A SEMINAL FLUID SERINE PROTEASE, *TRY-5*, SIGNALS

CAENORHABDITIS ELEGANS SPERM ACTIVATION

by

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ABSTRACT

During mating males transfer not only sperm, but also protein and other substances in their seminal fluid. The functions of some seminal fluid components have been identified and these include aiding in sperm motility and protecting sperm from the harsh environment of the female reproductive tract. However, the function of most seminal fluid components are unknown. In this dissertation, I identify a seminal fluid protease, TRY-5, in *Caenorhabditis elegans* and show that it functions in sperm activation, a necessary step in production of functional sperm.

C. elegans is a nematode that consists of two sexes: males, which produce only sperm, and hermaphrodites, which produce both sperm and eggs. During activation, each sperm cell develops a pseudopod, which allows sperm to migrate and fertilize oocytes. Male sperm are stored as nonmotile, nonactivated sperm, and little is known about the extracellular signals required for their activation. SWM-1, a protein with two trypsin inhibitor-like domains and a secretion signal, was identified as an inhibitor of male sperm activation. Here, I show a trypsin-like serine protease, TRY-5, is an extracellular signal for sperm activation necessary for sperm activation in *swm-1* mutant males. While *swm-1* mutant males contain prematurely activated sperm, *swm-1 try-5* males contain nonactivated sperm. Despite being a signal for activation, *try-5* is not required for male fertility, suggesting the existence of additional sperm activation signals. This alternative

signal likely comes from hermaphrodites, since a known hermaphrodite sperm activation pathway is required for male sperm activation in the absence of TRY-5.

To test whether TRY-5 functions in seminal fluid, I examined the localization of TRY-5 protein. A TRY-5::GFP fusion expressed under a *try-5* promoter localized to several tissues of the somatic gonad that are involved in the storage and transfer of sperm, including the vas deferens and valve region. I followed TRY-5::GFP localization during mating and found that TRY-5::GFP was transferred to hermaphrodites, making TRY-5 a likely seminal fluid component. Transfer occurred in a consistent pattern, suggesting this process is well regulated. Overall, these results suggest that TRY-5 is a protease seminal fluid signal of sperm activation.

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CHAPTER 1

INTRODUCTION

Infertility, defined as being unable to conceive despite trying for one year, affects as many as 24% of couples in the United States (Greenhall and Vessey, 1990). This definition does not consider whether the inability to conceive is due to male factors, female factors, or a combination of the two. However, it has been estimated that 40-60% of cases have a male component (reviewed in Niederberger, 2007). The causes of male infertility are poorly understood. When investigating cases of inability to conceive, attention is placed mainly on sperm number, motility, and appearance (World Health Organization, 2010). These factors fail to explain approximately 25% of male infertility cases (Lipshultz, 1997). A better understanding of the other components of transferred semen may provide opportunities for treating male infertility.

Sperm motility is of particular interest in studying fertility. In internally-fertilizing animals, sperm must migrate through the female reproductive tract to reach an oocyte. Therefore, improper regulation of motility can negatively impact sperm success. For instance, late, reduced, or no motility in sperm can reduce fertility, since fewer or no sperm would be able to reach an oocyte (World Health Organization, 2010). Since motility plays such a key role in male fertility, much study has been dedicated to understanding how it is regulated.

Mammals and *Drosophila* share a similar mode of reproduction; both are gonochoristic (male-female). In mammals, including humans, sperm become active in the epididymis (reviewed in Gatti et al., 2004), and then become even more active after transfer in a process termed hyperactivation (reviewed in Salicioni et al., 2007). In *Drosophila*, sperm become active within cysts shortly after development (Fabrizio et al., 1998), but no difference in motility has been observed after transfer (Perotti, 1969). In contrast to mammals and *Drosophila*, *C. elegans* is an androdioecious species; it has males that produce only sperm and hermaphrodites that produce both sperm and oocytes. In this species, within the male, sperm are stored in a nonmotile state, and sperm gain motility during mating in a process termed sperm activation (reviewed in L'Hernault, 2006). In hermaphrodites, sperm activate within the spermathecae, the sites of sperm storage and fertilization. If sperm do not activate, fertilization does not occur (L'Hernault et al., 1988). In this introduction, I will describe common mechanisms that exist to regulate sperm development and motility in mammals, including humans, as well as the model organisms *Drosophila melanogaster* and *C. elegans*, focusing on seminal fluid components.

Changes in motility over time

Between species, sperm vary greatly in their appearance and how they generate motility. In humans, other mammals, and *Drosophila*, a flagellum on the sperm generates force to move the cell. In nematodes, sperm have a significantly different appearance from flagellated sperm. Instead of having a flagellum, they have a pseudopod, and *C. elegans* sperm move by crawling in an amoeboid fashion (reviewed in L'Hernault, 2006).

Motility is not constant during a sperm cell's existence but instead is tightly regulated. In mammals, after the overall sperm structure has developed, sperm move to the epididymis. Within the epididymis, the seminal fluid that surrounds the sperm changes significantly. Here, sperm gain initial motility as they undergo further maturation and are stored until mating occurs (reviewed in Sullivan et al., 2005). The signals responsible for this increase in motility are unknown; however, this process has been shown to be dependent on cAMP, calcium signaling, and increased levels of protein phosphorylation (reviewed in Gatti et al., 2004).

Once sperm have been transferred to the female, motility changes again. Sperm become hyperactivated as their tails beat more strongly and form more bends. In mammals, hyperactivation is one step of capacitation, during which sperm gain the ability to fertilize an oocyte. Hyperactivation occurs in the uterus or the oviduct, possibly in response to signals from both the oviduct and the egg (reviewed in Suarez, 2008). While it is apparent that extracellular signals regulate mammalian sperm motility, those signals remain poorly understood.

In *Drosophila* sperm development occurs differently than in mammals. Similar to mammals, during spermatogenesis, sperm gain the structures necessary for motility and sperm begin to move shortly after development, but before mating occurs (Fabrizio et al., 1998). However, once sperm are transferred to the female, no further morphological changes are observed, either immediately or several days after mating (Perotti, 1969). Movement of sperm within the female occurs continuously, with sperm constantly moving between the sperm storage organs and the uterus (Yang and Lu, 2011). While

Drosophila sperm motility has been studied on the level of its role in sperm competition, less attention has been paid to regulation of motility.

During *C. elegans* sperm activation, many physiological changes occur within sperm. Overall, cell morphology changes from being round to having a pseudopod, polarizing the cell in the process. Membranous organelles fuse with the plasma membrane, releasing proteins into the extracellular matrix and introducing new proteins to the sperm surface (Washington and Ward, 2006). Within the pseudopod major sperm protein (MSP), the only cytoskeleton protein in sperm, polymerizes, allowing for the formation of the pseudopod and for movement to occur (Smith, 2006). Sperm also gain the ability to fertilize oocytes.

Intracellular regulators of sperm motility

Since sperm motility in mammals and *C. elegans* changes as the cells enter different tissues, changes in motility are likely to be regulated. In mammals, a significant change to motility occurs during capacitation, during which sperm become hyperactivated and become fully competent for fertilization. Several proteins have been found to have a role in regulation of sperm motility. One such protein is hydrogen voltage-gated channel 1 (Hv1), which has roles in proton transport. Hv1 has been implicated in increasing pH levels in human sperm after transfer, but it is not expressed in mouse sperm (Lishko et al., 2010). The increase in intracellular pH is hypothesized to open CatSper channels (cation channel, sperm associated), increasing levels of intracellular Ca^{2+}, which is thought to increase motility (reviewed in Lishko and Kirichok, 2010). pH has been implicated in the process of *C. elegans* sperm activation; as sperm activate, intracellular pH increases (Ward et al., 1983). No functional relevance

for this increase has been shown. Another protein that regulates motility in mouse sperm and is conserved in humans is A-kinase anchor protein 4 (AKAP4). This protein localizes cyclic AMP dependent kinases. Mice mutant for *Akap4* produce sperm that have significantly reduced motility, suggesting that proper regulation of phosphorylation is necessary for sperm motility (Miki et al., 2002). cAMP has also been shown to increase sperm motility in a concentration-dependent manner (Gorus et al., 1982; Liu et al., 2003). cAMP has been suggested to stimulate protein kinase A (PKA), which then phosphorylates other proteins that signal capacitation (Krapf et al., 2010). In *C. elegans*, two different kinases have been shown to be necessary for normal sperm activation, *spe-8* (L'Hernault et al., 1988) and *spe-6* (Muhlrad and Ward, 2002). These various means of signaling capacitation or motility show that this is a tightly regulated process.

Several molecules necessary for human sperm motility are also present in *Drosophila*; however their contributions to sperm motility may differ. cAMP may have roles in male fertility. A deficiency that reduced cAMP phosphodiesterase activity resulted in infertile males; however, this deficiency also removed additional genes (Kiger, 1977). Phosphatases also have an indirect role in *Drosophila* sperm motility. Phosphatidylinositol 4,5-bisphosphate (PI4,5P) is necessary for the proper formation of flagella, since overexpression of PI4,5P phosphatases result in sperm that do not properly form flagella (Wei et al., 2008). However, no direct relationship between phosphatases and motility has been shown. In addition, Ca^{2+} has been shown to have roles in mediating sperm motility, as a loss-of-function cation channel (*Pkd2*) mutation results in sperm that are unable to enter sperm storage organs in the female after transfer (Yang and Lu, 2011).

In *C. elegans*, several regulators of sperm motility have been identified. Some of these components are conserved in mammals, but others are found only in nematodes. Activation factors include a group of five genes, termed the *spe-8* group, consisting of *spe-8*, *-12*, *-19*, *-27*, and *-29*. Most of these genes encode novel proteins, except for *spe-8*, which encodes a tyrosine kinase (Geldziler et al., 2005; L'Hernault, 2009; L'Hernault et al., 1988; Minniti et al., 1996; Nance et al., 2000; Nance et al., 1999). Some of the SPE-8 group proteins have transmembrane domains, suggesting that they are present at the surface of the sperm cell. These genes are a group, since a mutation in any one of these genes results in the same phenotype: hermaphrodites that are sterile because their sperm do not activate. Interestingly, mutant males are fully fertile and are capable of rescuing hermaphrodite fertility, which suggests that a separate activator might be present in the male seminal fluid. *In vitro*, wild-type male sperm activates in the presence of Pronase, a mixture of proteases. *spe-8* group mutant sperm do not activate in the presence of Pronase, although they can be fully activated by triethanolamine (TEA), a weak base that also activates sperm *in vitro* (Shakes and Ward, 1989). These results suggest that proteases may act as a male-derived activation signal. An additional kinase, *spe-6*, necessary for proper regulation of sperm activation has been identified downstream of the *spe-8* group. This gene encodes a casein kinase I. *spe-6* functions during multiple steps of sperm development: early in sperm development, *spe-6* is necessary for the packaging of MSP in sperm (Varkey et al., 1993). After spermatids develop, *spe-6* is required for the inhibition of sperm activation, since males with certain alleles of *spe-6* contain activated sperm (Muhlrad and Ward, 2002).

Seminal fluid production

Males transfer sperm in a medium termed seminal fluid. Since seminal fluid components change during sperm development (reviewed in Sostaric et al., 2008) or are added during sperm transfer, this fluid would be an ideal candidate for containing sperm regulators.

In mammals, several tissues produce seminal fluid: the vas deferens, the prostate, and the seminal vesicles (reviewed in Bronson, 2011). Seminal fluid from the fruit fly *Drosophila melanogaster* is also produced by several tissues. These secretory tissues include the accessory glands, seminal vesicles, ejaculatory duct, ejaculatory bulb, and testes (reviewed in Avila et al., 2011). As in mammals, some of these tissues modify seminal fluid during mating and have roles in maximizing fertility.

Much less is known about the source, or even existence, of seminal fluid in *C. elegans*. The only previously identified seminal fluid protein was PLG-1, a mucin that forms a mating plug over the hermaphrodite's vulva (Hodgkin and Doniach, 1997; Palopoli et al., 2008). *plg-1* was shown to be expressed in the vas deferens, a tube through which sperm pass during mating. However, the vas deferens represents only one of three tissues that sperm come in contact with after finishing meiosis; the other two are the seminal vesicle, the storage site for sperm, and the valve region, a structure that regulates the release of sperm during mating (Lints and Hall, 2009).

Seminal fluid regulators of fertility and motility

In mice and humans, many components of seminal fluid have been identified. Proteins and other components have been identified using mass spectroscopy (Kumar et al., 2009; Utleg et al., 2003). Some of the earliest identified compounds protect sperm

from the harsh environment encountered after transfer and provide nutrients to sperm.

The highest concentration of a number of components derived from the prostate have

been identified as citric acid, spermine, zinc, prostate specific antigen (PSA), and

prostatic acid phosphatase (reviewed in Burden et al., 2006). The precise roles of these

factors in fertility are not known. For instance, citric acid will bind metal ions, such as

zinc, which inhibits several proteases in sperm (reviewed in Burden et al., 2006; Veveris-

Lowe et al., 2007). However, the binding of metal ions by citric acid and subsequent

inhibition of proteases havesot been shown to have functional relevance in altering a

sperm cell's fertilization ability or motility. Furthermore, many components of

mammalian seminal fluid may have roles in modulating female immunity. For example,

TGFβ and prostaglandins, known immunosuppressors, are present at a high concentration

in seminal fluid. The high level of these molecules may protect sperm from the female

immune response (reviewed in Bronson, 2011), although a clear function has not been

demonstrated.

A class of proteins that has been shown to have a function in mammalian seminal

fluid regulating motility is the semenogelins. Semenogelins are proteins that form

seminal clots after mating and inhibit sperm motility. These clots persist for several

minutes before being degraded by proteases. Once the clots have liquefied, sperm

motility once again increases. Temporary inhibition of motility is thought to increase

fertility, since males with a high proportion of prematurely hyperactivated sperm have

reduced fertility (reviewed in de Lamirande, 2007). A direct connection between the

reduced motility and fertility has not been shown. Within seminal fluid, exosomes, small

vesicles that are 50 to 500 nm in size, are present (Ronquist and Brody, 1985). These

exosomes are released from epididymis and prostate and are called epididymosomes and prostasomes, respectively. Prostasomes can fuse with sperm, allowing for modification of the sperm membrane and the proteins that it contains (Ronquist et al., 1990). Epididymosomes, small vesicles, are used to transfer proteins and lipids to the sperm membrane. One such protein is a cholesterol transporter, HE1 (Thimon et al., 2008).

Perhaps the best characterized organism for seminal fluid compounds and their functions is *D. melanogaster*. Components of seminal fluid have been determined using multiple methods, including detecting expressed sequence tags in tissues that express seminal fluid and using mass spectroscopy to identify proteins that have been transferred during mating (reviewed in Avila et al., 2011). The functions of proteins present in seminal fluid are better understood in flies due to the genetic techniques available.

Seminal fluid proteins in the fly have been shown to have diverse functions. While some components have been identified as having roles in sperm storage, other factors affect female behavior or physiology. One of the most well-characterized seminal fluid proteins is sex peptide (Acp70A), a 36 amino acid protein. Functions for sex peptide include altering gene expression in the female, increasing female egg production, and reducing female receptivity to remating (Chen et al., 1988; Peng et al., 2005). For long-term female responses to mating, sex peptide must associate with sperm, a process that is modulated by four other seminal fluid proteins: CG9997, CG1652, CG1656, and CG17575. After sperm are stored in the female, sex peptide is necessary to release sperm from the sperm storage organs (Ram and Wolfner, 2007). Another well-characterized seminal fluid protein is ovulin (Acp26Aa), which also stimulates egg production (Herndon and Wolfner, 1995). For this stimulation to occur, ovulin must be cleaved by a

protease, CG11864 (Ravi Ram et al., 2006). A third well-characterized protein is Acp36DE, which is necessary for the progression of changes in the female sperm storage organ. These changes are thought to aid in sperm storage, such that fewer sperm from Acp36DE mutant males are stored in the female after mating (Avila and Wolfner, 2009). Despite the identified roles for these proteins, the mechanisms that affect the observed changes are unknown. In addition, no protein has been shown to act directly on sperm to modulate sperm motility.

In the worm *C. elegans*, little is known about seminal fluid. Prior to this dissertation, *plg-1* was the only suspected seminal fluid component. While *plg-1* was shown to be expressed in the vas deferens of worms, it was not been shown directly to be transferred. Instead, mating plugs were labeled using a lectin that labels a carbohydrate modification that is common to mucins (Palopoli et al., 2008).

Despite this lack of knowledge about specific components of seminal fluid, there is some evidence for a role of seminal fluid in worm fertility. One potential role is regulation of sperm motility. Sperm activation can be signaled *in vitro* using a variety of compounds. These compounds include Pronase, a mixture of proteases; triethanolamine (TEA) a weak base; and monensin, an ionophore (Nelson and Ward, 1980; Shakes and Ward, 1989; Ward et al., 1983). Combined with the observation that spermatids lack the machinery necessary to make proteins (Ward, 1986), activation of sperm by these components suggests that extracellular compounds *in vivo* signal sperm activation. These signals could come from either the hermaphrodite or the male. If the signals are male based, the signal would likely be in seminal fluid, which is released at mating. However, evidence exists that seminal fluid might not be necessary for full male fertility. If sperm

are removed from the seminal vesicle, washed, and injected into a hermaphrodite's uterus, sperm activate and successfully fertilize the hermaphrodite's oocytes (LaMunyon and Ward, 1995). Due to the difficulty of introducing sperm into a hermaphrodite, it was not possible in these experiments to get an estimate of the percentage of sperm that fertilized an oocyte. Thus, while seminal fluid is not strictly necessary for male fertility, seminal fluid might still improve male fertility.

Seminal fluid proteases

Proteases have been shown to be common major components of seminal fluid in both flies and mammals. However, protease involvement in fertility has been difficult to study in mammals due to redundancy. For instance, there are at least 11 kallikrein proteases in human seminal fluid, ten of which are predicted to have trypsin-like activity. The exception is prostate-specific antigen (PSA), which has chymotrypsin-like activity and cleaves preferentially after tyrosine. PSA has been implicated in several aspects of seminal fluid biology; most significantly in the cleavage of semenogelins, where PSA cleavage of semenogelins after mating degrades these proteins, so that sperm regain motility. Furthermore, the products of semenogelin cleavage have been implicated in such processes as signaling an increase in sperm motility and increasing antibacterial activity (reviewed in Veveris-Lowe et al., 2007). However, this process may also be dependent on other kallikreins, as they are also able to cleave semenogelins.

Fly seminal fluid has also been found to have multiple proteases. Some of these proteases are major components of seminal fluid. One protease, CG11864, has been identified as being necessary for the processing of ovulin and Acp36DE (Ravi Ram et al., 2006). However, CG11864 must be activated, which is accomplished by another

protease, seminase (CG10586). Without CG11864 or seminase, an increase in egg laying is delayed. Without seminase, sperm release, but not sperm storage, from the spermatheca is reduced (LaFlamme et al., 2012). This is the first protease cascade identified in seminal fluid. However, 15 proteases are in seminal fluid, suggesting that proteases may have more roles in modulating fertility (Findlay et al., 2008).

Seminal fluid protease inhibitors

Where there are proteases, protease inhibitors are often present to regulate their activities. Premature or inappropriate cleavage of proteins can produce negative effects. For instance, a protease inhibitor in mice, seminal vesicle protease inhibitor (SVI), associates with the acrosomal region of sperm (Irwin et al., 1983). This association has suggested that protease inhibitors might block the acrosome reaction from occurring prematurely, since inhibition of SVI with an antibody results in the acrosome reaction (Aarons et al., 1991).

A seminal fluid protease inhibitor has also been found in flies, Acp62F. While this inhibitor has not been shown to have a function in fertility, it has been found in the female's hemolymph after mating (Lung and Wolfner, 1999). Ectopic expression of this inhibitor shortens the fly's life, and thus has been proposed to signal the shortened lifespan of females after mating (Lung et al., 2002). Thirteen additional protease inhibitors are present in seminal fluid, which suggests that tight regulation of proteases occurs in seminal fluid (Findlay et al., 2008).

In *C. elegans*, a protease inhibitor that is likely to be associated with seminal fluid proteins has been characterized. This protease inhibitor, SWM-1, encodes a protein with two trypsin inhibitor-like domains. The pattern of expression for *swm-1* has not been

determined; however, the SWM-1 protein does have a secretion signal and seems to be expressed in somatic tissue (Stanfield and Villeneuve, 2006). *swm-1* mutant sperm are activated, suggesting that SWM-1 protein inhibits a protease signal of sperm activation.

