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RESEARCH ARTICLE

Soma-germ line interactions and a role for muscle in the regulation of *C. elegans* sperm motility

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ABSTRACT

The development of highly differentiated sperm cells that are specialized for navigating to and fusing with an oocyte is essential for sexual reproduction. As a major part of differentiation, sperm undergo extensive post-meiotic maturation en route to the oocyte. This is regulated largely by soma-derived cues. In *Caenorhabditis elegans*, this process is called sperm activation, and it transforms immotile spermatids into migratory fertilization-competent cells. Here, we show that the negative regulator of sperm activation, SWM-1, is produced in an unexpected cell type: body wall muscle. SWM-1 is secreted into the body cavity and enters the gonad; there, it is present with its likely target, TRY-5, a spermiogenesis activator. We show that, in addition to SWM-1, the somatic gonad and body fluid can exchange other factors, suggesting that soma-germ line transfer could affect other reproductive processes. In addition, we show that SWM-1 may have a separate role in the sperm migratory environment, to which it is contributed by both males and hermaphrodites. These findings reveal that late stages in gamete differentiation can be regulated at the whole-organism level by broadly secreted factors.

This article has an associated 'The people behind the papers' interview.

KEY WORDS: Reproduction, Sperm, Cell differentiation, Intercellular signaling, Protease inhibitor, Seminal fluid, *Caenorhabditis elegans*

INTRODUCTION

Sexual reproduction requires the development and differentiation of sperm cells that are capable of migrating to and fusing with an oocyte. To become fertilization competent, sperm undergo significant post-meiotic morphological and physiological changes. This differentiation process is triggered in response to extracellular cues that are generated by specialized support cells of the male and female reproductive tracts. In mammals, sperm progressively mature as they pass along the considerable length of the male reproductive tract. Within the labyrinthine epididymis and the vas deferens, different cell types secrete distinct repertoires of factors including proteins, sugars and lipids (Zhang et al., 2006; Belleannée et al., 2012). These induce changes to sperm membrane fluidity and cell morphology, and also trigger biochemical modifications that are required for fertilization (reviewed by Gervasi and Visconti, 2017). Within the female, additional male and female factors promote further maturation. One key set of events, sperm capacitation, comprises a set of physiological changes that include induction of maximum forward movement as well as the acrosome reaction, a specialized membrane fusion occurring at the sperm head that is required for fertilization. The end result is a cell that is highly specialized to locate and fuse with an oocyte (reviewed by Abou-Haila and Tulsiani, 2009; de Lamirande et al., 1997; Salicioni et al., 2007).

In all species that have been investigated, a significant proportion of factors that regulate post-meiotic sperm development are secreted proteolysis regulators (Jodar et al., 2017; Laflamme and Wolfner, 2013). For example, in mice, proteolysis-regulated processes include hyperactivation of flagella by the degradation of proteins that inhibit motility, as well as proper localization of sperm in the female reproductive tract (Christensson et al., 1990; Yamaguchi et al., 2009; Robert et al., 1997; Jonsson et al., 2005). In *Drosophila*, seminal fluid contains multiple proteases and protease inhibitors that compose a proteolytic cascade that is important for many aspects of post-mating reproductive success, including egg laying, sperm storage and release, and processing of the ovulation-inducing seminal fluid protein ovulin (Acp26Aa) (Laflamme and Wolfner, 2013). Specific examples include the trypsin-like protease seminase, the astacin metalloprotease CG11864 (Semp1), and the trypsin inhibitor-like protein Acp62F, which also has a role in sperm competition (Ram et al., 2005, 2006; Laflamme et al., 2012; Mueller et al., 2008).

The nematode *Caenorhabditis elegans* represents a simple model to study how protease signaling regulates spermiogenesis and post-translational cell motility. In *C. elegans*, both males and hermaphrodites produce amoeboid sperm (Ward, 1977; Ward and Carrel, 1979). Meiotic cell division results in haploid spermatids, which are initially immotile (Ward and Carrel, 1979). Upon receiving a signal, spermatids complete differentiation into motile, fertilization-competent spermatozoa. During this process, which is termed activation, cells polarize and generate pseudopods that are used to migrate towards oocytes (Ward and Carrel, 1979; Bottino et al., 2002). Activated spermatozoa are fully motile and competent for fertilization. Within hermaphrodites, 'self' spermatid activation occurs after meiotic division, within the spermatheca and uterus. This is regulated by a signaling process, involving zinc, which occurs independently of males (Liu et al., 2013, 2014; Zhao et al., 2018). Within males, spermatids are stored in their non-activated form. During mating, cells of the vas deferens release seminal fluid components at specific times and locations, and these are transferred, along with spermatids, to the hermaphrodite uterus, in which they can signal to sperm (Smith and Stanfield, 2011). Rapid activation into functional spermatozoa ensues.

Two important regulators of sperm activation are the serine protease TRY-5 and the trypsin inhibitor-like protein SWM-1 (Smith and Stanfield, 2011; Stanfield and Villeneuve, 2006). TRY-5 is thought to be the sperm activation-promoting signal in seminal fluid. Conversely, SWM-1 inhibits activation; *swm-1* mutant males contain prematurely activated sperm and are frequently infertile because of

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the inefficient transfer of spermatozoa (Stanfield and Villeneuve, 2006). TRY-5 is required for this premature activation, and it is visible at elevated levels near activated sperm in *swm-1* mutants (Smith and Stanfield, 2011). Thus, by their opposing activities, SWM-1 and TRY-5 ensure male fertility by signaling for sperm differentiation to occur only at the right place and time. However, how SWM-1 inhibits TRY-5 from prematurely activating sperm in the male has been unknown, both in terms of where SWM-1 originates and its relationship to TRY-5 localization and/or activity.

Here, we describe how this crucial developmental transition is regulated by somatic tissues that generate the extracellular environment surrounding sperm. Using analysis of protein localization in mutants that are defective for regulators of sperm activation, we determine where the sperm activation inhibitor SWM-1 localizes and functions in conjunction with the activator TRY-5. We show that, surprisingly, SWM-1 is produced in and secreted from extragonadal muscle cells into the body cavity that surrounds the gonad. SWM-1 then enters the seminal vesicle, in which it can colocalize with TRY-5. Interestingly, SWM-1 also is produced in the gonad, from where it is released into the body cavity. Indeed, we find evidence for a bidirectional exchange of proteins in addition to SWM-1 between the body cavity and the somatic gonad, suggesting that this may be a general phenomenon. Lastly, we find that SWM-1 is present in the sperm migratory environment, contributed both by hermaphrodites, via a muscle-to-gonad pathway, and by males, via seminal fluid transfer. Although SWM-1 is not required for hermaphrodite fertility, when it is present at high levels in the hermaphrodite uterus, it can negatively affect male fertility.

RESULTS

swm-1 is expressed in the male somatic gonad and in extragonadal muscle cells

We sought to determine where SWM-1, the negative regulator of activation, is expressed in males. Based on its amino acid sequence, SWM-1 is predicted to be a secreted protein. Thus, to identify cells that produce SWM-1, we used Mos-mediated Single-Copy Insertion (MosSCI) to generate strains that carried a transcriptional reporter, *Pswm-1*::mCherry::H2B, in which 1.3 kb of sequence 5′ to the *swm-1* coding region controls expression of an mCherry-tagged histone protein (Merritt et al., 2008; Frøkjær-Jensen et al., 2008). This strategy allowed us to identify cells that express *swm-1* based on the position of mCherry-positive nuclei. Importantly, control strains with this promoter driving expression of full-length SWM-1 showed rescue of the null *swm-1(−)* mutation, *me87* (Fig. S1) (Stanfield and Villeneuve, 2006).

We found that *swm-1* is transcribed in both gonadal and extragonadal somatic tissues. In *C. elegans* males (Fig. 1A), the gonad tube contains distally located germline stem cells that proliferate and then move proximally to undergo meiotic division and give rise to haploid spermatids. Proximal to the meiotic zone, spermatids are enclosed by a thin sheath of somatic cells, the seminal vesicle (Kimble and Hirsh, 1979; Lints and Hall, 2009). Lastly, the vas deferens forms a complex secretory tube through which sperm pass during mating and contains at least three cell types: valve cells, cuboidal cells and elongated cells (Lints and Hall, 2009), the first two of which have been shown to produce seminal fluid (Smith and Stanfield, 2011; Hodgkin and Doniach, 1997; Palopoli et al., 2008). Somatic gonad development occurs throughout the larval stages and is completed during the L4 stage. Spermatid production begins in L4 larvae and continues during adulthood.

We observed *Pswm-1*::mCherry::H2B reporter expression in six to eight of the 12 vas deferens cuboidal cells (Fig. 1B,C; Fig. 2). We also observed *Pswm-1*::mCherry::H2B in posterior body wall muscle cells and in the male-specific diagonal muscle cells, both of which overlie the seminal vesicle and vas deferens region (Fig. 1D,E) (Sulston et al., 1980). We confirmed the identity of these cells by colocalization with a GFP::H2B reporter under the control of the widely used *myo-3* promoter (*Pmyo*), which drives expression in male and hermaphrodite body wall muscle and in male-specific diagonal muscles (Fig. 1F,G) (Fire et al., 1990). Expression of the *Pswm-1* reporter in both gonadal and muscle cells began at late larval stages and persisted in adult animals until at least 48 h post L4 (Fig. 2). In addition, we observed reporter expression at low levels in two cells in the head, which were likely neurons (unpublished). These data indicate that *swm-1* is not expressed in sperm. Instead, it is produced in somatic tissues: the vas deferens, which is involved in the production, release, and transfer of seminal fluid, and muscle cells, outside the gonad. These data suggest that somatic tissues are important for the regulation of a crucial step of sperm development, terminal differentiation required for the acquisition of motility.

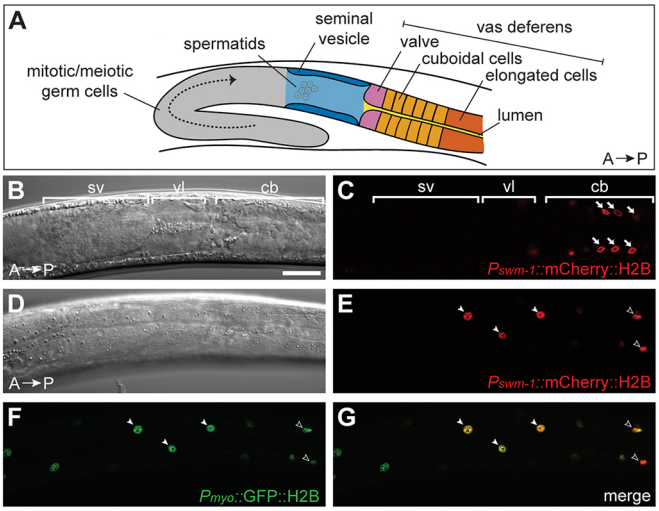

Fig. 1. *swm-1* is expressed in the somatic gonad and posterior muscle cells. (A) Schematic of structures in the male gonad. Somatic gonad cell types are the seminal vesicle, valve, cuboidal cells and elongated cells. Dashed arrow indicates the axis along which gametes differentiate in the gonadal tube. (B-G) Transmitted-light and confocal images of 24 h adult males. (B,C) *swm-1* is expressed in vas deferens cuboidal cells. Genotype is *jnSi130[Pswm-1::mCherry::H2B]*. Ventral view; arrows indicate cuboidal cell nuclei. (D-G) The *swm-1* reporter (E) is expressed in body wall muscle (white arrowheads) and male-specific diagonal muscle (open arrowheads), evidenced by colocalization with the muscle-specific transcriptional reporter *jnSi226[Pmyo::GFP::H2B]* (F,G). Genotype shown: *jnSi130/jnSi226*. *n*=30 animals/genotype at each time point. A, anterior; cb, vas deferens cuboidal cells; P, posterior; sv, seminal vesicle; vl, valve. Scale bar: 25 µm.

