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Article

Regulation of Sperm Activation by SWM-1 Is Required for Reproductive Success of *C. elegans* Males

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Summary

Background: Sexual reproduction in animals requires the production of highly specialized motile sperm cells that can navigate to and fertilize ova. During sperm differentiation, nonmotile spermatids are remodeled into motile spermatozoa through a process known as spermiogenesis. In nematodes, spermiogenesis, or sperm activation, involves a rapid cellular morphogenesis that converts unpolarized round spermatids into polarized amoeboid spermatozoa capable of both motility and fertilization.
Results: Here we demonstrate, by genetic analysis and in vivo and in vitro cell-based assays, that the temporal and spatial localization of spermiogenesis are critical determinants of male fertility in *C. elegans*, a male/hermaphrodite species. We identify *swm-1* as a factor important for male but not hermaphrodite fertility. We show that whereas in wild-type males, activation occurs after spermatids are transferred to the hermaphrodite, *swm-1* mutants exhibit ectopic activation of sperm within the male reproductive tract. This ectopic activation leads to infertility by impeding sperm transfer. The SWM-1 protein is composed of a signal sequence and two trypsin inhibitor-like domains and likely functions as a secreted serine protease inhibitor that targets two distinct proteases.
Conclusions: These findings support a model in which (1) proteolysis acts as an important in vivo trigger for sperm activation and (2) regulating the timing of proteolysis-triggered activation is crucial for male reproductive success. Furthermore, our data provide insight into how a common program of gamete differentiation can be modulated to allow males to participate in reproduction in the context of a male/hermaphrodite species where the capacity for hermaphrodite self-fertilization has rendered them nonessential for progeny production.

Introduction

To accomplish sexual reproduction, males and females make haploid gametes that must join to generate diploid offspring with a genetic complement derived from both parents. These gametes are highly specialized for the functions of locating one another, fusing together, and directing the initial development of the zygote. In animals, sexual reproduction typically involves the production of large gametes (ova) by females and small motile gametes (sperm) by males.

While sexual reproduction is very widespread, sexual systems tend to be highly plastic and evolve rapidly. For example, in the nematode genus *Caenorhabditis*, hermaphroditism has arisen independently more than once [1, 2], with anatomical females acquiring the ability to make sperm for use in self-fertilization. When both sexes make sperm, how do males participate in reproduction when they are essentially expendable? One method used by the male/hermaphrodite species *C. elegans* is modulation of sperm cell function. *C. elegans* male sperm are used preferentially [3], and this precedence pattern is due to the competitive superiority of male sperm cells correlating with a larger average cell size [4, 5].

Unlike the sperm of most metazoan species, the sperm of *C. elegans* and other nematodes lack flagella. Instead, they move by using a lamellopodium-like structure, the pseudopod [6]. This structure is generated during spermiogenesis, or activation, when round spermatids undergo rapid cellular morphogenesis to form polarized spermatozoa that are capable of both directed movement and fertilization (reviewed in [7, 8]). The activated sperm cell is organized with a pseudopod containing the motility apparatus at one end and a cell body containing the nucleus and mitochondria at the other end. Some sperm proteins, such as SPE-9 and SPE-38, which are important for fertilization interactions between sperm and oocytes, localize in a polarized fashion to the pseudopod [9, 10]. Notably, during the meiotic divisions, spermatids shed their transcriptional and translational machinery, so they must undergo the cellular morphogenesis of activation and perform their migration and fertilization functions without new gene expression.

C. elegans males and hermaphrodites differ in the timing and spatial context of sperm activation [3]. Hermaphrodites first make "self" sperm and then permanently switch to making oocytes; their spermatids become activated soon after they are produced. In contrast, adult males make sperm continuously and store them as inactive spermatids until they are transferred to a hermaphrodite; upon transfer, they become activated immediately. Further, genetic analysis of a set of genes collectively known as the "*spe-8* group" (*spe-8*, *spe-12*, *spe-27*, *spe-29*, and the recently identified *spe-19*) has revealed that during mating, males transfer a factor that can activate a hermaphrodite's self sperm. The *spe-8* group genes are largely dispensable for male fertility but are essential for hermaphrodite self fertility; mutations in these genes prevent spermatid activation in hermaphrodites, rendering them self-sterile [11–14]. However, mating of a male to a *spe-8* group mutant hermaphrodite induces the "*trans*"-activation of her self sperm (presumably via some seminal fluid factor transferred by the male) and rescues her self fertility. Thus, *C. elegans* possesses three distinct modes of activation

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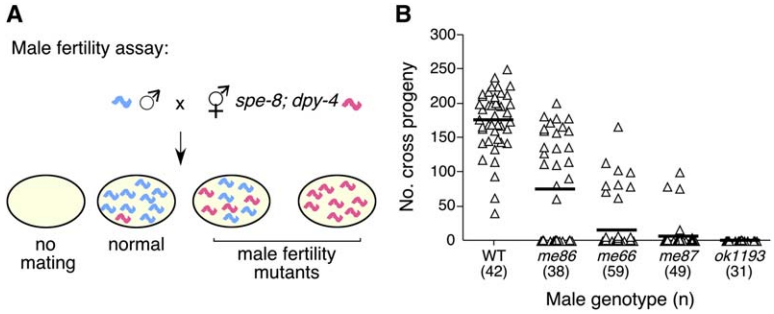


Figure 1. *swm-1* Mutant Males Have Fertility Defects

(A) Scheme of male fertility assay used to isolate the *swm-1(me66)* mutant (see text for description). Blue indicates worms carrying a wild-type allele(s) of the marker *dpy-4*; pink indicates worms homozygous for *dpy-4*. Ovals depict the appearance of plates showing the four possible outcomes of the male fertility assay.

(B) Reduced male fertility in different *swm-1* mutants. The null alleles *me87* and *ok1193* show the strongest reduction in fertility and are not significantly different from one another ($p > 0.05$, Mann-Whitney rank sum test). Triangles indicate the number of cross progeny produced by individual *spe-8; dpy-4* hermaphrodites after mating to individual males of the indicated genotypes. Only successful crosses for which mating could be confirmed are shown. Lines, average number of cross progeny; n, number of crosses.

(hermaphrodite activation of self sperm, male mating-dependent activation of male sperm, and *trans*-activation of hermaphrodite sperm), which represent sex-specific controls on the timing and spatial localization of spermatid activation.

What signals are used to initiate spermatid activation, and how is this process regulated to occur at different times in the different sexes? Spermatids can be activated in vitro by treatment with proteases [15], inhibitors of chloride channel activity [16], or compounds that increase their intracellular pH [15, 17], but the physiological relevance of these in vitro triggers is not known. The in vivo triggers of spermatid activation (by any of the above-described pathways) have not yet been identified.

We set out to investigate the requirements for *C. elegans* male fertility, focusing on the level of sperm cell function and differentiation. Our strategy was designed to identify factors that are important for male fertility but dispensable for hermaphrodite self fertility. Here we report the identification of one such factor, *swm-1*, which encodes a secreted serine protease inhibitor-like protein that functions as a negative regulator of spermatid activation in males. Our data provide the first evidence that multiple proteases act in vivo as triggers of spermatid activation. Further, our analysis demonstrates that the correct timing and spatial localization of spermatid activation are critical determinants of male reproductive success.

