Protein & Cell

# MINI-REVIEW

# Transformation: how do nematode sperm become activated and crawl?

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## ABSTRACT

**Nematode sperm undergo a drastic physiological change during spermiogenesis (sperm activation). Unlike mammalian flagellated sperm, nematode sperm are amoeboid cells and their motility is driven by the dynamics of a cytoskeleton composed of major sperm protein (MSP) rather than actin found in other crawling cells. This review focuses on sperm from *Caenorhabditis elegans* and *Ascaris suum* to address the roles of external and internal factors that trigger sperm activation and power sperm motility. Nematode sperm can be activated *in vitro* by several factors, including Pronase and ionophores, and *in vivo* through the TRY-5 and SPE-8 pathways. Moreover, protease and protease inhibitors are crucial regulators of sperm maturation. MSP-based sperm motility involves a coupled process of protrusion and retraction, both of which have been reconstituted *in vitro*. Sperm motility is mediated by phosphorylation signals, as illustrated by identification of several key components (MPOP, MFPs and MPAK) in *Ascaris* and the characterization of GSP-3/4 in *C. elegans*.**

**KEYWORDS**    spermiogenesis, major sperm protein, sperm motility

## INTRODUCTION

Fertilization is achieved by the fusion of a mature sperm with an oocyte to produce a diploid zygote. In nematode species, the primary sperm cells undergo spermatogenesis to produce spermatids, which remain in a non-motile state and are stored in the seminal vesicles. After ejaculation, spermatids are activated in the uterus through a process called spermiogenesis, and become motile spermatozoa. Spermatozoa are responsive to the chemotactic signals derived from the oocyte (Kubagawa et al., 2006; Edmonds et al., 2011) and are competent for fertilization with oocytes. Here we review two processes, spermiogenesis and major sperm protein (MSP)-based sperm motility, in the nematode species *Caenorhabditis elegans* and *Ascaris suum* (hereafter *Ascaris*).

## NEMATODE SPERM CAN BE ACTIVATED BY DIVERSE FACTORS

*C. elegans* and *Ascaris* have an evolutionary divergence of 350 million years (Vanfleteren et al., 1994). *C. elegans* is androdioecious; i.e., it has two sexes, hermaphrodite and male. The hermaphrodite form is able to produce self-progeny but, when mated with a male his sperm are used preferentially for fertilization, producing out-crossed sexual progeny. In contrast, *Ascaris* is dioecious, being either male or female. In both species, the mature spermatids must sense extracellular signals to become activated, a critical step for sperm to become motile. During this process, sperm extend pseudopods driven by the assembly of a cytoskeleton composed of MSP, instead of actin, which more typically forms the basis of amoeboid cell motility. At this time, membranous organelles (MOs), a type of intracellular vesicle similar to lysosomes (Zhu et al., 2009), fuse with the plasma membrane and secrete specific components required for motility and fertilization (L'Hernault, 2006).

Early *in vitro* assays documented a wide range of factors that induce sperm activation in both species. In *Ascaris*, spermatids can be activated using trypsin, α-chymotrypsin, *Streptomyces griseus* protease, and vas deferens extract

(Abbas and Cain, 1979; Sepsenwol and Taft, 1990). In contrast, *C. elegans* spermatids can be activated by Pronase, the cationic ionophore monensin, a weak base triethanolamine (TEA) and an anion channel inhibitor 4,4′-diisothiocyano-2,2′-stilbenedisulfonic acid (DIDS) (Nelson and Ward, 1980; Ward et al., 1983; Shakes and Ward, 1989; Machaca et al., 1996). Pseudopod extension involves membrane rearrangement, during which ionic fluxes play essential roles. In *C. elegans*, sperm can be activated by blockage of the inward-rectifying chloride ($Cl_{ir}$) channels or by accumulation of $HCO_3^-$ in the cytoplasm, leading to an increase of intracellular pH (Machaca et al., 1996). In *Ascaris*, an optimal intracellular pH, possibly buffered by $HCO_3^-$, is necessary for pseudopod integrity (Fraire-zamora and Cardullo, 2010). The cytosolic alkalization is associated with the exchange of protons with $Na^+$ and $K^+$ ions, as in the cases of monensin and valinomycin, both of which induce pseudopod extension (Nelson and Ward, 1980; Roberts and King, 1991). Extracellular $Ca^{2+}$ was shown not to trigger sperm activation but three lines of evidences indicate intracellular $Ca^{2+}$ is involved during spermiogenesis, e.g., in *C. elegans*, the $Ca^{2+}$-binding chaperone calreticulin (CRT-1) (Park et al., 2001), a $Ca^{2+}$/calmodulin-dependent protein phosphatase (PP2B) (Bandyopadhyay et al., 2002) and the ferlin protein (FER-1), which contains multiple calcium-sensing C2 domains (Washington and Ward, 2006), mediate the processes of pseudopod extension and MO fusion.