Summary

Seminal fluid has been shown to have important roles in male fertility, but the roles of seminal fluid remain poorly understood. Functional redundancy within mammalian seminal fluid has hindered understanding of its individual components of seminal fluid. While some seminal fluid components, such as Hv1 and CatSper channels, are not conserved from humans to model organisms, model organism have allowed for understanding of how different proteins can function in seminal fluid. The classes of proteins involved in sperm fertility are conserved; however, they frequently have different roles in ensuring fertility when compared between organisms. Thus, study of model organism seminal fluid may provide an understanding of human fertility and how sperm motility can be regulated.

In this dissertation I describe a protease, TRY-5, that has roles in male sperm activation in *C. elegans*. I have shown that this protein is necessary for the *swm-1* premature sperm activation phenotype. The TRY-5 protein acts as a likely signal for the male sperm activation pathway, since TRY-5 is capable of bypassing the SPE-8 group activation pathway. TRY-5 is a seminal fluid protein that is transferred during mating. A transgenic copy of a TRY-5 and GFP fusion allows for observing the transfer of TRY-5. With this transgene, an additional tissue of seminal fluid origination has been found: TRY-5 is first secreted from the vas deferens and then the valve region, followed by sperm transfer. These results suggest a model in which TRY-5 is a seminal fluid protease

and acts as a male sperm activator that is partially redundant with the unidentified

hermaphrodite activator.

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CHAPTER 2

TRY-5 IS A SPERM-ACTIVATING PROTEASE IN

CAENORHABDITIS ELEGANS SEMINAL FLUID[1]

Abstract

Seminal fluid proteins have been shown to play important roles in male

reproductive success, but the mechanisms for this regulation remain largely unknown. In

Caenorhabditis elegans, sperm differentiate from immature spermatids into mature,

motile spermatozoa during a process termed sperm activation. For *C. elegans* males,

sperm activation occurs during insemination of the hermaphrodite and is thought to be

mediated by seminal fluid, but the molecular nature of this activity has not been

previously identified. Here we show that TRY-5 is a seminal fluid protease that is

required in *C. elegans* for male-mediated sperm activation. We observed that TRY-

5::GFP is expressed in the male somatic gonad and is transferred along with sperm to

hermaphrodites during mating. In the absence of TRY-5, male seminal fluid loses its

potency to transactivate hermaphrodite sperm. However, TRY-5 is not required for either

hermaphrodite or male fertility, suggesting that hermaphrodite sperm are normally

activated by a distinct hermaphrodite-specific activator to which male sperm are also

competent to respond. Within males, TRY-5::GFP localization within the seminal

vesicle is antagonized by the protease inhibitor SWM-1. Together, these data suggest

that TRY-5 functions as an extracellular activator of *C. elegans* sperm. The presence of

TRY-5 within the seminal fluid couples the timing of sperm activation to that of transfer

of sperm into the hermaphrodite uterus, where motility must be rapidly acquired. Our

results provide insight into how *C. elegans* has adopted sex-specific regulation of sperm

motility to accommodate its male-hermaphrodite mode of reproduction.

Author summary

Sexual reproduction requires the generation of highly specialized gametes, eggs

and sperm, that must encounter one another and fuse together to form a zygote. Males

provide not only sperm but also seminal fluid, which contains a variety of factors that

promote male fertility through effects on sperm and on female physiology. We have

identified a *C. elegans* seminal fluid protease, TRY-5, which regulates sperm activation,

the process by which immature spermatids complete their differentiation to a motile form

capable of fertilizing an oocyte. We observed release of TRY-5 that coincided with

transfer of sperm, coupling the onset of sperm motility to transfer during mating.

Although TRY-5 functions only in males, both male and hermaphrodite sperm are

capable of responding to it. TRY-5 is not required for fertility, and we propose that a

hermaphrodite activator compensates in its absence. Our results reveal how sperm

development can be differentially modulated by males and hermaphrodites to promote

fertility in each sex and identify a novel function for a seminal fluid protein.

Introduction

A general feature of sexual reproduction is the generation of motile sperm that can navigate to an egg. To assist this process, males transfer their sperm along with seminal fluid, which enhances their reproductive success in a variety of ways (reviewed in Pitnick et al. (2009) and Poiani (2006)). Seminal fluid factors promote sperm survival, motility and fertilizing ability both by directly interacting with sperm and by interacting with tissues of the female to make her reproductive tract a more permissive environment. These factors include seminal fluid-specific proteins, a variety of hormones, and energy sources (Poiani, 2006). In mammals, roles for seminal fluid factors include the regulation of sperm motility and capacitation and the modulation of immune function (Poiani, 2006; Yoshida et al., 2008). Extensive analysis in *Drosophila* has identified many seminal fluid proteins and uncovered roles for several of these factors in sperm storage, sperm competition, female reproductive behavior and physiology, and other processes (Avila et al.). Due to their potential for influencing reproductive success, components of seminal fluid represent a forum for both conflict and cooperation between the sexes (Pitnick et al., 2009; Wolfner, 2009).

The androdioecious nematode *Caenorhabditis elegans* provides an opportunity to analyze sperm development and function in a context where both sexes produce sperm and can differentially regulate gamete function to promote their fertility. Hermaphrodites are self-fertilizing; during development, they produce a store of "self-" sperm, which can be used to fertilize their eggs. Males mate with and transfer sperm to hermaphrodites. Males are not required for reproduction to occur, and in their absence self-sperm are used

with extremely high efficiency; more than 99% of self-sperm are used. However, if male

sperm are present, then they preferentially fertilize eggs (Ward and Carrel, 1979).

C. elegans sperm, like those of other nematodes, lack flagella; instead, they move

by crawling using a pseudopod (Justine, 2002; Smith, 2006; Ward, 1977; Ward and

Carrel, 1979). Motility is acquired during sperm activation, a process analogous to

spermiogenesis in flagellate sperm, in which haploid spermatids undergo a dramatic

cellular rearrangement to become competent for both directional motility and fertilization

of an oocyte (Ward and Carrel, 1979). While most aspects of sperm development are

similar in males and hermaphrodites, the timing and context of activation differ in the

two sexes. In hermaphrodites, spermatids activate when they move into the

spermathecae, regions of the gonad where sperm are stored and fertilization occurs. In

males, sperm are stored in a nonactivated form and become activated after mating and

transfer to a hermaphrodite ((Ward and Carrel, 1979) and unpublished observations).

Sperm also can be activated *in vitro* in response to treatment with a variety of factors,

including an ionophore (monensin), proteases (Pronase), a weak base (triethanolamine/

TEA), and an ion channel inhibitor (4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid/

DIDS) (Machaca et al., 1996; Nelson and Ward, 1980; Shakes and Ward, 1989; Ward et

al., 1983). This ability, together with the observation that sperm generally activate *in vivo*

in response to a change in location, suggests that activation is controlled by extracellular

signals.

Genes that regulate sperm activation show distinct requirements in

hermaphrodites and males. The activity of a set of five genes termed the "*spe-8* group"

(*spe-8, -12, -19, -27,* and *-29*) is required specifically for hermaphrodites to activate their

self-sperm; hermaphrodites mutant for any one of these genes are self-sterile, while

mutant males are fertile (Geldziler et al., 2005; L'Hernault et al., 1988; Minniti et al.,

1996; Muhlrad and Ward, 2002; Nance et al., 2000; Nance et al., 1999; Shakes and Ward,

1989). Mating of *spe-8* group mutant hermaphrodites with males results in self-sperm

activation ("transactivation") and can restore self-fertility, suggesting that males provide

their own activator to which *spe-8* group hermaphrodite sperm can respond (Shakes and

Ward, 1989). *spe-8* group functions are dispensable for production of this activator, since

both wild-type and *spe-8* group males are competent for transactivating hermaphrodite

sperm. While these analyses indicate that there are differences in the intracellular

pathways by which sperm are activated in the two sexes, the functions of individual

activation genes are not strictly limited to a specific sex. *spe-8* group mutant male sperm

show some defects, failing to activate in response to Pronase *in vitro* (Geldziler et al.,

2005; Minniti et al., 1996; Nance et al., 2000; Shakes and Ward, 1989). Furthermore,

some *spe-8* group activity is likely required for sperm to transactivate, since animals

harboring *spe-8* group null alleles appear to be insensitive to male activator (Geldziler et

al., 2005; Nance et al., 2000). While most analysis has focused on hermaphrodite sperm

activation, a gene with a male-biased effect has been identified as well. Activity of an

extracellular trypsin inhibitor-like protein, SWM-1, is required in males to prevent

premature activation from occurring prior to mating, and *swm-1* mutant males are

infertile due to failure to transfer activated sperm (Stanfield and Villeneuve, 2006).

swm-1 activity is dispensable in hermaphrodites, though loss of *swm-1* improves fertility

in a sensitized *spe-8* group mutant background (Stanfield and Villeneuve, 2006). The

finding that a protease inhibitor regulates activation in males, combined with the ability

of proteases to activate sperm *in vitro*, suggested that protease activity could signal

activation *in vivo*. However, the endogenous activator has not been identified as yet in

either sex.

Here, we report the identification of a trypsin-family serine protease, TRY-5,

which has the properties expected of a male sperm activator. Loss of *try-5* suppresses

mutations in *swm-1*. Furthermore, during mating, TRY-5 is released from the somatic

gonad and transferred along with sperm, thus coupling the onset of sperm motility to the

time of their transfer to a hermaphrodite. Within the male gonad, TRY-5 activity must be

held in check to ensure male fertility. Strikingly, TRY-5 is not required for male fertility,

but strains lacking both *try-5* and *spe-8* group activation functions are totally sterile,

confirming that while male and hermaphrodite sperm motility is induced by distinct

signals, the two pathways are redundant. In summary, TRY-5 is the first factor

demonstrated to be a transferred component of seminal fluid in *C. elegans*, where it plays

a key role in male-specific regulation of sperm function.

Results

C. elegans male sperm activation is regulated by a protease

In wild-type males, sperm are stored in the inactive form within the seminal

vesicle (Figure 2.1A) and become activated after transfer to a hermaphrodite ((Ward and

Carrel, 1979) and unpublished data). Mutations in the secreted protease inhibitor SWM-1

result in premature sperm activation within males (Figure 2.1B, Figure 2.S1A, (Stanfield

and Villeneuve, 2006)). We predicted that loss of activation-promoting factors should

suppress this phenotype. To identify such factors, we performed genetic screens for

suppressors of premature sperm activation caused by the partial loss-of-function alleles

swm-1(me86) or *swm-1(me66)* (G.M.S., unpublished; (Stanfield and Villeneuve, 2006)).

Among the *swm-1* suppressor mutants, we identified three alleles of the serine protease

gene *try-5* (Figure 2.1E and 2.1F). We subsequently obtained *tm3813,* a deletion

affecting the 5' end of the *try-5* coding region (gift of S. Mitani, National Bioresource

Project, Japan), and showed that it also suppressed *swm-1(me86)* (Figure 2.1F).

Suppression of the premature activation phenotype in *swm-1 try-5* double mutants was

rescued by a genomic fragment containing the full-length *try-5* gene (Figure 2.1G, Tables

2.S1, 2.S2, 2.S3 and data not shown), confirming that *try-5* was responsible for this

effect.

In parallel to our forward genetic screen, we also tested individual serine proteases for

a role in sperm activation. We used RNA interference to reduce the function of

individual protease genes in a *swm-1* mutant background and screened for effects on

premature activation in males. Among the tested proteases, only reduction of *try-5*

resulted in strong suppression (Materials and Methods and data not shown), consistent

with our finding that *try-5* is a regulator of sperm activation.

Based on conservation of its sequence and domain structure (Marchler-Bauer et al.,

2009) with those of the trypsin-like superfamily, *try-5* is predicted to encode a trypsin-

class serine protease. This family of proteases contains numerous members in eukaryotes

and regulates many processes, including blood coagulation, developmental signaling and

fertilization (Di Cera, 2009). Specific residues that form the protease active site are

conserved in TRY-5, and the presence of a signal sequence on the N terminus of the

protein suggests that it is secreted (Figure 2.S2). While TRY-5 has clear orthologs in

other closely related nematodes, it is divergent from serine proteases in more distantly

related species (data not shown). In addition, its substrate-binding region is divergent

from those of trypsin family members with characterized substrate specificities (Perona

and Craik, 1995).

We initially identified *try-5* using partial loss-of-function alleles of *swm-1*. To

determine whether mutations in *try-5* are capable of suppressing a *swm-1* null, we

examined animals harboring both the null allele *swm-1(me87)* and an allele of *try-5*. We

found that whereas *swm-1(me87)* mutant males contain activated sperm (Stanfield and

Villeneuve, 2006), *swm-1(me87) try-5(jn2)* and *swm-1(me87) try-5(tm3813)* males

contained nonactivated sperm like those found in the wild type or in a *try-5* mutant

((Stanfield and Villeneuve, 2006), Figure 2.1A-2.1D and 2.1F, Figure 2.S1).   In

summary, these results indicate that the protease TRY-5 is responsible for the premature

sperm activation and associated loss of fertility that occur in *swm-1* mutant males and

suggest that the function of SWM-1 is to inhibit TRY-5 activity within the male.

Male sperm can activate in the absence of *try-5*

To see if *try-5* is required for male sperm to activate, we assessed the ability of

try-5 mutant sperm to respond to treatments that bypass normal activation signals. Wild-

type sperm can be activated *in vitro* by treatment with any of a variety of compounds

(Machaca et al., 1996; Nelson and Ward, 1980; Shakes and Ward, 1989; Ward et al.,

1983). Since TRY-5 is predicted to be a protease, we first assayed the ability of *try-5*

mutant spermatids to activate in response to Pronase treatment. In the absence of

Pronase, both wild-type and *try-5* mutant sperm remained nonactivated (Figure 2.2A and

2.2B). Within 5 to 10 min after addition of Pronase, the majority of sperm cells

developed a pseudopod, consistent with activation (Figure 2.2A and 2.2C, Video

PronaseAct). These cells were capable of motility, as they were observed crawling across the microscope slide (note altered positions of some cells in Figure 2.2B vs. Figure 2.2C). There was no significant difference in either the level of activation (Figure 2.2A; P=0.89, Student's t test) or the rate of activation (data not shown) of *try-5* mutant sperm as compared to the wild type. We then tested the ability of *try-5(tm3813)* spermatids to activate in response to treatment with a second known activator, the weak base TEA. When treated with TEA, *try-5* mutant spermatids activated at levels similar to wild-type sperm (data not shown). Thus, *try-5* is not required for sperm activation initiated *in vitro* either by exogenous proteases or TEA. This result distinguishes *try-5* mutants from the previously-characterized *spe-8* group mutants, for which sperm activate normally when treated with TEA, but arrest at a partially-activated, "spiky" stage in response to Pronase (Geldziler et al., 2005; Minniti et al., 1996; Nance et al., 2000; Shakes and Ward, 1989).

We next determined if *try-5* is required for activation induced by loss of the intracellular activation inhibitor *spe-6*. SPE-6 is a sperm casein kinase 1-like protein that functions at two points during spermatogenesis: during spermatogenic cell divisions (Varkey et al., 1993) and later during sperm activation (Muhlrad and Ward, 2002). Specific mutations in *spe-6* allow spermatogenesis to occur but lead to premature sperm activation in males, a phenotype that is thought to be independent of extracellular signaling (Muhlrad and Ward, 2002). To determine whether *try-5* function is required for the premature sperm activation phenotype of *spe-6*, we assayed sperm activation in *spe-6(hc163); try-5(tm3813)* and *spe-6(hc163); try-5(jn2)* mutant males. We found that, like *spe-6(hc163)* mutant males, *spe-6(hc163); try-5* males contained activated sperm (Table 2.1) and their appearance was indistinguishable from that of the *spe-6* mutant (data not

shown).   Thus, TRY-5 activity does not function downstream of the sperm protein SPE-6. Together, the ability of *try-5* sperm to activate in response to either *in vitro* activators or loss of an intracellular inhibitor indicates that TRY-5 is not required for the subcellular rearrangements of sperm activation. Rather, these data suggest a regulatory role for this protease in signaling sperm to initiate the activation process.

try-5 is not required for fertility

Since activation is necessary to generate mature, motile spermatozoa that are competent for fertilization, failure to activate results in infertility. If *try-5* is required for sperm activation, then loss of *try-5* should result in decreased fertility. To test this idea, we assayed fertility in *try-5* and *swm-1(me87) try-5* hermaphrodites and males, using the *try-5* alleles *jn2* and *tm3813*. In self-fertilizing hermaphrodites, sperm is the limiting gamete for offspring production; nearly every self-sperm in a hermaphrodite will fertilize an oocyte (Ward and Carrel, 1979), so the total self-brood size is a sensitive measure of the number of functional, activated sperm produced. We found no significant difference between the number of progeny produced by *try-5* or *swm-1 try-5* mutant hermaphrodites as compared to wild-type and *swm-1* controls (Figure 2.3A, Figure 2.S3A). Thus, *try-5* is not required for hermaphrodite sperm activation or fertility.

We next measured male fertility in crosses of individual males to *spe-8(hc40); dpy-4* recipient hermaphrodites. While there was a great deal of variation in the number of cross progeny produced even by wild-type males, as observed previously (Ward and Miwa, 1978), *try-5* mutant males showed a high level of fertility and no significant difference with the wild type was observed (Figure 2.3B). In addition, *swm-1 try-5* males showed high levels of fertility, in some cases equivalent to that of the wild type (Figure

2.S3B), along with suppression of the *swm-1* transfer defect (data not shown). While our assays detected no obvious fertility defects in *try-5* animals, it is possible that they might exhibit reduced fertility in other situations, *e.g.*, outside the laboratory or under conditions of sperm competition. However, these results suggest that *try-5* is not required for sperm activation or other aspects of fertility in either sex.

try-5 and the _spe-8_ group define two pathways

for sperm activation

Although *try-5* is not required for either male or hermaphrodite fertility, there is previous evidence for distinct pathways of sperm activation in males vs. hermaphrodites (L'Hernault et al., 1988; Shakes and Ward, 1989), raising the possibility that the effect of *try-5* loss is masked by functional redundancy. Therefore, we tested genes in the hermaphrodite pathway for redundancy with *try-5*. The activities of a set of five genes termed the "*spe-8* group" (*spe-8*, *-12*, *-19*, *-27*, and *-29*) are required for self-sperm activation in the hermaphrodite but not for activation of male sperm (reviewed in (L'Hernault, 2006)). To test whether *try-5* and the *spe-8* group function in independent, redundant activation pathways, we assayed sperm activation and male fertility in worms lacking both *try-5* and *spe-8* group activity, using the *spe-8* group mutations *spe-27(it110)* and *spe-29(it127)*. While *spe-27* mutant males are fertile and capable of generating cross progeny, we found that *spe-27; try-5* mutant males were completely infertile (Figure 2.3C). Similarly, while *spe-29* mutant males are fertile, *spe-29; try-5* fertility was greatly reduced as compared to the wild type (Figure 2.3C).

