Sperm Development and Motility are Regulated by PP1 Phosphatases in *Caenorhabditis elegans*

Jui-ching Wu, Aiza C. Go, Mark Samson, Thais Cintra, Susan Mirsoian, Tammy F. Wu, Margaret M. Jow, Eric J. Routman, and Diana S. Chu¹

Department of Biology, San Francisco State University, San Francisco, California 94132

ABSTRACT Sperm from different species have evolved distinctive motility structures, including tubulin-based flagella in mammals and major sperm protein (MSP)-based pseudopods in nematodes. Despite such divergence, we show that sperm-specific PP1 phosphatases, which are required for male fertility in mouse, function in multiple processes in the development and motility of *Caenorhabditis elegans* amoeboid sperm. We used live-imaging analysis to show the PP1 phosphatases GSP-3 and GSP-4 (GSP-3/4) are required to partition chromosomes during sperm meiosis. Postmeiosis, tracking fluorescently labeled sperm revealed that both male and hermaphrodite sperm lacking GSP-3/4 are immotile. Genetic and *in vitro* activation assays show lack of GSP-3/4 causes defects in pseudopod development and the rate of pseudopodial treadmilling. Further, GSP-3/4 are required for the localization dynamics of MSP. GSP-3/4 shift localization in concert with MSP from fibrous bodies that sequester MSP at the base of the pseudopod, where directed MSP disassembly facilitates pseudopod contraction. Consistent with a role for GSP-3/4 as a spatial regulator of MSP disassembly, MSP is mislocalized in sperm lacking GSP-3/4. Although a requirement for PP1 phosphatases in nematode and mammalian sperm suggests evolutionary conservation, we show PP1s have independently evolved sperm-specific paralogs in separate lineages. Thus PP1 phosphatases are highly adaptable and employed across a broad range of sexually reproducing species to regulate male fertility.

SPERM from different species undergo dramatic morphological changes to enable the streamlined delivery of paternal DNA to the oocyte. DNA is tightly compacted by replacing somatic histones with sperm nuclear basic proteins, resulting in global transcriptional repression after meiosis (Sassone-Corsi 2002; Tanaka and Baba 2005). Further, the bulk of cytoplasmic materials, including ribosomes, are discarded (Miller and Ostermeier 2006). Because sperm morphogenesis and motility occur during a period of diminished global transcription and translation, post-translational regulators, like kinases and phosphatases, play key roles.

An example is PP1gamma2, a testis-specific PP1 phosphatase required for male fertility in mammals, which have flagellar sperm. In mice, deletion of the *Ppp1cc* gene, which encodes PP1gamma2, results in defective sperm development and motility (Varmuza *et al.* 1999). *Ppp1cc*-deficient

mice exhibit polyploid cells, suggesting defects in sperm meiosis (Oppedisano *et al.* 2002). Although most of these defective sperm are culled by apoptosis, the few escapers exhibit deformed head, midpiece, and tail morphologies, indicating *Ppp1cc* is required for sperm development (Chakrabarti *et al.* 2007). PP1gamma2 localizes at the posterior and equatorial head regions and along the flagellum (Huang and Vijayaraghavan 2004) and motility of *Ppp1cc*-deficient sperm is also defective (Soler *et al.* 2009). The basis for PP1 function in such disparate processes of spermatogenesis is unclear.

Strikingly, RNA interference against either of two 98% identical PP1 phosphatases, *gsp-3* or *gsp-4* (Glc-seven-like phosphatase), causes incompletely penetrant male infertility in *Caenorhabditis elegans* (Chu *et al.* 2006). Unlike mammalian sperm that use microtubule-based flagella, nematode amoeboid sperm use a cytoskeletal component called major sperm protein (MSP) (Burke and Ward 1983; Sepsenwol *et al.* 1989). Assembly of MSP filaments at the leading edge of pseudopodia and disassembly at the pseudopod–cell body interface provide the protrusive force for actin-independent motility (Varkey *et al.* 1993; Stewart and Roberts 2005). Although phosphorylation regulates *Ascaris* amoeboid sperm

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¹Corresponding author: Department of Biology, San Francisco State University, Hensill Hall 538, 1600 Holloway Ave., San Francisco, CA 94132. E-mail: chud@sfsu.edu

motility (Yi *et al.* 2009), homologs to *Ascaris* regulators are unknown in *C. elegans*. Given the dependence of male fertility on phosphorylation, even in species with morphologically distinct sperm, we were interested to determine roles of PP1 phosphatases in sperm development.

The use of *C. elegans* allows the molecular characterization of spermatogenesis not currently possible in other organisms (L'Hernault 2006; Shakes *et al.* 2009). In contrast to mammals, defective cells are not removed by apoptosis during sperm formation in *C. elegans* (Gumienny *et al.* 1999; Jaramillo-Lambert *et al.* 2010). This allows observation of both normal and defective meiosis and postmeiotic events like morphogenesis and motility *in vivo* (Sadler and Shakes 2000). Sperm development can be visualized both *in vivo* through the transparent cuticle of males or staged hermaphrodites and *in vitro* with isolated sperm (L'Hernault and Roberts 1995; Miller 2006; Shakes *et al.* 2009). The use of genetic mutants defective in specific reproductive processes also allows analysis of PP1 function in distinct stages of sperm development. Our goal in this study was to identify processes that are mediated by PP1 phosphatases and required for male fertility in *C. elegans*.

Materials and Methods

Strains

C. elegans strains (listed in Supporting Information, Table S1) were cultured using standard conditions (Brenner 1974) at 20°, except for TY0119 *fem-1(hc17)* and JK0816 *fem-3(q20)*, which were maintained at 15°. Strains were crossed into a *him-8(e1489)* genetic background (high incidence of males: a mutation that causes X chromosome nondisjunction, yielding ~30% male progeny compared to 0.1% in a predominantly hermaphrodite population) to facilitate assessment in males (Hodgkin *et al.* 1979).

The *gsp-3(tm1647)* mutant was generated by the National Bioresource Project (Mitani 2009). *gsp-3(tm1647)* contains a 1045-bp deletion that removes over half of the 3' end of the gene and the 3' untranslated region (Figure S1A). The *gsp-4(y418)* mutant was isolated from a *C. elegans* deletion library constructed in the Meyer laboratory at the University of California, Berkeley, using the Koelle laboratory's gene knockout protocol (<http://info.med.yale.edu/mbb/koelle/>) (Ahringer 2006). The *gsp-4(y418)* allele harbors a 385-bp deletion with an insertion of 14 bp that removes a portion of the 5'-untranslated region and the translation start site (Figure S1, A and B). Mutant strains were backcrossed at least four times.

The *gsp-3* and *gsp-4* genes are on chromosome I (LG1) (Figure S1A). To construct the *gsp-3(tm1647) gsp-4(y418)* double mutant, two strains, *gsp-3(tm1647) unc-11(e47)* and *gsp-4(y418) dpy-5(e61)*, were constructed. These were crossed to one another and their progeny screened for recombination between *unc-11* and *dpy-5*. The *gsp-3(tm1647) gsp-4(y418)* mutant was confirmed via PCR analysis. Homozygous *gsp-3(tm1647) gsp-4(y418)* animals are sterile; thus,

the balancer *hT2[bli-4(e937) let-?(q782) qIs48]* that contains a dominantly expressed *myo-2::GFP* fusion transgene was introduced to facilitate maintenance of the strain as a heterozygote.

Analysis of GSP-3/4 protein levels

Large-scale culturing of worms and isolation of males were conducted as previously described (L'Hernault and Roberts 1995; Chu *et al.* 2006; Gent *et al.* 2009).

For whole worm lysates, a synchronous population of worms was collected and then frozen in liquid nitrogen. Frozen worms were ground with a mortar and pestle and then resuspended with lysis buffer [50 mM HEPES, pH 7.4, 1 mM EGTA, 1 mM MgCl₂, 100 mM KCl, 10% glycerol, 0.05% NP-40, 1 µl Protease Inhibitor Cocktail (Calbiochem, San Diego)/5 ml lysis buffer] and sonicated at 30% amplitude for 15 sec with 1-min intervals on ice until bodies were no longer visible using a stereomicroscope. Lysates were centrifuged at 20,000 relative centrifugal force (rcf) for 20 min at 4°. KCl was added to a final concentration of 300 mM to the supernatant.

For sperm purifications, synchronous populations of starved *fem-3(q20)* L1s were grown at a restrictive temperature of 25° for 5 days and sperm was isolated in monovalent free sperm medium (L'Hernault and Roberts 1995; Chu *et al.* 2006). Isolated sperm were pelleted at 20,000 rcf for 20 min at 4°, resuspended with lysis buffer, and sonicated (100 mg/1 ml lysis buffer) (Giresi *et al.* 2007). Sperm lysate was centrifuged at 20,000 rcf for 20 min at 4° and the supernatant collected. KCl was added to a final concentration of 300 mM.

Fifty micrograms of lysates of sperm purified from *fem-3(q20)* animals or whole bodies of *fem-3(q20)* or *fem-1(hc17)* were separated by SDS-PAGE on 4–20% polyacrylamide gels (Bio-Rad, Hercules, CA). The following antibodies were used for Western blot analysis: rabbit anti-GSP-3/4 [the epitope used for antibody generation (Chu *et al.* 2006) is shown in blue text in Figure S1C] at 1:4000, mouse anti-MSP (Greenstein laboratory) at 1:1000 dilution (Kosinski *et al.* 2005), mouse anti- α -tubulin (clone B-5-1-2; Sigma, St. Louis) at 1:500 dilution, and HRP-conjugated goat anti-rabbit at a 1:5000 dilution. HRP signal was detected using SuperSignal Chemiluminescent Substrate (Pierce Chemical, Rockford, IL). Western blots were analyzed using a Kodak (Rochester, NY) Imager.