Molecular genetic studies of *C. elegans* mutants have identified key components that regulate spermiogenesis. SPE-8 pathway members (SPE-8, SPE-12, SPE-19, SPE-27 and SPE-29) are major players in the control of this process (Minniti et al., 1996; Nance et al., 1999; Nance et al., 2000; Geldziler et al., 2005; L'Hernault, 2009). The *spe-8* group hermaphrodite mutant is self-sterile. However, when mated with wild type or *spe-8* males, *spe-8* partial loss-of-function (*lf*) hermaphrodites can produce self-progeny. This phenomenon is called "transactivation". Different levels of transactivation have been observed, i.e. *spe-8* hypomorphic mutants can be transactivated, whereas *spe-8* null mutants can rarely be transactivated (Nance et al., 1999). Transactivation is due to the transfer of the male activator in the seminal fluid to the hermaphrodite. TRY-5 is a typical male activator (see later sections). It was proposed that the targets of TRY-5 contain both non-SPE-8 group proteins (*spe-8* partial *lf* mutants can be fully transactivated) and SPE-8 group members (*spe-8* null mutants cannot be transactivated) (Smith and Stanfield, 2011). Transactivation is induced mostly via SPE-8 independent pathway, although SPE-8 group proteins retain some of their activities during this process.

SPE-6 and SPE-4 are two currently well-characterized suppressors of the SPE-8 pathway. SPE-6 is a casein I kinase (Varkey et al., 1993), whereas SPE-4 is a presenilin, an aspartyl protease that localizes within fibrous body-MOs (Arduengo et al., 1998). SPE-6 is thought to function down-

stream of the SPE-8 pathway and the onset of spermiogenesis requires reduction of SPE-6 activity (L'Hernault, 2009; Nishimura and L'Hernault, 2010). SPE-4 may participate in proteolytic processing of SPE-8 group proteins to inhibit spermiogenesis (L'Hernault, 2009; Nishimura and L'Hernault, 2010).

Another crucial component found in the male reproductive tract, SWM-1, containing two trypsin inhibitor-like (TIL) domains, inhibits premature activation of sperm in the seminal vesicle. Premature sperm activation would lead to the failure of sperm transfer during insemination because of the adherent nature of spermatozoa. Thus, SWM-1 ensures male reproductive success by keeping sperm in a non-activated state in the male body (Singson, 2006; Stanfield and Villeneuve, 2006).

