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A seminal fluid protease activates sperm motility in *C. elegans* males

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Seminal fluid factors have been shown to play a significant role in fertility in many animals. However, little is known about the contributions of seminal fluid to male fertility in *C. elegans*. In this commentary, we summarize our recent finding of a seminal fluid sperm activator, the serine protease TRY-5. TRY-5 is required for males to activate sperm, yet surprisingly it is not required for male fertility, likely due to redundancy with an activator present in hermaphrodites. TRY-5 is transferred to hermaphrodites during mating in a series of distinct release events just prior to transfer of sperm. Thus, we propose a model in which TRY-5 cleaves sperm cell surface proteins to trigger sperm maturation. We discuss other possible roles for seminal fluid factors in *C. elegans* and prospects for using TRY-5 as a marker for studies of male mating behavior and seminal fluid secretion.

Keywords: reproduction, sperm motility, spermiogenesis, serine protease, seminal fluid

Abbreviations: DIDS, 4,4'-Diisothiocyanatostilbene-2,2'-disulfonic acid; PMSF, phenylmethanesulfonylfluoride

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Introduction: Regulation of Sperm Motility

Sexual reproduction requires male and female animals to produce gametes that are highly specialized for the processes of meeting and fusing with one another. In particular, sperm cells are designed for motility and delivery of their cargo, the paternal genome, to the egg. Spermatogenesis involves an elaborate process of subcellular morphogenesis that culminates in polarized, motile spermatozoa. As sperm travel toward the egg, their motility is influenced by environmental stimuli. The successful spermatozoon must navigate and survive a variety of environments while adjusting its physiology and responding with an appropriate rate and direction of movement. To promote their reproductive success, males release accessory factors along with their sperm that can have effects on sperm motility and viability.

C. elegans is a male-hermaphrodite species in which both sexes make sperm: hermaphrodites can use their self sperm to fertilize their eggs, generating self progeny, or males can mate with hermaphrodites, resulting in cross progeny. Like those of other nematodes,[1] *C. elegans* sperm are amoeboid and move by crawling using a pseudopod.[2,3] Spermatocytes undergo meiotic divisions to generate haploid spermatids, which are transcriptionally and translationally inert. These cells are initially immotile, but they respond to external cues by undergoing a process called sperm activation in which they undergo rapid reorganization to become polarized, motile spermatozoa. Activation is required for sperm to become competent for migration to and fertilization of oocytes. Many genes important for spermatogenesis have been identified, primarily using genetic screens for sterile hermaphrodites whose fertility could be rescued by mating to a wild-type male (reviewed in ref. 4). Analysis of these mutants, along with observations of the wild-type pattern of sperm development,[5-10] have defined a cellular and genetic pathway for spermatogenesis in *C. elegans*.

While most steps of sperm development occur similarly in the two sexes and require the same gene functions, many mutants with sperm activation defects show sex-specific phenotypes, suggesting that this final step of sperm maturation is regulated differentially by males and hermaphrodites. Activation factors include a set of five genes termed the "*spe-8* group," which

are required for self-sperm activation within hermaphrodites.[11-16] However, the characterized members of the *spe-8* group all act within sperm, so none of them are candidates for signaling molecules that might initiate sperm activation. *spe-8* group hermaphrodite sperm can be activated by mating with a male (either the wild type or a *spe-8* group mutant), suggesting that males transfer an activator in their seminal fluid to which *spe-8* mutant sperm remain competent to respond.[11] Consistent with this model, a sperm-activating substance has been identified biochemically in male gonadal extracts from *Ascaris suum*.[17,18] In *C. elegans*, one seminal fluid component had been previously identified, the mucin PLG-1, which is required for deposition of a mating plug but does not appear to have a role in sperm activation.[19,20] *C. elegans* sperm can be activated in vitro by treatment with a number of different compounds, including Pronase, a mixture of proteases; triethanolamine, a weak base; DIDS, an ion channel inhibitor; and monensin, an ionophore.[8,12,21,22] Thus, a variety of different activities could be involved in activating sperm in vivo.

The Seminal Fluid Protease TRY-5 Promotes Sperm Activation

By searching for mutants with defects in male rather than hermaphrodite fertility, we previously identified a gene, swm-1, which regulates sperm activation in males.[23] Whereas male sperm normally delay their activation until after transfer to a hermaphrodite, *swm-1* males show precocious activation within the male gonad, associated with failure to transfer sperm to hermaphrodites. *swm-1* was found to encode a serine protease inhibitor, fitting with previous results that protease treatment in vitro was a potent activator[8] and that the Ascaris sperm-activating substance was inhibited by PMSF.[24] Together, these data strongly suggested that the male sperm activator would be a serine protease. The Swm-1 phenotype provided an opportunity to search for this activator using suppressor screens. Using a combination of RNAi to test candidate proteases, together with chemical mutagenesis screens to hunt more broadly for activation-promoting factors, we found that the serine protease TRY-5 is required for the premature sperm activation observed in *swm-1* males. Males doubly mutant for both *try-5* and *swm-1* contained non-activated sperm, as in the wild type, and both sperm transfer ability and male fertility were restored.[25]

Although it is critical for males to prevent TRY-5 activity within the male gonad in order to remain capable of transferring their sperm, the role of TRY-5 in normal fertility was not immediately clear. Since motility is required for sperm function, it might be expected that loss of a sperm activator would lead to reduced fertility. However, *try-5* mutant males and hermaphrodites showed fertility levels indistinguishable from those of the wild type. To uncover a defect in *try-5* males, it was necessary to use a specific assay for male activator: the ability of male mating to induce sperm activation and restore self fertility to *spe-8* group mutant hermaphrodites. This "trans" activation is assayed using males with defective sperm as seminal fluid donors to avoid generation of cross progeny.[12] *try-5* males were incapable of transactivating *spe-8* group hermaphrodite sperm, indicating that *try-5* males do not transfer activator. In addition, *spe-8* group; *try-5* double mutants were found to be completely infertile. These results suggested that the absence of fertility defects in *try-5* males is due to rescue of male sperm activation by an activator present in hermaphrodites. Furthermore, since *try-5* hermaphrodites support the fertility of sperm from *try-5* mutant males, this activator is distinct from TRY-5 itself. Thus, the control of sperm activation in *C. elegans* involves an unusual form of redundancy. Since sperm cells are transferred from one individual to another, redundant signaling pathways need not operate in the same tissues; instead one sex can compensate for a factor missing in the other.