Fertility assessment

Hermaphrodite progeny production assay: Individual N2, *gsp-3(tm1647)*, *gsp-4(y418)*, or *gsp-3(tm1647) gsp-4(y418)* hermaphrodites ($n = 30$) were transferred daily to nematode growth medium (NGM) agar plates containing fresh spots of the *Escherichia coli* strain OP50 for 4 days beginning with fourth larval stage (L4) animals. The numbers of unfertilized oocytes, viable progeny, and dead embryos were counted daily. *gsp-3(tm1647) gsp-4(y418)* hermaphrodites occasionally laid clutches of broken oocytes, which were

difficult to quantify because cell boundaries in these masses were often not discernible. Statistical comparisons were conducted using a standard Student's *t*-test.

Male progeny production assay: Virgin (L4) males [*him-8(e1489)* and *gsp-3(tm1647) gsp-4(y418); him-8(e1489)*] were mated with three virgin (L4) *fog-2(q71)* females and transferred to new NGM agar plates for 3 days. *fog-2(q71)* females lack sperm and can produce progeny only when mated with a fertile male (Schedl and Kimble 1988). Progeny were quantified by counting the number of objects laid that were unfertilized oocytes, embryos, and larvae. Eggs not hatched by 48 hr were scored as dead embryos.

Hermaphrodite fertility rescue assay: Virgin L4 staged *him-8* and *gsp-3(tm1647) gsp-4(y418); him-8* double-mutant hermaphrodites either unmated or mated to *him-8* mutant males for 24 hr at 20°. Viable progeny were counted over a 3-day period.

Transfer assays

Fluorescently labeled male sperm transfer/migration assay and fluorescently labeled hermaphrodite sperm displacement assay: Two to five plates containing five L4 stage virgin hermaphrodites and 50–60 males of indicated genotypes were mated at 20°. Three to eight animals with the patterns shown in Figures 2 and 3 were observed per experiment. At least three experiments were conducted for each type of assay. After mating for 24 or 48 hr the hermaphrodites were anesthetized with 200 μ M levamisole in M9 for 5 min, mounted onto a 3% agarose pad, and examined under a Zeiss (Thornwood, NY) Cell Observer spinning disc confocal microscope. Still DIC and fluorescent images were reassembled in Adobe Photoshop.

Live imaging

Chromosome segregation during sperm meiosis: mCherry-histone H2B-expressing worms were picked into 3.2 μ l of SM buffer [50 mM Hepes, pH 7, 50 mM NaCl, 25 mM KCl, 1 mM MgSO₄, 5 mM CaCl₂, 1 mg/ml BSA (Nelson and Ward 1980)] and dissected to release the gonad (see Table S1 for strains used). An 18 $\times$ 18 coverslip was laid on top of the droplet containing dissected worms to help release spermatocytes from the gonad. The coverslip was sealed with Vaseline to prevent evaporation. Time-lapse imaging was conducted under a 100 $\times$ objective, using a Zeiss Axiovert 200M fluorescent microscope with DIC optics.

Determination of sperm pseudopod treadmilling rate: *swm-1(me66) him-5(e1490)* and *gsp-3(tm1647) gsp-4(y418); swm-1(me66) him-5(e1490)* males were dissected in SM buffer to release activated sperm. Rapid membrane movements were captured by time-lapse recording with a Zeiss Cell Observer spinning disc confocal microscope. Movement of membrane vesicles traveling toward the cell body was tracked using ImageJ with Manual Tracking plugin. Vesicle movement rates obtained from 12 sperm from

five individual *swm-1(me66) him-5(e1490)*, *gsp-3(tm1647) gsp-4(y418)*; *swm-1(me66) him-5(e1490)*, or *fer-1(hc1); swm-1(me66) him-5(e1490)* worms were analyzed and plotted using Microsoft Excel.

In vitro sperm activation: L4 virgin *him-8(e1489)* and *gsp-3(tm1647) gsp-4(y418); him-8(e1489)* males were picked and transferred to NGM plates with OP50 bacteria and incubated overnight in the absence of hermaphrodites to prevent premature spermatid activation. Animals were dissected in activation solution [200 μ g/ml pronase (Sigma; P-6911) in SM buffer with 1 mg/ml BSA]. Sperm activation was recorded on time-lapse videos, using a Zeiss Axiovert 200M microscope equipped with Nomarski optics at $\times$ 100 magnification. Three experiments were conducted in which two to three worms were dissected to obtain a sufficient number of sperm for activation studies.

Immunohistochemistry

L4 virgin *him-8(e1489)* and *gsp-3(tm1647) gsp-4(y418); him-8(e1489)* males were picked and transferred to NGM plates with OP50 bacteria and incubated overnight in the absence of hermaphrodites to help prevent activation. Antibody staining on young adult animals followed a protocol devised by A. Chan (Howe *et al.* 2001; Shaham 2006). Adult worms were dissected into sperm salts (50 mM Pipes, pH 7, 25 mM KCl, 1 mM MgSO₄, 45 mM NaCl, and 2 mM CaCl₂) on a slide and an equal volume of 3% paraformaldehyde was added. The slide was then incubated in a humid chamber for 5 min, freeze cracked on dry ice, placed into 95% ethanol for 1 min, and washed with PBST (1 $\times$ PBS, 0.5% Triton X-100, and 1 mM EDTA, pH 8). Primary antibody incubation was done overnight and secondary antibody from 2 hr to overnight. Slides were then stained with DAPI (10 μ g/ml) and mounted with Vectashield.

The following primary antibodies were used: rat anti-GSP-3/4 (rt1494) at 1:50 and rat anti-GSP-3/4 (rt1495) at 1:25; rabbit anti-GSP-3/4 (rb1496) at 1:2000 and rabbit anti-GSP-3/4 (rb1497) at 1:1000 (Chu *et al.* 2006); monoclonal antibody 1CB4 at 1:500 or a AlexaFluor 488-conjugated wheat-germ agglutinin at 5 μ g/ml [W11261 from Invitrogen (Carlsbad, CA)], which recognizes membranous organelles (MO) in sperm (Ward *et al.* 1983; Okamoto and Thomson 1985; Kelleher *et al.* 2000); and mouse anti-MSP 4A5 antibody from the Developmental Studies Hybridoma Bank at 1:5 or the Greenstein laboratory at 1:50 (Kosinski *et al.* 2005). Secondary antibodies include anti-rabbit AlexaFluor 488-labeled IgG, anti-rat AlexaFluor 488-labeled IgG, and anti-rabbit AlexaFluor 546-labeled IgG at 1:100. DNA was stained using DAPI at 0.1 μ g/ml and mounted with VectaShield antifade. A Zeiss LSM710 confocal microscope was used to conduct immunofluorescence analysis.

Phylogenetic analysis

Nucleotide sequences from genes encoding PP1 proteins from 16 species representing a wide taxonomic distribution

(Table S2) were translated and aligned using TranslatorX (<http://translatorx.co.uk/>). Alignments were examined by eye to correct misaligned amino acids. Because N- and C-terminal sequences were too divergent to align, sequences were truncated to remove those regions as well as those with multiple insertions/deletions. The core phosphatase region analyzed corresponded to amino acids 14–301 of GSP-3. Corrected alignments were converted to NEXUS format with Mesquite (version 2.73; mesquiteproject.org). Bayesian phylogenetic analyses were conducted using parallel MRBAYES version 3.1.2 (Huelsenbeck and Ronquist 2001) on Cornell University's Computational Biology Service Unit computing cluster (cbsuapps.tc.cornell.edu/mrbayes.aspx). The final phylogeny estimation of 69 proteins (Figure 7) was conducted with a GTR+ Γ +I model with a variable Dirichlet rate prior, a fixed state prior, and unconstrained branch lengths. All other priors were uniform. Two runs of four chains were run for 3 million generations with a sample frequency of 1000. The first 1 million trees were discarded as burn-in. The *Saccharomyces cerevisiae* PP1 sequence was used as an outgroup.

Results

The function of the PP1 phosphatases GSP-3 and GSP-4 is specific to sperm

Given that RNAi targeted to either or both of the *gsp-3* and *gsp-4* genes resulted in infertility in hermaphrodites (Chu *et al.* 2006), we sought to define requirements for GSP-3 and GSP-4 in sperm and oocytes. Antibodies that recognize the nearly identical GSP-3 and GSP-4 (GSP-3/4) proteins (Figure S1) were used to show that, similar to the sperm-specific MSP protein, GSP-3/4 are expressed only in sperm-producing *fem-3(q20gf)* but not in oocyte-producing *fem-1(hc17lf)* mutant hermaphrodites (Figure 1A) (Hodgkin 1986; Ward *et al.* 1986; Barton *et al.* 1987; Chu *et al.* 2006). Likewise, immunolocalization of GSP-3/4 in wild-type males revealed their expression is limited to sperm-producing *germlines* (Figure 1B and data not shown). We previously observed that GSP-3/4 is localized around sperm chromatin during meiotic divisions (Chu *et al.* 2006). Here we found that GSP-3/4 strongly accumulate after meiosis in *spermatids* (Figure 1B). GSP-3/4 were undetectable in male *germlines* from a mutant strain with deletions in both *gsp-3* and *gsp-4* genes, which we subsequently refer to as the *gsp-3/4* mutant (see *Materials and Methods*), indicating the mutations are null alleles (Figure 1C). In contrast, staining was detectable in single-mutant male *gonads* similar to levels observed in wild type (data not shown). The detection of GSP-3/4 only in sperm-producing *germlines* implies that GSP-3/4 have a sperm-specific function.