To investigate the cause of this infertility, we labeled males with MitoTracker (Chen et al., 2000) and crossed them to unlabeled recipient hermaphrodites to assay

sperm transfer and migration (Stanfield and Villeneuve, 2006). We found that *spe-27; try-5* males were able to transfer sperm to hermaphrodites, but the transferred sperm did not migrate. Similarly, for *spe-29; try-5* males, we observed only rare instances of successful migration (Table 2.S4 and data not shown). To determine if the migration defect was due to improper activation or a defect in migration after sperm activation, we dissected hermaphrodites immediately after their mating to *spe-27*; *try-5* males and examined transferred, MitoTracker-labeled sperm. We found that whereas *spe-27* sperm activate within 15 minutes after transfer to a hermaphrodite, *spe-27*; *try-5* sperm fail to activate (data not shown). Thus, *spe-27; try-5* males are infertile due to failure to activate sperm upon transfer to hermaphrodites. Our findings of residual fertility and sperm migration in *spe-29; try-5* males are consistent with previous observations (Nance et al., 2000) that the single known mutation in *spe-29* leads to a weaker phenotype as compared to known null mutations in other *spe-8* group genes. These results suggest that *try-5* activity is the source of fertility in *spe-8* group mutant males; *i.e.*, the *spe-8* group and *try-5* function in two separate pathways for sperm activation, and either pathway is normally sufficient for full male fertility.

try-5 mutant males do not transfer activator to hermaphrodites

To determine whether *try-5* indeed functions in the male-derived activation pathway, we used a specific assay to measure transfer of functional male activator. Wild-type male seminal fluid is capable of activating *spe-8*-group mutant hermaphrodite sperm during mating; this process is termed "transactivation" and is generally assayed using *fer-1* mutant males, which are defective for producing functional sperm, to prevent cross-progeny production (Shakes and Ward, 1989). We crossed either *fer-1* (Ward and Miwa,

1978) or *fer-1; try-5* males to *spe-8(hc53); dpy-4* hermaphrodites and counted the number

of self-progeny generated. Crosses with *fer-1* control males resulted in transactivation

approximately 58% of the time. However, *fer-1; try-5* males were rarely if ever capable

of transactivating hermaphrodite sperm (Figure 2.4). To exclude the possibility that *fer-1; try-5* males simply might harbor a behavioral defect that reduced their mating

frequency, we used MitoTracker to label males and assessed their ability to transfer

sperm. We observed similar frequencies of hermaphrodites containing labeled sperm

after incubation with *fer-1* males, *fer-1; try-5(jn2)* males, or *fer-1; try-5(tm3813)* males

(43%, 57%, or 63%, respectively). These data indicate that *fer-1; try-5* mutants mate and

transfer sperm with similar success rates as compared to the control. Thus, *try-5* mutant

males are defective in transfer of the male activator responsible for transactivation of *spe-8* group hermaphrodite sperm.

TRY-5 is expressed in and secreted

from the male somatic gonad

To determine how TRY-5 functions in male sperm activation, we sought to

determine where it is expressed and localized. Since we predicted that TRY-5 protein is

secreted, we generated both a *Ptry-5::GFP::H2B* transcriptional reporter, a histone-H2B

fusion that localizes to cell nuclei and facilitates identification of cells, and a *Ptry-5::TRY-5::GFP* translational reporter for assessing TRY-5 protein localization and

function. We created stable transgenic worm strains using MosSCI (Mos1-mediated

Single Copy gene Insertion (Frokjaer-Jensen et al., 2008), Tables 2.S1 and 2.S2) and

confirmed that the *Ptry-5::TRY-5::GFP* transgene restored a premature sperm activation

phenotype to *swm-1 try-5* mutants (Materials and Methods, Tables 2.S1, 2.S2, 2.S3).

Using the *Ptry-5::GFP::H2B* reporter, we found that the primary site of *try-5* expression was in the male somatic gonad, in particular within tissues involved in storing sperm and tissues through which sperm pass during transfer to a hermaphrodite. The *C. elegans* male gonad is essentially a long tube. At the distal end of this tube, germline stem cells reside and proliferate, and as they move proximally, they undergo meiosis and differentiate into spermatids. A subset of somatic gonadal cells surround spermatids to form a storage organ, the seminal vesicle; a more proximal set forms a channel, the vas deferens, through which sperm move during transfer. A valve structure regulates movement of sperm between the seminal vesicle and vas deferens. The vas deferens contains at least two distinct cell types, based on shape: cuboidal and elongated cells (Lints and Hall, 2009). Beyond an obvious structural role, other functions of these different cell types are not known, although some of them appear to be involved in secretion (Lints and Hall, 2009). Starting at the L4 larval stage, when sperm production initiates, we observed *Ptry-5::GFP::H2B* expression in several regions of the male gonad (Figure 2.5A): the seminal vesicle (up to 7 of the 23e total cells in this tissue (Kimble and Hirsh, 1979)), the valve region (four cells), and the 12 cuboidal cells of the vas deferens (Lints and Hall, 2009). This overall pattern persisted into adulthood until at least 72 hr post-L4; highest expression levels were present consistently in the valve region. We observed no expression in the hermaphrodite gonad, so gonadal expression of *try-5* is sexually dimorphic. However, we also observed low levels of expression in a few cells within the head and tail of both males and hermaphrodites (data not shown).

In worms carrying the *Ptry-5::TRY-5::GFP* reporter, the TRY-5::GFP fusion protein exhibited a localization pattern consistent with secretion from the vas deferens.

Within the valve and cuboidal cells, TRY-5::GFP was localized to globular foci. In L4 larvae, most globules aligned with the apical domain that lines the developing sperm channel (Figure 2.5B). In mature adults, very large globules were present that tended to cluster apically, and additional small globules were present throughout the cytoplasm (Figure 2.5C and 2.5D). Such large globular structures are generally visible in adult males by DIC microscopy and diagnostic of vas deferens tissue, including within wild-type animals lacking a transgene. Based on their size and location, these large globules are likely to represent the "secretory globules" observed by electron microscopy (Lints and Hall, 2009).

We sometimes observed TRY-5::GFP within the lumen of the seminal vesicle, likely as a result of release from the adjacent, highly-expressing valve cells (Figure 2.5D and 2.5E, Table 2.S5). The timing and extent of TRY-5::GFP expansion into the seminal vesicle was dependent on activity of the protease inhibitor SWM-1, the level of expression, and male age. In animals wild-type for *swm-1*, TRY-5::GFP was usually restricted to the valve cells or regions close by; when present near sperm cells, TRY-5::GFP was usually localized to a few discrete foci (data not shown). However, in animals lacking *swm-1* activity, we often observed large zones of TRY-5::GFP extending from the valve and surrounding sperm in the seminal vesicle (compare Figure 2.5C and 2.5D; see Table 2.S5). Even in *swm-1*(+) animals, when high levels of TRY-5::GFP were present in the seminal vesicle, we almost always observed that sperm were activated (Figure 2.5E, Tables 2.S3 and 2.S5). Together, these data suggest that TRY-5 is produced by cells of the male somatic gonad and can induce sperm activation within males if it is released into the seminal vesicle. It has been observed previously that older

wild-type males sometimes contain activated sperm (Stanfield and Villeneuve, 2006), and the finding that TRY-5::GFP is released into the seminal vesicle in older males provides a basis for this phenotype. Thus, these results support a model in which TRY-5 acts locally on sperm, either to signal their activation or to generate such a signal, and SWM-1 acts to inhibit the accumulation and/or activity of TRY-5 in the seminal vesicle.

TRY-5 is transferred to hermaphrodites during mating

Since TRY-5 localization is consistent with secretion from the male gonad, we sought to determine whether TRY-5 is transferred during mating. We placed individual MitoTracker-labeled *Ptry-5::TRY-5::GFP; try-5(tm3813)* males with *unc-52* hermaphrodites, monitored the males for mating behavior (Barr and Garcia, 2006), and acquired fluorescence images starting at or just before spicule insertion. We observed that TRY-5::GFP was transferred to hermaphrodites during mating (Figure 2.6 and Video TRY5transfer). Shortly after spicule insertion, TRY-5::GFP was released from the vas deferens and transferred to the hermaphrodite (Figure 2.6A and 2.6B). A brief pause without obvious transfer then occurred (Figure 2.6C). Next, TRY-5::GFP was released from the valve cells and travelled rapidly through the vas deferens into the hermaphrodite (Figure 2.6D and 2.6E). Movement of this valve pool was immediately followed by transfer of sperm (data not shown). After transfer, the TRY-5::GFP signal dispersed throughout the uterus (Figure 2.6F) and remained visible near the vulva for several minutes, if eggs were not laid immediately. This stereotypical series of events occurred for all cases (n=5) in which the entire process was observed from spicule insertion to sperm transfer. We also observed a partial time course of five other matings, all of which were consistent with this sequence of events.

To confirm that this behavioral sequence is not unique to this specific hermaphrodite genotype, we mated *Ptry-5::TRY-5::GFP; try-5(tm3813)* males to either *unc-31* (n=4) or *him-5 unc-76* (n=3) hermaphrodites. We were unable to observe vas deferens TRY-5::GFP transfer in these cases due to excess hermaphrodite movement. However, we did observe that valve TRY-5::GFP transfer initiated approximately 15-55 sec after spicule insertion, which is similar to the time observed for mating with *unc-52* hermaphrodites (Figure 2.6, Video TRY5transfer and data not shown) and consistent with the reported timing for sperm transfer from 14.4 to 90.2 sec after spicule insertion as determined by Schindelman (2006). In summary, our data suggest that TRY-5 is a seminal fluid protein that is transferred to the hermaphrodite during copulation. Furthermore, our observations indicate that seminal fluid is released in discrete pools from specific tissues of the male gonad and that these events occur largely prior to and coincident with transfer of sperm.

Discussion

Activation of *C. elegans* sperm motility by a protease signal

We have identified a serine protease, TRY-5, which functions in *C. elegans* male sperm activation, the process by which amoeboid sperm cells become motile and competent to fertilize an egg. Based on our analysis of the defects of *try-5* mutants and the dynamic localization of a TRY-5 reporter, we propose that TRY-5 is a sperm activating signal (Figure 2.7A and 2.7C). TRY-5 function is required for premature activation of stored sperm in males lacking the protease inhibitor SWM-1. TRY-5::GFP is expressed by the male somatic gonad within secretory cells. When observed outside these cells, localization of TRY-5::GFP protein strongly correlates with the localization

of activated sperm. We have directly observed the transfer of TRY-5::GFP to hermaphrodites during copulation, and *try-5* mutant males are incapable of transferring sperm activator to hermaphrodites. Together, these data strongly support a model in which TRY-5 is a component of seminal fluid that is transferred during copulation to signal sperm activation. Coupling the exposure of sperm to TRY-5 to the timing of transfer serves to ensure that sperm motility is rapidly induced at the time of - but not before - entry into a hermaphrodite's reproductive tract, thereby promoting male fertility.

Our discovery of a seminal fluid serine protease provides a mechanistic explanation for previous results linking extracellular protease activity with sperm activation in *C. elegans* and in other nematodes. *C. elegans* sperm can be activated *in vitro* by incubation with Pronase, a protease preparation that primarily contains trypsin-like activity at the pH used for these assays (Ward et al., 1983). In *C. elegans* males, loss of the SWM-1 protease inhibitor, which should result in increased protease activity, results in increased activation (Stanfield and Villeneuve, 2006). Recent studies of sex determination in *C. remanei*, a male-female species, showed that females could be transformed into sperm-producing "pseudohermaphrodites," but their sperm were not motile; production of functional, activated sperm could be achieved through additional inhibition of the *C. remanei* orthologue of *swm-1* (Baldi et al., 2009). Finally, the somatic gonad of males from the related nematode *Ascaris suum* contains a protease activity, which can activate sperm (Burghardt and Foor, 1978; Foor and McMahon, 1973). Thus, a role for protease activity in promoting sperm motility appears to be conserved among nematodes.

Here we describe a novel role for a protease as a signaling molecule for differentiation of sperm to a motile form. Why would a protease be used in this context?

The onset of motility in *C. elegans* sperm, as in flagellate sperm, occurs at a stage subsequent to meiotic cell division and the compaction of the haploid genome. At this stage, *C. elegans* sperm cells no longer express new protein products (L'Hernault, 2006). Therefore, to alter their behavior they must either reorganize their cellular contents in response to their environment or take in external factors. In addition, the timing of activation must be tightly controlled: *C. elegans* sperm must become motile rapidly upon entry into the hermaphrodite to avoid being lost due to the continuous outward passage of eggs (Ward and Carrel, 1979), but early activation of motility precludes transfer of sperm from the male (Stanfield and Villeneuve, 2006). A protease activator provides a mechanism to trigger irreversible changes in the sperm cell surface that is readily coupled to mixing of sperm with seminal fluid. This type of activator also provides a simple mechanism to hold activation in check: the use of specific protease inhibitors such as SWM-1. We propose that the balance of TRY-5 and SWM-1 activities controls the likelihood of activation in specific locations and times within the male and hermaphrodite (Figure 2.7). For example, within the male gonad, SWM-1 may directly inhibit TRY-5 activity to prevent activation, allowing for sperm transfer and maintaining male fertility (Figure 2.7A). It is likely that additional proteases and/or inhibitors also function in this process. *try-5* mutant hermaphrodites are fertile, suggesting that hermaphrodites have an activator that is independent of TRY-5 (Figure 2.7B). This activator could be a protease, though its identity is not known. Male sperm sometimes activate prematurely in *try-5* mutants, suggesting that males could contain a second activator. However, if it exists, such a secondary male activator must not be competent to activate male or hermaphrodite *spe-8* group sperm.

Genetic analysis of *swm-1* had suggested that it functions to inhibit two distinct protease activities that act in parallel to promote sperm activation within males (Stanfield and Villeneuve, 2006). This model was based on the result that partial loss-of-function mutations affecting each of the two TIL domains of SWM-1 partially complement one another. By this model, loss of a single protease would not be expected to block sperm activation. However, we find that all SWM-1 activity works through TRY-5 in males, suggesting that both domains of SWM-1 inhibit TRY-5. The apparently separable activities of the SWM-1 TIL domains could arise from interactions with factors other than proteases. Alternatively, these results can be reconciled by a regulatory model in which SWM-1 inhibits two distinct proteases, both of which act upstream of TRY-5. It is also possible that SWM-1 might inhibit both TRY-5 and a second, TRY-5-activating protease. Consistent with these ideas, many well-known protease pathways consist of sequential cascades of activator and effector functions (e.g., (Ovaere et al., 2009; Pampalakis and Sotiropoulou, 2007)).

As an extracellular protease, TRY-5 likely signals activation by cleaving sperm cell surface proteins and altering their activity. Some of the targets of TRY-5 may be SPE-8 group proteins, based on the fact that TRY-5 is required for transactivation, a process dependent on having some *spe-8* group activity (sperm from hermaphrodites harboring null alleles of these genes are essentially incapable of being transactivated (Geldziler et al., 2005; Nance et al., 1999)). However, *spe-8* group mutant males are fertile, suggesting that SPE-8 group proteins are not essential for activation in all contexts. Thus, other targets may not be members of the SPE-8 group. The existence of such targets is further supported by our finding of additional *swm-1* suppressors (distinct from

try-5) that show full fertility in hermaphrodites and so do not fall into the *spe-8*

phenotypic class (G.M.S., unpublished data).

Could TRY-5 be functioning in some role other than as a direct activator? Sperm

from *try-5* mutant males can be activated within hermaphrodites after mating, in *spe-6*

mutants, or by exogenous activators *in vitro*. Thus, other activators can bypass TRY-5,

and *try-5* is not required for the cellular rearrangements that occur after activation is

triggered. These data support the idea that TRY-5 functions in a regulatory step of the

activation process. It is clear that TRY-5 is essential for transfer of sperm activator by *C.*

elegans males and its localization correlates strongly with that of activated sperm. These

data strongly suggest that if TRY-5 is not the signaling molecule *per se*, its activity is

intimately associated with generation of the sperm activation signal.

TRY-5 as a component of seminal fluid

Production and transfer of seminal fluid is an important aspect of male reproduction

(Gillott, 2003; Poiani, 2006; Wolfner, 2007). TRY-5 is one of the first seminal fluid

proteins identified in *C. elegans*. Indeed, it is the first directly demonstrated to be

transferred at mating, and the first with a specific role in promoting gamete function.

Previously, *plg-1* was identified as a seminal fluid factor required for production of a

copulatory plug (Hodgkin and Doniach, 1997) and shown to encode a mucin-like protein

with a function in male mate guarding (Palopoli et al., 2008). *plg-1* is expressed within

the male somatic gonad in a subset of cells that express *try-5*; interestingly, *plg-1* is not

expressed within the valve region (Palopoli et al., 2008), from which most TRY-5

appears to be released during mating (Video TRY5transfer). Thus, as in other animals

(Cornwall and van Horsten, 2007; Gatti et al., 2004), different regions of the *C. elegans*

male gonad appear to be specialized to produce specific components of seminal fluid. Furthermore, our data reveal considerable complexity in the timing of release of seminal fluid from specific tissues during the mating behavioral program.

Regulatory logic of sperm activation

in a male-hermaphrodite species

We have found that *try-5* is functionally redundant for fertility in *C. elegans*. Although *try-5* mutant males fail to transfer activator, they are fertile; however, loss of both *try-5* and *spe-8*-group function leads to complete infertility for both hermaphrodites and males (tested here with mutations in two of the *spe-8*-group genes, *spe-27* and *spe-29*). These data can be explained by the following model: *spe-27; try-5* and *spe-29; try-5* animals (1) make sperm that do not respond to hermaphrodite activator (due to loss of *spe-8*-group function) and (2) do not produce male activator (due to loss of *try-5*). In other words, *try-5* males may be fertile due not to the presence of additional activators provided by the male, but rather due to rescue of male sperm activation by a signal within the hermaphrodite (Figure 2.7C).

These findings of redundancy raise the question: why does *C. elegans* have *try-5*? At least part of the answer might lie in the evolutionary history of this species, which evolved from a gonochoristic (male-female) ancestor (Cho et al., 2004; Kiontke et al., 2004). As the male activator, *try-5* may represent the ancestral mode of activating sperm. Baldi et al. (2009) have shown that the transition from gonochorism to androdioecy in the related species *C. remanei* requires only two steps: making sperm and activating it. Acquisition of the ability to make sperm could be advantageous, even in the initial absence of a robust self-sperm activation mechanism, as long as it tended to increase

fertility. Chance encounters with a male would potentially activate hermaphrodite self-sperm, as long as hermaphrodite sperm remained capable of responding to male activator. In turn, the male may have developed mechanisms to ensure his sperm were used preferentially; indeed, *C. elegans* male sperm show strong precedence over those of the hermaphrodite (LaMunyon and Ward, 1995; Ward and Carrel, 1979). Eventually, the hermaphrodite might evolve her own mechanism for activating sperm. The self-sperm activator in *C. elegans* is not known, but it may be a serine protease. Indirect evidence for this idea is provided by data indicating that the inhibitor SWM-1 functions in hermaphrodites: while animals mutant for the *spe-8* class gene *spe-29* have very low levels of self-sperm activation and fertility, this phenotype is partially suppressed by mutations in *swm-1* (Stanfield and Villeneuve, 2006). However, this protease is likely distinct from TRY-5, since we have found that *try-5* is not required for either normal hermaphrodite fertility or increased activation in *spe-29; swm-1* hermaphrodites (Figure 2.3A, Figure 2.S4).

Alternatively, production of TRY-5 would be advantageous for males if it is a more efficient activator than that of hermaphrodites. While our fertility assays revealed no difference between fertility of wild-type and *try-5* males, those assays were performed under highly permissive conditions: young adult animals were provided with many opportunities for mating to occur under conditions of unlimited food resources. TRY-5 might be important to increase reproductive fitness in less-than-ideal conditions. For example, activation by TRY-5 might occur more rapidly than that mediated by the hermaphrodite activator. If so, its transfer would decrease the chance that transferred sperm would be lost before they have the opportunity to migrate away from the vulva.

In summary, our work has identified a serine protease in *C. elegans* male seminal fluid that regulates the timing of sperm activation to promote male fertility. TRY-5 is transferred along with sperm during mating to couple sperm motility to entry into the hermaphrodite reproductive tract. While TRY-5 appears to be necessary for males to signal activation, hermaphrodites contain their own activator. Interestingly, these redundant pathways are competent to activate sperm from either sex, providing insight into the strategies used by *C. elegans* to adopt a male-hermaphrodite mode of reproduction. Further dissection of these signaling pathways will require identifying targets of TRY-5 and determining the nature of the hermaphrodite activator.