SWM-1 is secreted into the body cavity and surrounds sperm within the seminal vesicle

To determine where the SWM-1 protein localizes, we generated worms that expressed a SWM-1::mCherry fusion protein. Using a knock-in CRISPR strategy adapted from Arribere et al. (2014), Kim et al. (2014) and Ward (2015), we inserted mCherry coding sequences at the endogenous *swm-1* locus (Materials and Methods, Fig. S2A). We isolated two independent lines: *swm-1(jn60)* and *swm-1(jn62)*. To determine whether these strains had the desired alterations, we sequenced the *swm-1* gene and repair template region in both lines. The *jn62* allele had repaired as designed. However, the *jn60* allele contained a silent mutation in codon 22 and mutations that were predicted to alter amino acids 24 and 25 from two glutamate residues

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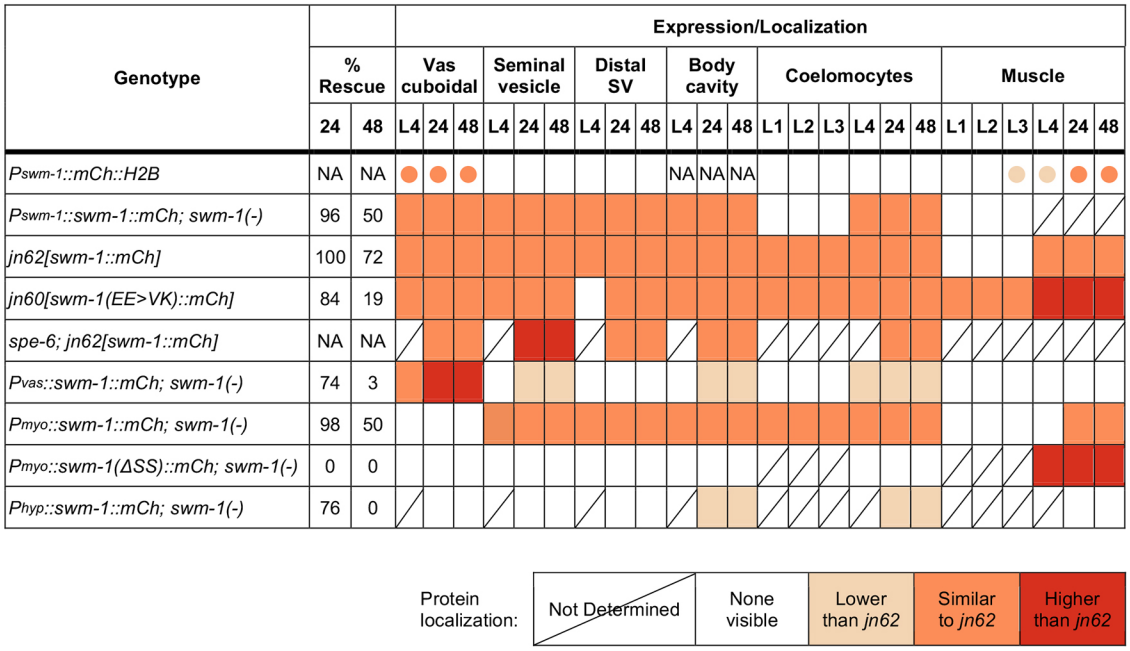

Fig. 2. Rescue and expression/localization of *swm-1* mCherry reporters. Quantification of sperm activation levels and patterns of expression and/or localization in *swm-1* mCherry reporter strains. *jn60* and *jn62* are CRISPR-induced knock-in alleles. All other transgenes are integrated at the *ttTi5605* site on *LGII* or the *cxTi10816* site on *LGIV*. For rescue, 24 h post L4 (young adult) males and 48 h post L4 (older adult) males were scored for the presence or absence of activated sperm; the percentage of rescued animals in a representative line is shown. For expression/localization, fully shaded cells indicate localization of SWM-1::mCherry protein and circles indicate expression of the transcriptional reporter. Darker colors indicate that expression was observed at higher levels and/or in a larger number of cells. NA, not applicable; diagonal line, not determined. *n*=21-56 animals/genotype.

(EE) to valine-lysine (VK). To test whether these SWM-1::mCherry fusion proteins were functional, we quantified sperm activation in *jn62[swm-1::mCherry]* and *jn60[swm-1(EE>VK)::mCherry]* males (Fig. 3A). For *jn62[swm-1::mCherry]* males, sperm were non-activated, as in the wild type, although there were slightly elevated levels of sperm activation in older adults. Thus, SWM-1::mCherry was functional and capable of inhibiting activation *in vivo*. Furthermore, western blot analysis of *jn62[swm-1::mCherry]* showed that adult males contain full-length SWM-1::mCherry (Fig. S2B). However, the *jn60[swm-1(EE>VK)::mCherry]* line showed sperm activation, especially in 48 h adults, indicating that the introduced mutations impaired SWM-1 function. Therefore, we primarily used the *jn62* line to analyze SWM-1 localization. We examined protein localization throughout postembryonic development (Fig. 2), but largely focused on L4 larvae and adults at 24-48 h post L4, when inhibition of activation is crucial.

In adult males, the SWM-1::mCherry protein was present, as expected, in cells in which we observed the transcriptional reporter: vas deferens cuboidal cells, diagonal muscles and body wall muscles (Fig. 3B-E and unpublished). Within cuboidal cells, SWM-1::mCherry was contained within large vesicles that were closely associated with the apical membrane (Fig. 3B,C), which are not well-characterized but have been shown to contain seminal fluid (Smith and Stanfield, 2011; Lints and Hall, 2009; Kimble and Hirsh, 1979). In muscle cells, we observed SWM-1::mCherry at low levels that appeared to be concentrated near the cell cortex (Fig. 3D,E). Interestingly, in *jn62[swm-1::mCherry]*, SWM-1::mCherry was also visible in anterior body wall muscles, rather than being restricted to the posterior region (unpublished), corresponding to slightly higher levels of rescue and protein expression as compared with the *Pswm-1* MosSCI reporter strain (Fig. S1 versus Fig. 3A; Fig. S2B). In *jn60* males, SWM-1(EE>VK)::mCherry was visible in the same tissues as *jn62*, but at varying levels. Within muscle cells, SWM-1(EE>VK)::mCherry was present at higher levels and at an earlier developmental stage compared with the wild-type fusion protein (Fig. S2C,D; Fig. 2), suggesting that the mutant form of the protein is stable but its secretion might be impaired. Although we focused on the wild-type fusion protein for the majority of our studies, this mutant allele supports the expression pattern of the knock-in and mosSCI transgenic strains.

The SWM-1::mCherry protein also localized to tissues in which we did not detect *swm-1* transcriptional expression. It was present throughout the body cavity (pseudocoelom) (Fig. 3F,G) and in coelomocytes (Fig. 3H,I), which are mesodermal cells that reside within the body cavity and take up soluble material (Zhang et al., 2001; Fares and Greenwald, 2001). Most notably, SWM-1::mCherry was present in the seminal vesicle lumen, surrounding sperm (Fig. 3F,G; Fig. 2). Concentrations were visible surrounding spermatocytes in the final stages of meiosis, which reside in the distal region. Lower levels were present throughout the seminal vesicle surrounding haploid spermatids (Fig. 3F,G), though this pool of SWM-1::mCherry was often difficult to detect. We surmised that this might be because of lack of space between spermatids, which are tightly packed together. To facilitate visualization of SWM-1::mCherry, we analyzed its localization in a *spe-6(hc163)* mutant, which contains sperm that activate prematurely, but independently of *swm-1* (Muhlrad and Ward, 2002). When activated sperm are present, the contents of the seminal vesicle are more disorganized, with empty spaces between cells. In *spe-6* males, SWM-1::mCherry was clearly evident in the seminal vesicle (Fig. S2E,F), indicating that it can spread among sperm.

Together with analysis of the transcriptional reporter, these data indicate that SWM-1 is produced in the somatic muscle and in vas deferens cuboidal cells. Furthermore, it is secreted from one or both of these tissues and can access the extracellular space around stored sperm.

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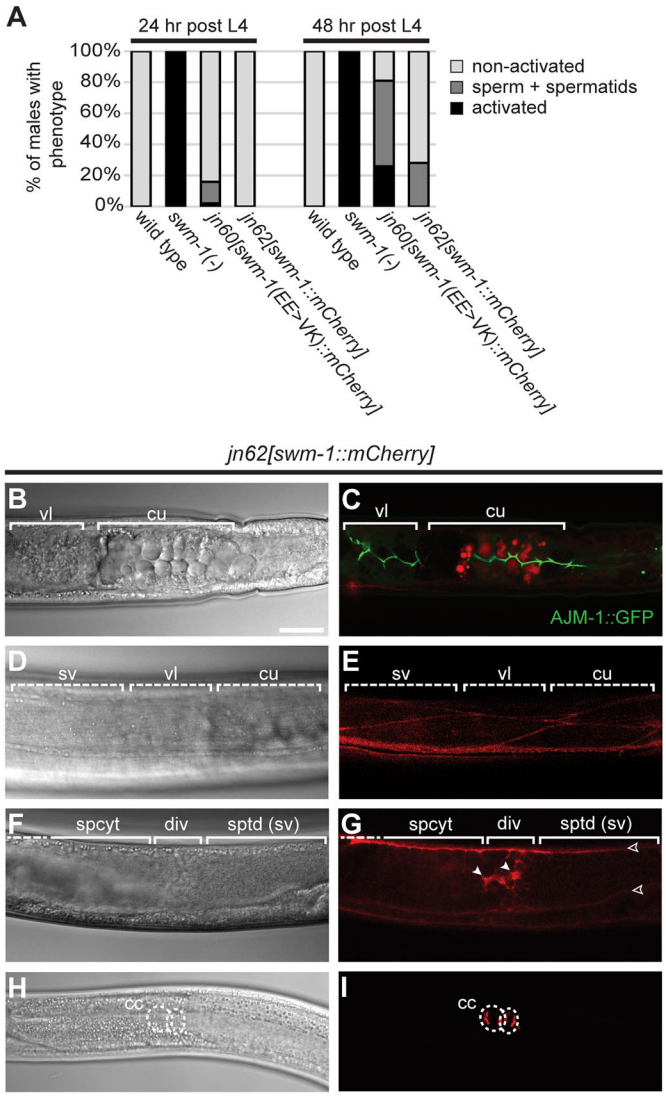