Recently our group and the group led by Standfield made intriguing progress in the identification of new components during sperm activation in *Ascaris* and *C. elegans*, respectively. Smith and Stanfield (Smith and Stanfield, 2011) identified a seminal fluid protease TRY-5 as a sperm activator in *C. elegans*. They devised an elaborate model for sperm activation. In the male seminal vesicle, SWM-1 inhibits leaky TRY-5 to prevent sperm from activating and thus keeps seminal vesicle in an environment unfavorable for sperm activation. However, during ejaculation, the vas deferens secretes TRY-5 that induces sperm activation. In hermaphrodites, sperm activation can be triggered by an unknown factor, which is not TRY-5. When mating occurs, both the seminal fluid-derived TRY-5 and the hermaphrodite activator are able to initiate sperm activation, a process dependent on SPE-8 signaling pathway (Fig. 1A). Our group reported comparable findings (Zhao et al., 2012). We performed extensive analyses on As_SRP-1, a serine protease inhibitor belonging to the serpin superfamily, originally localized in MOs of spermatids in *Ascaris*. We showed that As_SRP-1 released from activating spermatids, *in trans,* inhibits the activation of other surrounding spermatids, by blocking the vas deferens-derived sperm activator As_TRY-5 (a trypsin-like serine protease) through a suicide substrate mechanism of serpin. Meanwhile, As_SRP-1 functions *in cis* to support MSP-based cytoskeletal assembly in the spermatid that releases it, thereby facilitating sperm motility and enhancing the competitiveness of the resulting spermatozoon. These dual functions of As_SRP-1 facilitate the success of the activated sperm in sperm cooperation (in the male body to prevent premature activation) and sperm competition (in the female reproductive tract) (Fig. 1B). Sperm competition has been widely recognized as one of most potent driving forces of evolution. Previous studies on sperm competition mechanisms have focused on physical traits of the sperm and of the seminal fluid produced by several accessory glands in the male body. Our study demonstrates for the first time that, besides components secreted from accessory glands in male body, sperm also contributes a protein (As_SRP-1) to the seminal fluid,
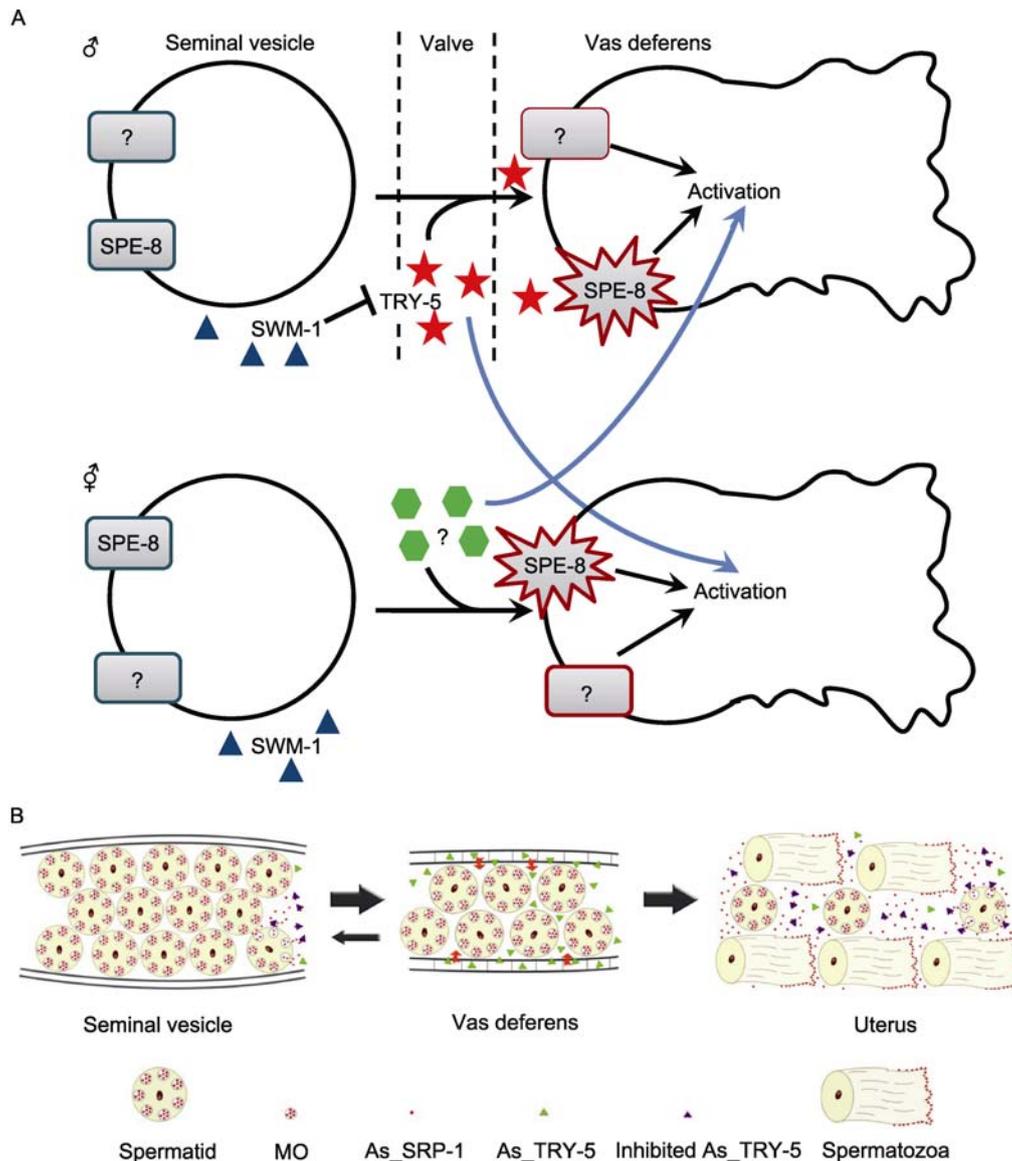