Materials and methods

C. elegans genetics

C. elegans strains were grown as described by Brenner (1974) at 20°C, except where otherwise noted. All strains were derived from the wild-type isolate Bristol N2. To ensure a ready supply of males, a strain harboring the mutation *him-5(e1490)* (Hodgkin et al., 1979) was used as the wild type and *him-5(e1490)* was present in all other strains unless explicitly noted. The *try-5* alleles *jn2* and *jn13* were isolated as suppressors of *swm-1(me86)* and *jn21* was isolated as a suppressor of *swm-1(me66)* (G.M.S., unpublished results). Ethyl methanesulfonate (EMS) mutagenesis was performed as in (Wood, 1988). *try-5(tm3813)* was a gift of S. Mitani (National Bioresource Project, Japan). Other alleles (described in Wood (1988) unless otherwise noted) were: *spe-8(hc40, hc53) I*, *fer-1(hc1*ts*) I*, *ttTi5605 II* (Frokjaer-Jensen et al., 2008), *unc-52(e444) II*, *dpy-18(e364) III*, *spe-6(hc163) III* (Muhlrad and Ward, 2002), *unc-119(ed3, ed9) III* (Maduro and Pilgrim, 1995), *spe-27(it110) IV* (Minniti et al., 1996),

spe-29(it127) IV (Nance et al., 2000), dpy-20(e1282) IV, mIs11[myo-2::GFP, pes-10::GFP, gut::GFP] IV, dpy-4(e1166) IV, unc-31(e169) IV, swm-1(me66, me86, me87) V (Stanfield and Villeneuve, 2006), unc-76(e911) V and *nT1[unc-?(n754) let-? qIs50](IV, V).*

Strains containing mutations in both a *spe-8* group gene and *try-5* were maintained as heterozygotes using the balancer *nT1*. Homozygous *spe-8* group; *try-5* males were generated by transactivation crosses of homozygous self-sterile hermaphrodites to *swm-1* mutant males, which are competent for transferring seminal fluid but rarely transfer sperm (Stanfield and Villeneuve, 2006). For example, for the *spe-27 dpy-20/nT1; try-5 him-5/nT1* strain, homozygous *spe-27 dpy-20; try-5 him-5* hermaphrodites were selected and crossed to either *swm-1(me87) him-5* or *mIs11; swm-1(me87) him-5* males to induce production of self-progeny, which can be recognized as being phenotypically Dumpy.

To screen *C. elegans* proteases for a function in sperm activation, RNAi against individual protease genes was performed on *swm-1 him-5* worm strains by feeding on agar plates essentially as described by (Ahringer, 2006). Bacteria containing inducible RNAi clones (described in (Fraser et al., 2000; Kamath et al., 2003)) were obtained from Source BioScience. Genes tested by RNAi were *try-1*, *try-2*, *try-3*, *try-5*, *try-6*, *try-7*, *try-8*, *F25E5.3*, *F25E5.4*, *F25E5.7*, and *F48E3.4*. For each gene, *swm-1(me66) him-5* and *swm-1(me86) him-5* eggs were collected on RNAi plates and allowed to grow to the L4 stage; L4 males were then transferred to a fresh RNAi plate and scored either 24 hr or 48 hr later for sperm activation.

Microscopy

Sperm activation was assayed in virgin males collected as L4 larvae and incubated at 20°C for 48 hr, unless otherwise indicated. To examine individual sperm cells, males were dissected in sperm medium (SM) (5mM HEPES sodium salt pH 7.4, 50 mM NaCl, 25 mM KCl, 5 mM $CaCl_2$, 1 mM $MgSO_4$) supplemented with 10 mM dextrose (Nelson and Ward, 1980). Samples were observed using differential interference contrast (DIC) microscopy and sperm were scored based on cell shape as either nonactivated, if spherical, or activated, based on the presence of a pseudopod. Samples were observed using an AxioImager M1 equipped with an AxioCam MRm (Zeiss). Confocal imaging was performed using a TCS SP2 (Leica). Images were processed using ImageJ (Rasband, 1997-2009) and Photoshop (Adobe Systems).

Fertility assays

Hermaphrodite self-fertility was measured by picking individual hermaphrodites, transferring them to fresh plates every 1-2 days until no more eggs were laid, and counting the total progeny after worms reached the L4 stage. Cases in which hermaphrodites failed to lay oocytes or died less than 4 days after adulthood were excluded from analysis.

Male fertility was measured in 1:1 crosses to *spe-8(hc40); dpy-4* hermaphrodites. L4 stage animals were placed together for 48 hr; hermaphrodites were then transferred to fresh plates without males and transferred again every 1-2 days until no more eggs were laid. All cross progeny, identifiable by their nonDumpy phenotype, were counted after worms reached the L4 stage. Use of the *spe-8* mutation in recipient hermaphrodites allows for detection of mating even in cases where functional sperm are not transferred,

since transfer of seminal fluid leads to production of self-progeny (L'Hernault et al., 1988; Stanfield and Villeneuve, 2006). Cases in which mating was not confirmed or the hermaphrodite died less than 3 days after adulthood were excluded from analysis.

For all fertility assays, wild-type broods were measured in parallel to those of the strain being assayed to control for variations in temperature, media quality and other factors that can affect progeny production or mating efficiency.

Sperm and seminal fluid transfer assays

To assay sperm transfer and migration, males were labeled with 1 µg/mL MitoTracker CMXRos (Invitrogen) as described by Chen et al. (2000) and observed as described previously (Stanfield and Villeneuve, 2006).

Seminal fluid transfer (transactivation, (Shakes and Ward, 1989)) was assayed using males harboring the *fer-1(hc1*ts*)* mutation, which results in nonfunctional sperm at the restrictive temperature of 25°C (Ward and Miwa, 1978). L4 males were crossed in a 4:1 ratio to L4 *spe-8(hc53); dpy-4* hermaphrodites for 48 hr at 25°C. The number of self-progeny (Dumpy offspring) produced during the mating period was determined after 3 additional days. Any crosses resulting in cross progeny (nonDumpy offspring) were excluded from analysis. All other crosses with recipient worms surviving to the end of the mating period were included, because no marker for successful mating is available for this assay. To assess mating frequency in different *fer-1* mutant strains, males were labeled with MitoTracker and incubated with hermaphrodites in 1:1 crosses. Hermaphrodites were then examined after 5 hr for the presence of labeled sperm. This assay likely underestimates the total mating frequency in transactivation assays, since 1) *fer-1* sperm cannot migrate and are only retained within hermaphrodites for a short time

period, and 2) a higher ratio of 4 males:1 hermaphrodite was used for transactivation assays.

Assays of *in vitro* sperm activation

Activation assays were performed essentially as in (Shakes and Ward, 1989). Adult virgin males were dissected to release sperm in a drop of SM on a glass slide; a chamber was formed over the cells using a coverslip supported by a thin layer of Vaseline; additional SM either with activator (200 µg/mL Pronase or 60 mM TEA) or without it (control) was wicked through this chamber; and the coverslip was completely sealed with Vaseline. An image was obtained immediately upon wicking through activator and subsequent images were obtained every 5 min for at least 25 min. Activation was scored at each time point based on cell shape. To obtain time-lapse videos, activation assays were performed as described except that images were obtained once per minute.

Observation of TRY-5::GFP transfer

For each trial, one to two 24 hr post-L4 *Ptry-5::TRY-5::GFP; try-5(tm3813) him-5* males were placed at the center of a circle of ten *unc-52*, *unc-31* or *unc-76 him-5* virgin adult hermaphrodites. Males were observed for 10 min under transmitted light using a Leica MZ16FL microscope. Prior to or shortly after spicule insertion occurred, the light source was switched to epifluorescence and images were collected at maximum speed (an exposure time of approximately 300 msec) using an AxioCam MRm (Zeiss) until spicules were removed. If copulation was not attempted within 10 min, males were removed and replaced with fresh males.

Molecular biology

Standard molecular biology protocols were used (Sambrook et al., 1989). RNA was extracted from mixed-stage *him-5* worms using TRIzol (Invitrogen). Reactions for 5'- and 3'-RACE (rapid amplification of cDNA ends) were performed using GeneRacer (Invitrogen). The MultiSite Gateway Three-fragment Vector Construction Kit (Invitrogen) with pCFJ150 as the destination vector (Frokjaer-Jensen et al., 2008) was used to generate MosSCI donor constructs (Tables 2.S1 and 2.S2). Plasmid pCM1.35 was a gift from G. Seydoux (Merritt et al., 2008). For TRY-5::GFP, fusion PCR was performed as in (Hobert, 2002). Details of Gateway plasmid construction are listed in Table 2.S1 and Table 2.S2. To generate pJRS17, the 279 bp KpnI-XhoI fragment from pPD95.85 was ligated into the 4855 bp KpnI-XhoI fragment from pJRS11, thereby replacing the Ser65Cys variation present in GFP derived from pPD95.75 with the Ser65Thr variation from pPD95.85.

Transgenic strains

To generate transgenic strains harboring extrachromosomal arrays, constructs were injected (Mello et al., 1991) into the strain *unc-119; swm-1(me86) try-5(jn2) him-5* and transgenic lines were selected based on rescue of the Unc-119 phenotype (Frokjaer-Jensen et al., 2008; Maduro and Pilgrim, 1995). Single-copy insertion (MosSCI) strains were generated by the direct insertion technique into the Mos1 insertion site *ttTi5605* as described by Frokjaer-Jensen (2008). Targeting constructs were coinjected with *Pglh-2::transposase* as the source of Mos transposase and coinjection markers labeling pharyngeal muscle (*Pmyo-2::mCherry*), body wall muscle (*Pmyo-3::mCherry*), and neurons (*Prab-3::mCherry*) (Frokjaer-Jensen et al., 2008).

Quantification of *spe-29* suppression

Single L4 hermaphrodites were placed on individual plates and transferred on day 2, 4 and 6. Dumpy self-progeny were counted once all worms had reached at least the L4 stage. Brood counts were performed on all strains in parallel. Cases in which hermaphrodites died prior to the sixth day were excluded. The *spe-29 dpy-20; swm-1 try-5 him-5* strain was maintained by trans-activation crosses in which *swm-1* males were crossed to Dumpy self-progeny in each generation.

Figure 2.1. Mutations in the serine protease gene *try-5* suppress premature sperm activation in *swm-1* mutant males. Sperm activation was examined in staged 48 hr post-L4 adult virgin males. (A-D) Differential interference contrast (DIC) images showing sperm morphology in the indicated strains. Top row: Images of intact males in the region of the seminal vesicle (SV), where sperm are stored. Strains shown in A, C and D have round spermatids, which pack together in the SV of intact animals to form a uniformly grainy appearance. The strain shown in B contains activated sperm, resulting in a rough appearance. Arrowheads indicate the anterior (white) and posterior (black) boundaries of the SV. Scale bar, 25 μm. Bottom row: DIC images of dissected sperm. Arrow indicates a pseudopod. Scale bar, 5 μm. (See Figure 2.S1 for high-resolution versions of the images in B, C.) (E) Schematic of the *try-5* region. We used RACE and RT-PCR to characterize *try-5* transcripts, generating updated gene models as compared to the WormBase prediction ((WormBase); accession numbers JN651275, JN651276 and JN651277). On gene models, darker shading indicates predicted coding regions and arrow indicates the direction of transcription. The positions of mutations in *try-5* are indicated along with their predicted effects. (See also Figure 2.S2.) (F) Quantitation of suppression of *swm-1* by mutations in *try-5*. Stacked columns indicate the percent of males containing either only activated sperm (black), a mixture of spermatids and activated sperm (hatched), or only nonactivated spermatids (grey). At least 30 animals were scored for each genotype. (G) A *try-5* transgene restores sperm activation in *swm-1* mutant males. Sperm activation was assayed in *unc-119; swm-1(me86) try-5(jn2) him-5* males bearing extrachromosomal arrays of pJRS14, which contains *try-5*(+) and *C. briggsae unc-119*(+) (Tables 2.S1 and 2.S2). Data from three independent transgenic lines are shown. Key for stacked columns as in (F). Between 22 and 31 animals were scored for each genotype.

A Wild type

B *swm-1(me87)*

C *swm-1(me87) try-5(tm3813)*

D *try-5(tm3813)*

E

500 bp

K07B1.7

jn21
M1T

jn2
C29Y

jn13
Q308Stop

try-5

tm3813
Δ311bp

F

% of males

Males containing:
□ unactivated spermatids
▨ spermatids + sperm
■ activated sperm

Wild type
try-5(tm3813)
try-5(+)
try-5(jn21)
try-5(+)
try-5(jn2)
try-5(tm3813)
try-5(jn13)
try-5(+)
try-5(jn2)
try-5(tm3813)

*swm-1
(me66)*
*swm-1
(me86)*
*swm-1
(me87)*

G

% of males

try-5(+)
array:
none
jnEx62
jnEx63
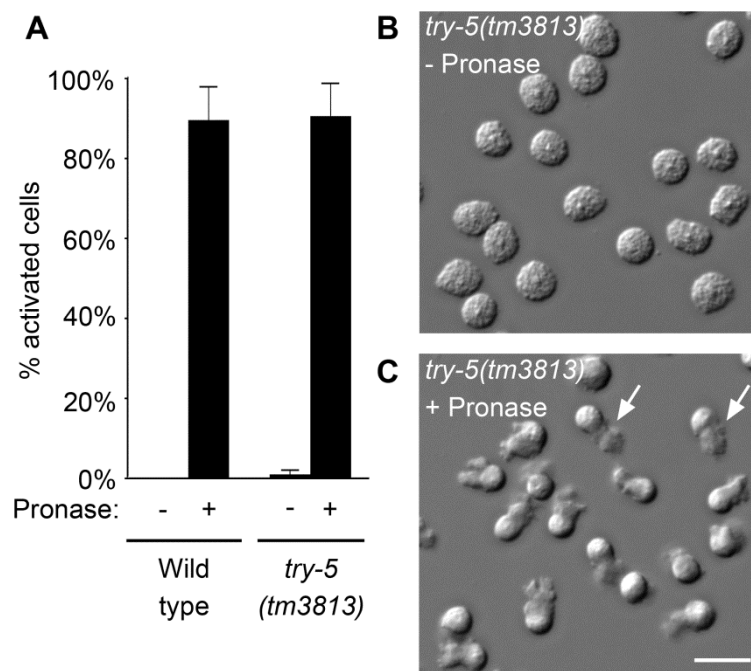*jnEx64*

swm-1(me86) try-5(jn2)

Figure 2.2. *try-5* mutant sperm are capable of activation. Sperm were assayed for activation in response to protease treatment. Wild-type or *try-5(tm3813)* mutant sperm were dissected and incubated in 200 μg/mL Pronase for 25 min; activation was scored every 5 min based on the presence or absence of a pseudopod. (A) Maximal activation observed at a single time point during the assay; average of 3 repeats. Error bars represent standard error of the mean. (B,C) DIC images of *try-5(tm3813)* sperm prior to Pronase treatment (B) and the same field of cells at 20 min (C). See also Video PronaseAct. Arrows indicate the pseudopodia for two of the activated cells. Scale bar, 10 μm.
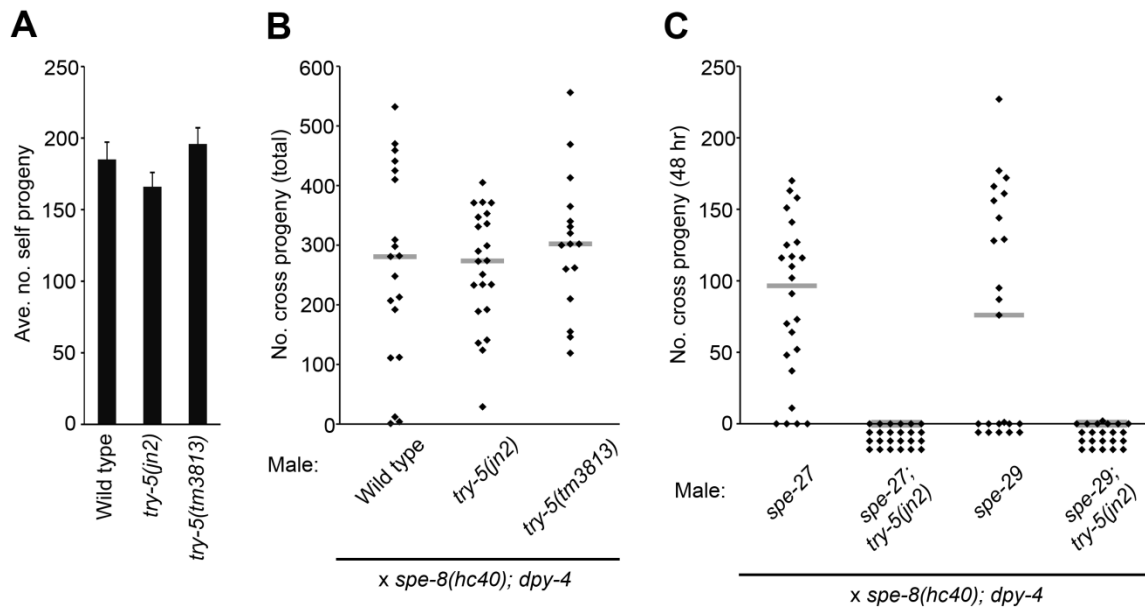
Figure 2.3. *try-5* is not required for fertility and functions in parallel to the *spe-8* group. Hermaphrodite and/or male fertility was measured for *try-5* mutant strains. (A) *try-5* hermaphrodites show normal fertility. Columns indicate average brood size of self-fertilizing hermaphrodites. Error bars represent standard error of the mean. (B) *try-5* males show normal fertility. Males were placed with *spe-8(hc40); dpy-4* hermaphrodites in 1:1 crosses for 48 hr and the entire brood size was measured by counting the total number of nonDumpy cross progeny. *try-5* mutants were not significantly different from the control (*try-5(jn2)*, p=0.95; *try-5(tm3813)*, p=0.40; Mann-Whitney U Test). (C) *try-5* is required for fertility in *spe-8* group mutant males. Males were placed with *spe-8(hc40); dpy-4* hermaphrodites in 1:1 crosses for 48 hr and the number of nonDumpy cross progeny produced during the mating period was counted. Infertility of *spe-8* group; *try-5* males was due to failure of sperm to activate or migrate (Table S4). (B,C) Each point represents the result of an individual cross; gray lines represent medians. Sets of crosses with each genotype were repeated at least twice and a representative set of data is shown. (See also Figure 2.S3.)
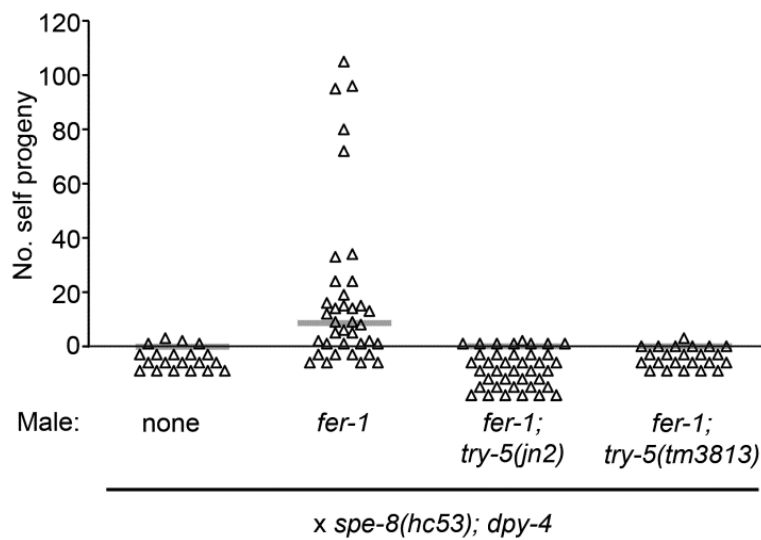
Figure 2.4. TRY-5 is required for the activation of hermaphrodite sperm by male seminal fluid. Transactivation was assayed for *try-5* males. L4 males were mated to L4 *spe-8(hc53); dpy-4* hermaphrodites in 4:1 crosses for 48 hr, and the number of Dumpy self-progeny produced during the mating period was counted. Each point represents the result from one cross. Gray bars represent medians.