We thus ruled out the possibility that GSP-3/4 are required in oocytes. To do this we utilized hermaphrodites carrying the *fog-2(q71)* mutation, which are “feminized” because they do not produce sperm. When crossed with wild-type males, *gsp-3/4; fog-2(q71)* females generate com-

parable levels of viable progeny to *fog-2(q71)* females (Figure S2). Together, these results support the conclusion that GSP-3/4 function is exclusive to sperm.

GSP-3/4 are required for sperm meiotic chromosome segregation

A role for GSP-3/4 in sperm meiosis was revealed by cytological examination of *gsp-3/4* mutant male *gonads*, which exhibit chromatin bridges during sperm meiotic divisions, suggesting the incomplete separation of chromosomes (Figure 1, D and E). PP1 homologs GSP-1 and GSP-2, which are 52–53% identical at the amino acid level to GSP-3/4 (Figure S1C), function in oocyte meiosis and mitosis by working counter to the aurora-like kinase AIR-2 (Rogers *et al.* 2002; De Carvalho *et al.* 2008). Loss of GSP-1 or GSP-2 causes premature separation of chromosomes during meiosis I in oocytes (Rogers *et al.* 2002). To investigate at which division defects arise in *gsp-3/4* mutants, we conducted live imaging of wild-type and *gsp-3/4* male *spermatocytes* expressing mCherry-histone H2B. In wild-type males, *spermatocytes* undergo two sequential rounds of chromosome segregation, first separating homologous chromosomes and then sister chromatids to generate four haploid cells that subsequently bud off of the residual body (Figure 1F, File S2). In cells lacking GSP-3/4, chromosomes appear to successfully separate in the first meiotic division but fail to separate in the second division and form bridged nuclei (Figure 1G, File S3), a phenotype distinct from loss of GSP-1 or GSP-2 (Rogers *et al.* 2002). Incompletely separated chromosomes in *gsp-3/4* mutants either segregate to one cell or are partitioned into the residual body, resulting in either aneuploid or anucleate sperm, respectively. Thus sperm meiosis requires GSP-3/4 for partitioning the correct haploid complement of paternal DNA.

After meiosis, GSP-3/4 are required for sperm movement

GSP-3/4 are also redundantly required for roles in postmeiotic processes required for fertility. *gsp-3* and *gsp-4* single mutants produce progeny at levels comparable to wild type (Figure 2A, Table 1). However, *gsp-3/4* double-mutant hermaphrodites produce no viable progeny and few unfertilized oocytes or dead embryos (Figure 2A, Table 1). This is unlike most previously characterized spermatogenesis-defective (*spe*) mutant hermaphrodites that are infertile but lay unfertilized oocytes (L'Hernault *et al.* 1988; McCarter *et al.* 1999), indicating *spe* mutant sperm still generate an MSP-based signal for oocyte maturation and ovulation. We thus sought to determine whether *gsp-3/4* mutants are capable of MSP-based sperm signaling (Miller *et al.* 2001; Nabeshima *et al.* 2005; Shakes *et al.* 2009). First, we found that MSP could be detected in distinct small vesicles in both wild-type and *gsp-3/4* mutant hermaphrodites (Figure S3, A and B). We next assessed the presence of oocyte maturation markers, including AIR-2 recruitment to chromosomes in late diakinesis

Figure 1 GSP-3/4 are sperm-specific proteins required for sperm meiosis. (A) Immunoblots of 50 μ g of extracts from sperm-producing *fem-3(q20)* and oocyte-producing *fem-1(hc17)* whole worms or purified sperm indicate GSP-3/4 are detected only in sperm and sperm-producing animals. (B and C) Immunostaining of (B) *him-8(e1489)* and (C) *gsp-3(tm1647) gsp-4(y418); him-8(e1489)* male gonads with anti-GSP-3/4 antibody shows GSP-3/4 protein is absent in the double mutant. A faint level of nonspecific staining was observed evenly distributed in karyosome nuclei in *gsp-3(tm1647) gsp-4(y418); him-8(e1489)* mutants. Bar, 10 μ m. (D and E) Comparison of DNA staining of the proximal end of gonads from (D) *him-8(e1489)* and (E) *gsp-3(tm1647) gsp-4(y418); him-8(e1489)* males shows chromosome segregation defects caused by loss of GSP-3/4 proteins. Examples of chromatin bridges and incompletely separated chromosomes are indicated with yellow arrowheads. The white dashed square shows the region containing postmeiotic sperm. Bar, 5 μ m. (F and G) Still frames from live-imaging analysis of (F) *him-8(e1489)* (File S2) and (G) *gsp-3(tm1647) gsp-4(y418); him-8(e1489)* (File S3) male spermatocytes expressing mCherry-histone (red) progressing through successive stages of meiotic divisions.

oocytes and MAP kinase activation, two key responses to MSP signaling (Schumacher *et al.* 1998; Miller *et al.* 2001; Rogers *et al.* 2002; Nabeshima *et al.* 2005). Both could be detected, though at reduced levels, indicating MSP signaling is intact (File S1, File S6, and Figure S3, C–F). Consistent with a response to MSP, ovulation rates of *gsp-3/4* mutant hermaphrodites were comparable to wild type (Figure S4A). Ovulated oocytes accumulated in the uterus, with small clutches of broken oocytes occasionally being laid (Figure S4, B–D). Therefore, egg laying, a process that is genetically separable from MSP-based sperm signaling for oocyte maturation and ovulation (McGovern *et al.* 2007), may be defective in *gsp-3/4* mutants (Table 1, Table S3, and data not shown). This suggests a possible role for GSP-3/4 in non-MSP-based sperm signaling pathways that are required for egg laying.

GSP-3/4 are also distinct from conditional mutants in genes that encode proteins that similarly fail in sperm meiotic chromosome segregation (Golden *et al.* 2000). Such mutants

produce anucleate spermatids that can activate and fertilize (Sadler and Shakes 2000). Oocytes fertilized by these anucleate sperm develop eggshells, are laid, and then die. The lack of dead embryo production in the *gsp-3/4* double mutant suggests *gsp-3/4* anucleate sperm do not fertilize. In fact, we found *gsp-3/4; him-8(e1489)* males crossed to *fog-2(q71)* hermaphrodites produced no viable progeny, oocytes, or dead embryos (Figure 2B, Table S3). To investigate why sperm lacking GSP-3/4 do not fertilize oocytes, we tracked wild-type or *gsp-3/4* male sperm expressing GFP-histone after mating to unlabeled wild-type hermaphrodites (Figure 2C). After mating for 24 hr, wild-type sperm are transferred and accumulate at the spermatheca and adjacent uterus (Figure 2, D and E). In contrast, *gsp-3/4* male sperm remained near the vulva (Figure 2, F–H). Therefore, although *gsp-3/4* sperm are transferred from the male to the hermaphrodite, they fail to migrate, suggesting *gsp-3/4* sperm do not fertilize because they fail to move.

Figure 2 GSP-3/4 are required for male fertility and male sperm movement. (A) Viable self-progeny from wild type (N2), *gsp-3* (*tm1647*), *gsp-4*(*y418*), and *gsp-3*(*tm1647*) *gsp-4*(*y418*) hermaphrodites (error bars are standard deviation from $n = 30$). P -values were calculated using Student's t -test. (B) Viable progeny are produced by *fog-2*(*q71*) hermaphrodites mated to *him-8*(*e1489*) but not *gsp-3*(*tm1647*) *gsp-4*(*y418*); *him-8*(*e1489*) males. Error bars are the standard deviation based on the data obtained from five crosses. (C) Schematic. After mating, male sperm expressing histone-GFP can be tracked in unlabeled hermaphrodites. Sperm have low levels of cytoplasmic autofluorescence. Oocytes are outlined in orange, spermatheca are shown by a yellow dashed line, uterus is blue, and vulva is magenta. (D–H) Male sperm lacking GSP-3/4 do not migrate to the spermatheca. (D) DIC and (E) fluorescence images are shown of GFP-histone wild-type labeled male sperm that have migrated to the spermatheca in a wild-type unlabeled hermaphrodite after mating (green arrows). (F) DIC and (G) fluorescence images show GFP-histone-labeled *gsp-3* (*tm1647*) *gsp-4*(*y418*) male sperm (green arrows) are transferred to the wild-type unlabeled hermaphrodite gonad after mating but do not migrate. The boxed region enlarged in H shows sperm nuclei from *gsp-3*(*tm1647*) *gsp-4*(*y418*). The yellow arrow indicates abnormal chromatin content indicative of *gsp-3*(*tm1647*) *gsp-4*(*y418*) male sperm. Bar, 20 μ m.

We suspected that *gsp-3/4* mutant hermaphrodite sperm could also have motility defects. We thus tested the ability of wild-type male sperm to rescue *gsp-3/4* hermaphrodite sterility and found that *gsp-3/4* hermaphrodites produced half the levels of viable cross-progeny of wild-type hermaphrodites after 24 hr of mating (Figure 3A). This is in

contrast to the complete rescue observed when wild-type males are crossed to *gsp-3/4*; *fog-2* hermaphrodites that lack hermaphrodite mutant sperm (Figure S2). One possible explanation is that the presence of mutant hermaphrodite sperm interferes with the fertilization by wild-type male sperm.

Table 1 GSP-3/4 are redundantly required for fertility

Strain ($n = 30$)	Total laid		Viable progeny			Dead embryos			Oocytes		
	No.	P -value	No.	P -value	% of total	No.	P -value	% of total	No.	P -value	% of total
N2	208.3	—	204.0	—	97.9	0.1	—	0.1	4.2	—	2.0
<i>gsp-3</i>	187.9	0.02	186.4	0.04	99.2	0.3	0.40	0.2	1.2	0.01	0.6
<i>gsp-4</i>	195.8	0.09	186.5	0.02	95.3	0.3	0.3	0.2	9.0	0.004	4.6
<i>gsp-3/4</i>	5.7	$5e^{-28}$	0.0	$2e^{-26}$	0.0	0.0	0.10	0.0	5.7	0.45	100

Hermaphrodite progeny production assay: The numbers of viable progeny, dead embryos, and unfertilized oocytes were quantified for N2 (wild type), *gsp-3* [*gsp-3*(*tm1647*)], *gsp-4* [*gsp-4*(*y418*)], and *gsp-3/4* [*gsp-3*(*tm1647*) *gsp-4*(*y418*)] hermaphrodites.