Fig. 3. SWM-1::mCherry localizes to the muscle, somatic gonad and sperm storage region. (A) Quantification of sperm activation levels of *jn62[swm-1::mCherry]*, *jn60[swm-1(EE>VK)::mCherry]* and control males at 24 h and 48 h post L4. Males were scored as having either only non-activated spermatids (light gray), a mixture of sperm and spermatids (dark gray) or only activated sperm (black). *n*=24-50 animals/genotype at each time point. (B-I) Transmitted-light and confocal images of *jn62[swm-1::mCherry]* 24 h post L4 males. *jn62[swm-1::mCherry]* adult male expressing the apical membrane marker AJM-1::GFP (Liu et al., 2005); SWM-1::mCherry is present within a subset of vas deferens cuboidal cells and localizes to intracellular vesicles that are closely associated with the apical membrane (B,C). SWM-1::mCherry localizes to muscle cells that overlie the gonad, which is not visible in the focal plane shown; positions of gonadal structures are indicated with dashed brackets (D,E). SWM-1::mCherry is present in the body cavity surrounding the seminal vesicle (open arrowheads). Within the seminal vesicle, it is concentrated near newly developed spermatids (white arrowheads) (F,G). SWM-1::mCherry accumulates in coelomocytes (outlined) (H,I). cc, coelomocytes; cu, vas deferens cuboidal cells; div, region with dividing spermatocytes; spcyt, gonad region with developing spermatocytes; sptd, region with haploid spermatids; sv, seminal vesicle; vl, vas deferens valve cells. Scale bar: 25 µm.

Muscle-derived SWM-1 regulates sperm activation within the gonad

To understand how males prevent premature sperm activation, we sought to determine which source(s) of SWM-1 are required for this regulation. We performed tissue-specific expression of SWM-1 in *swm-1(−)* animals and scored for rescue of the premature activation phenotype. We generated transgenic strains expressing SWM-1::mCherry driven by either the *clec-197* promoter (*Pvas*), which drives expression specifically in vas deferens cuboidal cells (Fig. 1A; Thoemke et al., 2005; data not shown) or by the muscle-specific *Pmyo* promoter (Fire et al., 1990; Moerman and Fire, 1997; Maryon et al., 1998). As a control, we also expressed untagged SWM-1 using these promoters, which yielded similar results.

When SWM-1::mCherry was expressed only in the vas deferens cuboidal cells, it rescued the premature activation of young adult 24 h post L4 *swm-1(−)* mutants (Table 1). However, by 48 h post L4, sperm were activated, which indicated that this expression was not sufficient to inhibit activation later in adulthood (Fig. 4A). To determine whether partial rescue activity correlated with differential localization of SWM-1 in the seminal vesicle at early versus later stages, we examined localization of *Pvas*::SWM-1::mCherry. At the L4 stage, *Pvas*::SWM-1::mCherry was visible in the gonad lumen between the cuboidal cells and seminal vesicle, where it contacted spermatids (Fig. 2; Fig. 4B-E). Interestingly, we also observed it in coelomocytes (Fig. 2; Fig. S3A,B), which indicated that SWM-1::mCherry derived from the vas deferens can enter the body cavity. However, we only observed *Pvas::*SWM-1::mCherry in the seminal vesicle lumen at low levels and at a low frequency (5-20% of animals) (Fig. 2). This was consistent with the lower rate of rescue we observed for this transgene, and we suspect that the rescue we observed at 24 h post L4 was due to SWM-1 being in contact with sperm early, at the L4 stage (Fig. 4B,C).

Surprisingly, when expressed in muscle, SWM-1 and SWM-1::mCherry rescued activation in *swm-1(−)* mutant males until at least 48 h post L4 (Fig. 2; Fig. 4A). This increased rescue was not simply due to expression levels, as *Pmyo*-driven expression was lower than that of *Pvas* (Fig. S3C). *Pmyo*::SWM-1::mCherry was present in body wall muscle, the body cavity and coelomocytes (Fig. 2; Fig. 4F,G). Similar to *jn62* knock-in animals, *Pmyo*::SWM-1::mCherry was present around developing and stored sperm in the seminal vesicle lumen (Fig. 4F,G); as levels were difficult to detect near stored sperm, we confirmed this using *spe-6* (data not shown). Removing the secretion signal resulted in the accumulation of SWM-1(ΔSS)::mCherry inside muscle cells, the absence of SWM-1(ΔSS)::mCherry from the seminal vesicle and the elimination of rescue, which confirmed a requirement for secretion as well as the muscle specificity of the *Pmyo* promoter (Fig. 2; Fig. 4A,H,I). Furthermore, SWM-1::mCherry was not visible in either the body cavity or in coelomocytes, which is consistent with SWM-1(ΔSS)::mCherry remaining within muscle cells (data not shown). Thus, these data suggest that muscle secretes SWM-1 into the body cavity, from where it enters the gonad to inhibit activation.

We reasoned that if the gonad takes up SWM-1 from its surroundings, then secretion into the body cavity from an ectopic source might be sufficient to inhibit activation. To test this, we used a *dpy-7* promoter (*Phyp*) to express SWM-1::mCherry from hypodermis, the epithelial tissue that encases the worm (Gilleard et al., 1997). We found that *Phyp*::SWM-1::mCherry could rescue activation, although at reduced levels (Fig. 2; Fig. 4A). It is likely that SWM-1::mCherry was secreted at lower levels from hypodermis compared with muscle, as we observed *Phyp*::SWM-1::mCherry only at low levels in coelomocytes within the body cavity and not at all in the seminal vesicle (Fig. 2; Fig. 4J-M).

Together, these experiments strongly support the idea that extragonadal muscle-derived SWM-1 is sufficient to inhibit precocious sperm activation by being secreted into the body cavity where it can enter the seminal vesicle. On the other hand,

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Table 1. Localization of secreted proteins expressed within or outside the gonad

					% Rescue‡	
	Tissue of origin	Secreted protein	Coelomocytes*	Seminal vesicle*	24 h	48 h
Extragonadal	Muscle	SWM-1::mCherry	+	+	98%	48%
	Muscle	TRY-5::GFP	+	+	100%	100%
	Muscle	mCherry	+	+§	NA¶	NA
	Muscle	GFP	+	+**	NA	NA
	Hypodermis	SWM-1::mCherry	+	–**	76%	0%
	Neurons‡‡	TRY-5::GFP	–§§	–§§	100%	100%
Gonadal	Cuboidal cells	SWM-1::mCherry	+	–**	74%	2%
	Cuboidal cells	TRY-5::GFP	–§§	–**	0%	0%
	Cuboidal cells	mCherry	+	–**	NA	NA
	Cuboidal cells	GFP	–§§	–**	NA	NA
	Valve cells	mCherry	+	–**	NA	NA
	Valve cells	GFP	–§§	–	NA	NA

*+, localization to indicated tissue; –, undetected in indicated tissue.
‡For secreted proteins other than TRY-5::GFP, percent of males that contained only non-activated sperm; for TRY-5::GFP, percent of males that contained only activated sperm.
§A *swm-1(me87)* null mutation was used to assess localization in seminal vesicles with activated sperm.
¶NA, not applicable.
**A *spe-6* mutation was used to assess localization in seminal vesicles with activated sperm.
‡‡A *rab-3* promoter was used for pan-neuronal expression (Hobson et al., 2011).
§§Low levels of GFP may not be detectable above gut or sperm autofluoresence.

expression in the vas deferens contributes to inhibiting sperm activation but is not sufficient throughout adulthood.

SWM-1 and TRY-5 localize near sperm independently of each other

In the absence of SWM-1, TRY-5 is visible at elevated levels in the seminal vesicle lumen (Smith and Stanfield, 2011). As both proteins are secreted into the extracellular space, a simple regulatory mechanism to envision is that SWM-1 inhibits TRY-5 activity via a direct interaction. However, an alternative possibility is that SWM-1 might affect the release of TRY-5. In either case, SWM-1 localization might be altered in the absence of TRY-5.

To address these models, we first compared the distributions of SWM-1::mCherry and TRY-5::GFP in males that expressed both transgenes. SWM-1::mCherry was localized broadly, completely surrounding the seminal vesicle in the pseudocoelom. By contrast, TRY-5 appeared to be largely restricted to the valve. It was not visible or present at lower levels in the posterior of the seminal vesicle, and only spread among sperm when they were activated (Fig. 5A,B) (Smith and Stanfield, 2011). However, we observed SWM-1::mCherry and TRY-5::GFP together in the seminal vesicle, in spaces created by activated sperm in *spe-6* mutants (Fig. 5C,D).

We then tested whether SWM-1 and TRY-5 regulate one another's localization. As *try-5* is tightly linked to *swm-1*, we used the integrated transgene *jnSi193[Pswm-1::swm-1::mCherry]* to examine SWM-1 localization in a *try-5* mutant background. The distribution of SWM-1::mCherry in the male gonad (Fig. 5E-H) showed no obvious differences compared with that in *jn62[swm-1::mCherry]* (Fig. 2; Fig. 3B,C,F,G). We then tested whether the apparent expansion of TRY-5::GFP into the seminal vesicle is due to the loss of *swm-1*, or whether TRY-5::GFP protein is simply more evident when activated sperm are present. In a *spe-6* activated-sperm background, we found that TRY-5::GFP spreads into the seminal vesicle of *swm-1(+)* males, just as it does in *swm-1(−)* males (Fig. 5I-L). Therefore, SWM-1 does not prevent the spread of TRY-5. Rather, these data suggest a model in which TRY-5 is continually present in the seminal vesicle, though presumably at levels low enough to be inhibited by SWM-1.

Proteins move between the body cavity and gonad

As we observed movement of SWM-1::mCherry between the soma and germ line, we sought to determine whether this might be a more general phenomenon. To test whether secreted proteins could enter and exit the gonad, we expressed secreted mCherry or secreted GFP in specific gonadal and extragonadal tissues. To assess entry into the gonad, we scored for localization to the seminal vesicle lumen in *spe-6* males, and to assess entry of proteins into the body cavity, we scored for localization in coelomocytes. We also scored for rescue of sperm activation phenotypes, which, based on our other results, strongly suggests entry of functional SWM-1 (or TRY-5) protein.