Figure 2.5. *try-5* is expressed in the male somatic gonad. Confocal and transmitted-light (TL) images of transgenic males bearing *try-5* reporter insertions. In TL images, boundaries of regions of the somatic gonad are labeled as sv, seminal vesicle; vl, valve; and vas, vas deferens. Scale bars, 10 µm. (A) *jnSi49[Ptry-5::GFP::H2B]; try-5(tm3813)* L4 male. In focal planes shown, GFP is visible in the four GFP-positive seminal vesicle cells, two of four GFP-positive valve cells, and six of twelve GFP-positive cuboidal cells. (B) *jnSi62[Ptry-5::TRY-5::GFP]; try-5(tm3813)* L4 male. TRY-5::GFP is concentrated at the apical side of seminal vesicle, valve and cuboidal cells. (C) *jnSi62[Ptry-5::TRY-5::GFP]; try-5(tm3813)* 48 hr adult male. Both large and small TRY-5::GFP globules are present in the cuboidal cells and valve region. In the seminal vesicle, TRY-5::GFP is present in the proximal region near the valve. (D) *jnSi62[Ptry-5::TRY-5::GFP]; swm-1(me87) try-5(tm3813)* 48 hr adult male. TRY-5::GFP has expanded into the seminal vesicle lumen and sperm are activated. (C', D') Images of seminal vesicle regions outlined in C and D with intensity levels optimized for the fainter GFP signal in these tissues. Arrows indicate GFP in the distal sheath-like cells of the seminal vesicle. (E) Paired DIC, epifluorescence and merged images of a *jnSi62[Ptry-5::TRY-5::GFP]; try-5(tm3813)* 72 hr adult male in the region of the seminal vesicle. Localization of TRY-5::GFP is correlated with activated sperm (also see Table 2.S3 and 2.S5).

jnSi49[Ptry-5::GFP::H2B]

jnSi62[Ptry-5::TRY-5::GFP]

jnSi62[Ptry-5::TRY-5::GFP]

swm-1(+)

swm-1(me87)

jnSi62[Ptry-5::TRY-5::GFP]
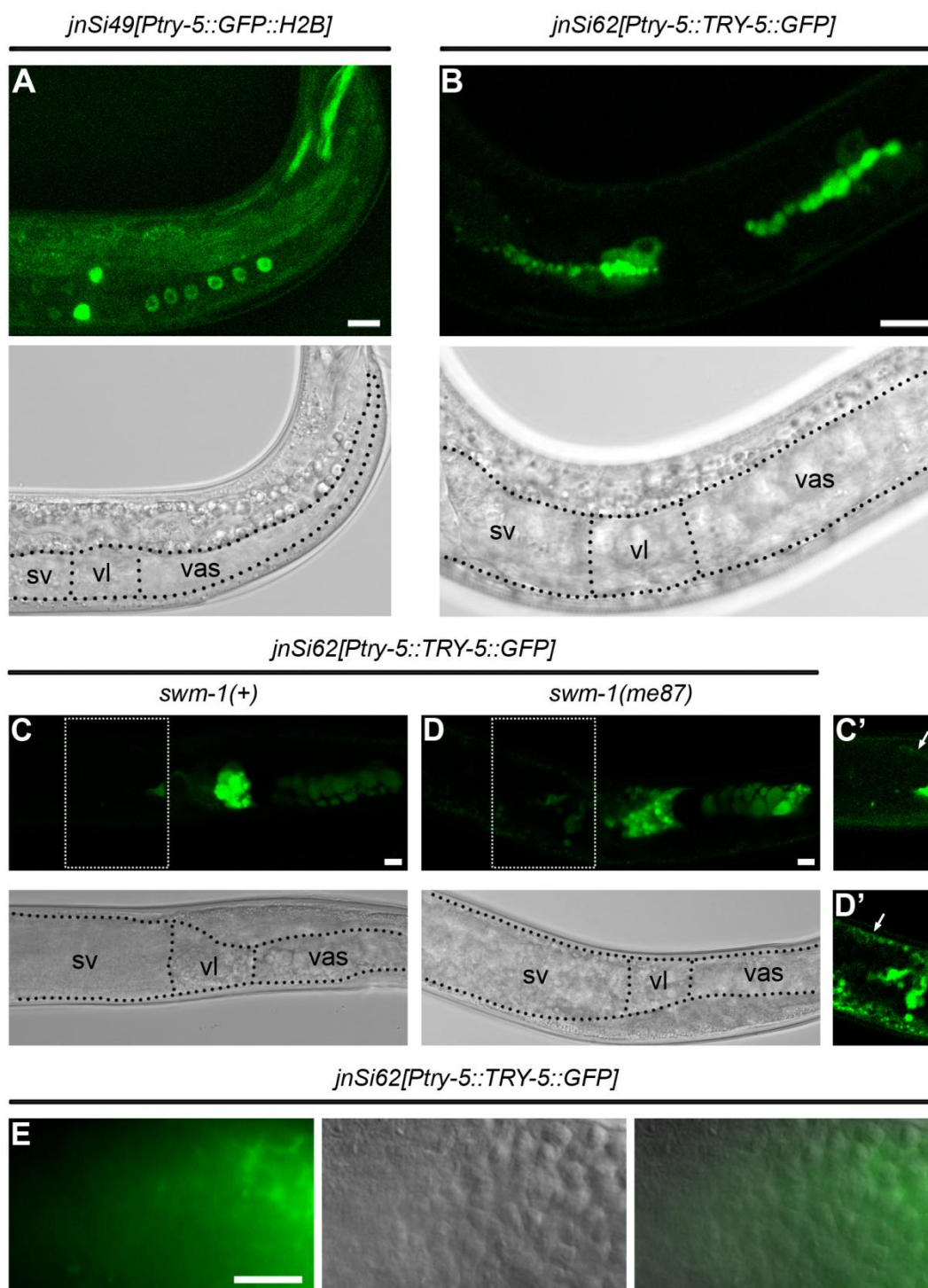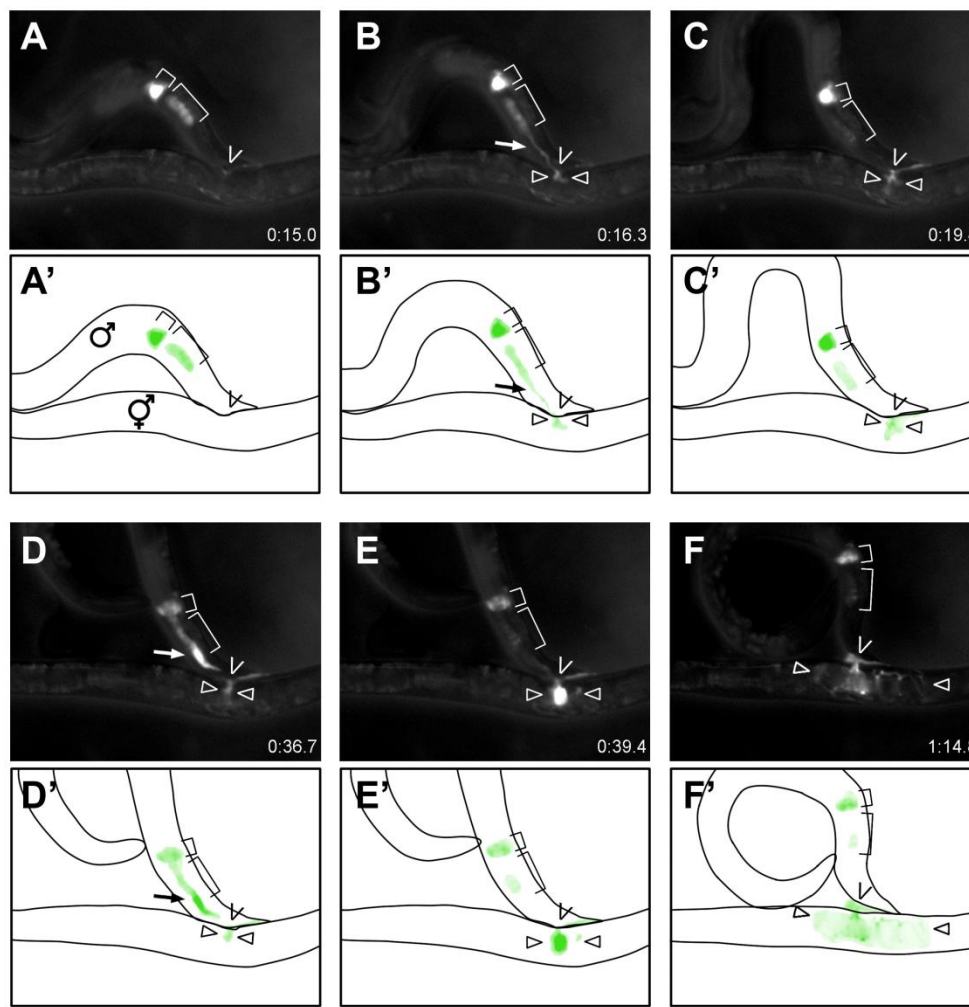
Figure 2.6. TRY-5 is transferred to hermaphrodites during mating. Selected still images (A-F) and schematics (A'-F') depicting the time course of TRY-5::GFP transfer to an *unc-52* hermaphrodite (see Video TRY5transfer). (A) TRY-5::GFP is visible within its source cells in the valve (small bracket) and vas deferens (large bracket). (B) Transfer of TRY-5::GFP from the vas deferens into the hermaphrodite. (C) Pause between transfer of the two pools. (D, E) Transfer of TRY-5::GFP from the valve into the hermaphrodite. (F) Spread of TRY-5::GFP within the uterus concomitant with transfer of sperm (data not shown). Arrows indicate TRY-5::GFP during transfer, "V" symbols indicate position of the hermaphrodite vulva, and arrowheads indicate TRY-5::GFP within the uterus. Time shown is relative to the beginning of Video TRY5transfer.

Figure 2.7. Models for the protease regulation of sperm activation in males and hermaphrodites. (A) Model: Male pathway for sperm activation, shown prior to mating when SWM-1 inhibits TRY-5 activity within the male gonad to prevent p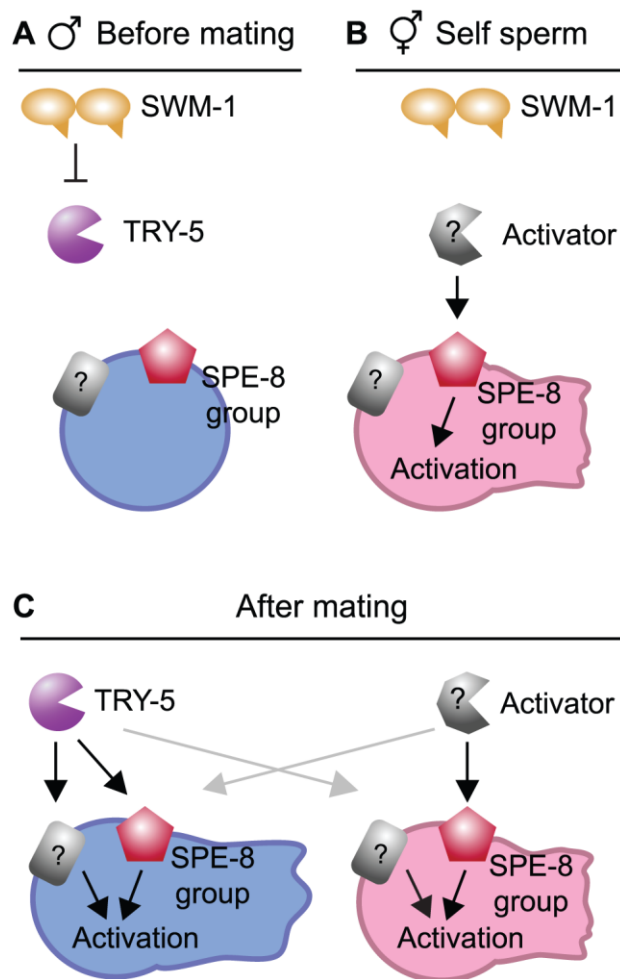remature activation. In the absence of SWM-1, activation is signaled through interaction between TRY-5 and spermatids, likely through cleaving a target(s) on the cell surface (black arrows). Potential targets of TRY-5 include both members of the SPE-8 group and one or more additional sperm surface proteins. (B) Model: Hermaphrodite pathway for self-sperm activation. Activation is signaled through interaction between hermaphrodite activator and spermatids in the spermathecae (black arrow). This process is dependent on activity of the SPE-8 group. SWM-1 weakly antagonizes self-sperm activation, suggesting that the hermaphrodite activator may be a protease. (C) Model: Pathways for activation after mating and seminal fluid transfer. When mating occurs, components of male and hermaphrodite pathways are both present and can promote sperm activation. Either pathway can activate both male and hermaphrodite sperm (grey arrows). However, hermaphrodite activator may function solely through the SPE-8 group.
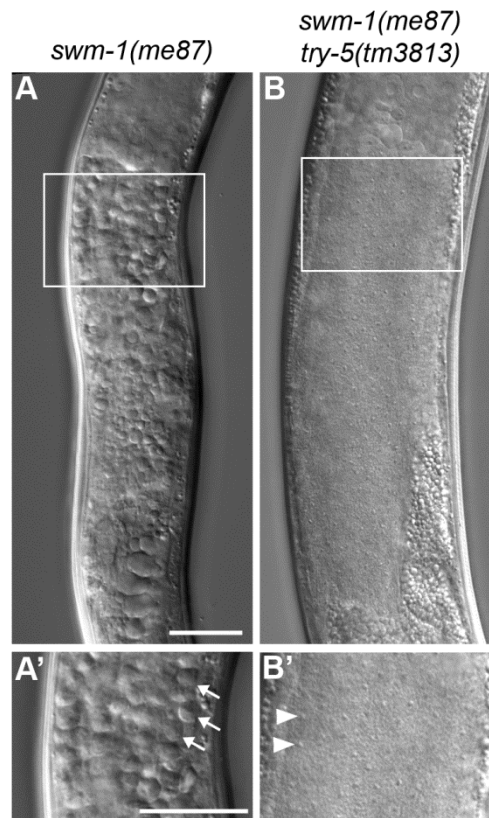
Figure 2.S1. High-resolution images of adult males showing suppression of *swm-1(me87)* premature sperm activation by *try-5(tm3813)*. (A and A') *swm-1(me87)* male from Figure 2.1B. Prematurely activated sperm within the seminal vesicle result in a disorganized appearance. Arrows indicate a subset of individual spermatozoa for which pseudopods are visible. (B and B') *swm-1(me87) try-5(tm3813)* male from Figure 2.1C. Nonactivated spermatids, containing condensed nuclei and distinctive grainy cytoplasm, are present throughout the seminal vesicle. Individual cell boundaries are often not visible by DIC; to convey packing together of these cells, arrowheads indicate the nuclei of two adjacent spermatids.

Figure 2.S2. TRY-5 is a serine protease. Alignment of TRY-5 with the serine proteases trypsin, chymotrypsin and elastase (accession numbers NP_002760, NP_001897, and NP_031378). The signal sequence was predicted for TRY-5 using SignalP 3.0 (Bendtsen et al., 2004). Positions of *try-5* alleles are shown. Shading corresponds to identities (black) or similarities (grey) among two or more family members. Arrows indicate residues of the active site. Arrowheads indicate residues important for substrate binding.

jn21
M1T

jn2
C29Y

Signal sequence

tm3813

jn13
Q308Stop

```
                    1                                                                                82
TRY-5            1  -MRPRIIVFLFQVLVIKGTKLKYYNDELCGRQSTYTS-FMLTDAAGNTGNPTHLAPWAVQIRVKARKGDFEVICGGTLITLKH  82
Trypsinogen      1  -MNPLLILTFVAALA------------APFDDDDKIVGGYNCEENSVPYQVSLN---ISG--YHFCGGSLINEQW            57
Chymotrypsinogen 1  MASLWLLSCFSLVGAAFG-----CGVPAIHPVLSGLSRIVNGEDAVPGSWPWQVSLQ--DKTG--FHFCGGSLISEDW         69
Elastase         1  -MMLRLLSSLLLVAVASG-----YGPPSSRVVNGEDAVPYSWPWQVSLQY-EKSGSFYHTCGGSLIAPDW                 67

TRY-5           83  VLTAAHCFQKHFGAKEGGEENSMSGRYCESNQRFTDSEILTRTVVTVGAMCTRLEQKYGCVNEKQNGKTLKISR---FAIGDF  163
Trypsinogen     58  VVSAGHCYKSR-IQVRLGEHNIEV------------LEGNEQFINAAK--IIRHPQY                             99
Chymotrypsinogen 70 VVTAAHCGVRT-SDVVVAGEFDQGS------------DEENIQVLKIAK--VFKNPKF                           112
Elastase        68  VVTAGHCISSSRTYQVVLGEYDRAV------------KEGPEQVIPINSGDLFVHPLW                           113

TRY-5          164  YKTHCEQGNDIVILELESTIDDVEGANYACLPFLPEVNIQSGANVTSFGWGSDPGKGFDNAAFPMIQVLTLATETLATCEENWG 247
Trypsinogen    100  -DRKTLNNDIMLIKLSSRAVINARVSTISLPTAPP-ATGTKCLISGWGNTASSGADYPDELQCLDAPVLSQAKCEAS--YP    176
Chymotrypsinogen 113 --SILTVNNDITLLKLATPARFSQTVSAVCLPSADDD-FPAGTLCATTGWGKTKYNANKTPDKLQQAALPLLSNAECKKS--WG 191
Elastase       114  NRSCVACGNDIALIKLSRSAQLGDAVQLASLPPAGDI-LPNETPCYITGWGR-LYTNGPLPDKLQEALLPVVDYEHCSRWNWWG 195

TRY-5          248  TSIPFDSFCTAE-EEDKNVCSGDSGGGLTFHQSDSAREFIAIVSYGSDCVQLIGGSEPRSQINTDVRKHQKFIVNFIINQA-   327
Trypsinogen    177  GKITSNMFCVGFLEGGKDSCQGDSGGPVVCNG---QLQGVVSWG---DGCAQKNKPGVYTKVYNYVKWIKNTIAANS         247
Chymotrypsinogen 192 RRIITDVMICAG--ASGVSSCMGDSGGPLVCQK---DT-CSTS-SPGVYARVTKLIPWVQKILAAN-                  263
Elastase       196  SSVKKTMVCAG--GDIRSGCNGDSGGPLNCPTE-DGGWQVHGVTSFVS---AFGCNTRRKPTVFTRVSAFIDWIEETIASH-   270
```
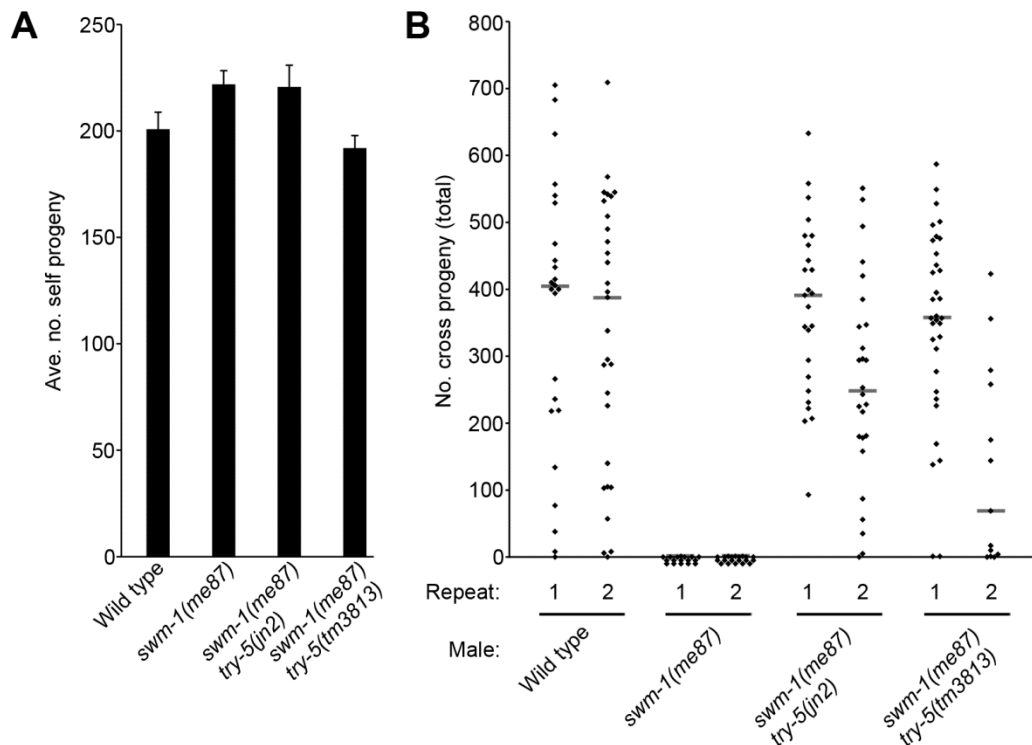
Figure 2.S3. *swm-1 try-5* double mutant hermaphrodites and males are fertile.   Assays of hermaphrodite self-fertility and male fertility. (A) *swm-1 try-5* double mutant hermaphrodites have wild-type fertility levels. Columns indicate average brood size of self-fertilizing hermaphrodites. Error bars represent standard error of the mean. (B) *swm-1 try-5* males have improved fertility as compared to *swm-1* males. Although the fertility of double mutants was always significantly higher than that of *swm-1*, variable levels of suppression were observed for the *swm-1 try-5(tm3813)* strain. The results of two representative experiments are shown. Each point represents the result of an individual cross; gray lines represent medians. For Repeat 1, *swm-1 try-5(tm3813)* fertility did not differ from that of wild-type males (p=0.67, Mann-Whitney U Test), a result obtained twice. For Repeat 2, *swm-1 try-5(tm3813)* fertility did differ from that of wild-type males (p=0.003, Mann-Whitney U Test), a result that was also obtained twice. For both repeats, *swm-1 try-5(jn2)* fertility did not differ from that of wild-type males (Repeat 1: p=0.97, Repeat 2: p=0.12; Mann-Whitney U Test).
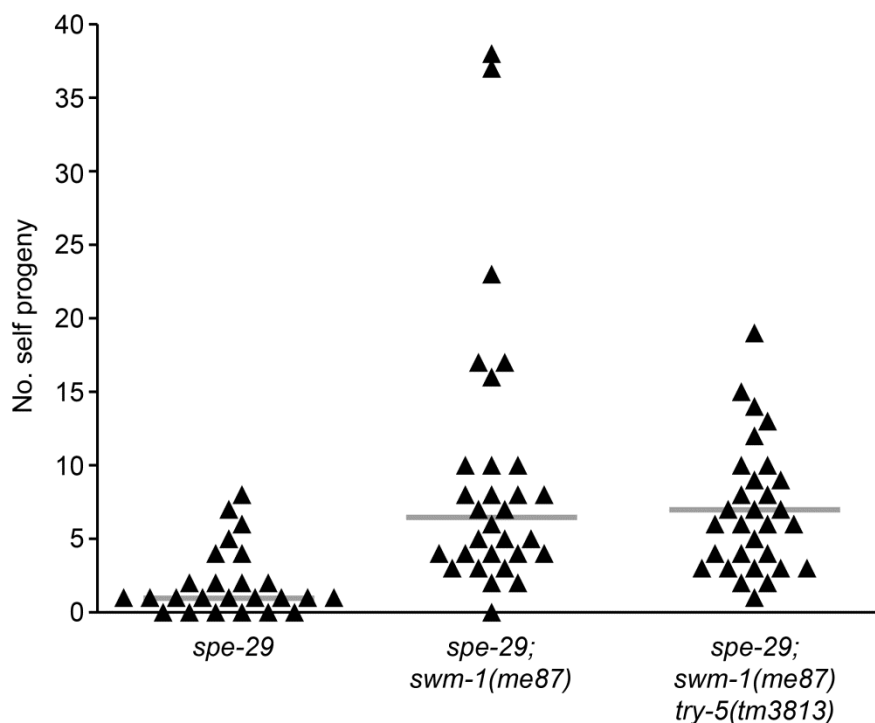
Figure 2.S4. *try-5* activity is not required in hermaphrodites for suppression of *spe-29* sterility by *swm-1*. Assay of hermaphrodite self-fertility. Total self-progeny broods from individual hermaphrodites were counted for each strain (Protocol S1). Each point represents the total self-progeny from an individual hermaphrodite; lines indicate the median for each set. Three replicates of the experiment were performed, with equivalent results; data from one such replicate are shown. *spe-29; swm-1* and *spe-29; swm-1 try-5* hermaphrodite fertility were each significantly different when compared to *spe-29* fertility (p<10[-6], Mann-Whitney U test). Their fertility was not significantly different when compared to each other (p=0.65). In addition to the listed genotypes, all strains also contained the mutation *dpy-20(e1282)*.