Figure 3 Displacement of hermaphrodite-derived sperm lacking GSP-3/4 by wild-type male-derived sperm is slowed. (A) Progeny production of wild-type and *gsp-3/4* hermaphrodites after mating with *him-8(e1489)* males. The number of viable progeny was averaged from eight *gsp-3/4* hermaphrodites relative to eight wild-type hermaphrodites for 24 hr of mating to *him-8(e1489)* males (standard deviations are shown). (B) Schematic. After mating, male sperm expressing histone-GFP (green) can be tracked in histone-mCherry-expressing (red) hermaphrodites. Sperm have low levels of cytoplasmic autofluorescence. Oocytes are outlined in orange, spermatheca are outlined in a yellow dashed line, and cells in the uterus are in blue. (C) DIC and fluorescence images of hermaphrodite histone-mCherry-labeled (red, marked with red arrows) wild-type sperm and male histone-GFP-labeled (green, marked with green arrows) wild-type sperm that come together at the spermatheca (yellow dotted line) and uterus. (D and E) DIC and fluorescence images of hermaphrodite histone-mCherry-labeled (red, marked with red arrows) *gsp-3/4* mutant sperm and male histone-GFP-labeled (green, marked with green arrows) wild-type sperm (D) before and (E) during ovulation within the same hermaphrodite. The orange bracket marks the position in the gonad where the oocyte was located before ovulation (D) in relation to after passing into the spermatheca (E). The spermatheca is outlined by the yellow dotted line. Bar, 20 μ m.

To test GSP-3/4 function in hermaphrodite sperm motility, we tracked the displacement of mCherry-histone-labeled hermaphrodite sperm by GFP-histone-labeled male sperm (Figure 3B). After 24 hr, wild-type hermaphrodite sperm intermingled with wild-type male sperm within the spermatheca and adjacent uterus (Figure 3C). Ovulation

pushes some sperm into the adjacent uterus. In contrast, *gsp-3/4* hermaphrodite sperm did not intermingle with wild-type male sperm, instead remaining packed within the spermatheca or adjacent ovary (Figure 3D). Even after ovulation, *gsp-3/4* hermaphrodite sperm remained at the distal end of the spermatheca and were not pushed into

Figure 4 GSP-3/4 are required for pseudopod formation and dynamics. (A) Still frames from time-lapse analysis of pseudopodial treadmilling in *swm-1* (*me66*) *him-5*(*e1490*) (File S4) and *gsp-3*(*tm1647*) *gsp-4*(*y418*); *swm-1*(*me66*) *him-5*(*e1490*) male sperm (File S5). Positions of selected membrane vesicles (outlined in white) were tracked over 7 sec. (B) Average pseudopod length and treadmilling speed of *swm-1*(*me66*) *him-5*(*e1490*), *gsp-3* (*tm1647*) *gsp-4*(*y418*); *swm-1*(*me66*) *him-5*(*e1490*), and *fer-1*(*hc11*); *swm-1*(*me66*) *him-5*(*e1490*) male sperm. (C) Distribution of recorded retrograde movement speeds for *swm-1*(*me66*) *him-5*(*e1490*) (black bars), *gsp-3*(*tm1647*) *gsp-4*(*y418*); *swm-1*(*me66*) *him-5*(*e1490*) (white bars), and *fer-1*(*hc11*); *swm-1*(*me66*) *him-5*(*e1490*) (gray bars) male sperm. (D) Quantitation of sperm morphologies after 10 min of *in vitro* activation with Pronase shows that *gsp-3*(*tm1647*) *gsp-4*(*y418*) do not activate properly. (E) The progression of morphological changes of a *him-8*(*e1489*) male sperm during *in vitro* activation with Pronase. Time elapsed is indicated. (F) Abnormal morphologies of *gsp-3*(*tm1647*) *gsp-4*(*y418*); *him-8*(*e1489*) sperm after *in vitro* activation with Pronase for time periods >30 min. Percentages are derived from quantitation of 40 sperm cells. From left to right: no pseudopod, short single pseudopod-like protrusion, short multiple pseudopod-like protrusion, short membrane protrusions all over the cell, and thick projections.

the adjacent ovary (Figure 3E). However, over time mutant *gsp-3/4* hermaphrodite sperm can eventually become displaced. After 48 hr, animals that had successfully mated accumulated large numbers of male sperm in and around their spermathecae (data not shown). In these animals both wild-type and *gsp-3/4* hermaphrodite sperm were largely absent, indicating both wild-type and *gsp-3/4* mutant hermaphrodite sperm are eventually displaced by passing oocytes or sperm from males (Lamunyon and Ward 1998). Overall, these results demonstrate that GSP-3/4 are re-

quired for the ability of both male- and hermaphrodite-derived sperm to move.

Pseudopod treadmilling and formation are dependent on GSP-3/4

We questioned whether GSP-3/4 function in regulating pseudopod dynamics. Amoeboid spermatozoa move by membrane treadmilling from the pseudopod tip toward the cell body (Roberts and Ward 1982). To analyze membrane treadmilling, we employed strains mutated for *swm-1*,

Figure 5 MSP localization dynamics are dependent on GSP-3/4. (A) GSP-3/4 (green) colocalizes with MSP (red) in the cytoplasm and oblong stripes in the mutant sperm from *fem-3(q20)* hermaphrodites raised at 25°, which are inactive. DNA (blue) is present in cell bodies. (B) GSP-3/4 (green) and MSP (red) concentrate in pseudopodial projections in *swm-1(me66) him-5(e1490)* activated sperm. DNA (blue) is found in cell bodies. (C and D) The distribution of GSP-3/4 (green) and MSP (red) localization is plotted along the length (white line) of the *swm-1(me66) him-5(e1490)* mutant sperm cell in C. (E) MSP is mislocalized in *gsp-3(tm1647) gsp-4(y418); swm-1(me66) him-5(e1490)* mutant sperm, remaining in oblong stripes. (F) MSP localizes to pseudopods in *swm-1(me66) him-5(e1490)* activated spermatozoa. (G) Although MSP polarizes in pseudopods of spermatozoa in *fer-1(hc11); swm-1(me66) him-5(e1490)* mutants similar to that observed in *swm-1(me66) him-5(e1490)*, MSP is also found at the cell body membrane. MOs dock at the cell periphery of *fer-1(hc11); swm-1(me66) him-5(e1490)* mutants but do not fuse. GSP-3/4 colocalize with MSP in pseudopods. Bars, 2 μ m.

which normally inhibits male sperm activation until after transfer to hermaphrodites. Thus, prematurely activated spermatozoa are easily observable in *swm-1* mutant males (Stanfield and Villeneuve 2006). Analysis of the treadmill rate, which directly correlates to sperm movement rate (Nelson *et al.* 1982), of *swm-1(me66) him-5(e1490)* spermatozoa is $20.72 \pm 7.08 \mu\text{m}/\text{min}$ (Figure 4, A–C, and File S4),

a rate comparable to that in previous reports (Nelson *et al.* 1982; Stanfield and Villeneuve 2006). In *gsp-3/4; swm-1(me66) him-5(e1490)* males we found fully activated spermatozoa, whose projections moved 5.5-fold slower than *swm-1(me66) him-5(e1490)* spermatozoa (Figure 4, A–C, and File S5). Lack of GSP-3/4 also caused pseudopods to be ~26% shorter than control sperm (Figure 4B). Short

Figure 6 Model for GSP-3/4 function in regulating MSP during sperm formation. GSP-3/4 function in release of MSP at distinct subcellular sites during different stages of postmeiotic sperm development. GSP-3/4 are shown in green. MSP is shown in red. Unfused MOs are shown as gray circles. Fused MOs are gray-outlined semicircles.

pseudopod length and decreased motility are phenotypes also exhibited by mutants defective for *FER-1*, a membrane protein required for MO fusion (Figure 4, B and C) (Ward *et al.* 1981; Roberts and Ward 1982). However, loss of *GSP-3/4* results in more severe motility defects than those caused by loss of *FER-1*. Projections on *fer-1(hc11); swm-1(me66) him-5(e1490)* spermatozoa moved almost 3-fold faster than *gsp-3/4; swm-1(me66) him-5(e1490)* (Figure 4, B and C). The severity of the motility defects of *gsp-3/4; swm-1* mutants compared to *fer-1; swm-1* mutants suggests *GSP-3/4* likely function in distinct aspects of motility generation in comparison to *FER-1*. Thus, *GSP-3/4* are required for modulating pseudopodial treadmilling.

In *swm-1* mutant backgrounds, we observed either non-activated spermatids or fully activated spermatozoa, but not spermatids in intermediate stages of activation. This may be because either *swm-1* sperm activate rapidly or they do not activate after the gonad has been dissected for observation. Therefore, to visualize the process of pseudopod development, we employed live imaging of *in vitro* Pronase-activated sperm in either *him-8(e1489)* or *gsp-3/4; him-8(e1489)* males. Quantitation of the number of sperm that activated over 10 min showed that >60% of *him-8(e1489)* sperm form pseudopods (Figure 4, D and E). In contrast, 10% of *gsp-3/4; him-8(e1489)* male sperm formed pseudopods after 10 min (Figure 4D). Even after 30 min, <40% of *gsp-3/4; him-8(e1489)* male sperm formed pseudopods and instead exhibited abnormal morphologies, including short pseudopods, multiple pseudopods, projections that extended around the cell periphery, and long thick projections (Figure 4F). These results indicate *GSP-3/4* function in timely pseudopod development.