The expression pattern of TRY-5 was consistent with its role in male sperm activation. A TRY-5::GFP reporter was expressed in the male somatic gonad in structures involved in storing sperm and supporting its transfer to the hermaphrodite: the seminal vesicle, the vas deferens and the intervening valve region.[26,27] Within the valve and vas deferens, TRY-5::GFP localized to globular vesicle-like structures, likely correlating with previously-described secretory globules in these cells.[26,27] Prior to mating, TRY-5::GFP was usually excluded from the lumen of the seminal vesicle, except in *swm-1* mutants where TRY-5::GFP spread into the sperm region and sperm activation was also observed. During mating, TRY-5::GFP was released from the valve and vas deferens and transferred to hermaphrodites. Furthermore, this release occurred in a stereotypical, stepwise fashion from specific tissues, in a manner timed to precede and correspond with transfer of sperm. After spicule insertion, TRY-5::GFP was first released from the vas deferens and transferred to the hermaphrodite; a pause in obvious transfer then occurred; TRY-5::GFP was then released from valve cells and transferred to the hermaphrodite concomitant with movement of sperm. Thus, TRY-5 is a seminal fluid protein that is transferred in a regulated fashion from males to hermaphrodites during mating.

Together, these data suggest a model in which males produce TRY-5 as a seminal fluid factor and transfer it during mating to promote activation of their sperm. Since TRY-5 is a secreted serine protease, it likely cleaves sperm cell-surface proteins, which could alter their activity and thereby induce sperm activation. Aspects of this model remain to be tested directly. Efforts are currently underway in our lab to determine whether TRY-5 is sufficient to activate sperm in vitro and to identify potential targets of TRY-5 from among the other genes identified in the *swm-1* suppressor screen. RNAi is generally inefficient for sperm-expressed genes,[28] and given the range of different activities that can induce sperm activation in vitro, the list of potential targets of TRY-5 is long. Thus, a forward genetic approach seems preferable in the absence of a strong reason to favor a particular subset of candidate molecules.

C. elegans as a Model for Seminal Fluid Biology

Beyond identifying a function for a specific seminal fluid factor, the discovery

of TRY-5 suggests a number of new directions to be taken in seeking to understand why—and how—male nematodes transfer seminal fluid factors, with respect to both specific mechanisms of reproduction and the contribution of these factors to male reproductive fitness. *C. elegans* should be a useful model for studying these processes given the genetic tools available to test the function of specific factors and the ease of imaging their transfer.[25]

Only two seminal fluid proteins have so far been identified in *C. elegans*, but there are likely to be more. Analyses of seminal fluid in other animals and in humans have identified a large number of components that encompass a wide range of biological activities (e.g., refs. 29–33; reviewed in refs. 34 and 35). Using genetic analysis in Drosophila, specific seminal fluid proteins have been shown to act on sperm and on female physiology to promote sperm storage and increase ovulation and egg-laying rates, among other functions.[35] Thus, it would be unusual if *C. elegans* males did not also use such factors to enhance their reproductive success. On the other hand, there is evidence that seminal fluid is somewhat dispensable in *C. elegans*. In experiments addressing the role of seminal fluid in sperm competition, male sperm were removed from donors, washed and activated in vitro, then transferred to recipient hermaphrodites by artificial insemination. These sperm fertilized oocytes and outcompeted hermaphrodite sperm, indicating that seminal fluid has no essential role in sperm competition or indeed in other aspects of male fertility.[36] However, artificial insemination tends to result in low offspring numbers that are produced over a relatively short time period as compared with normal broods. Parameters such as long-term sperm storage and use, egg-laying rates and male-male competition were not addressed in this assay but could have a significant effect on reproductive fitness. Nevertheless, these data suggest that the contribution of seminal fluid to male success might be weak, at least under lab conditions.

It is possible that variation exists among wild strains of *C. elegans* with respect to seminal fluid production or function. Differences among strains have been observed with respect to reproduction as well as other traits (reviewed in ref. 37). Indeed, males from several wild strains show greater success than the standard Bristol N2.[38] Strains have been shown to vary with respect to mating ability, sperm size, and other specific reproductive traits.[39,40] With the exception of *plg-1*,[41] the contributions of seminal fluid to male success have not been evaluated. Expanding such comparative analyses to other nematodes with different modes of reproduction should provide an interesting opportunity to examine seminal fluid evolution.

Finally, the release of TRY-5::GFP from discrete regions of the gonad subdivides the previously-defined "ejaculation" or "sperm transfer" step of male mating behavior (reviewed in ref 42) into a number of distinct events that can be easily observed. As for other regulated behaviors of *C. elegans*, the stereotypical nature of these events should permit analysis of their cellular basis and genetic control. One step will be to elucidate the neuronal circuits involved, candidates for which already exist from descriptions of the male nervous system anatomy [43] and from genetic studies.[42,44] It will also be interesting to determine how neuronal activity feeds into gonadal cell physiology to trigger regulated secretion. By using TRY-5::GFP as a marker to observe seminal fluid transfer, these and other questions can be addressed.

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