Results

Isolation of *swm-1* Mutants with Reduced Male Fertility

We obtained the original *swm-1* mutant, *me66*, in a genetic screen for males with reduced fertility (G.M.S. and A.M.V., unpublished results). The details of the screen will be reported elsewhere; however, here we highlight the key features of the screen that are essential for understanding subsequent assays and analyses. Following EMS mutagenesis of a male-producing *C. elegans* strain (*him-5(e1490)*) [18], a series of lines was generated by plating individual F2 hermaphrodites that were potentially homozygous for new mutations and allowing them to self-fertilize, thereby selecting for hermaphrodite fertility. Each of these lines was then assayed for male fertility by mating males to tester hermaphrodites containing the recessive marker *dpy-4(e1166)*, which allowed us to distinguish cross progeny from self progeny (Figure 1A). Since matings with wild-type males produce broods composed almost exclusively of cross progeny, male fertility defects were readily detected by any increase in the number of self progeny. Importantly, tester hermaphrodites also carried the *spe-8(hc40)* mutation; unmated *spe-8* hermaphrodites cannot activate their sperm and are self-sterile [19], but their spermatids can be activated in *trans* by male seminal fluid, so mating by a male results in the production of fertilized eggs. Thus, the use of *spe-8* tester hermaphrodites allowed us to distinguish crosses where mating occurred but male fertility was impaired from crosses where mating simply failed and no progeny were generated (Figure 1A).

We subsequently obtained two additional alleles, *me86* and *me87*, based on failure to complement *me66* for its visible Swm phenotype (see below and Experimental Procedures), and a deletion, *ok1193*, from the *C. elegans* Knockout Consortium. Characterization of both the phenotypes of these mutants and the molecular lesions associated with the *swm-1* locus in these strains suggests that *me66* and *me86* result in a reduction of *swm-1* function and that *me87* and *ok1193* are null alleles that completely eliminate *swm-1* function.

For all four alleles, we found that the fertility assay used in the screen showed a profound reduction in male fertility (Figure 1B). Among males carrying the same *swm-1* allele, individuals differed with respect to their level of reproductive success: while most *swm-1* males failed to produce any cross progeny, a subset of males were capable of siring offspring. For *swm-1* null alleles, these semifertile males were very infrequent and sired far fewer progeny than did control males.

Sperm Are Activated within the Reproductive Tracts of Virgin *swm-1* Males

To determine the nature of the *swm-1* fertility defect, we used Nomarski microscopy to examine the reproductive tracts of wild-type and *swm-1* males. In wild-type males, spermatid activation is delayed until mating, so virgin males accumulate unactivated round spermatids. We observed large numbers of such unactivated spermatids within the seminal vesicle in wild-type virgin males (Figure 2D). By contrast, age-matched *swm-1* males contained many cells that exhibited the asymmetrical

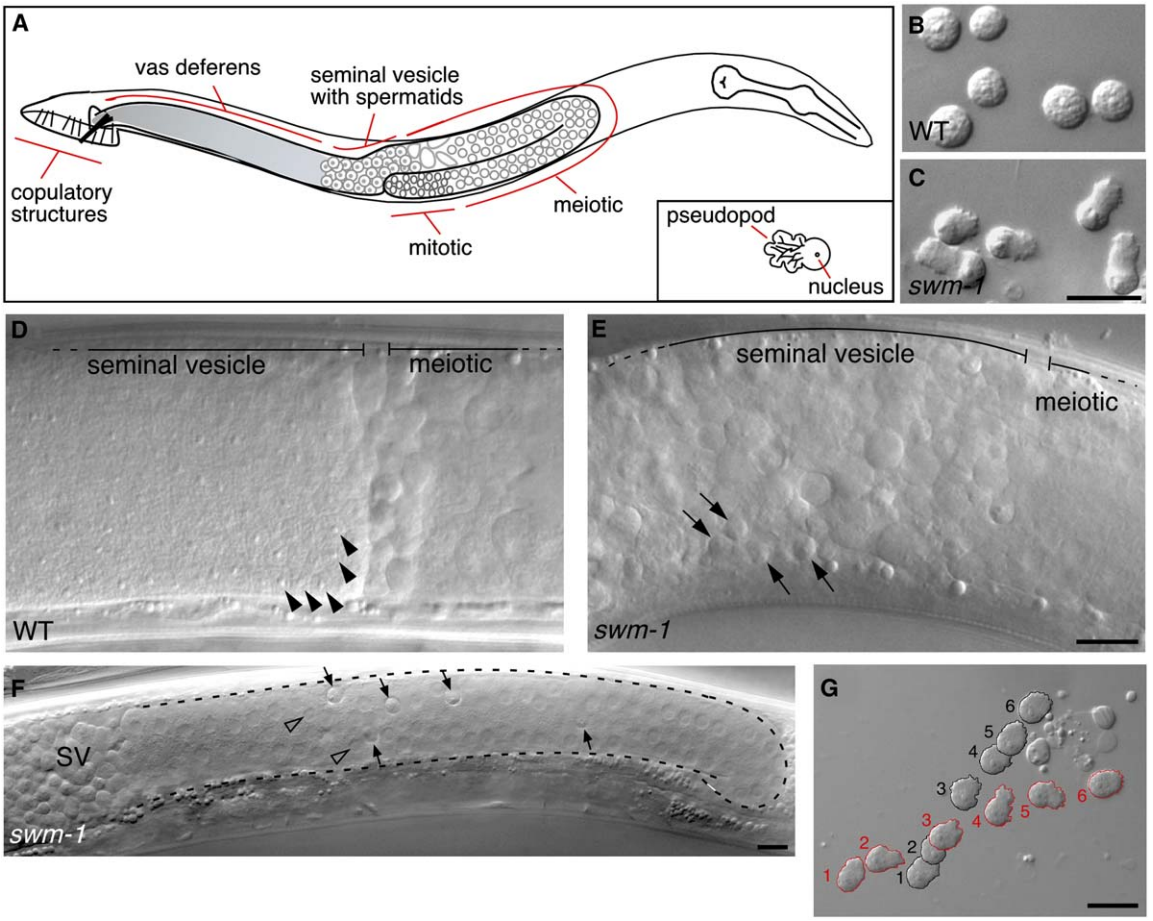


Figure 2. *swm-1* Virgin Males Contain Activated, Motile Sperm

(A) Diagram of wild-type *C. elegans* male showing gonadal and copulatory structures. Inset, diagram of activated sperm showing polarized morphology.

(B and C) Sperm cells dissected from 48 hr post-L4 virgin males.

(B) *swm-1*(+) spermatids from a *dpy-20(e1282); him-5(e1490)* control male appear round and unpolarized. (The *dpy-20* mutation does not affect spermatid activation; data not shown.)

(C) *swm-1*(-) activated sperm from a *dpy-20(e1282); swm-1(me87) him-5(e1490)* male exhibit pseudopodia.

(D and E) DIC images of males showing portions of the seminal vesicle and proximal meiotic region.

(D) Wild-type *him-5(e1490)* male with densely packed round spermatids filling the seminal vesicle. Cell-cell boundaries of packed-together spermatids are difficult to distinguish by DIC microscopy, but sperm chromatin is distinct; arrowheads mark a subset of visible nuclei to indicate the density of sperm present.

(E) *swm-1(me87) him-5(e1490)* male containing activated sperm (arrows) within the seminal vesicle. As compared to unactivated spermatids, activated sperm do not pack together as densely, leading to a nonuniform, irregular appearance of the tissue.

(F) DIC image of a *swm-1(me66) him-5(e1490)* male showing the proximal gonadal arm (dashed line). Activated sperm (arrows) are visible among meiotic nuclei (open arrowheads). SV, seminal vesicle.