***GSP-3/4* regulates MSP dynamics that modulate pseudopod development and motility**

Pseudopod development and dynamics rely on changes in the organization of the sperm cytoskeletal component MSP (Nelson *et al.* 1982). We thus employed immunofluorescence to analyze where *GSP-3/4* and MSP localize before and after activation. Before activation, MSP is sequestered in sperm-specific organelles called fibrous bodies (FBs) that are closely associated with MOs. To analyze inactive sperm, we

used *fem-3(q20gf)* hermaphrodites, which do not produce oocytes (Barton *et al.* 1987); thus, spermatids remain inactive because they are not pushed into the spermatheca by passing oocytes (L'Hernault 2006). In *fem-3(q20gf)* spermatids, a portion of MSP is found in oblong stripes, which represents assembled MSP in FBs (Figure 5A, Figure S5) (Ward and Klass 1982; Varkey *et al.* 1993; L'Hernault 2009). A distinct portion is diffuse in the cytoplasm, perhaps representing free MSP dimers (Figure 5A, Figure S5). Thus, before activation *GSP-3/4* colocalize with MSP at FBs and in the cytoplasm. Upon activation, MSP releases from FBs and is assembled into nonpolarized fibers, first forming thin projections that coalesce into a polarized pseudopod (Wolf *et al.* 1978). MSP assembly at the pseudopod tip and disassembly at the base induce treadmilling forces (Stewart and Roberts 2005). *GSP-3/4* concentrate with MSP in the pseudopods of fully activated *swm-1(me66) him-5(e1490)* sperm (Figure 5B). However, when fluorescence intensity is plotted along the pseudopod length, MSP levels are highest at the edge of the pseudopod while *GSP-3/4* fluorescence is concentrated at the pseudopod base (Figure 5, C and D). These results show *GSP-3/4* shift localization patterns in concert with MSP for sperm activation and motility. Further, high levels of *GSP-3/4* at the cell–pseudopod interface and low levels at the pseudopod tip suggest a role for *GSP-3/4* in regulating MSP disassembly.

Indeed, immunolocalization of MSP in *gsp-3/4* mutant male germlines revealed *GSP-3/4* regulate MSP localization. Although a portion of MSP localizes in abnormal pseudopod-like extensions, MSP is not fully depleted from the cell body and remains in multiple oblong stripes well within the cell interior, suggesting MSP remains assembled within FBs (Figure 5E). The normal localization of FB-associated MOs evenly spaced and close to the plasma membrane, as seen in *swm-1(me66) him-5(e1490)* male sperm (Figure 5F), is also prevented. This localization of MSP and MOs within the cell interior is also distinct from that observed in *swm-1(me66) him-5(e1490)* male sperm that also lack *FER-1*, which show a less severe motility defect than *gsp-3/4; swm-1(me66) him-5(e1490)* mutant sperm (Figure 4, B and C). In these *fer-1(hc1); swm-1(me66) him-5(e1490)* male sperm, MSP is disassembled from FBs and MOs dock to the plasma membrane

but do not fuse (Figure 5G) (Ward *et al.* 1981; Roberts and Ward 1982; Washington and Ward 2006). This supports that GSP-3/4 function in MSP dynamics likely contributes to the more severe motility defects observed in *gsp-3/4*; *swm-1* (*me66*) *him-5(e1490)* sperm. MSP is similarly mislocalized in both *gsp-3/4* mutant hermaphrodite and male sperm (Figure S6). *gsp-3/4* mutant hermaphrodite sperm also exhibit thin projections that contain MSP that were not seen on wild-type hermaphrodite sperm (Figure S3B, Figure S6). It is possible that such abnormalities cause *gsp-3/4* hermaphrodite sperm to be more adherent and thus resist displacement by passing oocytes and wild-type sperm (Figure 3). These results support that GSP-3/4 are required for MSP dynamics that form normal pseudopods.

The spatial regulation of MSP disassembly may be a key aspect of GSP-3/4 function for motility. On the basis of our data, we propose a model in which GSP-3/4 facilitate MSP release from complexes during sperm development (Figure 6). In motility, GSP-3/4 localize at the pseudopod base to disassemble MSP fibers. Depletion of GSP-3/4 protein severely slows release at the pseudopodial base, causing a reduced rate of treadmilling. MSP release from FBs facilitated by GSP-3/4 is also important for pseudopod formation. For example, slowed release of MSP from FBs may cause defects in pseudopod formation. Malformed projections of *in vitro* activated sperm may also arise from slowed release from primary projections. In this model inefficient release of MSP at each stage can account for the multiple defects we observe in *gsp-3/4* mutants.

Sperm-specific PP1 phosphatase function in male fertility is not evolutionarily conserved

Given that GSP-3/4 regulate aspects of motility specific to nematode sperm, how have sperm-specific PP1 phosphatases come to play a central role in sperm motility in nematodes and mouse? One hypothesis is that they are orthologs of a common ancestor that adapted male-biased expression. To test this possibility we conducted Bayesian phylogenetic analysis of the core PP1 phosphatase domain in 16 species across a wide taxonomic distribution (Table S2). Surprisingly, this analysis shows PP1gamma2 is in the same weakly supported clade as mouse PP1alpha, which exhibits an unbiased expression pattern, and is significantly excluded from the clade containing *C. elegans* GSP-3/4 (Figure 7). Thus, GSP-3/4 and PP1gamma2 have likely evolved independently.

Further supporting their independent evolution, testis-specific expression of PP1s is achieved through distinct mechanisms in nematodes, flies, and mammals. *C. elegans* GSP-3/4 are encoded by autosomal genes that are transcriptionally upregulated during spermatogenesis (Reinke *et al.* 2000) and *Drosophila* PP1-Y1 and Y2 are located on the Y chromosome, which is not present in females (Carvalho *et al.* 2001). Although gene duplication is a commonly used strategy within species for diversifying genes important for fertility (Wyckoff *et al.* 2000; White-Cooper and Bauser 2010), it is intriguing that mouse PP1gamma2 results from

alternative splicing of the last exon of the *PPP1ccc* gene (Okano *et al.* 1997). Other PP1 proteins function in other male reproductive cell types, indicating PP1 phosphatases can also be adapted for other processes critical for male fertility as well (Armstrong *et al.* 1995). Therefore, animals from a broad taxonomic range have used distinct mechanisms to separately evolve PP1 phosphatases as critical regulators of male reproduction.

Discussion

This work has revealed multiple roles for PP1 phosphatases in sequential processes during spermatogenesis. Sperm-specific PP1 phosphatases GSP-3/4 are required for chromosome segregation during sperm meiosis. After meiosis, they are also necessary for the ability of sperm to fertilize because they mediate efficient pseudopod formation and the subsequent treadmilling of pseudopods required for motility. Further, our studies identify a novel role for PP1 phosphatases in regulating the dynamics of the sperm cytoskeletal component MSP in amoeboid sperm development and motility.

PP1 homologs have not yet been shown to function in MSP dynamics in other nematodes, although dephosphorylation is key to disassembly. In *Ascaris*, the PP2A phosphatase depolymerizes MSP *in vitro* and localizes at the pseudopod base (Yi *et al.* 2009), although the role of PP2A in *C. elegans* MSP dynamics is unknown. In terms of filament assembly in *Ascaris*, the kinase MPAK localizes at the pseudopod leading edge and regulates MSP filament disassembly *in vitro* via the MSP polymerization-organizing protein (MPOP) and MSP fiber protein 2 (MFP2) (Yi *et al.* 2007). However, homologs to MPAK, MPOP, or MFP2 have not been identified in *C. elegans*. Instead the casein kinase I homolog SPE-6 functions in MSP assembly (Varkey *et al.* 1993). Loss-of-function *spe-6* mutants show MSP distributing in the cytoplasm instead of assembling into FBs. Other *spe-6* alleles suppress defects in spermatogenesis-defective mutants that cannot undergo spermiogenesis, revealing a role for SPE-6 in sperm morphogenesis *in vivo* (Varkey *et al.* 1993; Muhlrads and Ward 2002). Although its localization is unknown, SPE-6 is thus a candidate to counteract GSP-3/4 in the MSP assembly/disassembly process in *C. elegans*. Additional studies are required to determine the extent to which *C. elegans* and *Ascaris* use different components in signaling pathways that regulate MSP assembly.

Overall, it is evident that PP1 phosphatases are key regulators of sperm development and function. In light of the independent evolution of sperm-specific PP1s in nematodes and mouse, there are striking parallels between sperm-specific PP1 function in *C. elegans* and that in mice. For example, GSP-3/4 and PP1gamma2 function in chromosome segregation during sperm meiosis (Varmuza *et al.* 1999; Oppedisano-Wells and Varmuza 2003), although additional experiments are necessary to determine the extent to which PP1 phosphatases function in similar aspects of sperm meiotic chromosome segregation in each species. Postmeiosis, GSP-3/4 and PP1gamma2 both localize at

Figure 7 Sperm-specific PP1 phosphatases in mammals and nematodes have evolved independently. Bayesian phylogeny estimate is shown from core phosphatase domains of PP1 proteins in the species indicated (Table S2). Red shading indicates known testis-biased expression. Red boxes are functionally validated sperm-specific expression. Green shading indicates functional evidence of nonbiased expression. Numbers above branches are Bayesian posterior probabilities.

and regulate the development and dynamics of motility structures (Soler *et al.* 2009). Such processes rely on post-translational regulation because replacement of histones with sperm nuclear basic proteins imposes global transcriptional repression (Sassone-Corsi 2002). Adaptation of phos-

phatases may optimize intracellular processes, such as relaying extracellular signals or activation of motility structures, which are common in all sperm. Gene duplicates of functionally constrained PP1 phosphatases can coadapt with rapidly evolving reproductive systems (White-Cooper and

Bausek 2010). In particular, diversification of PP1 C termini allows interaction with tissue-specific regulatory subunits (Cohen 2002). For example, testis-enriched endophilin B1t binds PP1gamma2, but not PP1gamma2 lacking its C terminus or PP1alpha (Hrabchak *et al.* 2007). The *C. elegans* and *Drosophila melanogaster* PP1 paralogs exhibiting male-biased expression also exhibit substantial variation in the C-terminal domains (Adam *et al.* 2010). Studies to find PP1 interacting proteins in organisms with sperm- or testis-specific PP1 phosphatases have potential to further define pathways required for regulation of sperm of all morphologies.