Table 2.1. TRY-5 is not required for activation in *spe-6* animals.

Genotype[1]	% Act[2]	n
wild type	97	32
spe-6(hc163)	100	40
try-5(jn2)	0	43
try-5(tm3813)	0	53
spe-6(hc163); try-5(jn2)	100	42
spe-6(hc163); try-5(tm3813)	100	61

[1]All strains also contained the mutation *dpy-18(e364)*.
[2]Percent of 48 hr post-L4 males containing activated sperm.

Table 2.S1. Primers used for construction of Gateway Donor plasmids.

Plasmid	Fragment description	Length	Gateway vector	Forward primer[1]	Reverse primer[1]
pJRS13	*try-5* promoter	1857 bp	pDONR P4-P1r	ggggacaactttgtatagaaaagttgTGCTTGTCCTCACACTGCTC	ggggactgcttttttgtacaaacttgTTGAATTTGAATTCCCGCTG
pJRS8	*try-5* 3' UTR	776 bp	pDONR P2r-P3	ggggacagctttcttgtacaaagtggCTTGATTCTTTGTTCACATTCAA	ggggacaactttgtataataaagttgAGCTGAGCATTTTGGGAGTCTGACTC
pJRS7	*try-5* coding region	1589 bp	pDONR 221	ggggacaagtttgtacaaaaaagcaggctATGCGTCCCCGAATAATTGTATTCCT	ggggaccactttgtacaagaaagctgggtAAGCTTGATTAATAAAATTCACG
pJRS11[2]	*try-5::GFP(S65C)*	2590 bp	pDONR 221	ggggacaagtttgtacaaaaaagcaggctATGCGTCCCCGAATAATTGTATTCCT	ggggaccactttgtacaagaaagctgggtCTATTTGTATAGTTCATCCATGCC
pJRS11	*try-5*	1631 bp	NA[3]	AGCGGGAATTCAAATTCAAATGCGT	AGTCGACCTGCAGGCATGCAAGCTAGCTTGATTAATAAAATTCACG
pJRS11	GFP(S65C)[4]	1892 bp	NA	AGCTTGCATGCCTGCAGGTCG	AAGGGCCCGTACGGCCGACTA

[1] For primers, lower-case type indicates spacer and *att* site sequences; upper-case type indicates gene-specific sequences. Genomic *him-5* DNA was used as a template unless noted.

[2] To generate the try-5::GFP(S65C) construct pJRS11, fusion PCR was performed using the 1631bp try-5 and 1892bp GFP fragments listed.

[3] NA, not applicable.

[4] The plasmid pPD95.75 (gift of A. Fire) was used as a template.

Table 2.S2. Donor plasmids used for construction of destination constructs.

Plasmid	Description	Position 1	Position 2[1]	Position 3	Vector[2]
pJRS14	*Ptry-5::try-5::try-5* 3' UTR	pJRS13	pJRS7	pJRS8	pCFJ150
pJRS18	*Ptry-5::try-5::GFP(S65T)::try-5* 3' UTR	pJRS13	pJRS17	pJRS8	pCFJ150
pJRS22	*Ptry-5::GFP::H2B::try-5* 3' UTR	pJRS13	pCM1.35	pJRS8	pCFJ150

[1] Plasmid pCM1.35 was a gift of of G.Seydoux (Merritt et al., 2008).
[2] Plasmid pCFJ150 was a gift of C. Frokjaer-Jensen (Frokjaer-Jensen et al., 2008).

Table 2.S3. Premature sperm activation depends on TRY-5 and SWM-1 and increases

with male age.

Genotype	Age[1]	nonAct	partAct	fullAct	Total
Wild type	24hr	56	0	0	56
	48hr	47	0	0	47
	72hr	51	5	1	57
swm-1(me87)	24hr	1	1	51	53
	48hr	0	0	49	49
	72hr	0	1	52	53
try-5(tm3813)	24hr	53	0	0	53
	48hr	44	0	0	44
	72hr	72	0	0	72
swm-1(me87) try-5(tm3813)	24hr	53	0	0	53
	48hr	50	0	0	50
	72hr	51	0	0	51
jnSi62[Ptry-5::TRY-5::GFP]; try-5(tm3813)	24hr	55	0	0	55
	48hr	51	0	0	51
	72hr	120	4	0	124
jnSi62[Ptry-5::TRY-5::GFP]; swm-1(me87) try-5(tm3813)	24hr	22	11	9	42
	48hr	0	0	54	54
	72hr	0	0	67	67

[1]L4 larval stage males were isolated from hermaphrodites and incubated at 20C for the indicated number of hours before observation.

Table 2.S4. *spe-8* group*; try-5* sperm do not migrate after transfer to a hermaphrodite.

Genotype	Total crosses	No. transfer[1]	No. migration[2]
spe-27	45	21	12
spe-27; try-5(tm3813)	42	19	0
spe-29	50	34	32
spe-29; try-5(tm3813)	50	35	1

[1]Number of crosses in which MitoTracker-labeled sperm were observed inside recipients.
[2]Number of crosses in which sperm migrated to the spermathecae.

Table 2.S5. Correlation between sperm activation and TRY-5::GFP localization.

Genotype	Age[4]	No TRY-5::GFP present within sperm zone[1]			TRY-5::GFP foci within sperm zone[2]			TRY-5::GFP around cells in sperm zone[3]			Total
		non Act	part Act	full Act	non Act	part Act	full Act	non Act	part Act	full Act	
jnSi62[Ptry-5::TRY-5 ::GFP]; try-5(tm3813)	24hr	5	0	0	50	0	0	0	0	0	55
	48hr	25	0	0	26	0	0	0	0	0	51
	72hr	43	0	0	75	0	0	2	4	0	124
jnSi62[Ptry-5::TRY-5 ::GFP]; swm-1(me87) try-5(tm3813)	24hr	12	0	0	10	2	0	0	9	9	42
	48hr	0	0	0	0	0	0	0	0	54	54
	72hr	0	0	0	0	0	0	0	0	67	67

[1]No TRY-5::GFP was visible adjacent to sperm cells in the seminal vesicle lumen.

[2]Discrete foci of TRY-5::GFP were observed adjacent to sperm cells within the seminal vesicle lumen.

[3]Areas of TRY-5::GFP were present in a dispersed honeycomb-like pattern surrounding sperm cells in the seminal vesicle lumen.

[4]L4 larval stage males were isolated from hermaphrodites and incubated at 20°C for the indicated number of hours before observation.

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CHAPTER 3

EXPRESSION AND TRANSFER OF TRY-5

Introduction

Seminal fluid contains many proteins, which are predicted to have varying

activities. For these proteins to affect sperm function, they need to enter the seminal fluid

by secretion from neighboring tissues. Secretion of proteins into seminal fluid occurs by

a variety of methods. Proteins can be secreted directly into the seminal fluid as soluble

proteins prior to mating. This secretion occurs in the seminal vesicle, epididymis, and

prostate. For example, HE1/CTP transports hydrophobic compounds such as cholesterol

in the seminal fluid to sperm (Kirchhoff et al., 1996). Another important group of

seminal fluid proteins are the semenogelins, which form seminal clots after mating that

restrict sperm movement (Lilja and Lundwall, 1992). Other factors are secreted only

after mating begins and may have roles only during the process of mating. One such

protease is prostate specific antigen (PSA), which is secreted from the prostate and added

to seminal fluid during mating (reviewed in Veveris-Lowe et al., 2007).

As an alternative to direct secretion, seminal fluid components may be transferred

in small, 50-500 nm vesicles that are called exosomes. Exosomes can be secreted from

several tissues. Within the male reproductive tissues, exosomes are secreted from the

epididymis (epididymosomes) and the prostate (prostasomes) (reviewed in Saez et al.,

2003). Once secreted, these vesicles can either remain within the seminal fluid or bind to

the sperm membrane, altering the components on the cell surface, and thus sperm function. In the cases where prostasomes remain unfused, no precise function is known. However, prostasomes are thought to have roles in multiple processes, including modulating immune responses, capacitation, and providing antioxidant protection (reviewed in Sullivan et al., 2005).

In TRY-5::GFP expressing male worms, I found that TRY-5 is secreted from several tissues of the somatic gonad. Transfer occurs in a series of discrete steps and always occurs in the same order. First, TRY-5 from the vas deferens is transferred. A brief pause in TRY-5 transfer occurs before TRY-5 from the valve region is transferred. The valve pool of TRY-5 is immediately followed by sperm transfer (Smith and Stanfield, 2011). The mechanisms for how TRY-5 is transferred in such an ordered fashion are unknown.

In this chapter, I focus on the secretion of TRY-5. I describe additional *try-5* alleles that suggest TRY-5 secretion in *swm-1* mutant males and during mating may occur through different processes, and I explore the expression and transfer of TRY-5 in more detail. These results suggest that TRY-5 release is dependent on a secretion signal and may originate in vesicles of the valve region.

Results

jn13 and *jn21* Swm-1 phenotype suppression

A nonsense mutation, *jn13*, in which the last 20 amino acids of the TRY-5 protein are not translated, was identified in a screen for suppressors of the premature sperm activation phenotype in males with the partial loss-of-function allele of *swm-1*, *me86* (A. Mulia, B. Duffy, and A. Duffy, personal communication). I found that *jn13* fully

suppressed the Swm-1 premature sperm activation phenotype (Smith and Stanfield, 2011). In addition, *jn21*, a mutation in the start codon of *try-5*, was identified in a premature sperm activation phenotype suppressor screen using males with a different partial loss-of-function allele of *swm-1*, *me66* (B. Duffy, and A. Duffy, personal communication). I examined the suppression of the Swm-1 premature sperm activation phenotype. I found that while all *swm-1(me66)* virgin males contained activated sperm 48 hr after the L4 stage, 60% of *swm-1(me66) try-5(jn21)* males did not contain activated sperm. Of the remaining 40% of males, about half contained activated sperm and the other half contained a mix of spermatids and spermatozoa (Smith and Stanfield, 2011). Since suppression is not complete, *jn21* represents a likely partial loss-of-function allele.

Timing and temperature sensitivity

of activation in *try-5(jn2)* males

Male sperm activates earlier at higher temperatures (G. Stanfield, personal communication). I tested if *try-5* mutant male sperm activated at higher temperatures than the standard maintenance temperature of 20°C (Figure 3.1). I used the *jn2* allele of *try-5*, since it is a mutation in a conserved cysteine and is a likely null (Smith and Stanfield, 2011). At 24 hr, 48 hr, and 72 hr after the L4 stage and at 15°C, 20°C, and 25°C for each time point, fewer *swm-1(me86) try-5(jn2)* males than either the wild-type control or the *swm-1(me86)* control contained activated sperm. This result suggests that while *try-5* is necessary for efficient activation in *swm-1* mutants, activation can still occur, although at a slower rate. Alternatively, *jn2* may be a partial loss-of-function allele of *try-5* and its residual activity activates sperm at later time points.

Time course of TRY-5::GFP expression

To learn more about the regulation of *try-5* expression and timing of expression, I examined the pattern of TRY-5::GFP localization in four independent *Ptry-5::TRY-5::GFP* extrachromosomal array lines in *unc-119; swm-1(me86) try-5(tm3813)* males. I found that expression started during the L4 stage in the vas deferens. Expression was observed in the valve region 4 to 9 hours later. TRY-5::GFP was observed initially only within the tissues of expression; however, beginning between 12 and 25 hr after L4, TRY-5::GFP was observed to spread into the lumen of the seminal vesicle (data not shown). The source of this TRY-5::GFP was not determined and activation was not scored for these worms. These results show that while TRY-5::GFP is present within the seminal vesicle starting at 48 hr after the L4 stage, prior to that time, TRY-5::GFP may only be in or immediately adjacent to the cells of expression.

Tissue specific rescue of *try-5*

TRY-5::GFP expression was previously observed in neurons in *Ptry-5::TRY-5::GFP* strains in which the transgene was stably inserted using the MosSCI technique (Mos1-mediated Single Copy gene Insertion) (Frokjaer-Jensen et al., 2008; Smith and Stanfield, 2011). I sought to determine if *try-5* expression in tissues other than the somatic gonad is sufficient for the rescue of the *try-5* phenotype, or if expression in the somatic gonad is necessary for rescue of the *try-5* phenotype. I generated transgenic worms with *Prab-3::TRY-5::GFP* (neuronal) and *PC49C3.12::TRY-5::GFP* (vas deferens) transgenes stably inserted. When present in a *try-5(tm3813)* mutant background, virgin adult males did not contain activated sperm, similar to *Ptry-5::TRY-5::GFP; try-5(tm3813)* males. However, in a *swm-1(me87) try-5(tm3813)* mutant

background, sperm activation did occur in males with neuronally expressed TRY-5::GFP at a rate similar to that of *Ptry-5::TRY-5::GFP* males (Figure 3.2). In males containing activated sperm, GFP was not visible within the seminal vesicle. In *PC49C3.12::TRY-5::GFP* males, fewer than 10% of virgin adult males aged 48 hr after the L4 stage contained activated sperm (Figure 3.2). To determine if activation was time dependent, I also scored these males 72 hr after the L4 stage. I found that the older males more frequently had activated sperm, but this level of rescue was lower than that of *Ptry-5::TRY-5::GFP* or *Prab-3::TRY-5::GFP* males. These results show that expression of TRY-5 in neurons, but not the seminal vesicle, can rescue sperm activation in a *swm-1* mutant background. While TRY-5 expression in neurons may rescue sperm activation, whether TRY-5 enters the seminal vesicle and signals sperm activation directly is unknown.

Transactivation is not rescued by TRY-5::GFP expression
in the somatic gonad or neurons

     try-5 has been shown to be necessary for activation in two different cases. One case is activation of sperm in *swm-1* males. Sperm activation has been rescued with a stable insertion of *Ptry-5::TRY-5::GFP* in *swm-1 try-5* mutant backgrounds (Smith and Stanfield, 2011). The other case is transfer of the male activator, as measured by transactivation of *spe-8* mutant hermaphrodite sperm. To show that *try-5* is necessary for transactivation, I expressed *try-5* in a *fer-1* mutant background, using the same transgene that rescues premature sperm activation. Despite observations that TRY-5::GFP can be transferred to hermaphrodites during mating, this transgene does not rescue transactivation to any large extent (Figure 3.3). In a few cases, some self-progeny was

observed; however, the number of self-progeny was not different when compared to that of the no mating (P=0.76, Mann-Whitney U Test) and *fer-1; try-5* (P=0.15, Mann-Whitney U Test) controls. However, 3 out of 22 crosses did produce more self-progeny than produced in any of the no male controls. Lack of rescue was confirmed in an additional two lines (A. Hansen, personal communication), although infrequent transactivation did occur after mating with males from these two lines. Overall, the *Ptry-5::TRY-5::GFP* transgene does not efficiently rescue the transactivation defect.

To test whether expression in other tissues is sufficient for transactivation, I expressed TRY-5::GFP in neurons (*Prab-3::TRY-5::GFP*) and the vas deferens (*PC49C3.12::TRY-5::GFP*) in a *fer-1* mutant background. Upon mating to *spe-8* mutant hermaphrodites, *fer-1; Prab-3::TRY-5::GFP; try-5(tm3813)* males did not transactivate *spe-8* mutant hermaphrodite sperm (Figure 3.3). *fer-1; PC49C3.12::TRY-5::GFP; try-5(tm3813)* male transactivation was similar to that of *fer-1; Ptry-5::TRY-5::GFP; try-5(tm3813)* transactivation (P=0.15, Mann-Whitney U Test) (Figure 3.3). Transactivation was observed in 5 of 13 crosses performed, although few self-progeny were produced in each case. I confirmed that TRY-5::GFP expression occurred at 25°C by examining males at the beginning and end points of the experiment and saw expression similar to that of males grown at 20°C (data not shown). These results suggest that transactivation cannot be rescued by expression of TRY-5::GFP, which may be due to the necessity of a high level of TRY-5 transfer for transactivation to occur. To test if GFP interferes with TRY-5 function, I generated a line consisting of a *Ptry-5::TRY-5* stable insertion in a *fer-1; try-5(tm3813) him-5* background. I found that these males were not capable of transactivation (Figure 3.3). However, this transgene has not been shown to be active by

rescuing *try-5* suppression of the Swm-1 premature sperm activation phenotype and only one line has been tested for rescue of the transactivation defect.

Transactivation is deficient in *try-5* transheterozygotes

Since transgene rescue of the Try-5 transactivation defect was unsuccessful, mutations in *try-5* may not be the cause of the transactivation defect, and instead another background mutation might be responsible for this phenotype. To test whether mutations in *try-5* result in a transactivation defect, I determined if *try-5* mutant transheterozygotes were able to transactivate *spe-8* hermaphrodite sperm. If the transactivation defect remains in *try-5* transheterozygotes, then background mutations are unlikely to be the causal mutations for the transactivation phenotype. I tested the *jn2* and *tm3813* alleles, since both of these mutations are likely nulls and were produced in different labs, and thus they were likely to have different background mutations. *try-5* heterozygous mutant males appeared to transactivate fewer *spe-8* mutant hermaphrodite sperm and induced transactivation less frequently than the *fer-1* control (Figure 3.4). The *try-5* transheterozygotes do not transactivate *spe-8* hermaphrodite sperm, which is similar to that of *try-5(jn2)* and *try-5(tm3813)* homozygous males. Since a temperature-sensitive allele of *fer-1* was used, cross progeny occasionally resulted from crosses. Cases in which cross progeny were produced were removed from analysis, since sperm competition could have reduced the number of self-progeny produced. Removal of these crosses from analysis resulted in a relatively small data set. In addition, mating was not confirmed for the transheterozygotes, although homozygotes for both alleles of *try-5* have been confirmed to mate. The reduced transactivation by heterozygous *try-5* mutants demonstrates that transactivation is dependent on the number of copies of *try-5* present in

the male. These results suggest that *try-5* is necessary for transactivation, and the phenotype is not a result of a background mutation.