**Figure 1. Models for the modulation of sperm activation by protease and protease inhibitors in *C. elegans* and *Ascaris*.** (A) The end of *C. elegans* male gonad is composed of a seminal vesicle and vas deferens connected by a valve. The presence of SWM-1 (blue triangle) in the seminal vesicle inhibits leaky TRY-5 (red star) derived from vas deferens and prevents premature sperm activation. During the insemination, the high level of TRY-5 secreted from the vas deferens is incorporated into seminal fluid and overcomes the inhibitory capacity of SWM-1, thus enabling sperm activation in the uterus via a SPE-8-independent pathway. The major targets of TRY-5 (rectangle with question mark) are unknown. Some receptors of TRY-5 may be SPE-8 group proteins. Blue and red rectangles indicate pre- and post-activated states. Hermaphrodite sperm activation is triggered by a currently unidentified factor (green hexagon) possibly solely via the SPE-8 pathway. When mating occurs, TRY-5 and the hermaphrodite activator are able to mutually activate both pathways, a phenomenon called "transactivations". (B) After the second meiosis and budding from the residual body, spherical inactive spermatids are stored in the seminal vesicle. When copulation occurs, the epithelia of the glandular vas deferens begin to secrete As_TRY-5 into the seminal fluid, causing a momentary increase of As_TRY-5 activity in the lumen. When passing through the narrow channel of the vas deferens, spermatids are exposed to and activated by As_TRY-5. The ensuing exocytosis releases the MO components, including non-membrane-bound As_SRP-1, into the seminal fluid. Sperm compete for activation and, later, for migration and fertilization. As_SRP-1 released from activated sperm inhibits As_TRY-5 in the seminal fluid by forming a covalent complex with it and thereby inhibiting activation of other spermatids. Meanwhile, As_SRP-1 associated with the inner leaflet of the MO membrane is displayed on the outer surface of spermatozoa following the fusion of MOs with the plasma membrane (PM). Alternatively, As_SRP-1 secreted into the seminal fluid could be recruited back to the cell surface of activated spermatozoa. In either case, we suggest that PM-bound As_SRP-1 facilitates MSP cytoskeleton dynamics and enhances sperm migration. When mating ends, the vas deferens stops secreting As_TRY-5 and the residual As_TRY-5 in the male reproductive tract may trigger marginal sperm activation, and therefore the release of As_SRP-1. This, in turn, immediately extinguishes the residual As_TRY-5 activity. This negative feedback loop thus functions to maintain the male reproductive tract an unfavorable environment for sperm activation and to guarantee the efficiency of sperm transfer when mating takes place again. We propose that this 'yin-yang' relationship between the protease and the protease inhibitor in the seminal fluid modulates nematode sperm cooperation, motility and competition.