Proteins expressed from multiple regions of the gonad were released into the body cavity. Both *Pvas*::SWM-1::mCherry and *Pvas*::mCherry that were derived from cuboidal cells were detected in coelomocytes (Table 1, Fig. S3A,B,D,E), as was valve-derived *Pins-31*::mCherry (Table 1, Fig. S3F,G). However, we did not observe gonad-derived GFP in coelomocytes, possibly because autofluorescence reduced our ability to detect low levels (Table 1; data not shown). Examining the opposite direction of movement, we found that multiple proteins secreted from extragonadal muscle were present in the body cavity and entered the gonad. *Pmyo*::TRY-5::GFP, *Pmyo*::mCherry and *Pmyo*::GFP were visible in both coelomocytes and the seminal vesicle (Fig. S3H-S). In addition, TRY-5::GFP that was secreted from either muscle or neurons rescued sperm activation in males that carried the null mutation *try-5(tm3813)* (Table 1), indicating that the protein was functional.

These data show that the exchange of proteins between the gonad and other tissues is bidirectional and neither specific to SWM-1 nor limited to the sperm activation pathway. In addition, these data further support our findings that if a protein is present in the body cavity, it can enter the gonad and influence sperm differentiation.

SWM-1 is produced in muscle and enters the reproductive tract in hermaphrodites

Previous work demonstrated that *swm-1* is not required for hermaphrodite fertility, but is apparently present and functional (Stanfield and Villeneuve, 2006), so we examined the expression and localization of SWM-1 in hermaphrodites (Fig. 6A). As in males, *swm-1* was expressed in posterior body wall muscle

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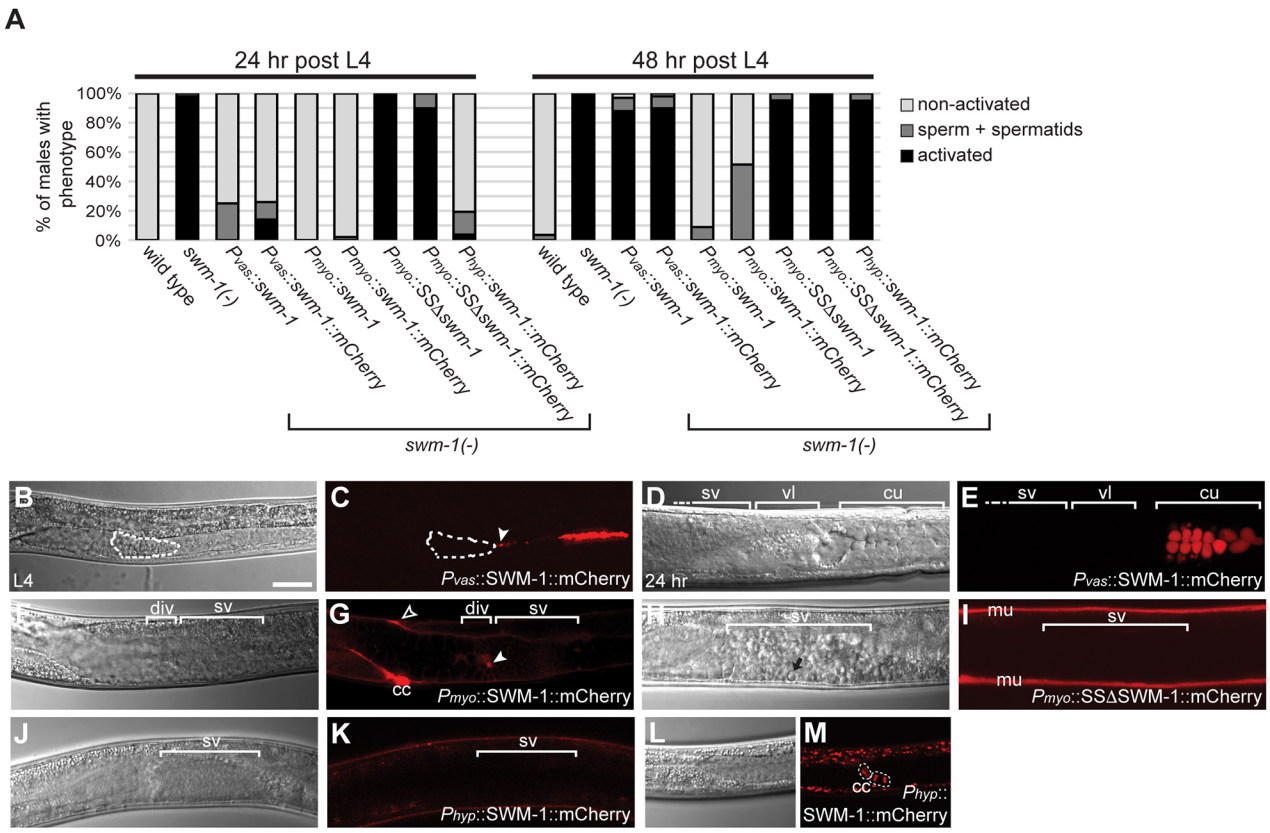

Fig. 4. Secretion of SWM-1 from muscle is sufficient for rescue of premature activation in males. (A) Quantification of sperm activation in 24 and 48 h post L4 males. Allele designations for strains are listed in Table S1. *n*=20-47 animals/genotype at each time point. (B-M) Transmitted-light and confocal images of males expressing SWM-1::mCherry in specific tissues. All genotypes include *swm-1(–)*. Age is 24 h post L4 unless indicated otherwise. (B-E) Males expressing SWM-1::mCherry in cuboidal cells. In L4s, SWM-1::mCherry is in close proximity (arrowhead) to sperm in the seminal vesicle (outlined) (B,C). In 24 h post L4 adults, no SWM-1::mCherry is visible near sperm (D,E). (F,G) Muscle cell-derived SWM-1::mCherry is present in the body cavity (open arrowhead) and in the seminal vesicle, concentrated near dividing sperm (white arrowhead). (H,I) Expression of SWM-1::mCherry lacking a signal sequence leads to sequestration in muscle and activation of sperm (arrow). (J-M) Hypodermis-derived SWM-1::mCherry is not detectable in the seminal vesicle (J,K) but is visible within hypodermal cells and in coelomocytes (L,M). cc, coelomocytes; cu, vas deferens cuboidal cells; div, region with dividing spermatocytes; mu, muscle; sv, seminal vesicle (dashed outline); vl, vas deferens valve cells. Scale bar: 25 µm.

(Fig. S4A,B) consistent with the non-sex-specific nature of this tissue. SWM-1::mCherry protein was detectable in muscle and secreted into the body cavity, as evidenced by its presence in coelomocytes (Fig. S4C,D; Fig. 6B,C). Interestingly, *swm-1* also was expressed in the spermathecae, structures that are unique to the hermaphrodite gonad, and SWM-1::mCherry surrounded sperm that were stored in the spermathecal lumen (Fig. 6D-G). Indeed, SWM-1::mCherry protein was present throughout the outer hermaphrodite reproductive tract and it surrounded fertilized eggs (Fig. 6H,I). As it can be difficult to distinguish between the body cavity and the uterus, we used GFP-labeled seminal fluid to mark the lumen of the uterus. *jn62[swm-1::mCherry]* hermaphrodites mated to males that expressed TRY-5::GFP (Fig. 6H-K′) showed clear colocalization of SWM-1::mCherry and TRY-5::GFP in the uterus. In similar experiments, we found that muscle-derived SWM-1::mCherry also colocalized with GFP transferred in seminal fluid (Fig. 6L-O′). Thus, as in males, SWM-1 is secreted into the hermaphrodite body cavity and then taken up into the gonad, in which it is present throughout the sperm migratory path.

SWM-1 in seminal fluid can modulate male fertility

As SWM-1 is produced in cuboidal cells of the vas deferens, which release TRY-5 during mating, we investigated whether SWM-1 might also be a component of seminal fluid. The cuboidal cells contain a population of variably sized membrane-bound vesicles, which can be observed either using differential interference contrast (DIC) or by visualizing TRY-5::GFP (Smith and Stanfield, 2011). We observed that SWM-1::mCherry and TRY-5::GFP were both present in these vesicles. In a subset, they colocalized, but their relative levels varied (Fig. 7A-D). To test for transfer, we crossed males that expressed SWM-1::mCherry to wild-type hermaphrodites. During mating, SWM-1::mCherry was released and transferred to the hermaphrodite uterus, as was previously observed for TRY-5::GFP (Fig. 7E,F) (Smith and Stanfield, 2011). Thus, SWM-1 is contributed to the migratory path of sperm both by hermaphrodites, via uptake into the gonad, and by males, via transfer in seminal fluid.

Our data suggested that SWM-1 might have a post-mating role within the hermaphrodite. Not only is it contributed to the uterus by both sexes, but its presence in the male gonad is dispensable for regulating activation within the male. Therefore, we sought to determine whether the presence of SWM-1 in the hermaphrodite uterus affects male fertility. We used our strains that expressed *swm-1* in specific tissues to manipulate the level provided by the hermaphrodite and/or male. We also took advantage of a *fog-2(q71)* mutation, which prevents sperm production in XX animals and results in ‘females’ (Schedl and Kimble, 1988), to eliminate possible complications of hermaphrodite self fertility.

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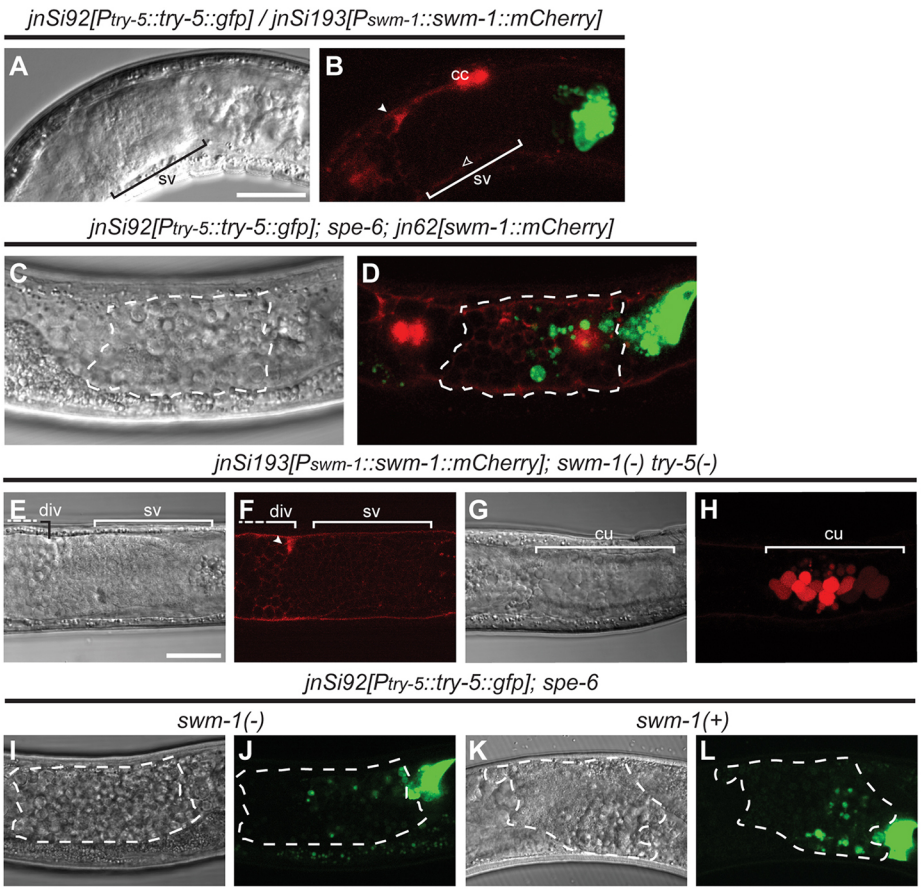