(G) Superimposed time-lapse images of two *swm-1(me87) him-5(e1490)* sperm (outlined in red and black, respectively) migrating across a microscope slide. Individual images were chosen to convey the full observed migration paths, which crossed one another; where overlap occurs between the two cells or successive images of the same cell, the later image is placed on top. Time points (min:s): 1 = 0:00, 2 = 0:46, 3 = 2:29, 4 = 4:12, 5 = 6:10, 6 = 10:03. Scale bars equal 10 μm.

polarized morphology typical of activated spermatozoa (Figure 2E). These activated sperm were present not only in the seminal vesicle, but also throughout the distal gonad (Figure 2F), suggesting that they were motile and crawling around inside the male. The name *swm-1* refers to this defect of sperm activated without mating. To examine *swm-1* sperm in more detail, we dissected males to release individual sperm and found that in contrast to the unactivated spermatids present in wild-type control males (Figure 2B), many normal-looking activated sperm were present in *swm-1* (Figure 2C). In addition, *swm-1* activated sperm were capable of migrating in vitro across a glass microscope slide (Figure 2G), further demonstrating their motility.

The onset of spermatid activation varies among males carrying different *swm-1* alleles (Figure 3A). First-day adult (24 hr post-L4 larval stage) *me86* or *me66* virgin males often contained only unactivated spermatids or a mixture of spermatids and activated sperm. These animals resembled nonvirgin wild-type males, which often contain a mixture of spermatids and activated sperm ([3] and data not shown). However, 100% of 24 hr post-L4 *me87* and *ok1193* virgin males contained only activated sperm. By 48 hr post-L4, all males carrying any of the

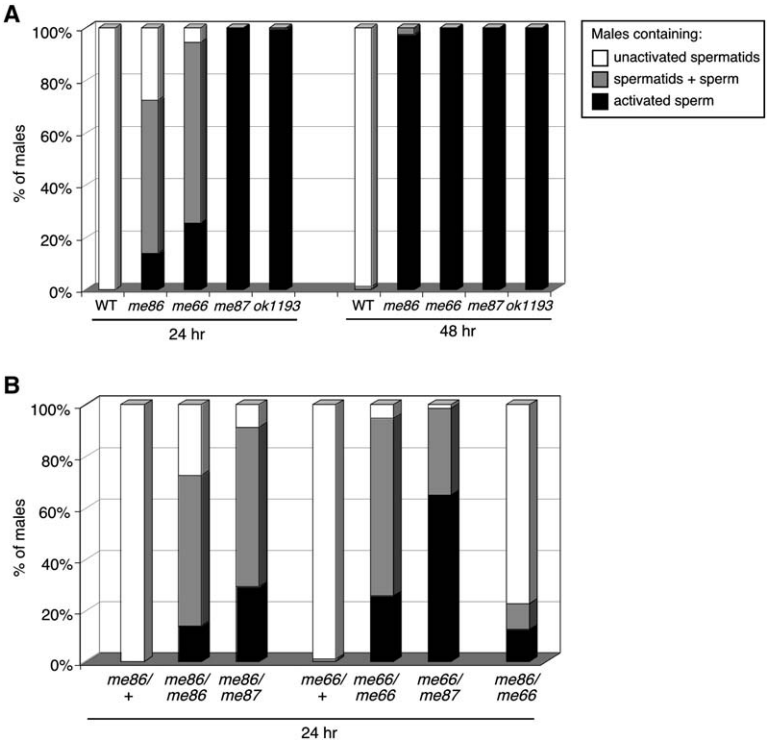


Figure 3. Activated Sperm Phenotype in *swm-1* Mutants

Stacked graphs in which different shading indicates the percentage of males containing either all unactivated spermatids (white), a mixture of spermatids and activated sperm (gray), or all activated sperm (black). At least 50 animals were examined for each genotype/time point.

(A) Control males and males homozygous for the different *swm-1* alleles were examined at 24 and 48 hr post-L4. Sperm are activated by 24 hr post-L4 in *me87* and *ok1193*, but many *me86* and *me66* animals still contain some unactivated spermatids at this time point.

(B) Data demonstrating partial complementation of the *me86* and *me66* alleles. As compared to *me86* or *me66* homozygous males, fewer *me86/me66 trans*-heterozygous males contain activated sperm. Males were examined at 24 hr post-L4.

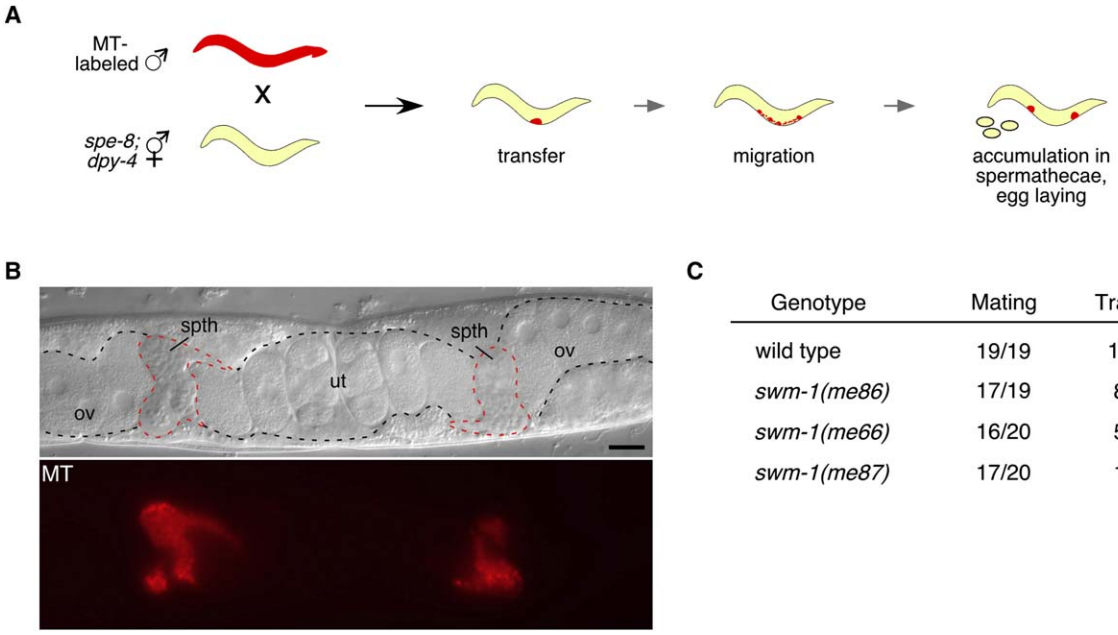
four *swm-1* mutant alleles contained at least some activated sperm, while most wild-type males still only contained unactivated spermatids (Figure 3A). To determine when sperm become activated in *swm-1* null mutants, we examined *swm-1(me87)* and *swm-1(ok1193)* males at the mid-L4 stage when the first sperm are being produced and found that they contained a mixture of activated and unactivated sperm (data not shown). These data indicate that the absence of *swm-1* activity results in rapid ectopic spermatid activation inside males within 3–5 hr of the meiotic divisions that generate haploid spermatids.