and that this protein coordinates both spermatozoon motility and sperm competition. In summary, these studies provide evidence that protease(s) and protease inhibitor(s) coordinately modulate spermiogenesis. This mechanism may be conserved and extend to other species, e.g., ~18% of the proteins in the *Drosophila* ejaculate are predicted to be proteases or protease inhibitors (Ram and Wolfner, 2007; Findlay et al., 2008), and the protease cascade acts during and following insemination (LaFlamme et al., 2012).

## MSP-BASED SPERM MOTILITY IS REGULATED BY PHOSPHORYLATION SIGNALING

Once the nematode sperm are activated in the uterus, sperm motility becomes critical for male fertility. Because *Ascaris* sperm can easily be obtained in large quantities and are large, this species is suited for biochemical and biophysical studies of amoeboid sperm motility. *Ascaris* sperm utilize MSP, rather than actin, for locomotion, providing a simplified model for the study of amoeboid cell motility. The pioneering work of Italiano et al. (1996) established an *in vitro* system for MSP polymerization. By adding ATP to the cell-free sperm extract, MSP-based motility was reconstituted *in vitro*. Vesicles derived from the plasma membrane at the leading edge triggered the assembly of a columnar meshwork of MSP filaments called fibers, resembling the *in vivo* MSP polymerization at the leading edge during pseudopod protrusion. Later, Miao et al. (Miao et al., 2003) used the same cell-free extract to reconstitute *in vitro* the MSP cytoskeleton retraction, like that observed at the rear end of cells *in vivo*, by adding *Yersinia* tyrosine phosphatase (YOP). Because MSP lacks ATP-binding site and MSP filaments are non-polar, this work

demonstrates that amoeboid cells can retract their posterior end and pull the cell body forward with no contractile input from conventional motor proteins such as myosin, and that protein phosphorylation/dephosphorylation functions as a molecular switch to modulate MSP cytoskeletal assembly and disassembly (Mogilner and Oster, 2003).

Biochemical studies identified several key proteins that regulate MSP cytoskeletal dynamics during amoeboid sperm motility. A 48-kDa MSP polymerization organizing protein (MPOP) was identified as the only membrane component that nucleates MSP assembly (LeClaire et al., 2003). Phosphorylation of MPOP is pH-sensitive and seems to require a yet undetermined tyrosine kinase. Phospho-MPOP binds to and recruits a serine/threonine kinase, MPAK (MSP polymerization-activating kinase), to the membrane at the leading edge (Yi et al., 2007). MPAK phosphorylates MFP2 (MSP fiber protein 2), which in turn increases the rate of fiber growth. In contrast, MFP1 negatively regulates fiber elongation (Buttery et al., 2003). A putative protein phosphatase 2A (PP2A) is present in the cell body and targets MFP3 (Yi et al., 2009). Phosphorylated MFP3 binds to and stabilizes the MSP filaments, whereas, dephosphorylation of MFP3 by PP2A releases it from the cytoskeleton, leading to the disassembly of MSP fibers (Fig. 2).

Currently the sequence of MPOP has not been elucidated but the other *Ascaris* proteins all have homologs in *C. elegans* (PP2A: LET-92; MPAK: C39H3.1/Y38H8A.3; MFP3: SSQ1 to SSQ4; MFP2: ZK265.3; MFP1: MSD-4/SSP-9 to SSP-32). According to microarray analysis, all of these *C. elegans* homologous genes except for *let-92* are highly enriched during spermatogenesis (Reinke et al., 2004). It was recently found that the *C. elegans* PP1 phosphatases GSP-3
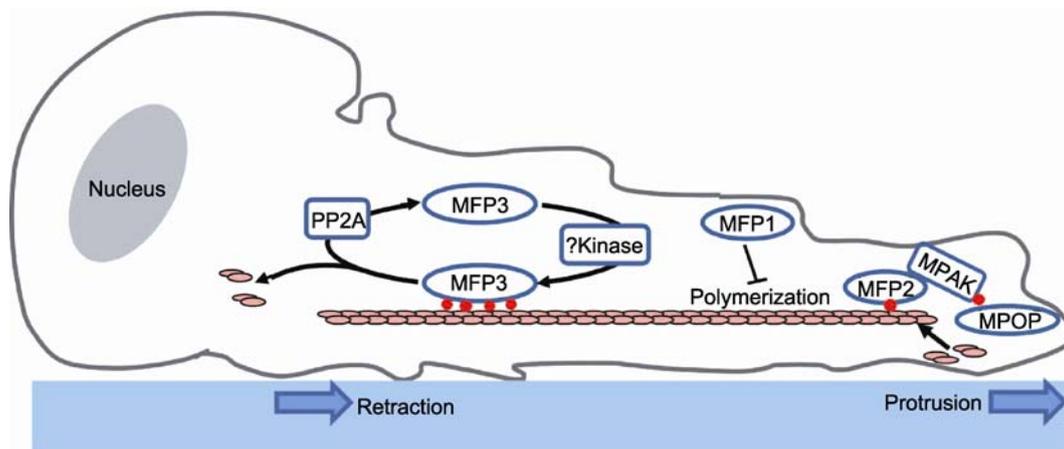