Fig. 5. SWM-1 and TRY-5 do not regulate localization of each other. Transmitted-light and confocal images of 24 h post L4 males. (A,B) In *jnSi92[Ptry-5::try-5::gfp]* males, TRY-5::GFP is primarily visible in valve cells. White arrowhead indicates SWM-1 in the seminal vesicle, open arrowhead indicates SWM-1 in the body cavity. (C,D) In *jnSi92[Ptry-5::try-5::gfp]; spe-6; jn62[swm-1::mCherry]* males, SWM-1::mCherry and TRY-5::GFP are both present in the seminal vesicle (outlined). (E-H) In *jnSi193[Pswm-1::swm-1::mCherry]; swm-1(−) try-5(−)* males, SWM-1::mCherry displays the wild-type localization pattern. It is near developing spermatocytes (white arrowhead) and in cuboidal cells. (I-L) TRY-5::GFP spreads into the seminal vesicle (outlined) independently of *swm-1*. cc, coelomocytes; cu, vas deferens cuboidal cells; div, region with dividing spermatocytes; sv, seminal vesicle. *n*=20-25 animals/genotype. Scale bars: 25 µm.

First, to create 'Vas(−)' males that do not transfer SWM-1 in seminal fluid, we used *Pmyo::swm-1; swm-1(−)* males, in which muscle-derived SWM-1 rescues the activation defect so that sperm can be transferred (Fig. 7G; Fig. 4A,F,G). We then crossed either these Vas(−) or wild-type males to *swm-1(−) fog-2* recipients. We found no difference in fertility, which suggests that the lack of seminal fluid SWM-1 does not alter male fertility (Fig. 7G).

Second, to create 'Vas-OE' (overexpression) males that transfer an elevated amount of SWM-1, we used *Pvas::swm-1; swm-1(+)* worms, in which SWM-1 is expressed from not only the wild-type locus, but also the *Pvas* transgene (Fig. 7H). When we crossed Vas-OE males to *swm-1(+)* recipients, they showed lower fertility compared with wild-type males (Fig. 7H, box plot 1 versus box plot 2). However, this difference between males was eliminated in crosses to *swm-1(−)* recipients (Fig. 7H, box plot 3 versus box plot 4). Similarly, Vas-OE males showed lower fertility in crosses to *swm-1(+)* hermaphrodites compared with crosses to *swm-1(−)* hermaphrodites (Fig. 7H, box plot 1 versus box plot 3). These results suggest that absence of *swm-1* in hermaphrodites can compensate for overexpression in males. Thus, high levels of SWM-1 can decrease sperm success, likely through a process that acts after sperm transfer. Although it is unclear how excess SWM-1 reduces sperm success, SWM-1 is clearly present throughout the sperm migratory tract and is an abundant component of the seminal fluid that males transfer to hermaphrodites during mating.

DISCUSSION

Regulation of sperm development by somatic tissues

Sperm maturation is an essential developmental process that is regulated by cues derived from multiple somatic cell types. Here, we characterized the expression and protein localization of SWM-1, a negative regulator of *C. elegans* sperm activation. We determined that it is produced by somatic body wall muscle and specific regions of the somatic gonad. We showed that the source of SWM-1 within the gonad, the vas deferens cuboidal cells, contributes to regulating motility but is not sufficient to inhibit activation throughout adulthood. Surprisingly, secretion of SWM-1 from extragonadal somatic muscle cells is sufficient to inhibit activation. This finding, that a factor that is crucial for fertility is derived from extra-gonadal muscle cells, is unexpected, though not unprecedented. The *C. elegans* hemicentin, HIM-4, is secreted from muscle and forms a scaffold within the gonad that is required to promote germ cell cytokinesis and chromosome segregation (Vogel and Hedgecock, 2001; Xu and Vogel, 2011). Whereas regulation of sperm development by factors secreted from the vas deferens or other somatic cells has been shown in *C. elegans* and other organisms, a regulatory role for muscle in sperm development has not, to our knowledge, been identified. Our data contributes to an emerging body of work showing that muscle is an important source of secreted factors that act on various tissues and that the role of muscle goes beyond movement and structure (Iizuka et al., 2014). Specifically, muscle cells release factors called myokines, which have been shown to be important in tissues such as vasculature and bone. Differentiation of *Drosophila* intestinal stem cells also is regulated by factors secreted by smooth muscle (Lin et al., 2008).

Muscle as a source of inhibitory signals may help to ensure male fertility by maintaining stores of non-activated sperm. Using tissue-specific expression, we showed that SWM-1::mCherry, when expressed from its native genomic locus or derived only from muscle, is concentrated near sperm that have recently completed

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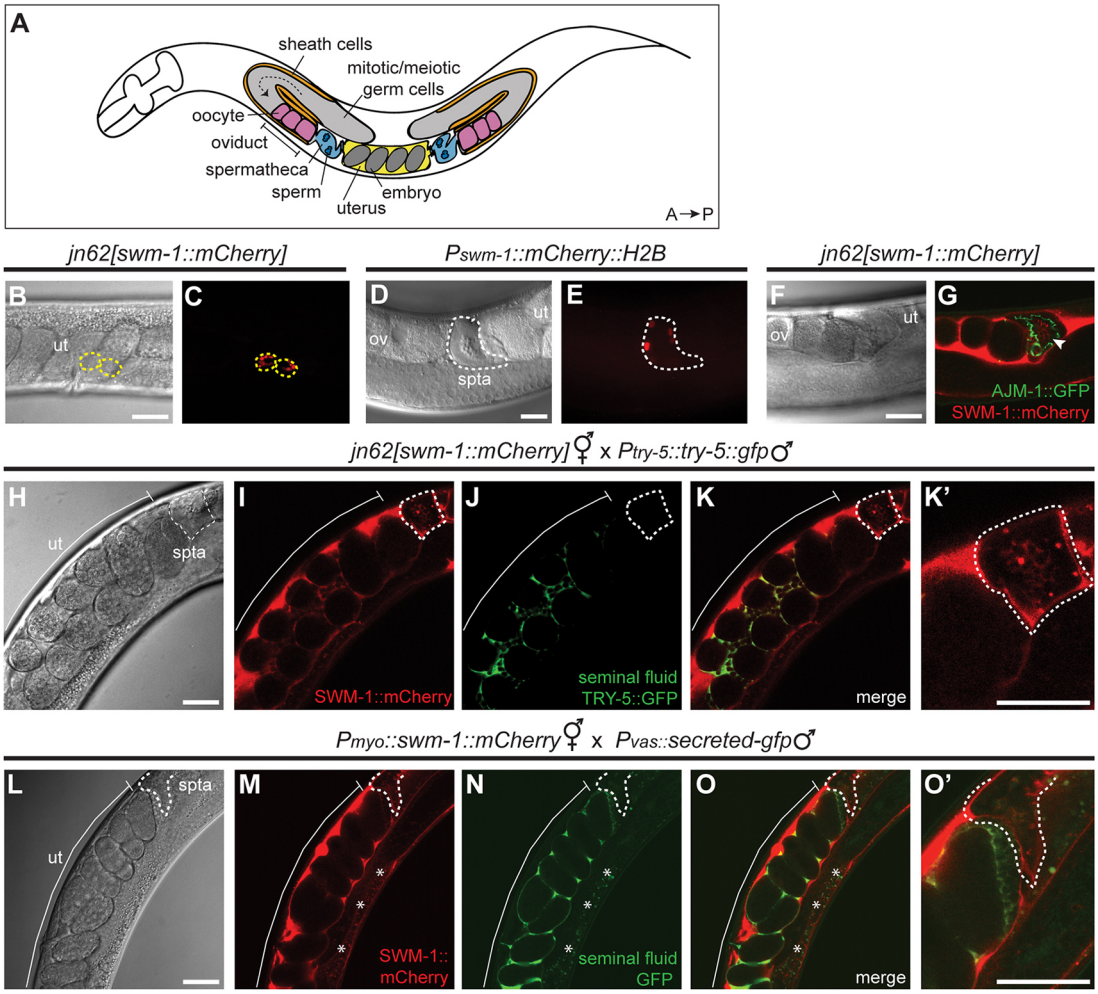

Fig. 6. SWM-1 is present in the hermaphrodite reproductive tract. (A) Schematic of structures in the hermaphrodite gonad. Dashed arrow indicates the axis along which gametes differentiate in the gonadal tube. The two gonadal arms have symmetrical structures; only the anterior arm is labeled for simplicity. (B-O′) Transmitted-light and confocal images of 24 h post L4 hermaphrodites expressing *swm-1* reporters. SWM-1::mCherry is visible in coelomocytes (outlined in yellow) (B,C). *jnSi130[Pswm-1::mCherry::H2B]* hermaphrodite showing expression in cells of the spermatheca (D,E). SWM-1::mCherry is visible within the spermatheca (white arrowhead) and in the body cavity. AJM-1::GFP marks the spermathecal lumen (F,G). Uterus of *jn62[swm-1::mCherry]* hermaphrodite after mating with a *jnSi92[Ptry-5::try-5::gfp]; swm-1(–) try-5(–)* male; SWM-1::mCherry colocalizes with male seminal fluid in the uterus and is present in the spermatheca surrounding stored sperm (H-K′). Uterus of *jnSi255[Pmyo::swm-1::mCherry]* hermaphrodite after mating with a *jnSi56[Pvas::secreted-GFP]* male; muscle-derived hermaphrodite SWM-1::mCherry colocalizes with male seminal fluid in the uterus (L-O′). Asterisks indicate intestinal autofluorescence. K′ and O′ are higher magnification images of spermatheca regions of K and O, respectively. spta, spermatheca (dashed white outline); ut, uterus. Scale bars: 25 µm.

meiosis. By contrast, cuboidal cell-derived SWM-1::mCherry is not present in these pools. Cuboidal cells are at the proximal end of the gonad, at some distance from newly developed spermatids, and do not complete development until after some sperm has accumulated. In addition, proteins with a secretion signal that are expressed in vas deferens cuboidal cells are sequestered in large apical vesicles that are depleted during mating, and little appears to be constitutively released. When the same proteins are expressed in muscle, they are not sequestered, but rather are continuously secreted. It has been shown that males that have mated display increased levels of activation compared with virgins, likely because of residual seminal fluid in the reproductive tract (Ward et al., 1983). We speculate that, by producing SWM-1 in a cell type that develops well before sperm are made and does not sequester or become depleted of secreted proteins, the male worm ensures that sufficient inhibitory signal is continuously present, starting at the time that the first sperm complete meiosis and become competent to activate.