Frequent Failure of Sperm Transfer by *swm-1* Mutant Males

Since activated sperm in *swm-1* males appeared to be morphologically normal and motile, the basis of the reduced fertility of *swm-1* males was unclear. We therefore sought to determine whether *swm-1* sperm can be transferred to hermaphrodites, and if so, whether they can migrate appropriately within the hermaphrodite reproductive tract. To fertilize eggs, male sperm must migrate from the vulva, where they are deposited, through the uterus into the spermathecae. To visualize male sperm, we labeled males with the vital dye mitotracker and mated individual mitotracker-labeled males to individual unlabeled *spe-8; dpy-4* hermaphrodites. We then examined the hermaphrodites periodically for (1) the presence of mitotracker-labeled male sperm, (2) movement of mitotracker-labeled sperm to the spermathecae, and (3) laying of fertilized eggs, indicative of mating accompanied by seminal fluid transfer (with or without sperm transfer) (Figures 4A and 4B).

swm-1 mutant males were capable of mating and transferring seminal fluid at wild-type or near-wild-type frequencies. By using males' ability to *trans*-activate *spe-8* hermaphrodite sperm as an assay, we were able to confirm that ~80%–89% of *swm-1* males had succeeded in mating and transfer of seminal fluid under conditions in which wild-type animals showed ~100% mating and transfer (Figure 4C and data not shown). However, *swm-1* mutants showed strong defects in transferring their sperm to hermaphrodites. For the *me87* null mutant, sperm were rarely transferred (Figure 4C), and when they were, the intensity of the fluorescent mitotracker signal was substantially lower than that seen in matings with wild-type males (data not shown), suggesting that many fewer sperm were transferred. *me66* and *me86* males also showed reduced sperm transfer frequency and quantity. Low frequencies of sperm transfer and low numbers of transferred sperm were already suggested by features of the data from initial fertility assays, in that the majority of *swm-1* males were completely infertile but some animals, depending on the *swm-1* allele, were capable of siring a small number of progeny (Figure 1B).

When sperm were transferred by *swm-1* mutant males, their migration behavior and fertilization ability were indistinguishable from those of wild-type sperm. Specifically, after transfer of either wild-type or *swm-1* sperm, we observed the first mitotracker-labeled sperm beginning to reach the spermathecae within 45 to 60 min; all mitotracker labeling became localized to the spermathecae within 2.5 to 3.5 hr. Moreover, for either wild-type or *swm-1* matings, cross-progeny eggs began to be laid within 2.5 to 5 hr after sperm transfer. Fertilized eggs must transit through the uterus before being laid, resulting in a delay of 2 to 3 hr between the time of fertilization and the time of egg laying [20], so these data are consistent with male-derived sperm being used



C

Genotype	Mating	Transfer
wild type	19/19	19/19
swm-1(me86)	17/19	8/17
swm-1(me66)	16/20	5/16
swm-1(me87)	17/20	1/17

Figure 4. A Mitotracker-Based Assay for Sperm Behavior Reveals Defects in Sperm Transfer but Normal Sperm Migration in *swm-1* Mutant Males

(A) Diagram of mitotracker assay (see Results and Experimental Procedures). Mitotracker-labeled cells are indicated in red.

(B) Paired DIC and fluorescence images of a *spe-8; dpy-4* hermaphrodite containing wild-type mitotracker-labeled male sperm successfully targeted to the spermathecae (spth). The images show a ventral view of a hermaphrodite with a large population of mitotracker-labeled sperm within both spermathecae (spth, red dashed lines; scale bar equals 20 μm). Labels: black dashed lines, boundary of gonad; ov, oviduct containing oocytes; ut, uterus containing embryos.

(C) *swm-1* males are capable of copulation but show sperm-transfer defects in the mitotracker assay. Table shows data for mitotracker assays performed in parallel for all genotypes in a single experiment; similar results were obtained in other experiments including other combinations of strains (data not shown).

immediately upon reaching the spermathecae. In summary, *swm-1* sperm migrate to the spermathecae and begin to fertilize eggs as rapidly as do wild-type sperm. These data suggest that if *swm-1* sperm are transferred by a mutant male, they are functional. Thus, the infertility of most *swm-1* males likely arises from their frequent failure to transfer sperm.

As an additional test of function for *swm-1* sperm, we used artificial insemination [21] to introduce sperm from either wild-type or *swm-1* mutant males into recipient hermaphrodites. Given the low frequency of transfer success by *swm-1* males, it was possible that those mutant males that were capable of sperm transfer represented a less defective subset of *swm-1* males. We found that whereas in mating experiments, only 1/17 *swm-1(me87)* males (Figure 4C) transferred sperm and produced cross progeny, 9/9 artificial inseminations by *swm-1(me87)* male sperm resulted in at least one cross progeny and four of these resulted in 15 or more cross progeny (see Figure S1 in the Supplemental Data available with this article online). This frequency of success was comparable to that obtained with wild-type male sperm, for which 7/9 artificial inseminations resulted in at least one cross progeny, and 4/9 resulted in 27 or more cross progeny. Our ability to bypass the *swm-1* transfer defect by experimental transfer of sperm and obtain high fertility rates reinforces our conclusion that the infertility of most *swm-1* males is a consequence of their sperm transfer defect.

SWM-1 Is Similar to Secreted Serine Protease Inhibitors from Parasitic Nematodes

We determined the molecular identity of the *swm-1* gene by a positional strategy, involving a combination of visible marker, SNP, and deficiency mapping [22] to localize the gene to a 200 kb interval followed by transformation rescue assays (see Supplemental Experimental Procedures and Figure S2). A PCR fragment containing the single predicted gene C25E10.9 was sufficient to rescue *swm-1* mutants, whereas a fragment containing the closely related neighboring gene C25E10.8 (Figure 5C) failed to rescue. Sequencing of the *swm-1* alleles revealed point mutations in the C25E10.9 coding region for *me66*, *me86*, and *me87* (Figure 5A and Figure S2C), confirming that C25E10.9 is *swm-1*. Consistent with its being a null allele, *me87* is a G-to-A transition in the splice acceptor for the second exon and should result in frameshift of the SWM-1 protein at amino acid 17 followed by a stop codon after two additional amino acids. The deletion *ok1193* eliminates the entire coding sequence of *swm-1* as well as a portion of C25E10.8 (Figure 5A and Figure S1C). We observed no additional defects in *ok1193* animals as compared to *me87* animals, and a C25E10.9 transgene was fully sufficient to rescue the *ok1193* sperm activation phenotype (Table S1). Thus, we find no evidence of a function for C25E10.8 in sperm activation, in spite of its high identity with *swm-1*.

The predicted SWM-1 protein is composed of a signal sequence and two trypsin inhibitor-like (TIL) domains

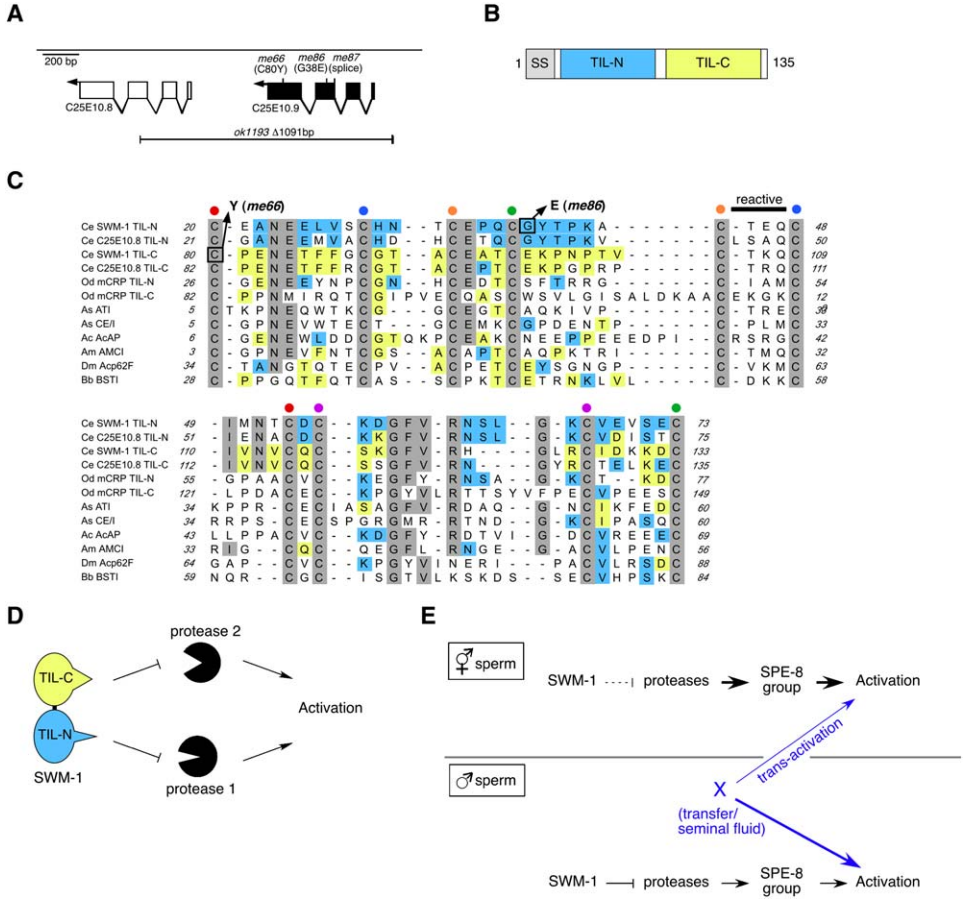


Figure 5. The SWM-1 Protein Is Likely to Function as a Secreted Inhibitor of Serine Proteases