**Figure 2. Schematic representation of MSP-based sperm motility in *Ascaris*.** MSP dimers constitute the basic unit of the MSP fibers. MSP-based sperm motility is a coupled process of protrusion and retraction. At the leading edge, tyrosine phosphorylated MPOP recruits MPAK, which in turn phosphorylates MFP2. Phospho-MFP2 promotes MSP assembly, generating a protrusion force. MFP1 inhibits MSP polymerization. The cytosolic MFP3 is phosphorylated by an unknown kinase, and then binds to and stabilizes MSP filaments. At the cell body-pseudopod junction, PP2A becomes activated by a phosphatase and dephosphorylates phospho-MFP3, resulting in the release of MFP3 from MSP fibers. Ultimately, MSP fibers disassemble generating a retraction force near the cell body.

and GSP-4 regulate MSP dynamics during sperm development (Wu et al., 2012). Lack of GSP-3/4 causes defects in pseudopod development and sperm motility. Taken together, these studies of both *C. elegans* and *Ascaris* are shedding lights on MSP-based amoeboid sperm motility.

## FUTURE PERSPECTIVES

Nematode sperm provides a simplified system in which to study morphological and physiological changes associated with activation. *In vitro* assays can be readily conducted to examine the responses of sperm to various chemical compounds, such as ion carriers or ion channel inhibitors. These assays have demonstrated that ion channels (e.g., $Cl^-$, $Na^+$, and $K^+$) that regulate intracellular pH are indispensable for nematode sperm activation. Through genetic screening, SPE-8 group proteins were shown to mediate sperm activation in both males and hermaphrodites. However, other pathways that respond to extracellular activators exist in both sexes. Early *in vitro* studies showed that a protease triggers sperm activation in *Ascaris*. Recent characterization of TRY-5 in *C. elegans* strongly supports the notion that the protease cleaves its receptors on the sperm surface to initiate sperm activation. This process is spatially regulated because SWM-1, a protease inhibitor, suppresses TRY-5 activity in the seminal vesicle. That protease and protease inhibitors function in pair to modulate spermiogenesis was also demonstrated in *Ascaris*. *Ascaris* sperm are activated by the seminal fluid-derived protease As_TRY-5. MOs of activated sperm secrete the protease inhibitor As_SRP-1, which then forms a covalent complex with As_TRY-5, to block TRY-5 from activating other sperm. Possibly, the roles of a protease and a protease inhibitor in sperm activation have been evolutionarily conserved; e.g., in *Drosophila* a seminal fluid protease regulates post-mating reproductive processes (LaFlamme et al., 2012) and the lack of a serine protease inhibitor nexin-1 causes male fertility defects in mice (Murer et al., 2001).

*Ascaris* sperm activation *in vitro* requires a supply of bicarbonate/$CO_2$, as observed during sperm capacitation in mammals (Fraser, 2010). Whether in nematodes, bicarbonate also stimulates a soluble adenylate cyclase that generates cAMP as a second messenger for the downstream signaling cascade, as reported in flagellated sperm capacitation (Buck et al., 1999; Chen et al., 2000), deserves further investigations. Elucidating the regulatory mechanisms of nematode sperm activation might therefore provide new insights into the factors essential for male reproductive success and help to develop novel strategies for male contraception.

With respect to MSP-based sperm motility, progress has been made into the characterization of several phosphorylation-associated proteins (i.e., MPOP, MFPs, MPAK and PP2A) in *Ascaris*. Additionally, the leading-edge protrusion and cell body retraction of *Ascaris* sperm have been reconstituted simultaneously *in vitro* (Shimabukuro et al., 2011).

Comparison of nematode sperm crawling with actin-based cell locomotion raises some interesting questions. For example, myosin, a motor protein, participates in eukaryotic cell motility; in contrast, MSP lacks detectable ATPase activity (Yi et al., 2009) and the energy source that powers nematode sperm motility remains elusive. Taken together, nematode sperm activation and motility are integrated events involving a complex signaling cascade that modulate cytoskeleton dynamics. This unique system could reveal some evolutionarily conserved mechanisms of cell motility.

## ACKNOWLEDGMENTS

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