Soma-germ line communication

Other proteins secreted from extragonadal tissues are essential for fertility. For example, long-distance soma-to-germline transfer has been demonstrated in mice, in which the protease inhibitor fetuin-B is secreted from the liver but required by oocytes to prevent premature zona pellucida hardening (Dietzel et al., 2013; Denecke et al., 2003). In *C. elegans*, production and loading of yolk proteins into oocytes occurs via uptake from the body cavity, similar to SWM-1. Yolk is produced in the intestine, secreted into the body cavity and taken up by receptor-mediated endocytosis via a yolk protein receptor (Kimble and Sharrock, 1983; Grant and Hirsh, 1999; Hall et al., 1999). Our experiments suggest that SWM-1, unlike yolk protein, does not require a specific transporter. Additional experiments to understand how SWM-1 physically enters the seminal vesicle would be interesting for understanding how factors are exchanged.

Our investigation of SWM-1 demonstrates a curious phenomenon: systemic factors present in the body cavity can

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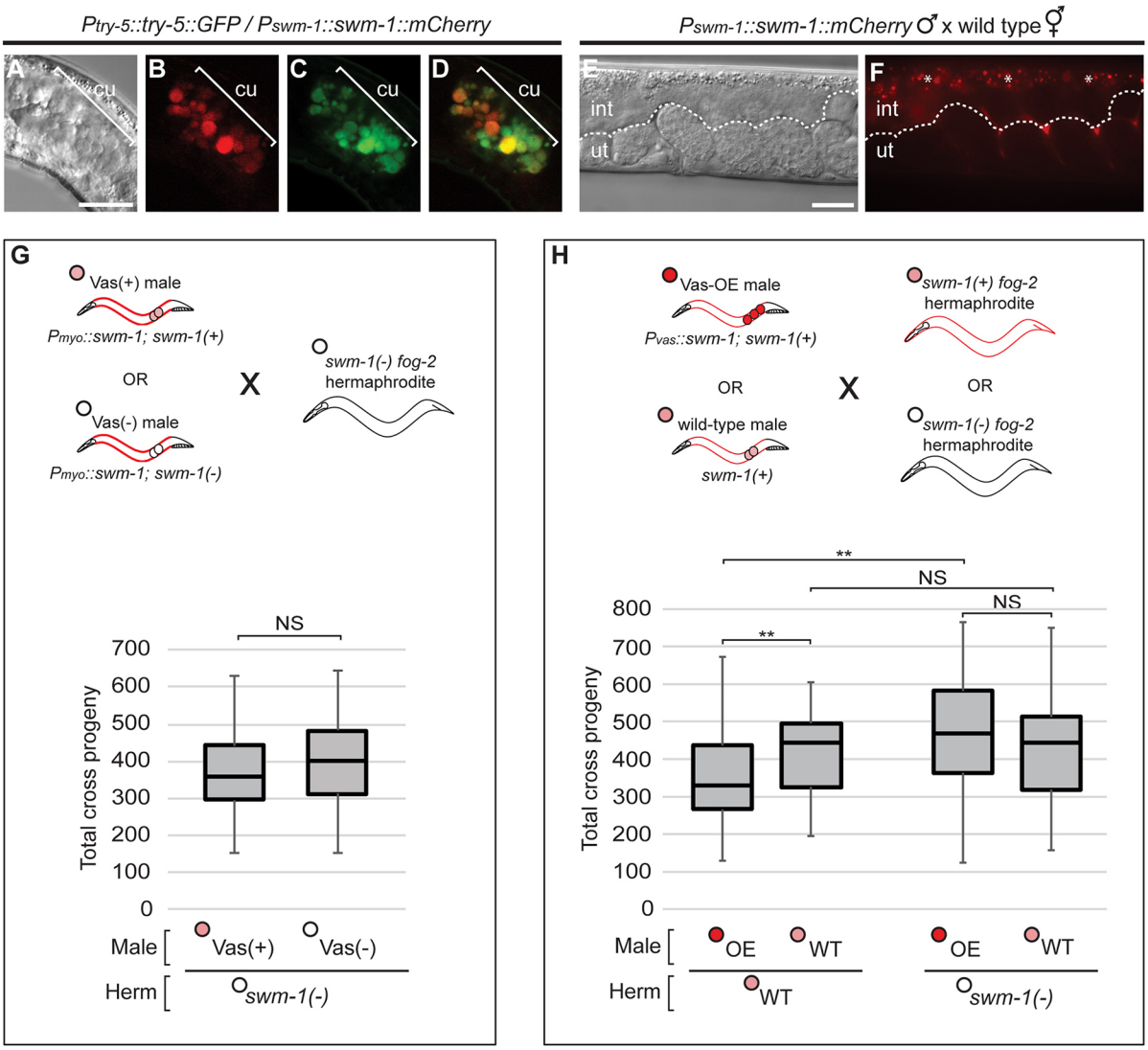

Fig. 7. SWM-1 contributed by the male or hermaphrodite can affect male fertility. (A-D) Transmitted-light and confocal images of *jnSi92[Ptry-5::try-5::gfp]; jn62[SWM-1::mCherry]* adult male. TRY-5 and SWM-1 partially colocalize in vas deferens cuboidal cells (cu). (E,F) Uterus of an adult hermaphrodite that has mated with a *jnSi193[Pswm-1::swm-1::mCherry]; swm-1(–)* male. Seminal fluid-derived SWM-1::mCherry is present among fertilized eggs throughout the uterus. Asterisks indicate intestinal autofluorescence. Dotted line indicates the edge of the uterus adjacent to the intestine. int, intestine; ut, uterus. (G) Male fertility is unaffected by lack of SWM-1 in seminal fluid. Vas(–) *[Pmyo::swm-1]; swm-1(–)* and control Vas(+) *[Pmyo::swm-1]; swm-1(+)* males were mated to *fog-2 swm-1(–)* hermaphrodites and total cross progeny were counted. Box and whisker plots show the total number of progeny sired by males. The central line is the median, the top and bottom of the box are the 75th and 25th percentiles, respectively, and the ends of the whiskers indicate the minimum and maximum data points. *n*=36-37 animals/set of crosses. (H) Overexpression (OE) of SWM-1 in seminal fluid reduces male fertility, but fertility is restored by eliminating SWM-1 in the hermaphrodite. Males overexpressing SWM-1 in the vas deferens were crossed to *swm-1(+) fog-2* or *swm-1(–) fog-2* hermaphrodites and total cross progeny were counted. Plot as in G. *n*=48-60 animals/set of crosses. ***P*<0.01 (Kolmogorov–Smirnov test). NS, not significant; WT, wild type.

enter the gonad and, vice versa, factors produced in the gonad can enter the body cavity. By analyzing mCherry and GFP secreted from within the gonad and from outside the gonad, we demonstrated that this exchange is not specific to sperm activation proteins and that other proteins can also act this way, which implies that such exchange is widespread. Kim et al. (2016) previously reported that INS-31::GFP, which is expressed in valve cells, can enter the body cavity and accumulate in coelomocytes. Our study expands on this finding by showing bidirectional movement of various secreted proteins. Double-stranded RNAs have previously been shown to move from *C. elegans* neurons to the germ line (Devanapally et al., 2015). These observations imply that sperm are exposed to a variety of factors from other tissues, which suggests that sperm cells are robust in their ability to encounter many signaling environments. This is reminiscent of a similar phenomenon in *Drosophila*, in which seminal fluid proteins enter the female circulation after transfer to the female during mating (Lung et al., 2002).

Regulation of sperm development by protease signaling

Our analysis of the localization of SWM-1 and TRY-5 in combination is a step toward understanding the molecular mechanism by which they regulate sperm activation. By using *spe-6* mutants, we showed that TRY-5 is able to spread into the seminal vesicle even in the presence of SWM-1. We also showed that the opposite is true: localization of SWM-1 does not depend on TRY-5. Although we did not investigate the mechanism involved, we presume that once in the seminal vesicle, these regulators spread by simple diffusion, as space among sperm permits. Visualizing

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SWM-1::mCherry in combination with TRY-5::GFP suggests that males regulate activation mainly by expressing the activator and inhibitor in different domains. Whereas TRY-5 is largely sequestered in valve cells, SWM-1 is widespread, surrounding the entire seminal vesicle in the body cavity from which it can freely enter the gonad and keep activation in check. It is interesting that there is a small degree of colocalization in the seminal vesicle. This suggests that where TRY-5 does encounter SWM-1, they might directly interact.

These data show that SWM-1 does not inhibit TRY-5 release. Instead, they support a model in which TRY-5 is secreted into the seminal vesicle at a low basal rate that is not sufficient to activate sperm, because of a predominantly inhibitory environment that is maintained by constant secretion of SWM-1 from somatic muscle cells that surround the gonad. Although these findings do not fully define the molecular mechanisms of SWM-1 and TRY-5, they are consistent with a direct interaction in the extracellular environment. Furthermore, these colocalization studies highlight the utility of *C. elegans* as a model for studying the sperm signaling environment and measuring the production and release of seminal fluid proteins in the context of live animals.

SWM-1 in the sperm migratory environment

SWM-1 is present at high levels in not only males, but also in hermaphrodites. Within the hermaphrodite uterus, it is contributed both by the hermaphrodite and by males in seminal fluid. It has previously been shown that *swm-1* is functional in the hermaphrodite, although it is dispensable for fertility (Stanfield and Villeneuve, 2006). Here, we further dissected the relative roles of male and hermaphrodite SWM-1. Using males that lack SWM-1 in the vas deferens but do have it in muscle to inhibit premature activation, we showed that male fertility is not affected if SWM-1 is absent from seminal fluid. Interestingly, providing excess SWM-1 reduced male fertility. The *in vivo* significance of this result is unclear, as overexpression within a single animal is not directly analogous to wild-type conditions. However, excess SWM-1 could conceivably be provided in a situation of repeated matings or the presence of multiple males. Given that SWM-1 plays an essential role for male but not hermaphrodite fertility, perhaps its effect in the uterus on fertility is subtle and may only be evident in more stringent conditions, such as when sperm from multiple males compete. In future studies, it would be interesting to analyze SWM-1 levels and kinetics in the uterus after mating to determine how levels at specific locations throughout the reproductive tract may affect sperm.

In summary, these studies have uncovered a crucial yet surprising role for muscle in the regulation of *C. elegans* sperm development. The presence of significant communication between extragonadal tissues and the gonad in both sexes has implications for understanding not only sperm activation but virtually every reproductive process in the adult. Further investigation of how protease signaling regulates sperm function in various species and contexts will provide a better understanding of male-factor infertility, the role of protease signaling in post-translational cellular processes and how tissues coordinate with one another to establish complex signaling environments.