(A) *swm-1* genomic region showing the four *swm-1* alleles mapped onto the splicing diagrams of *swm-1*/C25E10.9 and the neighboring gene C25E10.8, which is closely related (73% identity) to C25E10.9. Connected boxes indicate exons; arrows indicate direction of transcription.

(B) Diagram of the predicted SWM-1 protein indicating a signal sequence (SS) and two trypsin inhibitor-like (TIL) domains.

(C) Alignment of SWM-1 N-terminal and C-terminal TIL domain sequences with TIL domains from *C. elegans*, the parasitic nematodes *Oesophagostomum dentatum*, *Ascaris suum*, and *Ancylostomum caninum*, the insects *Apis mellifera* and *Drosophila melanogaster*, and the frog *Bombina bombina*; similar alignments have been previously reported [29, 33]. Blue shading marks residues identical with those of the SWM-1 N-terminal TIL domain, yellow marks residues identical with the SWM-1 C-terminal TIL domain, and gray marks residues identical with both the SWM-1 N- and C-terminal TIL domains. The reactive sites of these TIL domains are generally divergent from one another, including for SWM-1 versus C25E10.8, so identities have not been marked for that 3–4 aa region. The amino acid changes caused by the *me66* and *me86* missense alleles are indicated on the SWM-1 sequence. Pairs of circles of the same color denote disulfide-bonded cysteine residues based on the structures of the CE/I, ATI, AMCI, and BSTI proteins.

(D) Model for SWM-1 function in males. We suggest that the two TIL domains of SWM-1 function to inhibit two distinct proteases, either of which is sufficient to activate sperm.

(E) Model for differential usage of sperm activation pathways in males and hermaphrodites. See text for discussion. Lines with arrowheads indicate positive regulation and lines with flat ends indicate negative regulation. Thick, thin, and dashed lines are used to denote more to less important inputs into each pathway. X indicates a second, *spe-8*-independent activation pathway that is present in males and may also *trans*-activate hermaphrodite sperm.

(Figure 5B). The TIL domain was originally identified in a small serine protease inhibitor, ATI (*Ascaris* trypsin inhibitor), from the parasitic nematode *Ascaris suum* [23–25]. A single TIL domain comprises ~60 amino acids including 10 characteristic cysteine residues, which form five disulfide bonds and are required for proper folding of the inhibitor domain, and a short (3–4 aa) reactive site. While the general pattern of cysteine residues is well conserved, the sequences of intervening amino acids can be divergent [26–28] (Figure 5C). TIL structure and protease inhibitor function have subsequently been shown to be conserved in proteins from other nematodes [29], insects [30, 31], and frogs [32]; TIL-like modules are also present in many longer secreted and extracellular proteins including von Willebrand factor and zonadhesin [30]. Proteins like SWM-1 with a signal sequence followed by two TILs appear to make up a distinct class of TIL-containing proteins and are particularly abundant in nematodes, including *C. elegans* [29, 33]. Based on its structural features, it is highly likely that SWM-1 functions as a secreted serine protease inhibitor.

Consistent with the prediction that the SWM-1 protein is secreted, analysis of mosaic worms carrying extrachromosomal *swm-1(+)* rescuing arrays in a subset of their cells provided evidence that *swm-1* can function in a cell-nonautonomous manner (see Supplemental Results). These experiments ruled out several tissues, including the intestine and the somatic gonad, as required

sources of *swm-1(+)* activity. Further, they suggested that the germline is a likely source of *swm-1(+)* activity. Importantly, they also indicated that either: (1) *swm-1* functions in a nonautonomous manner within the germline itself, such that *swm-1(+)* activity provided by a subset of germ cells can prevent premature activation of spermatids derived from *swm-1(-)* germ cells; or (2) at least one additional tissue, possibly the somatic gonad, may be capable of providing *swm-1(+)* activity.

SWM-1 May Inhibit Two Distinct Proteases

As seen for other serine protease inhibitors, the specificity and activity of a TIL inhibitor depend largely on the sequence of its reactive site, which the target serine protease binds and cleaves [34, 35]. The two TIL domains of SWM-1 have different reactive sites (Figure 5B), suggesting that SWM-1 could function to inhibit two distinct proteases. We were able to obtain evidence for this idea by examining the kinetics of spermatid activation in males heterozygous for the two partial loss-of-function missense alleles *me86* and *me66*, which affect different TIL domains (Figure 5C). Whereas 72% of *me86* homozygous males and 95% of *me66* homozygous males contained at least some activated sperm by 24 hr post-L4, only 23% of age-matched *me86/me66* heterozygotes contained activated sperm (Figure 3B). Thus, the onset of spermatid activation in *trans*-heterozygous *me86/me66* males was substantially later than in either *me86* or *me66* homozygous males. This partial complementation indicates that the two TILs of SWM-1 are not functionally redundant, supporting the idea that they act upon different protease targets. These data further suggest a model in which two distinct protease activities normally function to promote spermatid activation, and loss of inhibition of either protease can be sufficient to trigger premature activation of male sperm (Figure 5D).

Ectopic Spermatid Activation in *swm-1* Males Mimics In Vitro Activation by Protease Treatment

Protease treatment of wild-type spermatids in vitro leads to their activation, converting round spermatids into amoeboid, motile spermatozoa. Robust activation by this treatment requires the function of the *spe-8* group of genes, which are required in vivo for hermaphrodite spermatid activation but are dispensable for male fertility [11–13]. In vitro protease treatment of mutant spermatids thought to lack *spe-8* group function does not lead to pseudopod formation or motility, but instead elicits a spiky morphology that may correspond to a transient intermediate stage of normal pseudopod formation [12, 13, 36]. Thus, *spe-8* group activity is required for full sperm activation by proteases in vitro. Since loss of *swm-1* would be predicted to increase protease activity in vivo, we sought to determine whether the ectopic activation observed in *swm-1* mutant males is similarly dependent on *spe-8* group activity.