Males with partial loss-of-function alleles

of *try-5* have defects in transactivation

Using the transactivation assay, I could test if *jn13* and *jn21* were indeed partial loss-of-function alleles of *try-5*. Since *try-5* is only 0.1 map unit from *swm-1*, I had not separated the *swm-1* and *try-5* mutations for either *try-5* allele. Instead, I tested *fer-1; swm-1(me86)* males and *fer-1; swm-1(me86) try-5(jn13)* males for their ability to transactivate *spe-8* hermaphrodite sperm (Figure 3.5A). I found that *fer-1; swm-1(me86)* males were able to transactivate sperm infrequently, and at levels greater than that of *fer-1; try-5(tm3813)* males (P=0.08, Mann-Whitney U Test). Transactivation of *spe-8* hermaphrodite sperm occurred very infrequently (4 crosses out of 134 performed) after mating with *fer-1; swm-1(me86) try-5(jn13)* males (Figure 3.5A and data not shown). While *fer-1* and *fer-1; swm-1(me66)* males were capable of transactivation of *spe-8* hermaphrodite sperm, *fer-1; swm-1(me66) try-5(jn21)* males never transactivated *spe-8* hermaphrodite sperm (Figure 3.5B). I found no transactivation even in three rounds of setting up 25 crosses each. These results suggest that *jn21* is a partial loss-of-function allele of *try-5* based on partial suppression of the Swm-1 premature activation phenotype, but is not capable of transactivation. The *jn13* allele results suggest that it may be a null allele. However, the occasional transactivation observed suggests that *jn13* may be a partial loss-of-function allele of *try-5*. Further repeats are needed to confirm this possibility.

Higher resolution imaging of TRY-5::GFP transfer

Transfer of TRY-5::GFP has been described previously (Smith and Stanfield, 2011); however, spatial and temporal resolution was limited due to the techniques used. A compound microscope subsequently was used to decrease exposure times and increase resolution. I found that exposure to the level of light necessary for GFP imaging disturbed mating. Males appeared to stop the process of mating and would attempt to crawl away, as has been previously described in response to blue light (Edwards et al., 2008). I did observe one successful case of mating (Figure 3.6). TRY-5::GFP was present at the tip of the male tail prior to imaging, thus vas deferens TRY-5::GFP transfer already may have occurred. The valve TRY-5::GFP transfer appeared similar to previous observation, including the timing, amount transferred, that a single pool was transferred, and spread to a similar extent once in the hermaphrodite. However, the male did not successfully terminate mating by removing his spicules from the hermaphrodite, which suggests other aspects of mating may be disturbed. In the valve region, only a fraction of TRY-5::GFP appeared to be released. Shortly before release, TRY-5::GFP appeared to form small foci similar in size to vesicle-like structures seen in the valve region prior to mating. Remaining TRY-5::GFP in the valve region also appeared to be in vesicle-like structures that appeared to be in cells of the valve region. These results suggest that membrane-bound TRY-5 may be transferred during mating. Membrane transfer from the vas deferens has been suggested based on transfer of GFP by males expressing *PC49C3.12::memGFP* (data not shown). In this case, a membrane-localization signal was fused to GFP, thus the GFP present in the hermaphrodite after mating likely was transferred with membrane from the vas deferens.

Discussion

try-5 expression appears to begin early during the L4 stage. Expression initially starts in a few cells of the vas deferens and expands to the valve region and seminal vesicle as the males age. During adulthood, expression of *try-5* appears to continue, since GFP continually becomes brighter until at least 75 hr after the L4 stage. Expression occurs in the three different tissues by early adulthood, with expression seen in the valve region and vas deferens. These results show that TRY-5 is expressed before the male is capable of mating. Expression continues through adulthood, allowing for a high level of TRY-5 protein to be present during mating. Also, TRY-5 may be replenished after mating by the continued expression; however, the recovery of TRY-5 after mating has not been analyzed. For the time course, the TRY-5::GFP transgene was present on an extrachromosomal array, and as a result an unusually high level of expression may have occurred. A higher level of expression may not accurately replicate the endogenous expression of TRY-5. Additionally, mosaicism may occur, since the array can be lost. To control for these cases, multiple lines were examined and a similar pattern of expression was found in each line. Finally, the experiment was performed under a dissecting microscope, which allowed for observation of the same worms at multiple times. However, a higher level of GFP is required to see expression. As a result, areas of low expression may not be visible.

In *Ptry-5::TRY-5::GFP* and *Ptry-5::GFP::H2B* transgenic males, GFP was observed in a small number of neurons within the tail and near the pharynx of the worm, both when expressed from extrachromosomal arrays and MosSCI insertions. To determine if expression outside the somatic gonad is sufficient for TRY-5 function, I

expressed TRY-5 in neurons and observed that sperm activation occurred in a *swm-1*

mutant background. This result shows that TRY-5 expression in neurons can result in

activation. Two models of how this activation occurs are possible. First, TRY-5 within

neurons is necessary for the signaling of activation. Alternatively, TRY-5 is secreted

from neurons and able to enter the seminal vesicle to signal sperm activation in a process

that does not replicate endogenous sperm activation. While TRY-5::GFP was not

observed in the lumen of seminal vesicles, expression was observed adjacent to cells of

the seminal vesicle. Since TRY-5 is likely to function as a protease, cell-cell contacts

may be disturbed by proteolytic cleavage of proteins necessary to maintain cell-cell

adhesion, allowing for TRY-5 to enter the seminal vesicle in low concentrations, which

would not be easily observable. This model would require that TRY-5 is able to signal

sperm activation at a low concentration within the male. Additional expression in other

somatic tissues needs to be performed in the future to determine if secretion from

anywhere outside of the somatic gonad can signal sperm activation in males.

To test whether the mutations I have identified in *try-5* are responsible for the

transactivation defect phenotype, I tried to rescue the defect using a known functional

TRY-5::GFP transgene. Surprisingly, I found that only a low level of transactivation

occurred. I confirmed that TRY-5::GFP is expressed at the experiment temperature. I

observed that a low level of transactivation also occurred after mating with males that had

a *PC49C3.12::TRY-5::GFP* transgene, which expresses TRY-5::GFP in the vas deferens.

However, this transactivation did not occur after mating with *Prab-3::TRY-5::GFP*

transgenic males. These results suggest that TRY-5::GFP expressed within the somatic

gonad may rescue the *try-5* transactivation defect. Since this rescue is not seen when

TRY-5::GFP is expressed in neurons, the level of transactivation is likely due to the expression of TRY-5::GFP in the vas deferens and also potentially the valve region. However, the low level of transactivation observed may be due to several complications inherent in this experiment. TRY-5::GFP must be secreted and transferred in sufficient levels to signal sperm activation in hermaphrodites. Any reduction in TRY-5 expression compared to endogenous TRY-5 expression or reduction in transfer of TRY-5 may reduce the concentration of TRY-5 within the hermaphrodite after transfer to levels below that which is necessary to signal activation. Furthermore, the TRY-5::GFP fusion may have reduced activity if GFP interferes with activity of the protease domain.

Since the *try-5* transactivation defect phenotype could not be strongly rescued by a transgene, I tested males mutant for different alleles of *try-5* for transactivation. I have found that males mutant for all alleles of *try-5* do have the same transactivation defect phenotype. To further show that the mutations in *try-5* are likely causal for this phenotype and not another background mutation, I tested *try-5* transheterozygotes for transactivation. I found that transheterozygotes could not activate *spe-8* mutant hermaphrodite sperm. These results suggest that the *try-5* mutation is responsible for the transactivation defect. The likelihood of *try-5* being necessary for transactivation is made stronger by the *jn2* and *tm3813* alleles being produced in independent labs. Independent production of these alleles reduces the number of background mutations theses two strains would have in common. By analyzing transheterozygotes, these background mutations are likely to be heterozygous and thus not causal for the elimination of transactivation observed in *try-5* transheterozygotes.

I tested *try-5(jn13)* using a transactivation assay to determine if *jn13* is a partial loss-of-function allele of *try-5*. I found that a low level of transactivation of *spe-8* hermaphrodite sperm did occur after mating with these males, based on the frequency of self-progeny produced after mating. However, this transactivation was not significantly different from that of the no male control. This result suggests that residual function of the TRY-5 protein does remain. While *jn13* appears to be a null of *try-5*, the infrequent transactivation observed suggests it may not be.

In addition, I observed that *swm-1(me86)* males are less capable of transactivation than wild-type males. This result was surprising, as TRY-5 function is expected to still be present in the male, since sperm activation still occurs within the male and the screen which identified *swm-1* was depended on mutants being capable of transactivation (Stanfield and Villeneuve, 2006). In *swm-1* mutant males, I observed that TRY-5 is more broadly localized, appearing to fill the lumens of the vas deferens and seminal vesicle. This localization may result in a lower concentration of TRY-5 that is not sufficient to signal sperm activation, since broader localization of TRY-5 may be less efficiently transferred than when TRY-5 is released from somatic gonad tissues during mating. To differentiate between these possibilities, observations of TRY-5 transfer from *swm-1* mutant males need to be made to determine how much TRY-5 is transferred. In addition, a better understanding of how SWM-1 and TRY-5 interact may reveal whether TRY-5 is degraded in the absence of SWM-1.

I found that the *jn21* allele of *try-5* only partially suppressed the *swm-1* premature sperm activation phenotype. This observation suggests that *jn21* is a partial loss-of-function allele of *try-5*. However, I observed that *jn21* males were not capable of

transactivation. This differs from the other likely potential partial loss-of-function allele of *try-5*, *jn13*, which does occasionally transactivate *spe-8* sperm. These results suggest that *jn21* may affect a different aspect of TRY-5 function than *jn13*. *jn21* alters the translational start site of TRY-5. Since TRY-5 activity is still present, based on the partial suppression of the *swm-1* phenotype, TRY-5 likely is translated, starting from a different translational start site. By starting at a different site, the secretion signal may be removed, while maintaining the protease domain. Several proteins, such as expression of a membrane-localization signal GFP fusion, ectopically expressed in the somatic gonad will still localize to secretory structures (A. Snow, personal communication). This suggests that a secretion signal may not be necessary for secretion to occur from the somatic gonad. Thus TRY-5 without a secretion signal may still be released from the somatic gonad, but perhaps at a lower level. If a low level of protease is sufficient for sperm activation in a *swm-1* mutant, but a high level is necessary for transactivation to occur, then this model would explain the phenotype seen. To confirm that this is the case, the localization and dynamics of TRY-5 prior to and during mating would need to be determined. A TRY-5::GFP construct that includes the *jn21* mutation would allow for such observations. Understanding how TRY-5 is secreted from the somatic gonad could allow for a better understanding of the regulation of secretion from the vas deferens and valve region.

Initial experiments to examine the process of TRY-5::GFP transfer to hermaphrodites during mating were performed using a dissecting microscope, which limited the resolution such that subcellular localization within cells could not be determined. By using conditions that improved imaging resolution, I found that only a

subset of the TRY-5::GFP pool within the valve region was transferred to hermaphrodites during mating. Upon further examination of images previously taken on the dissecting microscope, partial transfer does not appear to be a result of the imaging method, and instead is common amongst males tested. TRY-5::GFP that was transferred appeared to be from the apical side of the valve region. Whether this pool is intracellular has not been determined. Thus, two models of TRY-5::GFP transfer from the valve region can be proposed. One model is that TRY-5::GFP is secreted shortly after it is expressed. This pool may remain in the valve region either by creating barriers to diffusion or by a lack of flow within the lumen of the somatic gonad. The latter model could also be true if TRY-5::GFP release occurred shortly before the beginning of observations, thus not allowing enough time for it to spread within the somatic gonad. The other model is that TRY-5 is secreted only after mating has occurred. By further observing the process of TRY-5::GFP transfer, support for one of these two models may be found. More detail may be observable by using *lite-1* mutants, which do not respond to exposure of blue light (Edwards et al., 2008).

Overall, TRY-5 and seminal fluid secretion remains poorly understood with many remaining questions. Mechanisms for regulating secretion, especially how timing is accomplished, are not understood. Furthermore, how seminal fluid could be quickly released is not understood. Since TRY-5 represents only the second seminal fluid protein identified in *C. elegans*, whether most seminal fluid proteins are expressed in the same tissues and secreted simultaneously as TRY-5 is not known. Despite the remaining questions, identification of TRY-5 and understanding how its secretion is regulated may aid future studies of seminal fluid generation in worms.

Materials and methods

C. elegans **genetics**

Worms were maintained at 20°C as described in Brenner (1974) unless otherwise mentioned. *him-5(e1490)* worms were used as the wild type to ensure a ready supply of males and always present in male stocks (Hodgkin et al., 1979). Alleles used in this study were: *spe-8(hc53) I* (Wood and The Community of *C. elegans* Researchers, 1988), *fer-1(hc1*ts*) I* (Wood and The Community of *C. elegans* Researchers, 1988), *unc-119(ed3) III* (Maduro and Pilgrim, 1995), *dpy-4(e1166) IV* (Wood and The Community of *C. elegans* Researchers, 1988), *unc-31(e169) IV* (Wood and The Community of *C. elegans* Researchers, 1988)*, swm-1(me66, me86, me87) V* (Stanfield and Villeneuve, 2006), and *try-5(jn2, jn13, jn21, tm3813) V* (Smith and Stanfield, 2011).

Temperature and time course of suppression

L4 hermaphrodites were moved to plates placed at 15°C, 20°C, or 25°C. Males from subsequent generations were picked as L4s onto plates without hermaphrodites and scored 24, 48, or 72 hr later. At least 28 worms were observed at each time point and temperature combination.

Tissue specific rescue

Gateway MosSCI constructs were prepared as described previously (Smith and Stanfield, 2011). *Prab-3* was from pEGB05, which was a gift of the Jorgensen lab (Hobson et al., 2011). *PC49C3.12* (Thoemke et al., 2005) was prepared using primers GGGGACAACTTTGTATAGAAAAGTTGagcctcattcatcgctgcgtcagtg and GGGGACTGCTTTTTTGTACAAACTTGtgcctgaagtgccacaaagca amplified from *him-5*

worms and recombined into the pDONR P4-P1r vector (Invitrogen). The 3' UTR used was the *try-5 3'UTR* from plasmid pJRS8 (Smith and Stanfield, 2011). Injections into *ttTi5605; unc-119(ed3)* worms were performed as previously described (Frokjaer-Jensen et al., 2008). Scoring was performed in the indicated backgrounds. Transactivation assays were performed as previously described (Smith and Stanfield, 2011). Transactivation experiments were repeated at least three times each with 25-40 crosses performed each repeat mating four males to one *spe-8(hc53); dpy-4* hermaphrodite. Crosses in which more than two cross progeny were produced were excluded from analysis.

Transheterozygotes

Transheterozygous males were generated by crossing *fer-1; try-5 him-5* males grown at 20°C to *fer-1; him-5* or *fer-1; try-5 him-5* hermaphrodites that were grown at 25°C, so that only cross progeny would result. The crosses were performed at 25°C. Males were picked and grown at 25°C and transactivation assays were performed as described previously (Smith and Stanfield, 2011).

Time course of TRY-5::GFP expression

I generated four independent extrachromosomal array lines consisting of a *Ptry-5::TRY-5::GFP, Cbr-unc-119(+)* array in a *unc-119(ed3); swm-1(me86) try-5(tm3813)* background (Maduro and Pilgrim, 1995). I picked five L4 males from each of two independent extrachromosomal array lines and ten L4 males from each of the other two independent lines to individual plates. I observed regions of TRY-5::GFP localization on a dissecting Leica MZ16FL meicroscope immediately after picking and 35 min, 1 hr 30

min, 3 hr 30 min, 5 hr 30 min, 8 hr 30 min, 11 hr 30 min, 24 hr 30 min, 36 hr, 48 hr, 60

hr, and 75 hr after picking.

Transfer of TRY-5::GFP

Mating was performed on 30 mm NGM plates without bacteria. One or two

jnSi62[Ptry-5::TRY-5::GFP]; try-5(tm3813) males aged 24 hr after the L4 stage were

mated to eight *unc-31(e169)* aged 24 hr after the L4 stage. Male movement was observed

under DIC using minimal light under a 10X objective using an AxioImager M1 (Zeiss)

microscope. Once a male made contact with a hermaphrodite, male mating behaviors

were observed under a 20X objective until the male found the vulva, at which point

recording of GFP images began. Exposure was 200 ms at maximal speed, acquired with
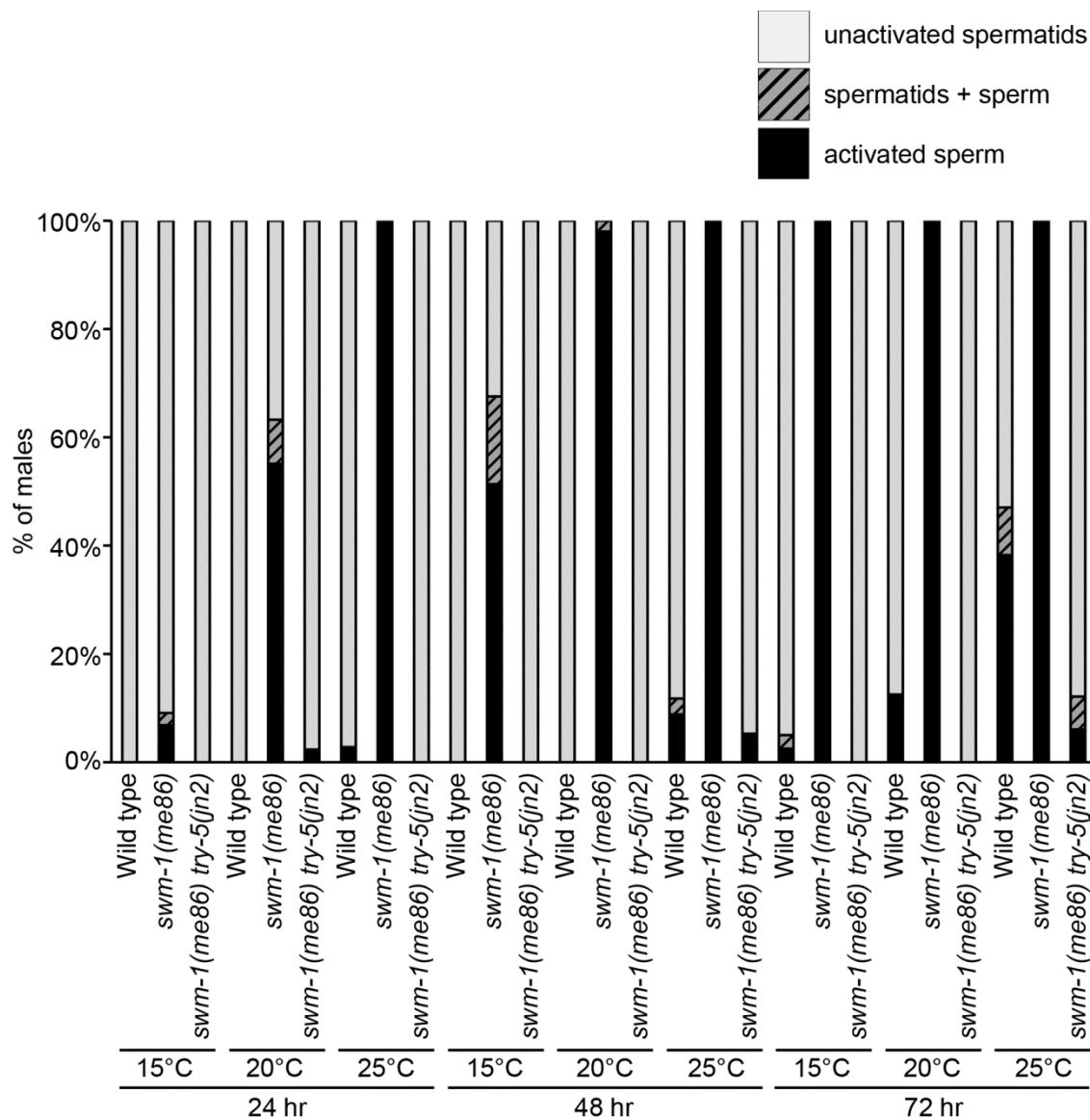
an AxioCam MRm (Zeiss) camera.