MATERIALS AND METHODS

C. elegans genetics

C. elegans were grown at 20°C on nematode growth medium seeded with *Escherichia coli* strain OP50 and were derived from the wild-type Bristol N2 strain (Brenner, 1974). To obtain males, a *him-5* allele was present in strains in which males were analyzed. For the *jn62[swm-1::mCherry]* strain, we used CRISPR/Cas9 to generate the allele *him-5(jn64)*, and for other strains we used *him-5(e1490)* (Hodgkin et al., 1979). *swm-1(me87)* was used as the null allele for all rescue experiments. Other alleles used were *ttTi5605*, *dpy-18(e364)*, *spe-6(hc163)*, *unc-119(ed3,ed9)*, *cxTi10816*, *dpy-11(e224)*, *try-5(tm3813)*, *fog-2(q71)* and *ncIs13[ajm-1::GFP]* (Frøkjær-Jensen et al., 2008, 2012; Lee et al., 2018; Liu et al., 2005). Full genotypes of strains analyzed in this work are listed in Table S1.

Transgenic strains

MosSCI was used to integrate transgenes at the *ttTi5605 II* and *cxTi10816 IV* loci as described by Frøkjær-Jensen et al. (2008, 2012). Two or more independent lines were obtained for each construct.

MosSCI donor constructs were generated using the MultiSite Gateway Three-fragment Vector Construction Kit (Thermo Fisher Scientific). Fragments were PCR amplified from wild-type worms with appropriate terminal *att* sites, recombined into the entry vectors pDONR P4-P1r, pDONR221 or pDONR P2r-P3, and verified by sequencing. Entry fragments were recombined into the pCFJ150 or pCFJ212 destination vectors for transgene insertion on chromosome II or IV, respectively. A description of fragments used for generating targeting constructs is listed in Table S2 and the primers used to generate donor vector fragments are listed in Table S3.

To identify a *swm-1* genomic region with optimal rescue activity, we generated transgenes that included different lengths of sequence 5′ and 3′ to the *swm-1* coding region and assayed their ability to rescue *swm-1(me87)*. Transgenes containing 1.3 kb of 5′ sequence and 0.7 kb of downstream sequence showed strong levels of rescue of the premature activation phenotype (Fig. S1). However, 0.7 kb of 5′ sequence and 0.2 kb of 3′ sequence regions showed greatly reduced rescue (data not shown).

CRISPR/Cas9 genome editing

The co-CRISPR technique described by Arribere et al. (2014), Kim et al. (2014) and Ward (2015) was adapted to insert mCherry at the endogenous *swm-1* locus. N2 hermaphrodites were injected with DNA fragments for expression of *swm-1* guide RNA5 and *C25E10.8* guide RNA3 along with the pJA42 and AF-JA-53 reagents for *rol-6* co-CRISPR selection (Table S3; Arribere et al., 2014). Simultaneously, we provided a linear DNA repair template, which included 114 bp of sequence 5′ to the *swm-1* gRNA cut site, mCherry fused to the C terminus of *swm-1*, 124 bp of sequence 3′ to the *C25E10.8* gRNA cut site and two silent protospacer adjacent motif (PAM) site mutations (Fig. S2A). pJW1259 was used for Cas9 expression and DNA fragments for guide RNA expression were generated by fusion PCR (Ward, 2015). The repair template was amplified with Phusion (New England Biolabs) and purified with the Thermo Fisher Scientific PureLink PCR purification kit. Rollers were selected and PCR assays were used to screen for integration of mCherry. *rol-6* mutations were removed by outcrossing before analysis of strains. For the *jn60* and *jn62* strains, the sequence was obtained from the *swm-1* locus from 3390 bp upstream of the *swm-1* gRNA5 cut site to 263 bp downstream of the *swm-1* gRNA3 cut site. The *him-5(jn64)* allele was generated using the *him-5* gRNA1 and *him-5* gRNA3 guide RNAs to induce a deletion at the *him-5* locus in the *jn62[swm-1::mCherry]* strain.

Quantification of sperm activation

Sperm activation was measured by collecting virgin males as L4 larvae, incubating them at 20°C for 24 h or 48 h, and examining the seminal vesicle region using DIC microscopy. Sperm were deemed activated if they had visible pseudopods. Each animal was scored as ‘non-activated’ if it contained only spermatids without visible pseudopods; ‘sperm+spermatids’ if it contained a mixture of sperm with and without pseudopods, and ‘activated’ if it contained only sperm with pseudopods. Animals were excluded from analysis if the full seminal vesicle region could not be clearly seen. For DIC and epifluorescence, we used an AxioImager M1 microscope equipped with an AxioCam MRm (Zeiss). Confocal images were acquired using Olympus FV1000 and Leica SP8 microscopes.

Western blots

Batches of 50 virgin 24 h post L4 males were picked into 5 µl M9 worm buffer and flash-frozen in liquid nitrogen. Samples were thawed on ice and SDS loading buffer was added to a final concentration of 100 mM Tris-HCL (pH 6.8), 2% SDS, 5% β-mercaptoethanol, 15% glycerol and 4 M

urea with bromophenol blue. Samples were boiled for 12 min, passed three times through a thin gel-loading needle, centrifuged at 21,000 ***g*** for 4.5 min and kept on ice. Pooled samples of 100 worms/well were loaded onto a 12% SDS-PAGE gel. Protein was transferred onto a polyvinylidene difluoride membrane (Immobulon-P, Millipore Sigma) using a semi-dry blotter (Bio-Rad) run at 14 V for 50 min. For blocking, the membrane was incubated with 4% bovine serum albumin in TBST [20 mM Tris (pH 7.4), 500 mM NaCl, 0.05% Tween-20] for 40-60 min. Primary and secondary antibody incubations were for 1 h at room temperature, followed by 3× 8 min washes with TBST. Detection was performed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) and exposure to X-ray film (Amersham Hyperfilm HCL, GE Healthcare). Primary antibodies were rabbit monoclonal anti-RFP (Rockland, 600-401-379) and mouse monoclonal anti-tubulin (Sigma, T6199) at 1:5000. Secondary antibodies were goat anti-rabbit IgG H+L HRP (Bio-Rad, 170-6515) and goat anti-mouse IgG (H+L) HRP (Bio-Rad, 170-6516) used at 1:2000.

Male fertility assays

Males and hermaphrodites were collected as L4 larvae, incubated at 20°C for 24 h and placed together in a 1:1 ratio for 24 h to allow mating. Males were then removed and hermaphrodites were transferred to fresh plates every 24 h for 4 additional days. Matings that failed and those for which offspring were not produced for at least three transfers were excluded from analysis. *dpy-11 swm-1(me87) fog-2* animals are both male- and hermaphrodite-sterile, so *dpy-11 swm-1(me87) fog-2/dpy-11 jn62[swm-1::mCherry] fog-2* balanced heterozygotes were maintained and hermaphrodites lacking mCherry were selected for assays. The *dpy-11 fog-2* homozygous strain was maintained by crossing males to hermaphrodites.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: D.R.C., J.R.S., G.M.S.; Methodology: D.R.C.; Validation: D.R.C.; Formal analysis: D.R.C., G.M.S.; Investigation: D.R.C., A.K.S., J.R.S., G.M.S.; Resources: A.K.S.; Writing - original draft: D.R.C.; Writing - review & editing: D.R.C., A.K.S., J.R.S., G.M.S.; Visualization: D.R.C., G.M.S.; Project administration: G.M.S.; Funding acquisition: G.M.S.

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Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.167734.supplemental

References

Abou-Haila, A. and Tulsiani, D. R. P. (2009). Signal transduction pathways that regulate sperm capacitation and the acrosome reaction. *Arch. Biochem. Biophys.* **485**, 72-81.

Arribere, J. A., Bell, R. T., Fu, B. X. H., Artiles, K. L., Hartman, P. S. and Fire, A. Z. (2014). Efficient marker-free recovery of custom genetic modifications with CRISPR/Cas9 in *Caenorhabditis elegans*. *Genetics* **198**, 837-846.

Belleannée, C., Thimon, V. and Sullivan, R. (2012). Region-specific gene expression in the epididymis. *Cell Tissue Res.* **349**, 717-731.

Bottino, D., Mogilner, A., Roberts, T., Stewart, M. and Oster, G. (2002). How nematode sperm crawl. *J. Cell. Sci.* **115**, 367-384.

Brenner, S. (1974). The Genetics of *Caenorhabditis elegans*. *Genetics* **77**, 71-94.

Christensson, A., Laurell, C.-B. and Lilja, H. (1990). Enzymatic activity of prostate-specific antigen and its reactions with extracellular serine proteinase inhibitors. *Eur. J. Biochem.* **194**, 755-763.

de Lamirande, E., Leclerc, P. and Gagnon, C. (1997). Capacitation as a regulatory event that primes spermatozoa for the acrosome reaction and fertilization. *Mol. Hum. Reprod.* **3**, 175-194.

Denecke, B., Gräber, S., Schäfer, C., Heiss, A., Wöltje, M. and Jahnen-Dechent, W. (2003). Tissue distribution and activity testing suggest a similar but not identical function of fetuin-B and fetuin-A. *Biochem. J.* **376**, 135-145.

Devanapally, S., Ravikumar, S. and Jose, A. M. (2015). Double-stranded RNA made in *C. elegans* neurons can enter the germline and cause transgenerational gene silencing. *Proc. Natl. Acad. Sci. USA* **112**, 2133-2138.

Dietzel, E., Wessling, J., Floehr, J., Schäfer, C., Ensslen, S., Denecke, B., Rösing, B., Neulen, J., Veitinger, T., Spehr, M. et al. (2013). Fetuin-B, a liver-derived plasma protein is essential for fertilization. *Dev. Cell* **25**, 106-112.

Fares, H. and Greenwald, I. (2001). Genetic analysis of endocytosis in *Caenorhabditis elegans*: coelomocyte uptake defective mutants. *Genetics* **159**, 133-145.

Fire, A., Harrison, S. W. and Dixon, D. (1990). A modular set of lacZ fusion vectors for studying gene expression in *Caenorhabditis elegans*. *Gene* **93**, 189-198.

Frøkjær-Jensen, C., Davis, M. W., Hopkins, C. E., Newman, B. J., Thummel, J. M., Olesen, S.-P., Grunnet, M. and Jorgensen, E. M. (2008). Single-copy insertion of transgenes in *Caenorhabditis elegans*. *Nat. Genet.* **40**, 1375-1383.

Frøkjær-Jensen, C., Davis, M. W., Ailion, M. and Jorgensen, E. M. (2012). Improved Mos1-mediated transgenesis in *C. elegans*. *Nat. Methods* **9**, 117-118.

Gervasi, M. G. and Visconti, P. E. (2017). Molecular changes and signaling events occurring in spermatozoa during epididymal maturation. *Andrology* **5**, 204-218.