The adaptation of PP1s for sperm function across different species may be facilitated by the presence of PP1alpha and -beta, which are evolutionarily constrained to function in basic cellular processes like chromosome segregation and glycogen metabolism from yeast to humans (Figure 7) (Cohen 2002). In fact, testis- or sperm-specific forms of PP1s have been revealed here in *C. elegans* and in other functional studies in *D. melanogaster* and *Mus musculus* (Shima *et al.* 1993; Chu *et al.* 2006; Adam *et al.* 2010). While four PP1 phosphatases (including PP1gamma2) are known in mammals, 6 of 10 PP1 homologs in *D. melanogaster* exhibit testis-specific expression (Adam *et al.* 2010). *C. elegans* has 15 putative PP1 homologs, 12 of which (including GSP-3/4) show sperm-enriched expression (Reinke *et al.* 2000). Thus, future analysis of PP1 function across species will likely reveal new fertility factors and mechanisms required for male reproductive success and improve our ability to trace their evolutionary origins.

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Literature Cited

- Adam, C., L. Henn, M. Miskei, M. Erdelyi, P. Friedrich *et al.*, 2010 Conservation of male-specific expression of novel phosphoprotein phosphatases in *Drosophila*. *Dev. Genes Evol.* 220: 123–128.
- Ahringer, J., 2006 *Reverse Genetics WormBook*, ed. The *C. elegans* Research Community. WormBook, doi/10.1895/wormbook.1.47.1, <http://www.wormbook.org>.
- Armstrong, C. G., D. J. Mann, N. Berndt, and P. T. Cohen, 1995 *Drosophila* PPY, a novel male specific protein serine/threonine phosphatase localised in somatic cells of the testis. *J. Cell Sci.* 108(Pt. 11): 3367–3375.
- Barton, M. K., T. B. Schedl, and J. Kimble, 1987 Gain-of-function mutations of *fem-3*, a sex-determination gene in *Caenorhabditis elegans*. *Genetics* 115: 107–119.
- Brenner, S., 1974 The genetics of *Caenorhabditis elegans*. *Genetics* 77: 71–94.
- Burke, D. J., and S. Ward, 1983 Identification of a large multigene family encoding the major sperm protein of *Caenorhabditis elegans*. *J. Mol. Biol.* 171: 1–29.
- Carvalho, A. B., B. A. Dobo, M. D. Vibrationovski, and A. G. Clark, 2001 Identification of five new genes on the Y chromosome of *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA* 98: 13225–13230.
- Chakrabarti, R., D. Kline, J. Lu, J. Orth, S. Pilder *et al.*, 2007 Analysis of Ppp1cc-null mice suggests a role for PP1gamma2 in sperm morphogenesis. *Biol. Reprod.* 76: 992–1001.
- Chu, D., H. Liu, P. Nix, T. Wu, E. Ralston *et al.*, 2006 Sperm chromatin proteomics identifies evolutionarily conserved fertility factors. *Nature* 443: 101–105.
- Cohen, P. T. W., 2002 Protein phosphatase 1–targeted in many directions. *J. Cell Sci.* 115: 241–256.
- De Carvalho, C., S. Zaaijer, S. Smolnikov, Y. Gu, J. Schumacher *et al.*, 2008 LAB-1 antagonizes the Aurora B kinase in *C. elegans*. *Genes Dev.* 22: 2869–2885.
- Gent, J. I., M. Schvarzstein, A. M. Villeneuve, S. G. Gu, V. Jantsch *et al.*, 2009 A *Caenorhabditis elegans* RNA-directed RNA polymerase in sperm development and endogenous RNAi. *Genetics* 183: 1297–1314.
- Giresi, P. G., J. Kim, R. M. Mcdaniell, V. R. Iyer, and J. D. Lieb, 2007 FAIRE (Formaldehyde-Assisted Isolation of Regulatory Elements) isolates active regulatory elements from human chromatin. *Genome Res.* 17: 877–885.
- Golden, A., P. L. Sadler, M. R. Wallenfang, J. M. Schumacher, D. R. Hamill *et al.*, 2000 Metaphase to anaphase (*mat*) transition-defective mutants in *Caenorhabditis elegans*. *J. Cell Biol.* 151: 1469–1482.
- Gumienny, T. L., E. Lambie, E. Hartwig, H. R. Horvitz, and M. O. Hengartner, 1999 Genetic control of programmed cell death in the *Caenorhabditis elegans* hermaphrodite germline. *Development* 126: 1011–1022.
- Hodgkin, J., 1986 Sex determination in the nematode *C. elegans*: analysis of *tra-3* suppressors and characterization of *fem* genes. *Genetics* 114: 15–52.
- Hodgkin, J., H. R. Horvitz, and S. Brenner, 1979 Nondisjunction mutants of the nematode *Caenorhabditis elegans*. *Genetics* 91: 67–94.
- Howe, M., K. L. McDonald, D. G. Albertson, and B. J. Meyer, 2001 HIM-10 is required for kinetochore structure and function on *Caenorhabditis elegans* holocentric chromosomes. *J. Cell Biol.* 153: 1227–1238.
- Hrabchak, C., H. Henderson, and S. Varmuza, 2007 A testis specific isoform of endophilin B1, endophilin B1t, interacts specifically with protein phosphatase-1cγ2 in mouse testis and is abnormally expressed in PP1cγ null mice. *Biochemistry* 46: 4635–4644.
- Huang, Z., and S. Vijayaraghavan, 2004 Increased phosphorylation of a distinct subcellular pool of protein phosphatase, PP1gamma2, during epididymal sperm maturation. *Biol. Reprod.* 70: 439–447.
- Huelsenbeck, J. P., and F. Ronquist, 2001 MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* 17: 754–755.
- Jaramillo-Lambert, A., Y. Harigaya, J. Vitt, A. Villeneuve, and J. Engebrecht, 2010 Meiotic errors activate checkpoints that improve gamete quality without triggering apoptosis in male germ cells. *Curr. Biol.* 20: 2078–2089.