We dissected *spe-8* group and *spe-8* group; *swm-1* double mutant males and examined the morphology of their sperm (Figure 6). The *spe-8* group mutant sperm were round and unactivated, consistent with previous reports of normal timing of male sperm activation in these mutants [11–13]. The *spe-8; swm-1*, *spe-12; swm-1*, and *spe-27; swm-1* double mutant sperm also lacked pseudopodia and instead exhibited a spiky

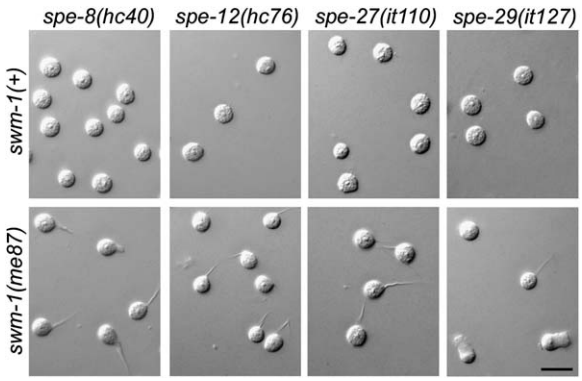


Figure 6. *spe-8* Group Function Is Required for Full Spermatid Activation in *swm-1* Mutant Males

All panels show sperm cells dissected from 2-day-old males. All *spe-8* group single mutant sperm (top row) appear round and unactivated. The *spe-8; swm-1*, *spe-12; swm-1*, and *spe-27; swm-1* double mutant sperm (bottom left) also lack pseudopodia, in contrast to the fully activated spermatozoa in *swm-1* single mutants (Figure 2C); instead, they display a spiky appearance indicative of partial activation. *spe-29; swm-1* double mutant sperm (bottom right) exhibit the full range of morphologies: unactivated, partially activated/spiky, and fully activated. All strains with mutations in either *spe-27* or *spe-29* also contain a *dpy-20* mutation. We obtained identical results with *swm-1(ok1193)* (data not shown). Scale bar equals 10 μm.

sperm morphology identical to that elicited by in vitro protease treatment of spermatids from the *spe-8*, *spe-12*, and *spe-27* single mutants [12, 13, 36]. From *spe-29; swm-1* males, we observed both spiky sperm and fully activated sperm with pseudopodia. Again, this range was identical to that seen upon in vitro protease treatment of *spe-29* spermatids [12]. Thus, loss of *swm-1* activity mimicked the effect of in vitro protease treatment of *spe-8* group mutant spermatids. These data further indicate that the *spe-8* group genes are required in males to promote the ectopic spermatid activation seen in *swm-1* mutants.

Correlation between Spermatid Activation and Sperm Transfer Defects

Having demonstrated that *spe-8* group mutations could largely suppress premature activation, we sought to determine whether *spe-8* group mutations would also suppress the sperm transfer defects of *swm-1* males. As before, we labeled males with mitotracker, crossed them to unlabeled *spe-8; dpy-4* hermaphrodites, and monitored sperm transfer, sperm migration, and egg production. The frequency of sperm transfer by *spe-8; swm-1* and *spe-27; swm-1* males was comparable to that of wild-type, *spe-8*, and *spe-27* control males, i.e., virtually all males were capable of transferring at least some sperm (Table 1 and data not shown). For *spe-29; swm-1*, we observed a partial suppression of the sperm transfer defect consistent with the partial suppression of activation we had observed by Nomarski microscopy. Thus, for these genotypes, sperm transfer success correlated with the presence of unactivated sperm. These data support the conclusion that ectopic activation is responsible for the sperm transfer defect.

We note that despite suppression of the sperm transfer defect, normal fertility levels were not restored: fewer males produced cross progeny and the number of

Table 1. Suppression of the *swm-1* Sperm Transfer Defect by *spe-8* Group Genes

Strain[b]	Mating[c]	Transfer[d]	Cross Progeny[e]	Male Fertility[a]		
				<10	10–100	>100
him-5	18/19	17/18	17/18	0	0	17
spe-8; him-5	18/20	16/18	10/18	7	2	1
swm-1 him-5	16/18	1/16	5/16	5	0	0
spe-8; swm-1 him-5	17/20	15/17	3/17	3	0	0
dpy-20; him-5	16/20	15/16	16/16	1	2	13
spe-27 dpy-20; him-5	16/20	16/16	16/16	4	3	9
dpy-20; swm-1 him-5	11/19	1/11	0/11	—	—	—
spe-27 dpy-20; swm-1 him-5	16/20	15/16	2/16	2	0	0
dpy-20; him-5	16/20	15/16	15/16	1	2	12
spe-29 dpy-20; him-5	11/18	10/11	10/11	1	1	8
dpy-20; swm-1 him-5	10/19	0/10	0/10	—	—	—
spe-29 dpy-20; swm-1 him-5	9/19	4/9	3/9	2	1	0

Single males of the indicated genotypes were mated to *spe-8; dpy-4* tester hermaphrodites and assayed as in Figure 4. Assays for each *spe-8* group; *swm-1* double mutant were performed in parallel with relevant controls (as grouped together above).

[a] Number of crosses giving rise to the indicated number of cross progeny.

[b] *swm-1(me87)* was used.

[c] Fraction of total crosses for which sperm transfer or progeny production indicated that copulation had occurred.

[d] Fraction of successful crosses for which transfer of mitotracker-labeled sperm was detected cytologically.

[e] Fraction of successful crosses that resulted in one or more cross progeny; in a few cases, a small number of cross progeny were produced even though mitotracker-labeled sperm were not detected cytologically.

progeny sired by each male was low as compared to control matings (Table 1). Our observations of *spe-27; swm-1* male sperm after transfer to hermaphrodites revealed a likely basis for this reduction in fertility. In the double mutant, transferred sperm rarely migrated to the spermathecae; instead, most remained near the vulva and were expelled by the hermaphrodite within 2–4 hr during egg laying. These data suggest that the partially activated *spe-27; swm-1* sperm have a reduced ability to complete normal transfer-dependent activation and/or migrate to the spermathecae in a timely fashion. We favor the idea that completing activation is the major defect, particularly since data suggesting a slow activation rate after transfer have previously been reported for another *spe-8* group mutant, *spe-12* [11].

swm-1 Activity Is Present in Hermaphrodites but Is Not Required for Hermaphrodite Spermatid Activation or Fertility

To determine whether spermatids are activated precociously in *swm-1* hermaphrodites like they are in *swm-1* males, we examined hermaphrodites at the late L4 to young adult stages during the transition from sperm to oocyte production when self spermatids move into the spermathecae and become activated. Both *swm-1 (me87)* and control L4/young adult hermaphrodites contained unactivated sperm in the oviducts as well as activated sperm in the spermathecae (Figure 7A). Thus, we found no evidence of altered timing or spatial location of hermaphrodite self-spermatid activation. Moreover, self-fertilizing hermaphrodites from different *swm-1* strains have brood sizes indistinguishable from those of the control (Figure 7B), indicating that *swm-1* mutant hermaphrodites make normal numbers of functional sperm. Whereas *swm-1* is crucial for male fertility, *swm-1* activity is dispensable for normal fertility in hermaphrodites.

However, examination of hermaphrodite self-fertility in a sensitized genetic background, the *spe-8* group mutant *spe-29*, provided evidence that *swm-1* activity is nevertheless present and functional in hermaphrodites. We found that *spe-29; swm-1* hermaphrodites had significantly higher brood sizes than did *spe-29* (Figure 7C), indicating that self-spermatid activation is improved in the double mutant. These data suggest that *swm-1* activity is normally present in hermaphrodites but does not play an important role in wild-type worms in which the pathway for self sperm activation is fully functional.

Discussion

Proteases as an In Vivo Trigger of Spermatid Activation

By searching for factors important for *C. elegans* male fertility, we have identified SWM-1, which functions as a negative regulator of spermatid activation. The identification of SWM-1 as a serine protease inhibitor suggests by extension that serine protease activity is a trigger for activation. Although it has been shown previously that protease treatment can result in activation in vitro, our findings represent the first evidence that proteolysis regulates spermatid activation in vivo.