Gilleard, J. S., Barry, J. D. and Johnstone, I. L. (1997). *cis* regulatory requirements for hypodermal cell-specific expression of the *Caenorhabditis elegans* cuticle collagen gene *dpy-7*. *Mol. Cell. Biol.* **17**, 2301-2311.

Grant, B. and Hirsh, D. (1999). Receptor-mediated endocytosis in the *Caenorhabditis elegans* oocyte. *Mol. Biol. Cell* **10**, 4311-4326.

Hall, D. H., Winfrey, V. P., Blaeuer, G., Hoffman, L. H., Furuta, T., Rose, K. L., Hobert, O. and Greenstein, D. (1999). Ultrastructural features of the adult hermaphrodite gonad of *Caenorhabditis elegans*: relations between the germ line and soma. *Dev. Biol.* **212**, 101-123.

Hobson, R. J., Liu, Q., Watanabe, S. and Jorgensen, E. M. (2011). Complexin maintains vesicles in the primed state in *C. elegans*. *Curr. Biol.* **21**, 106-113.

Hodgkin, J. and Doniach, T. (1997). Natural variation and copulatory plug formation in *Caenorhabditis elegans*. *Genetics* **146**, 149-164.

Hodgkin, J., Horvitz, H. R. and Brenner, S. (1979). Nondisjunction mutants of the nematode *Caenorhabditis elegans*. *Genetics* **91**, 67-94.

Iizuka, K., Machida, T. and Hirafuji, M. (2014). Skeletal muscle is an endocrine organ. *J. Pharmacol. Sci.* **125**, 125-131.

Jodar, M., Soler-Ventura, A., Oliva, R. and **Molecular Biology of Reproduction and Development Research Group** (2017). Semen proteomics and male infertility. *J. Proteomics* **162**, 125-134.

Jonsson, M., Linse, S., Frohm, B., Lundwall, A. and Malm, J. (2005). Semenogelins I and II bind zinc and regulate the activity of prostate-specific antigen. *Biochem. J.* **387**, 447-453.

Kim, H., Ishidate, T., Ghanta, K. S., Seth, M., Conte, D., Shirayama, M. and Mello, C. C. (2014). A Co-CRISPR Strategy for Efficient Genome Editing in *Caenorhabditis elegans*. *Genetics* **197**, 1069-1080.

Kim, B., Suo, B. and Emmons, S. W. (2016). Gene function prediction based on developmental transcriptomes of the two sexes in *C. elegans*. *Cell Rep.* **17**, 917-928.

Kimble, J. and Hirsh, D. (1979). The postembryonic cell lineages of the hermaphrodite and male gonads in *Caenorhabditis elegans*. *Dev. Biol.* **70**, 396-417.

Kimble, J. and Sharrock, W. J. (1983). Tissue-specific synthesis of yolk proteins in *Caenorhabditis elegans*. *Dev. Biol.* **96**, 189-196.

Laflamme, B. A. and Wolfner, M. F. (2013). Identification and function of proteolysis regulators in seminal fluid. *Mol. Reprod. Dev.* **80**, 80-101.

Laflamme, B. A., Ram, K. R. and Wolfner, M. F. (2012). The *Drosophila melanogaster* seminal fluid protease “seminase” regulates proteolytic and post-mating reproductive processes. *PLoS Genet.* **8**, e1002435.

Lee, R. Y. N., Howe, K. L., Harris, T. W., Arnaboldi, V., Cain, S., Chan, J., Chen, W. J., Davis, P., Gao, S., Grove, C. et al. (2018). WormBase 2017: molting into a new stage. *Nucleic Acids Res.* **46**, D869-D874.

Lin, G., Xu, N. and Xi, R. (2008). Paracrine Wingless signalling controls self-renewal of *Drosophila* intestinal stem cells. *Nature* **455**, 1119-1123.

Lints, R. and Hall, D. H. (2009). Male reproductive system, somatic gonad. In *WormAtlas*.

Liu, Z., Fujii, T., Nukazuka, A., Kurokawa, R., Suzuki, M., Fujisawa, H. and Takagi, S. (2005). *C. elegans* PlexinA PLX-1 mediates a cell contact-dependent stop signal in vulval precursor cells. *Dev. Biol.* **282**, 138-151.

Liu, Z., Chen, L., Shang, Y., Huang, P. and Miao, L. (2013). The micronutrient element zinc modulates sperm activation through the SPE-8 pathway in *Caenorhabditis elegans*. *Development* **140**, 2103-2107.

Liu, Z., Wang, B., He, R., Zhao, Y. and Miao, L. (2014). Calcium signaling and the MAPK cascade are required for sperm activation in *Caenorhabditis elegans*. *Biochim. Biophys. Acta.* **1843**, 299-308.
Lung, O., Tram, U., Finnerty, C. M., Eipper-Mains, M. A., Kalb, J. M. and Wolfner, M. F. (2002). The *Drosophila melanogaster* seminal fluid protein Acp62F is a protease inhibitor that is toxic upon ectopic expression. *Genetics* **160**, 211-224.
Maryon, E. B., Saari, B. and Anderson, P. (1998). Muscle-specific functions of ryanodine receptor channels in *Caenorhabditis elegans*. *J. Cell Sci.* **111**, 2885-2895.
Merritt, C., Rasoloson, D., Ko, D. and Seydoux, G. (2008). 3′ UTRs are the primary regulators of gene expression in the *C. elegans* germline. *Curr. Biol.* **18**, 1476-1482.
Moerman, D. G. and Fire, A. F. (1997). Chapter 16. Muscle: structure, function, and development. In *C. elegans, 2nd edn* (ed. D. L. Riddle, T. Blumenthal, B.J. Meyer, et al.), pp. 1-26. Cold Spring Harbor Laboratory Press.
Mueller, J. L., Linklater, J. R., Ravi Ram, K., Chapman, T. and Wolfner, M. F. (2008). Targeted gene deletion and phenotypic analysis of the *Drosophila melanogaster* seminal fluid protease inhibitor Acp62F. *Genetics* **178**, 1605-1614.
Muhlrad, P. J. and Ward, S. (2002). Spermiogenesis initiation in *Caenorhabditis elegans* involves a casein kinase 1 encoded by the *spe*-6 gene. *Genetics* **161**, 143-155.
Palopoli, M. F., Rockman, M. V., TinMaung, A., Ramsay, C., Curwen, S., Aduna, A., Laurita, J. and Kruglyak, L. (2008). Molecular basis of the copulatory plug polymorphism in *Caenorhabditis elegans*. *Nature* **454**, 1019-1022.
Ram, K. R., Ji, S. and Wolfner, M. F. (2005). Fates and targets of male accessory gland proteins in mated female *Drosophila melanogaster*. *Insect Biochem. Mol. Biol.* **35**, 1059-1071.
Ram, K. R., Sirot, L. K. and Wolfner, M. F. (2006). Predicted seminal astacin-like protease is required for processing of reproductive proteins in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA* **103**, 18674-18679.
Robert, M., Gibbs, B. F., Jacobson, E. and Gagnon, C. (1997). Characterization of prostate-specific antigen proteolytic activity on its major physiological substrate, the sperm motility inhibitor precursor/semenogelin. *Biochemistry* **36**, 3811-3819.
Salicioni, A. M., Platt, M. D., Wertheimer, E. V., Arcelay, E., Allaire, A., Sosnik, J. and Visconti, P. E. (2007). Signalling pathways involved in sperm capacitation. *Soc. Reprod. Fertil. Suppl.* **65**, 245-259.
Schedl, T. and Kimble, J. (1988). *fog-2*, a germ-line-specific sex determination gene required for hermaphrodite spermatogenesis in *Caenorhabditis elegans*. *Genetics* **119**, 43-61.
Smith, J. R. and Stanfield, G. M. (2011). TRY-5 is a sperm-activating protease in *Caenorhabditis elegans* seminal fluid. *PLoS Genet.* **7**, e1002375.
Stanfield, G. M. and Villeneuve, A. M. (2006). Regulation of sperm activation by SWM-1 is required for reproductive success of *C. elegans* males. *Curr. Biol.* **16**, 252-263.
Sulston, J. E., Albertson, D. G. and Thomson, J. N. (1980). The *Caenorhabditis elegans* male: postembryonic development of nongonadal structures. *Dev. Biol.* **78**, 542-576.
Thoemke, K., Yi, W., Ross, J. M., Kim, S., Reinke, V. and Zarkower, D. (2005). Genome-wide analysis of sex-enriched gene expression during *C. elegans* larval development. *Dev. Biol.* **284**, 500-508.
Vogel, B. E. and Hedgecock, E. M. (2001). Hemicentin, a conserved extracellular member of the immunoglobulin superfamily, organizes epithelial and other cell attachments into oriented line-shaped junctions. *Development* **128**, 883-894.
Ward, S. (1977). Use of nematode behavioral mutants for analysis of neural function and development. In *Approaches to the Cell Biology of Neurons Society for Neuroscience Symposia*, Vol. II Sixth Ann Meeting, Toronto, Nov. 1976, 1-26.
Ward, J. D. (2015). Rapid and precise engineering of the *Caenorhabditis elegans* genome with lethal mutation co-conversion and inactivation of NHEJ repair. *Genetics* **199**, 363-377.
Ward, S. and Carrel, J. S. (1979). Fertilization and sperm competition in the nematode *Caenorhabditis elegans*. *Dev. Biol.* **73**, 304-321.
Ward, S., Hogan, E. and Nelson, G. A. (1983). The initiation of spermiogenesis in the nematode *Caenorhabditis elegans*. *Dev. Biol.* **98**, 70-79.
Xu, X. and Vogel, B. E. (2011). A secreted protein promotes cleavage furrow maturation during cytokinesis. *Curr. Biol.* **21**, 114-119.
Yamaguchi, R., Muro, Y., Isotani, A., Tokuhiro, K., Takumi, K., Adham, I., Ikawa, M. and Okabe, M. (2009). Disruption of ADAM3 impairs the migration of sperm into oviduct in mouse. *Biol. Reprod.* **81**, 142-146.
Zhang, Y., Grant, B. and Hirsh, D. (2001). RME-8, a conserved J-domain protein, is required for endocytosis in *Caenorhabditis elegans*. *Mol. Biol. Cell* **12**, 2011-2021.
Zhang, J.-S., Liu, Q., Li, Y.-M., Hall, S. H., French, F. S. and Zhang, Y.-L. (2006). Genome-wide profiling of segmental-regulated transcriptomes in human epididymis using oligo microarray. *Mol. Cell. Endocrinol.* **250**, 169.
Zhao, Y., Tan, C.-H., Krauchunas, A., Scharf, A., Dietrich, N., Warnhoff, K., Yuan, Z., Druzhinina, M., Gu, S. G., Miao, L. et al. (2018). The zinc transporter ZIPT-7.1 regulates sperm activation in nematodes. *PLoS Biol.* **16**, e2005069.