp in lieu of a stop codon TAG, allowing the protein synthetic machinery to continue translating past the premature stop codon TAG in our *as38* allele. We tested our hypothesis by constructing a *sup-5(e1464);spe-45(as38)* double mutant. The *spe-45(as38)* essentially no progeny at 25°C. In contrast, the *sup-5(e1464);spe-45(as38)* double mutant showed a statistically significant increase in the number of progeny produced (Figure 1C), reaffirming that we have correctly identified the molecular lesion responsible for the *as38* phenotype.

spe-45 Encodes a Single-Pass Transmembrane Protein with a Single Immunoglobulin Domain

The gene F28D1.8 is originally predicted to have seven exons and six introns in Wormbase. However, close examination of the sequence upstream of this gene revealed a predicted start codon. Therefore, we analyzed RT-PCR products of F28D1.8 and found the existence of additional sequences upstream of the predicted transcript. We conclude that F28D1.8 is composed of eight exons and seven introns and is predicted to encode 492 amino acids (Figure S2).

We find that *spe-45* encodes a one immunoglobulin (OIG) transmembrane protein. This is a large family of proteins that is found in a broad range of species and most likely functions in many different tissues. In *C. elegans*, there are at least eight members of this gene family, *oig-1* through *oig-8* [11]. The *spe-45* gene was originally annotated as *oig-7*. Where functional and cell biological data exist, single-immunoglobulin proteins from various species are involved in cell-cell interactions [11]. Further, recent biochemical analysis suggests that OIG proteins can function in direct ligand-receptor interactions [1]. The OIG protein Izumo1 has been shown to bind to an oocyte-specific glycosylphosphatidylinisotol (GPI)-anchored protein Juno [1].

Izumo1 is a sperm-specific protein, and knockout mice have male specific infertility [13]. Izumo1 was originally identified by biochemical characterization of an antigen that upon inhibition by monoclonal antibody prevented sperm-egg fusion in vitro [13]. Izumo1 mutant sperm are morphologically normal, with normal motility, the ability to find the egg, and transit through the egg coat (zona pellucida). However, these sperm cannot fuse with the egg plasma membrane. As presented in this paper, *spe-45* mutants have the equivalent mutant phenotype in *C. elegans*. SPE-45 has a sperm-specific function. *spe-45* mutant sperm are morphologically normal but cannot fuse with the eggs despite direct contact at the site of fertilization in the reproductive tract. Based on this structural [5] and functional similarity, we propose that the *spe-45* encodes an Izumo-like function. A common ancestor for *C. elegans* and mammals existed about 700 million years ago [14], and our discovery of *spe-45* represents the most deeply conserved and ancient fertility function discovered to date.

Use of many model organisms continues to shape our current understanding of fertilization. Large quantities of sperm and egg can be obtained from the sea urchin, which makes it feasible to do a variety of biochemical assays. However, the current technology allows us to perform very limited genetic manipulation in this organism. In contrast, a variety of sophisticated genetic analyses are feasible in *C. elegans*. Since leveraging the strengths of all model organisms advances the field of fertilization, we have demonstrated that conducting an unbiased, forward genetic screen, a procedure particularly well suited in *C. elegans*, should aid in the discovery of new key components of fertilization pathways.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2015.10.055.

AUTHOR CONTRIBUTIONS

G.S. wrote the manuscript and helped to train and supervise undergraduate student S.R. G.S. with D.S. discovered the *as38* mutation. S.R. characterized the *spe-45* mutant strain and mapped and cloned the gene. A.K. characterized the *spe-45* transcript and assisted in phenotypic analysis. Undergraduate students A.R. and S.D. aided with phenotypic analysis. D.S. worked with G.S. on the genetic screen that identified the *as38* mutation in *spe-45.* H.S. and A.G. carried out whole-genome sequencing and data analysis for the *spe-45(as38)* mutant strain. A.S. was the project primary investigator. A.S. designed and supervised all aspects of the project, as well as assisted in phenotypic analysis, creation of transgenic animals, and manuscript editing.

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