Our data suggest that the SWM-1 target proteases are present in and can be used to trigger activation in both sexes, but they also imply that males and hermaphrodites differ in their relative reliance on protease-triggered activation and its regulation by SWM-1. We integrate our findings with the previous literature in a model depicted in Figure 5E that includes the following features. In hermaphrodites, proteases trigger self-sperm activation via a *spe-8*-dependent pathway; SWM-1 is present in hermaphrodites, but it is not sufficient to prevent activation unless the pathway is partially disrupted (i.e., by mutation of *spe-29*). In males, the protease-triggered *spe-8*-dependent activation pathway is also present, but prior to transfer, activation via this pathway is prevented by SWM-1. Males also contain a robust *spe-8*-independent activation pathway, which is coupled to sperm transfer. This transfer-dependent *spe-8*-independent pathway may involve factors provided in seminal fluid and may also be responsible for *trans*-activation of hermaphrodite sperm. The *spe-8*-independent pathway may be the predominant mode of activation used by wild-type males. Alternatively, both pathways may contribute to activation in wild-type males; sperm transfer might allow release from SWM-1-mediated inhibition of the protease/*spe-8*-dependent pathway while simultaneously triggering the *spe-8*-independent pathway. Coupling both modes of activation to transfer could help ensure male fertility by precipitating

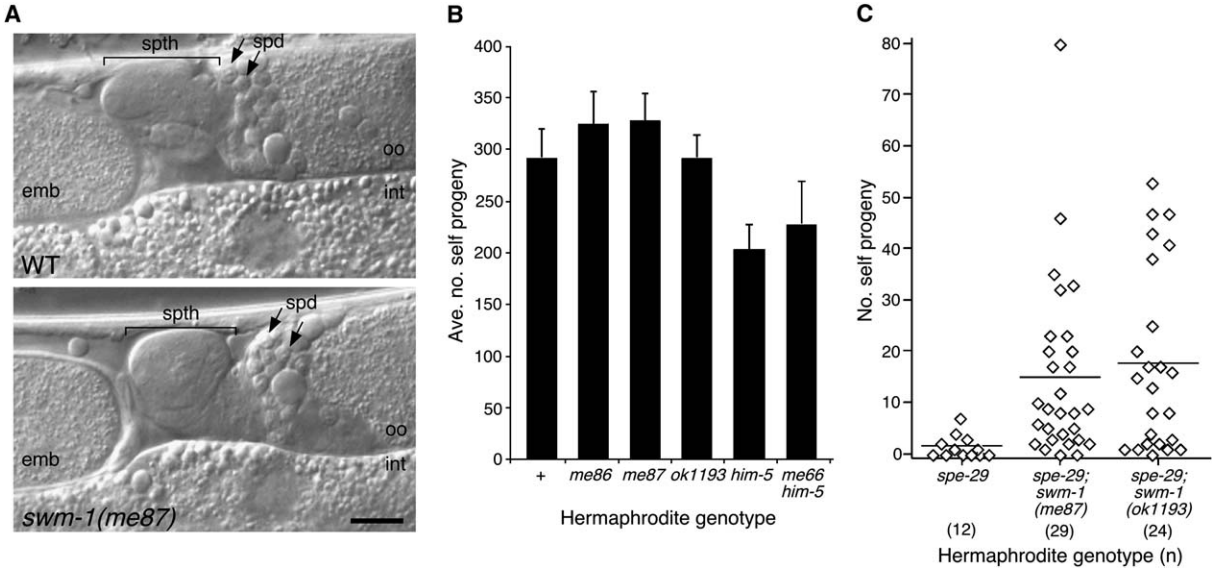


Figure 7. *swm-1* Is Functional in Hermaphrodites but Dispensable for Self Fertility

(A) DIC images of young adult virgin hermaphrodites at the time of self-spermatid activation showing the regions where self sperm are visible. Wild-type and *swm-1* mutant hermaphrodites are indistinguishable from one another: both contain unactivated spermatids (spd) in the oviduct adjacent to an oocyte (oo) and activated sperm, which are not readily visible in this focal plane, in the spermatheca (spth). The presence of a fertilized embryo (emb) proximal to the spermatheca also indicates that activated sperm are present. Int, intestine. Scale bar equals 10 μm.

(B) Bar graphs indicate the average number of self progeny (±standard deviation) produced by *swm-1* mutant hermaphrodites and the corresponding wild-type controls (N2 for the *me86*, *me87*, and *ok1193* strains and *him-5* for the *swm-1(me66) him-5* strain). *swm-1* mutant hermaphrodites have normal self-fertilizing brood sizes.

(C) Scatter plots showing that loss of *swm-1* suppresses the self sterility of *spe-29* hermaphrodites: *spe-29; swm-1* hermaphrodites have increased brood sizes as compared to *spe-29* hermaphrodites ($p < 0.001$, Mann-Whitney rank sum test). Diamonds indicate brood sizes produced by individual self-fertilizing hermaphrodites of the indicated genotypes; lines indicate average brood size for each strain.

an abrupt transition from a cellular organization that favors transfer into one that is competent for motility and fertilization (discussed below). We note that additional proteolysis targets (not shown in Figure 5E) may act in parallel to the SPE-8 group to promote the partial activation observed in both *spe-8* group; *swm-1* double mutants and protease-treated *spe-8* group single mutant sperm.

The presence of two TIL domains with different reactive sites suggests that SWM-1 acts on two proteases with distinct substrate specificities. This model fits with previous observations in which at least two proteases (either Pronase, a crude preparation of protease activities, or a mixture of trypsin and chymotrypsin [15]) were required to efficiently activate spermatids in vitro. Based on sequence comparisons with other serine protease inhibitors, it is likely that the SWM-1 TIL-C domain (reactive site sequence TKQ) inhibits a protease with a substrate specificity similar to that of trypsin itself [29]. However, the sequence of the TIL-N reactive site (TEQ) is not similar to TIL domains for which targets have been elucidated, so it is not clear what the other target of SWM-1 might be. Our analysis of the mutant alleles *me86* and *me66* indicates that loss of inhibition of either protease is sufficient to cause activation and thus either protease is independently capable of triggering sperm activation. This model provides a likely explanation for the failure so far to identify genes encoding proteases in genetic screens for fertility-defective mutants: it might be necessary to eliminate the function of more than one protease to elicit an activation failure.

The presence of a signal sequence in SWM-1 indicates that it is a secreted protein, implying that any relevant target proteases are also extracellular. An extracellular protease could function by cleaving sperm cell-surface proteins, leading to spermatid activation. Candidate targets for proteolysis include SPE-12, which is localized to the sperm plasma membrane, as well as SPE-29 and SPE-19, which also encode predicted transmembrane proteins [11, 12, 14]. An extracellular role for SWM-1 and its target proteases, acting upstream of the SPE-8 group, is also consistent with our observation that *spe-8* group mutations largely suppress ectopic spermatid activation in *swm-1* mutants. In contrast to this role for SWM-1 as an extracellular inhibitor of sperm activation, the protein kinase SPE-6 has been proposed to act downstream of the SPE-8 group as an intracellular inhibitor of activation based on the ability of *spe-6* mutations that cause ectopic sperm activation to suppress the self sterility of *spe-8* group mutant hermaphrodites [37].