- Kelleher, J. F., M. A. Mandell, G. Moulder, K. L. Hill, S. W. L'Hernault *et al.*, 2000 Myosin VI is required for asymmetric segregation of cellular components during *C. elegans* spermatogenesis. *Curr. Biol.* 10: 1489–1496.
- Kosinski, M., K. McDonald, J. Schwartz, I. Yamamoto, and D. Greenstein, 2005 *C. elegans* sperm bud vesicles to deliver a meiotic maturation signal to distant oocytes. *Development* 132: 3357–3369.
- L'Hernault, S. W., 2006 Spermatogenesis, *WormBook*, ed. The *C. elegans* Research Community. *WormBook*, doi/10.1895/wormbook.1.85.1, <http://www.wormbook.org>.
- L'Hernault, S. W., 2009 The genetics and cell biology of spermatogenesis in the nematode *C. elegans*. *Mol. Cell. Endocrinol.* 306: 59–65.
- L'Hernault, S. W., and T. M. Roberts, 1995 Cell biology of nematode sperm. *Methods Cell Biol.* 48: 273–301.
- L'Hernault, S. W., D. C. Shakes, and S. Ward, 1988 Developmental genetics of chromosome I spermatogenesis-defective mutants in the nematode *Caenorhabditis elegans*. *Genetics* 120: 435–452.
- Lamunyon, C. W., and S. Ward, 1998 Larger sperm outcompete smaller sperm in the nematode *Caenorhabditis elegans*. *Proc. R. Soc. Lond. B Biol. Sci.* 265: 1997–2002.
- McCarter, J., B. Bartlett, T. Dang, and T. Schedl, 1999 On the control of oocyte meiotic maturation and ovulation in *Caenorhabditis elegans*. *Dev. Biol.* 205: 111–128.
- McGovern, M., L. Yu, M. Kosinski, D. Greenstein, and C. Savage-Dunn, 2007 A role for sperm in regulation of egg-laying in the nematode *C. elegans*. *BMC Dev. Biol.* 7: 41.
- Miller, D., and G. C. Ostermeier, 2006 Towards a better understanding of RNA carriage by ejaculate spermatozoa. *Hum. Reprod. Update* 12: 757–767.
- Miller, M. A., 2006 Sperm and oocyte isolation methods for biochemical and proteomic analysis. *Methods Mol. Biol.* 351: 193–201.
- Miller, M. A., V. Q. Nguyen, M. H. Lee, M. Kosinski, T. Schedl *et al.*, 2001 A sperm cytoskeletal protein that signals oocyte meiotic maturation and ovulation. *Science* 291: 2144–2147.
- Mitani, S., 2009 Nematode, an experimental animal in the national BioResource project. *Exp. Anim.* 58: 351–356.
- Muhlrad, P. J., and S. Ward, 2002 Spermiogenesis initiation in *Caenorhabditis elegans* involves a casein kinase 1 encoded by the *spe-6* gene. *Genetics* 161: 143–155.
- Nabeshima, K., A. M. Villeneuve, and M. P. Colaiacovo, 2005 Crossing over is coupled to late meiotic prophase bivalent differentiation through asymmetric disassembly of the SC. *J. Cell Biol.* 168: 683–689.
- Nelson, G. A., and S. Ward, 1980 Vesicle fusion, pseudopod extension and amoeboid motility are induced in nematode spermatids by the ionophore monensin. *Cell* 19: 457–464.
- Nelson, G. A., T. M. Roberts, and S. Ward, 1982 *Caenorhabditis elegans* spermatozoan locomotion: amoeboid movement with almost no actin. *J. Cell Biol.* 92: 121–131.
- Okamoto, H., and J. N. Thomson, 1985 Monoclonal antibodies which distinguish certain classes of neuronal and supporting cells in the nervous tissue of the nematode *Caenorhabditis elegans*. *J. Neurosci.* 5: 643–653.
- Okano, K., H. Heng, S. Trevisanato, M. Tyers, and S. Varmuza, 1997 Genomic organization and functional analysis of the murine protein phosphatase 1c gamma (*Ppp1cc*) gene. *Genomics* 45: 211–215.
- Oppedisano-Wells, L., and S. Varmuza, 2003 Protein phosphatase 1c gamma is required in germ cells in murine testis. *Mol. Reprod. Dev.* 65: 157–166.
- Oppedisano, L., G. Haines, C. Hrabchak, G. Fimia, R. Elliott *et al.*, 2002 The rate of aneuploidy is altered in spermatids from infertile mice. *Hum. Reprod.* 17: 710–717.
- Reinke, V., H. E. Smith, J. Nance, J. Wang, C. Van Doren *et al.*, 2000 A global profile of germline gene expression in *C. elegans*. *Mol. Cell* 6: 605–616.
- Roberts, T. M., and S. Ward, 1982 Centripetal flow of pseudopodial surface components could propel the amoeboid movement of *Caenorhabditis elegans* spermatozoa. *J. Cell Biol.* 92: 132–138.
- Rogers, E., J. D. Bishop, J. A. Waddle, J. M. Schumacher, and R. Lin, 2002 The aurora kinase AIR-2 functions in the release of chromosome cohesion in *Caenorhabditis elegans* meiosis. *J. Cell Biol.* 157: 219–229.
- Sadler, P. L., and D. C. Shakes, 2000 Anucleate *Caenorhabditis elegans* sperm can crawl, fertilize oocytes and direct anterior-posterior polarization of the 1-cell embryo. *Development* 127: 355–366.
- Sassone-Corsi, P., 2002 Unique chromatin remodeling and transcriptional regulation in spermatogenesis. *Science* 296: 2176–2178.
- Schedl, T., and J. Kimble, 1988 *fog-2*, a germ-line-specific sex determination gene required for hermaphrodite spermatogenesis in *Caenorhabditis elegans*. *Genetics* 119: 43–61.
- Schumacher, J. M., A. Golden, and P. J. Donovan, 1998 AIR-2: an Aurora/Ipl1-related protein kinase associated with chromosomes and midbody microtubules is required for polar body extrusion and cytokinesis in *Caenorhabditis elegans* embryos. *J. Cell Biol.* 143: 1635–1646.
- Sepsenwol, S., H. Ris, and T. M. Roberts, 1989 A unique cytoskeleton associated with crawling in the amoeboid sperm of the nematode, *Ascaris suum*. *J. Cell Biol.* 108: 55–66.
- Shaham, S., 2006 Methods in cell biology, *WormBook*, ed. The *C. elegans* Research Community. *WormBook*, doi/10.1895/wormbook.1.49.1, <http://www.wormbook.org>.
- Shakes, D. C., J. C. Wu, P. L. Sadler, K. LaPrade, L. L. Moore *et al.*, 2009 Spermatogenesis-specific features of the meiotic program in *Caenorhabditis elegans*. *PLoS Genet.* 5: e1000611.
- Shima, H., T. Haneji, Y. Hatano, I. Kasugai, T. Sugimura *et al.*, 1993 Protein phosphatase 1 gamma 2 is associated with nuclei of meiotic cells in rat testis. *Biochem. Biophys. Res. Commun.* 194: 930–937.
- Soler, D. C., S. Kadunganattil, S. Ramdas, K. Myers, J. Roca *et al.*, 2009 Expression of transgenic PPP1CC2 in the testis of *Ppp1cc*-null mice rescues spermatid viability and spermiation but does not restore normal sperm tail ultrastructure, sperm motility, or fertility. *Biol. Reprod.* 81: 343–352.
- Stanfield, G. M., and A. M. Villeneuve, 2006 Regulation of sperm activation by SWM-1 is required for reproductive success of *C. elegans* males. *Curr. Biol.* 16: 252–263.
- Stewart, M., and T. M. Roberts, 2005 Cytoskeleton dynamics powers nematode sperm motility. *Adv. Protein Chem.* 71: 383–399.
- Tanaka, H., and T. Baba, 2005 Gene expression in spermiogenesis. *Cell. Mol. Life Sci.* 62: 344–354.
- Varkey, J. P., P. L. Jansma, A. N. Minniti, and S. Ward, 1993 The *Caenorhabditis elegans spe-6* gene is required for major sperm protein assembly and shows second site non-complementation with an unlinked deficiency. *Genetics* 133: 79–86.
- Varmuza, S., A. Jurisicova, K. Okano, J. Hudson, K. Boekelheide *et al.*, 1999 Spermiogenesis is impaired in mice bearing a targeted mutation in the protein phosphatase 1c gamma gene. *Dev. Biol.* 205: 98–110.
- Ward, S., and M. Klass, 1982 The location of the major protein in *Caenorhabditis elegans* sperm and spermatocytes. *Dev. Biol.* 92: 203–208.
- Ward, S., Y. Argon, and G. A. Nelson, 1981 Sperm morphogenesis in wild-type and fertilization-defective mutants of *Caenorhabditis elegans*. *J. Cell Biol.* 91: 26–44.

- Ward, S., E. Hogan, and G. A. Nelson, 1983 The initiation of spermiogenesis in the nematode *Caenorhabditis elegans*. *Dev. Biol.* 98: 70–79.
- Ward, S., T. M. Roberts, S. Strome, F. M. Pavalko, and E. Hogan, 1986 Monoclonal antibodies that recognize a polypeptide antigenic determinant shared by multiple *Caenorhabditis elegans* sperm-specific proteins. *J. Cell Biol.* 102: 1778–1786.
- Washington, N. L., and S. Ward, 2006 FER-1 regulates Ca²⁺-mediated membrane fusion during *C. elegans* spermatogenesis. *J. Cell Sci.* 119: 2552–2562.
- White-Cooper, H., and N. Bausek, 2010 Evolution and spermatogenesis. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 365: 1465–1480.
- Wolf, N., D. Hirsh, and J. R. Mcintosh, 1978 Spermatogenesis in males of the free-living nematode, *Caenorhabditis elegans*. *J. Ultrastruct. Res.* 63: 155–169.
- Wyckoff, G. J., W. Wang, and C. I. Wu, 2000 Rapid evolution of male reproductive genes in the descent of man. *Nature* 403: 304–309.
- Yi, K., S. M. Buttery, M. Stewart, and T. M. Roberts, 2007 A Ser/Thr kinase required for membrane-associated assembly of the major sperm protein motility apparatus in the amoeboid sperm of *Ascaris*. *Mol. Biol. Cell* 18: 1816–1825.
- Yi, K., X. Wang, M. R. Emmett, A. G. Marshall, M. Stewart *et al.*, 2009 Dephosphorylation of major sperm protein (MSP) fiber protein 3 by protein phosphatase 2A during cell body retraction in the MSP-based amoeboid motility of *Ascaris* sperm. *Mol. Biol. Cell* 20: 3200–3208.

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GENETICS

Supporting Information

<http://www.genetics.org/content/suppl/2011/10/31/genetics.111.135376.DC1>

Sperm Development and Motility are Regulated by PP1 Phosphatases in *Caenorhabditis elegans*

Jui-ching Wu, Aiza C. Go, Mark Samson, Thais Cintra, Susan Mirsoian, Tammy F. Wu, Margaret M. Jow,
Eric J. Routman, and Diana S. Chu

File S1

Supplemental Methods

Fertility Assessment

fog-2 Female Fertility Rescue Assay: A cross-fertility assay was carried out using *fog-2(q71)* and *gsp-3(tm1647) gsp-4(y418)*; *fog-2(q71)* mutant hermaphrodites either unmated or mated to *him-8* mutant males. *fog-2(q71)* animals do not produce self-sperm and thus are phenotypically female. Progeny, which can only be produced after a hermaphrodite successfully mates with males, were quantified over a 3-day period (n=5).

Immunohistochemistry

Hermaphrodite and male gonads were dissected and stained using a protocol devised by A. Chan and described in the Methods section (Howe *et al.* 2001; Shaham 2006). The following primary antibodies were used: rabbit-anti-AIR-2 at 1:200; and monoclonal Anti-MAP Kinase, diphosphorylated (M9692 from Sigma) at 1:200. For AIR-2 and MAP-kinase staining, hermaphrodites were picked at the L4 stage, incubated overnight at 19°C, and dissected.

Ovulation Assays

Determination of ovulation rates were conducted as in the following references (McCarter *et al.* 1999; Miller *et al.* 2001). Hermaphrodites were picked at the L4 stage, incubated overnight at 19°C, and observed as young adults.

Files S2-S5

Supporting Movies

Supporting movies are available for download at .

File S2 Movie of live-imaging analysis of *him-8* male spermatocytes expressing mCherry-histone (red) in different stages of meiotic divisions.

File S3 Movie of live-imaging analysis of *gsp-3(tm1647) gsp-4(y418); him-8(e1489)* male spermatocytes expressing mCherry-histone (red) in different stages of meiotic divisions.

File S4 Movie of pseudopodial treadmilling of *swm-1(me66) him-5(e1490)* spermatozoa.

File S5 Movie of pseudopodial treadmilling of *gsp-3(tm1647) gsp-4(y418); swm-1(me66) him-5(e1490)* spermatozoa.

Figure S2 Oocytes in GSP-3/4 null mutants are fertilization-competent. Viable progeny are produced by mating *him-8(e1489)* males to either *fog-2(q71)* or *gsp-3(tm1647) gsp-4(y418); fog-2(q71)* hermaphrodites. Error bars are the standard deviation derived from 5 crosses.

Figure S3 *gsp-3/4* mutant sperm can generate and respond to MSP signals. **(A and B)** MSP vesicles are released from *gsp-3(tm1647) gsp-4(y418)* mutant hermaphrodite sperm. **(A)** The majority of MSP staining (green) is associated with sperm in *him-8(e1489)* hermaphrodite sperm but can be detected in small foci around the -1 and -2 oocytes (white arrows) that likely represent MSP vesicles. DNA is shown in red. **(B)** In *gsp-3(tm1647) gsp-4(y418); him-8(e1489)* hermaphrodites MSP is also present in sperm and small foci (white arrows) are detected around the -1 oocyte. MSP staining can also be occasionally observed in thin tubules or lines (yellow arrow) in *gsp-3(tm1647) gsp-4(y418); him-8(e1489)* hermaphrodites, which may correspond to abnormal projections formed upon activation. **(C and D)** Sperm signaling induces AIR-2 (green) to localize to the short arms of the bivalents in the -1 oocyte in **(C)** *him-8(e1489)* and **(D)** *gsp-3(tm1647) gsp-4(y418); him-8(e1489)* hermaphrodites. DNA is shown in red. Relative levels of AIR-2 are lower in *gsp-3(tm1647) gsp-4(y418); him-8(e1489)* hermaphrodites compared to *him-8(e1489)*. **(E and F)** Activation of mitogen-activated protein kinase (MAPK) is a critical step in oocyte maturation that is dependent on receiving an MSP signal from sperm (Miller, 2001). The active diphosphorylated form of MAPK is recognized by the MAPK-YT antibody (green). DNA is shown in red. **(E)** MAPK activation is detected in the most distal oocytes in *him-8(e1489)* hermaphrodites. **(F)** In *gsp-3(tm1647) gsp-4(y418); him-8(e1489)* hermaphrodites, MAPK activation is detectable in the most distal oocytes but at lower levels compared to *him-8(e1489)*, particularly in the -1 oocyte.

Figure S4 *gsp-3/4* mutant hermaphrodites ovulate but retain oocytes in the uterus. **(A)** Lack of *GSP-3/4* do not affect oocyte ovulation rates. **(A)** *gsp-3(tm1647) gsp-4(y418)* hermaphrodites ovulate at frequencies comparable to wild-type. The time between two ovulation events in young adult wild type (N2) and *gsp-3(tm1647) gsp-4(y418)* hermaphrodites (n=10 each) are comparable. **(B-E)** DIC images of **(B-C)** *him-8(e1489)* and **(D-E)** *gsp-3(tm1647) gsp-4(y418); him-8(e1489)* hermaphrodites. **(B and C)** After 24 and 48 hours, individual embryos, which have developed eggshells, are distinguishable in the *him-8(e1489)* hermaphrodites uterus (white bracketed line), which flanks the vulva (marked with the letter V). The region of the proximal ovary of one gonad arm is indicated with the blue line. In contrast, **(D)** at 24 hours oocytes are visible in the *gsp-3(tm1647) gsp-4(y418); him-8(e1489)* hermaphrodite uterus. **(E)** After 48 hours, the uterus in *gsp-3(tm1647) gsp-4(y418); him-8(e1489)* hermaphrodites is distended through accumulation of oocytes, which are difficult to distinguish as individual entities. Also, we do not observe accumulation of oocytes within the proximal ovary.

Figure S5 MSP dynamics after meiotic divisions in *fem-3(q20ts)* hermaphrodites. **(A)** A dissected, fixed and immunostained gonad from a *fem-3(q20ts)* hermaphrodite grown at 25°C. MSP is shown in green. The MOs, labeled with wheat germ agglutinin, are shown in red. DNA is shown in blue. **(B)** Enlarged images of nuclei boxed by the white dotted lines shown in **(A)**. Region 1 highlights nuclei that have not yet undergone meiotic divisions. MSP (green) is sequestered in oblong stripes corresponding to FBs that are adjacent to MOs (red). Region 2 indicates nuclei that have completed meiotic divisions and budding. Most spermatids still have a large proportion of MSP localized in oblong stripes adjacent to fully formed MOs that are seen within the cell interior. Region 3 shows inactive spermatids that are stored. A much greater proportion of MSP is now cytoplasmic, though FBs have still not been fully disassembled. Most cells now have MOs that are more distributed to the cell periphery.

Figure S6 MSP is mislocalized in *gsp-3/4* mutant hermaphrodite sperm. **(A-B)** Dissected, fixed and immunostained gonads from a **(A)** *him-8(e1489)* or **(B)** *gsp-3(tm1647) gsp-4(y418); him-8(e1489)* hermaphrodite. MSP is shown in green. DNA is shown in red. The location of the -1 and -2 oocytes are indicated and outlined with a white dashed line. The regions where activated sperm are present are demarcated with a purple dashed line. Enlarged images of nuclei indicated with small case letters (a, b, and c) are shown in the right hand panels. The exposure in these panels has been increased to show small projections visible on *gsp-3(tm1647) gsp-4(y418); him-8(e1489)* hermaphrodite sperm.

Table S1 Strains used in this study.

Strain name	genotype	note
N2		Bristol strain
CB1489	<i>him-8(e1489)</i>	(3)
FX1647	<i>gsp-3(tm1647)</i>	this study
TY4192	<i>gsp-4(y418)</i>	this study
CB138	<i>unc-24(e138)</i>	(1)
TY3291	<i>hT2[qIs48 bli-4(e937)]</i>	
JK0816	<i>fem-3(q20ts)</i>	(7)
TY0119	<i>fem-1(hc17ts)</i>	(8)
XC21	<i>gsp-3(tm1647);him-8(e1489)</i>	constructed in this study
XC26	<i>gsp-3(tm1647) gsp-4(y418)/hT2[qIs48 bli-4(e937)]; him-8(e1489)</i>	constructed in this study
XC46	<i>gsp-3(tm1647) gsp-4(y418)/hT2[qIs48 bli-4(e937)]</i>	constructed in this study
TY2012	<i>fog-2(q71)</i>	
XC43	<i>fog-2(q71); gsp-3(tm1647) gsp-4(y418)/hT2[qIs48 bli-4(e937)]</i>	constructed in this study
XC38	<i>him-8(e1489) ItIs37[pAA64; pie-1::mCherry::his-58; unc-119(+)]IV</i>	constructed in this study
XC41	<i>zuIs178[pS0159; his-72 (1kb 5'UTR)::HIS-72::SRPVAT::GFP::HIS-72 (1kb 3'UTR)]; him-8(e1489)</i>	constructed in this study
XC52	<i>gsp-3(tm1647) gsp-4(y417)/hT2[bli-4(e937) qIs48]; him-8(e1489)ItIs37[pAA64; pie-1/mCherry::his-58; unc-119 (+)] IV</i>	constructed in this study
XC67	<i>gsp-3(tm1647) gsp-4(y418)/hT2[qIs48 bli-4]; swm-1(me66)him-5(e1490)</i>	constructed in this study
AV285	<i>swm-1(me87) him-5(e1490)</i>	

Table S2 Gene sequences from a broad range of species used for phylogenetic analysis.

Table S2 is available for download at <http://www.genetics.org/content/suppl/2011/10/31/genetics.111.135376.DC1> as an Excel file.

Table S3 *gsp-3/4* mutant males are infertile *fog-2(q71)* hermaphrodites, which are sterile because they lack hermaphrodite sperm, were crossed with *him-8(e1489)* or *gsp-3(tm1647) gsp-3(y418)*; *him-8(e1489)* males and resulting progeny, dead embryos and unfertilized oocytes laid were quantified.

Genotype hermaphrodite (n=15) x male	total laid	viable progeny		dead embryos		oocytes	
		#	%	#	%	#	%
<i>fog-2</i>	0	0	0	0	0	0	0
<i>fog-2</i> x <i>him-8</i>	64.9	64.3	99.1	0.1	1.0	0.6	0.9
<i>fog-2</i> x <i>gsp-3/4</i>; <i>him-8</i>	0.3	0	0	0	0	0.3	100

fog-2 hermaphrodites, which are sterile because they lack hermaphrodite sperm, were crossed with *him-8(e1489)* or *him-8(e1489); gsp-3/4 [gsp-3(tm1647) gsp-3(y418)]* males and resulting progeny, dead embryos and unfertilized oocytes laid were quantified.

Supplemental Information References

- Howe, M., K. L. McDonald, D. G. Albertson and B. J. Meyer, 2001 HIM-10 is required for kinetochore structure and function on *Caenorhabditis elegans* holocentric chromosomes. *J Cell Biol* **153**: 1227-1238.
- McCarter, J., B. Bartlett, T. Dang and T. Schedl, 1999 On the control of oocyte meiotic maturation and ovulation in *Caenorhabditis elegans*. *Dev Biol* **205**: 111-128.
- Miller, M. A., V. Q. Nguyen, M. H. Lee, M. Kosinski, T. Schedl *et al.*, 2001 A sperm cytoskeletal protein that signals oocyte meiotic maturation and ovulation. *Science* **291**: 2144-2147.
- Shaham, S., 2006 *Methods in Cell Biology*, WormBook: edited by The *C. elegans* Research Community. WormBook, doi/10.1895/wormbook.1.49.1, <http://www.wormbook.org>