Timing of Spermatid Activation Is an Important Determinant of Male Fertility

Several lines of evidence suggest that the main role of *swm-1* in male fertility is to regulate the timing of spermatid activation. In particular, our data suggest that premature activation of sperm within the reproductive tract of *swm-1* males results in failure to transfer sperm to the hermaphrodite. There is a strong correlation between ectopic activation and transfer failure throughout our observations of *swm-1* mutant males. The fraction of

males that failed to transfer sperm in mating assays was roughly proportional to the fraction of males that exhibited the Swm-1 activated sperm phenotype. The null mutants *me87* and *ok1193* nearly always contained activated sperm, which they failed to transfer; mutant *me66* and *me86* males exhibited intermediate levels of both the spermatid activation and sperm-transfer defects. In addition, *spe-8* group mutations partially suppress both activation and transfer defects. Finally, we observed a trend toward better transfer ability by younger *swm-1* males, which were less likely to contain activated sperm, as compared to older *swm-1* males (data not shown). When *swm-1* sperm were transferred, we found that they migrated to the spermathecae and fertilized eggs, suggesting that *swm-1* sperm have normal motility, taxis to the spermathecae and fertilization competence once they gain entry into a hermaphrodite's reproductive tract, implying that the transfer defect is the primary, if not sole, reason for reduced *swm-1* fertility.

Why aren't activated sperm transferred? One possibility is that, due to their asymmetrical shape, they are too large to exit the male gonad. If this were so, we might expect to see sperm accumulating within the vas deferens at the proximal end of the gonad in *swm-1* males. However, most sperm remain within the seminal vesicle; although some sperm crawl into other regions of the gonad, the proximal end remains largely free of sperm (data not shown; see Figure 2A for diagram). Thus, this "blockage" hypothesis seems unlikely. Another possibility is that activated, crawling sperm are more adhesive than inactive spermatids and thus may not be transferred readily (the "sticky cells" hypothesis). In support of this idea, we noticed during dissections that whereas spermatids were freely released from the gonad and dispersed throughout the medium (consistent with previous observations, e.g., [38]), activated sperm tended to remain clumped together with gonadal tissue. Finally, it is possible that the sperm-transfer defect does not arise entirely from a direct effect on sperm cells. Another effect of losing *swm-1* and thus allowing excess protease activity could be to change the environment inside the male gonad (the "sticky tissue" hypothesis). We cannot exclude the possibility that *swm-1* loss affects cell types other than sperm. However, our data suggest that sperm activation state is the major determinant of whether sperm are transferred and therefore whether males are fertile.

While maintenance of the inactive state might be necessary for the transfer of sperm from males to hermaphrodites, unactivated spermatids are neither capable of motility nor competent to fertilize eggs. If spermatids are not activated rapidly after transfer, they can be swept out of the hermaphrodite's reproductive tract by the movements of eggs toward the outside. To ensure that activation can occur efficiently upon entry into the hermaphrodite's reproductive tract, males appear to have adopted a strategy in which spermatids are poised to activate yet are held back by the constant presence of inhibitors (SWM-1) inside the male gonad.

Spermatid activation inside the male gonad is not limited to *swm-1* mutants: as they age, wild-type virgin males occasionally contain activated sperm, and wild-type nonvirgin males often contain activated sperm ([3] and data not shown). Thus, our observations of activation-induced transfer defects in *swm-1* males have relevance for wild populations as well. Since opportunities for males in the wild to mate may be few and far between, it may be advantageous to invest in a strategy—fast spermatid activation—that leads to a high probability of one-time reproductive success rather than in a strategy—slow spermatid activation—that relies on the availability of repeated mating attempts to compensate for an increased risk of sperm loss from the hermaphrodite's reproductive tract. We speculate that maintaining the balance between the inactive and activated states may be an important factor in determining the reproductive life span of males in wild populations. Different wild *C. elegans* strains can vary substantially with respect to when males cease to be fertile [39], and it will be interesting to determine the extent to which modulating spermatid activation contributes to this variation.

Experimental Procedures

Nematode Genetics

C. elegans were grown on NGM at 20ºC according to Brenner [40]. All strains with the exception of CB4856 (Hawaiian wild-type) were derived from the wild-type Bristol N2 strain. Mutations used in this study were *spe-8(hc40)* I, *spe-12(hc76)* I, *dpy-18(e364)* III, *spe-27(it110)* IV, *spe-29(it127)* IV, *dpy-20(e1282*ts*)* IV, *dpy-4(e1166)* IV, *unc-46(e177)* V, *sDf47* V, *mDf1* V, *nDf31* V, *him-5(e1490)* V, *swm-1(me66, me86, me87, ok1193)* V, *dpy-11(e224)* V, and *unc-76(e911)* V.

To ensure a ready supply of males, *him-5(e1490)* [18] was used as the wild-type and is included in all strain backgrounds unless explicitly stated otherwise.

swm-1(me66) was isolated as described in Results. To screen for new alleles that failed to complement *swm-1(me66)*, we mutagenized *him-5(e1490)* L4 males with EMS (ethylmethanesulfonate; Sigma) [20] and crossed them to *dpy-11 swm-1(me66) him-5* hermaphrodites. Non-Dpy hermaphrodite progeny were plated singly, and 4–10 of their non-Dpy male progeny were scored at 48 hr post-L4 by Nomarski microscopy for the Swm phenotype of sperm activation inside the male gonad.

Fertility and Activation Assays

To measure male fertility, L4 males were placed with *spe-8(hc40); dpy-4(e1166)* hermaphrodites in 1:1 crosses, the hermaphrodites were removed after 48 hr, and the numbers of Dpy (self progeny) and non-Dpy (cross progeny) were counted after 4 additional days. To measure hermaphrodite fertility, self-fertilizing hermaphrodite brood counts were performed by picking L4 hermaphrodites singly to plates and transferring them to new plates every 1–2 days until they stopped laying eggs; the number of progeny on each plate was counted after all worms were at least at the L4 stage.

The Swm-1 spermatid activation phenotype was scored in intact staged virgin males by Nomarski microscopy. To isolate staged virgin males, L4 larvae were placed on plates without hermaphrodites and incubated at 20ºC for the indicated amount of time (typically either 24 or 48 hr). For examining individual sperm cells, staged virgin males were dissected [17] in pH 7.4 sperm medium (50 mM HEPES, 50 mM NaCl, 25 mM KCl, 5 mM $CaCl_2$, 1 mM $MgSO_4$) supplemented with either 10 mM dextrose, 10 mg/ml PVP, or 1 mg/ml BSA.

Artificial insemination was performed as in [21]. Donors were virgin second day adult *him-5(e1490)* or *swm-1(me87) him-5(e1490)* males. For each insemination, sperm was collected from 1–3 donors in a single needle and transferred to a *dpy-4(e1166)* first day adult hermaphrodite.

Mitotracker Sperm Behavior Assay

Males were incubated with MitoTracker Red CMXRos (Invitrogen) at 0.5 to 2 μg/ml in NGM as in Chen [41]. Under this protocol, all sperm were labeled with mitotracker (data not shown). For time-course assays, L4 virgin males were moved to mitotracker plates seeded with

OP50 and stained for 14–24 hr, then incubated briefly on a clean plate before transfer to 1:1 crosses with age-matched *spe-8(hc40); dpy-4(e1166)* hermaphrodites. Each experiment included both wild-type and mutant males staged and crossed in parallel on freshly seeded (1–2 day of growth) plates. With a dissecting microscope fitted for epifluorescence, crosses were checked at 0.5 to 2 hr intervals for sperm transfer and sperm migration toward the spermathecae as well as for the appearance of fertilized eggs. Each male was removed from its plate after sperm transfer or laying of fertilized eggs became evident. Experiments were continued until >95% of wild-type males had mated (5–8 hr), and then any remaining males were removed from all plates. Only crosses for which mating could be confirmed were considered successful and included in analyses; confirmation of mating was provided by direct observation of mitotracker-labeled sperm within the hermaphrodite, production of cross progeny, or production of at least two self progeny (indicating seminal fluid transfer).

Supplemental Data

Supplemental Data include two figures, one table, and Supplemental Results and Experimental Procedures and can be found with this article online at http://www.current-biology.com/cgi/content/full/16/3/252/DC1/.

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