Figure 3.1. Sperm activation is time and temperature sensitive. Wild-type, *swm-1(me86)*, and *swm-1(me86) try-5(jn2)* males were examined for sperm activation 24 hr, 48 hr, or 72 hr after the L4 stage. For each genotype and time point, males were also scored for activation at 15°C, 20°C, or 25°C. Males were scored as containing only nonactivated sperm (gray bars), containing a mixture of activated and nonactivated sperm (hatched bars), or containing only activated sperm (black bars). At least 28 males were examined for each condition. All strains also contained the *him-5(e1490)* mutation to ensure a ready supply of males.
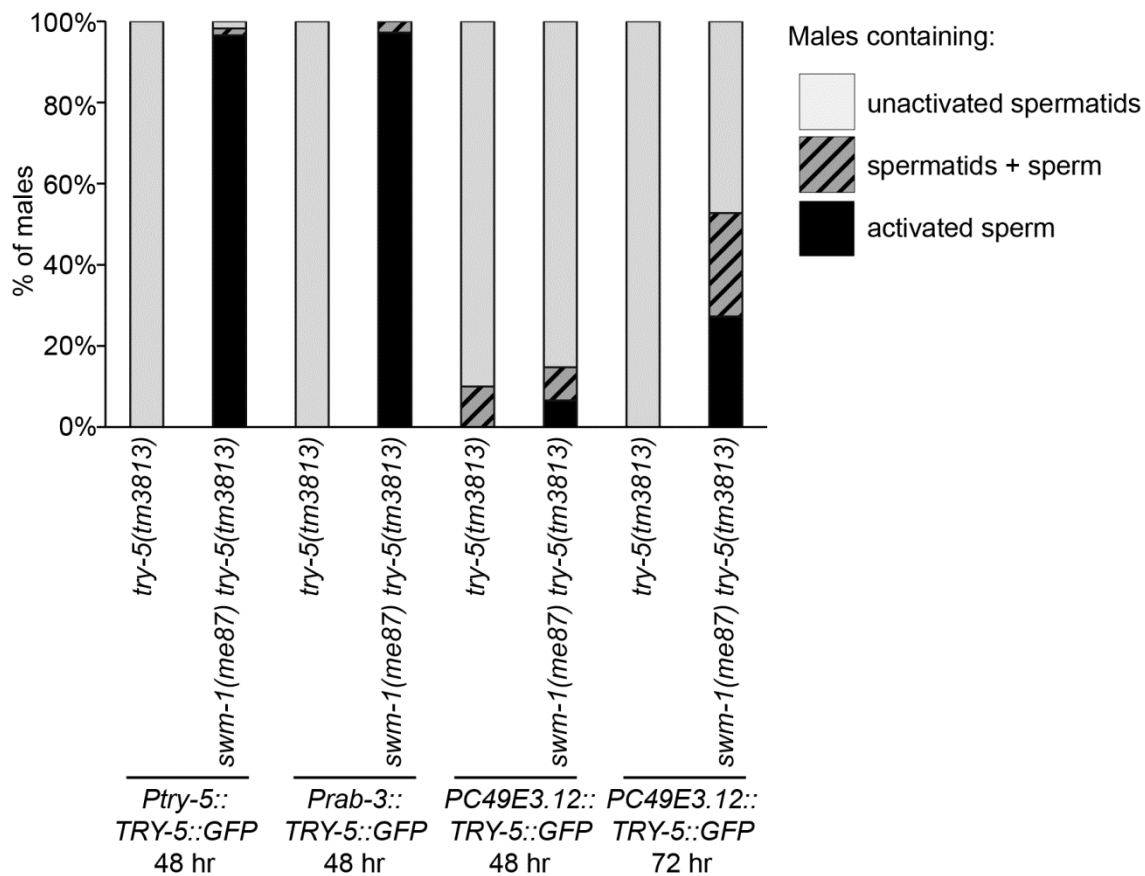
Figure 3.2. Activation of sperm in a *swm-1 try-5* mutant background can be rescued by expression of TRY-5 in several different tissues. TRY-5::GFP was expressed using different tissue-specific promoters: *Ptry-5* (vas deferens, valve region, seminal vesicle, and some neurons), *Prab-3* (neurons), and *PC49C3.12* (vas deferens). Activation was assessed in *try-5(tm3813)* and *swm-1(me87) try-5(tm3813)* backgrounds. Males were scored as containing only nonactivated sperm (gray bars), containing a mixture of activated and nonactivated sperm (hatched bars), or containing only activated sperm (black bars). At least 36 males were examined for each condition. All strains also contained the *him-5(e1490)* mutation to ensure a ready supply of males.
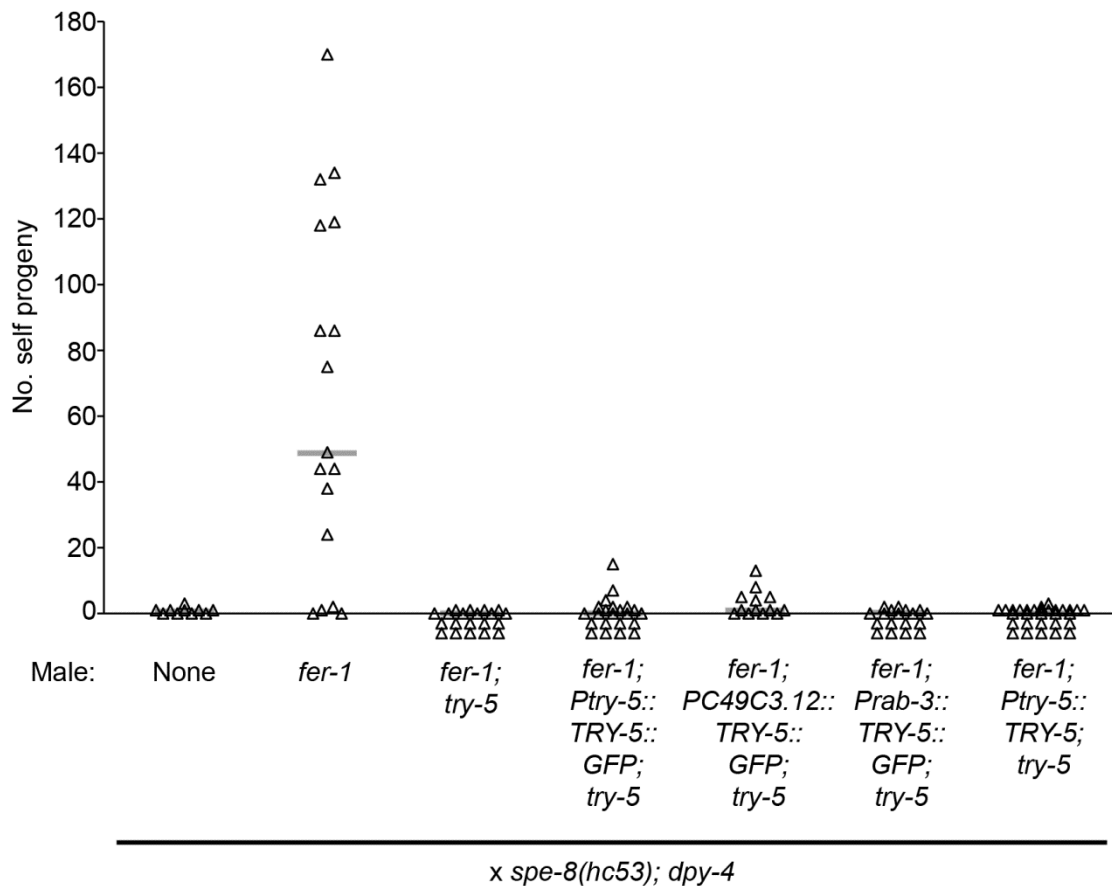
Figure 3.3. Transactivation by *try-5* mutant males cannot be efficiently rescued by transgenic expression of TRY-5::GFP. L4 males expressing TRY-5::GFP or TRY-5 under the endogenous *try-5* promoter, the *rab-3* neuronal promoter, or *C49C3.12* vas deferens promoter were mated 4:1 with L4 *spe-8(hc53); dpy-4* hermaphrodites for 48 hr. The number of self-progeny was scored. Gray lines indicate the median.

Figure 3.4. *try-5* transheterozygous males are not capable of transactivation. L4 males that were heterozygous for either the *jn2* or *tm3813* allele of *try-5* and transheterozygotes were mated 4:1 with L4 *spe-8(hc53); dpy-4* hermaphrodites for 48 hr. The number of self-progeny was scored. Gray lines indicate the median.
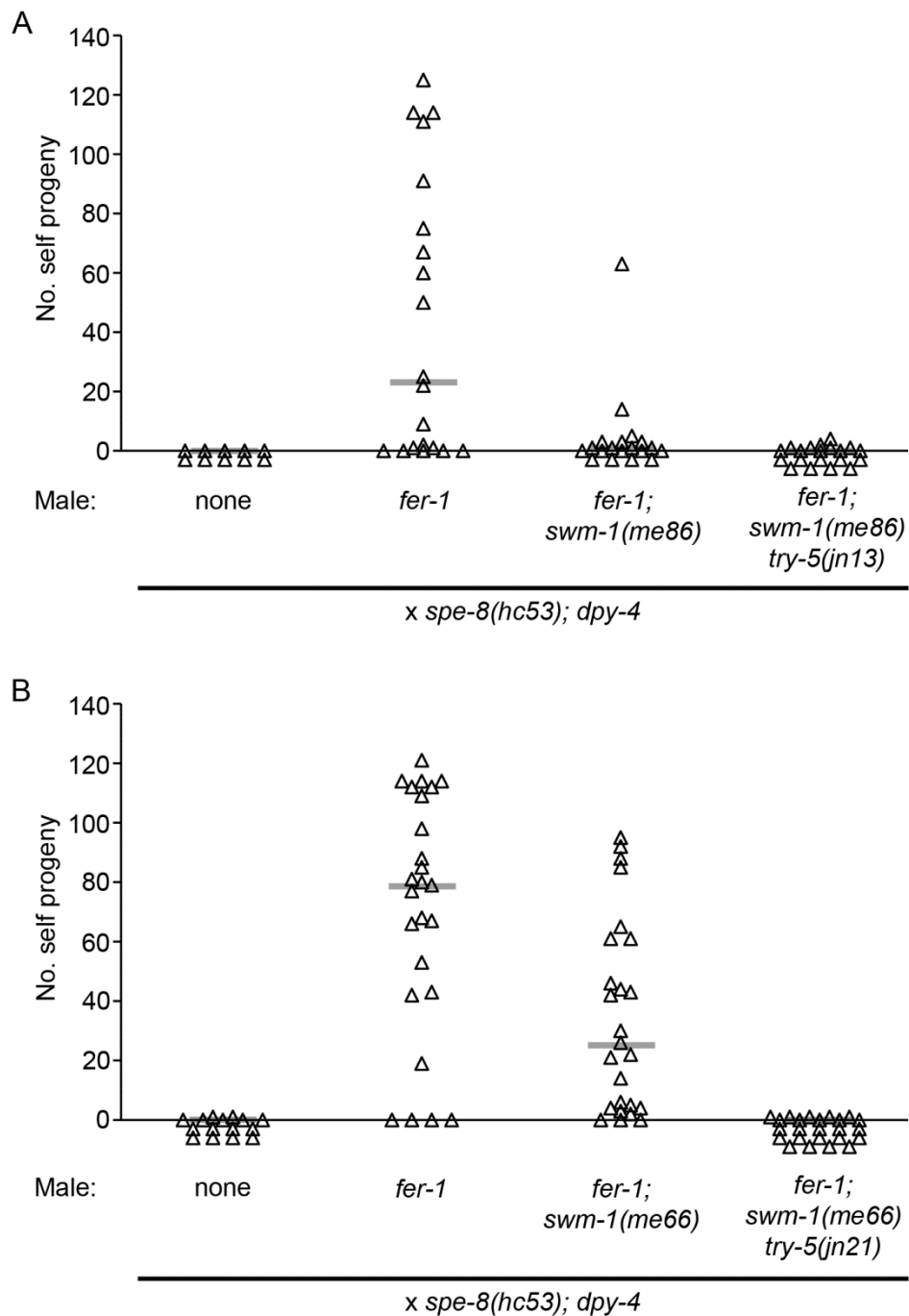
Figure 3.5. Transactivation by partial loss-of-function alleles of *try-5*. *try-5(jn13)* males transactivate *spe-8* hermaphrodite sperm infrequently and produces few self-progeny. *try-5(jn21)* males do not transactivate *spe-8* hermaphrodite sperm. L4 males that had the *jn13* (A) or *jn21* (B) allele of *try-5* were mated 4:1 with L4 *spe-8(hc53); dpy-4* hermaphrodites for 48 hr. The number of self-progeny was scored. Gray lines indicate the median.
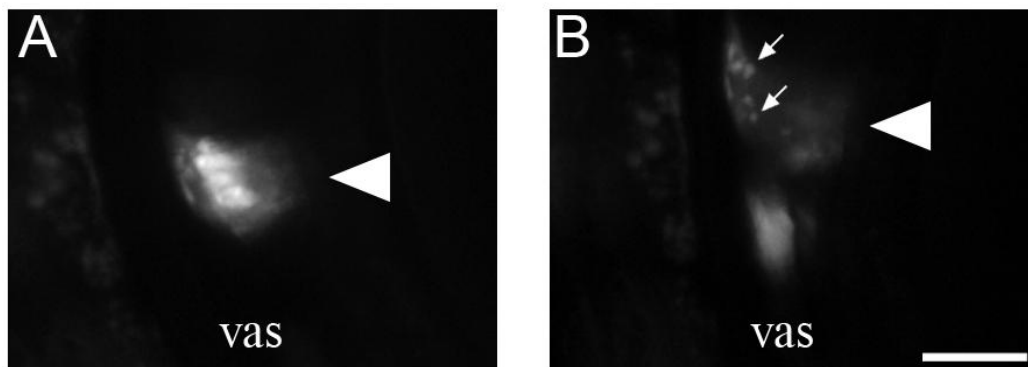
Figure 3.6. TRY-5::GFP appears to be transferred in vesicles. Males expressing TRY-5::GFP were mated to *unc-31* hermaphrodites and seminal fluid transfer was recorded. A) TRY-5::GFP is present within or adjacent to cells of the valve region (arrowhead) after the male has begun mating but before valve TRY-5::GFP begun transfer. TRY-5::GFP appears localized to vesicle-like structures. B) TRY-5::GFP in the valve region (arrowhead) after TRY-5::GFP transfer appears to be limited to the cells of the valve region in vesicle-like structures (arrows). vas represents the location of the vas deferens. Exposure is 200 ms; scale bars are 20 μm.

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CHAPTER 4

RECOMBINANT EXPRESSION AND *IN VIVO*

REGULATION OF TRY-5

Introduction

Proteases are frequently found in seminal fluid (reviewed in Veveris-Lowe et al.,

2007); however, in many cases, their functions remain unknown. Signals that increase or

decrease sperm motility are poorly understood. These signals may or not be dependent

on proteolysis. In some cases, likely signals have been identified (such as degraded

semenogelins), but how they function is not understood (reviewed in de Lamirande,

2007). Recently, a likely protease, TRY-5, that signals sperm activation in

Caenorhabditis elegans was identified; how it signals activation remains unknown

(Smith and Stanfield, 2011). By studying how TRY-5 can signal sperm motility in

nematodes, conserved mechanisms for regulation of sperm motility in other organisms

may be found.

Activity of TRY-5

Based on sequence homology, TRY-5 is predicted to be a trypsin-like serine

protease. However, TRY-5 has not been directly shown to have proteolytic activity.

Even if it does have protease activity, TRY-5 may have a different substrate specificity

than other well-characterized trypsin-like serine proteases. Purification of TRY-5 will

allow for testing of activity, which could be assayed using two methods. One assay would be a biological test of the ability of purified TRY-5 protein to activate sperm. This experiment would indicate that the purified protein is active, but would not determine whether TRY-5 is indeed a protease. To test if TRY-5 is a protease, a biochemical assay could be performed using fluorogenic peptides, which fluoresce after being cleaved, to test for proteolytic activity and determine whether TRY-5 shares the specificity of other trypsin-like serine proteases. The specificity of TRY-5 is of interest due to one of the predicted substrate binding residues differing from that of other serine proteases. This information could help predict the target of TRY-5 and whether it is on the sperm cell surface or another seminal fluid protein.

Genetics-based experiments have shown that *try-5* is necessary for several aspects of sperm activation, such as the premature sperm activation phenotype observed in *swm-1* mutant males and transactivation of *spe-8* mutant hermaphrodite sperm (Smith and Stanfield, 2011). However, those experiments did not indicate whether or not the TRY-5 protein functions directly on sperm, as opposed to an indirect mechanism, such as signaling through another protein. If TRY-5 acts directly on sperm, it would represent a protease pathway that signals quick, irreversible changes to activate sperm. If TRY-5 acts on another portion of the pathway, then a possible signal amplifying pathway, which may or may not be a protease cascade, would be identified. A cascade would indicate how a strong signal is produced, similar to earlier steps in platelet activation (reviewed in Roberts et al., 1998; Schenone et al., 2004). The simplest test of whether TRY-5 functions on sperm is to test if TRY-5 signals sperm activation *in vitro*.

The substrate specificity of TRY-5 and whether it directly signals sperm activation could aid in determining the downstream steps of activation. For instance, if TRY-5 is known to directly signal sperm activation, then sperm surface proteins would be expected to be cleaved. By knowing the substrate specificity and that TRY-5 has protease activity, target proteins can be predicted and assayed for cleavage by TRY-5. The ideal proteins would have the appropriate cleavage site, contain a transmembrane domain and be expressed in sperm. However, if TRY-5 does not activate sperm, potential targets can be identified by assaying secreted proteins that are more highly expressed in males.

Regulation of TRY-5 expression

In a *swm-1* mutant background, an upregulation of TRY-5 seemed to occur as compared to wild-type worms. TRY-5::GFP was present not only in the tissues of expression, but also in the lumen of the seminal vesicle. The cells of the valve region and vas deferens appeared to contain equal or more TRY-5::GFP when compared to *swm-1* wild-type males. A model for upregulation would be that depletion of TRY-5 from the cells of the somatic could be detected, and in response additional TRY-5 is produced. Upregulation of TRY-5 expression after mating could prepare the male for another round of mating. To quantify the levels of TRY-5 in wild-type and *swm-1* mutant males, Western blots can be performed on males that carry a stable TRY-5::GFP insertion using an anti-GFP antibody.

Exploring the biochemistry of TRY-5 will help determine how it functions in sperm activation. In this chapter, I describe initial steps in analyzing TRY-5

biochemically. This analysis includes optimization of Western blots to detect TRY-5::GFP and expression of TRY-5 in *E. coli* for purification.

Results

Comparison of TRY-5 levels in *swm-1* wild-type and mutant males

In virgin adult males with the TRY-5::GFP transgene in *swm-1* mutant backgrounds, the level of TRY-5::GFP seemed elevated (Figure 4.1). This elevated level may have been due to an actual increase of TRY-5::GFP, levels thus more TRY-5::GFP was likely present in the animal; or due to TRY-5::GFP having a more broad localization creating the appearance of more TRY-5::GFP. To differentiate between these two possibilities, I sought to compare protein levels between *swm-1* mutant and wild-type animals.

To measure protein levels of TRY-5::GFP, I performed Western blots on virgin males that were aged 48 hr after the L4 stage and OP50 bacteria, which is used to feed worms, as a control. I noted that many bands were present in each lane, including the OP50 bacteria only control and males not expressing GFP, suggesting that those bands are the result of nonspecific binding of the antibody to proteins present in bacteria and worms. The specific-binding band was slightly above the predicted TRY-5::GFP size (66.5 kDa) and was visible when 50 worms were loaded. The control GFP band was visible (27 kDa) when 15 *mIs11[Pmyo-2::GFP, Ppes-10::GFP, gut::GFP]* males were loaded (Figure 4.2). To reduce nonspecific binding of the antibody, I preabsorbed the anti-GFP antibody with acetone powder made from bacteria or *him-5* worms. Acetone powder consists of dehydrated proteins from which lipids and DNA have been removed.

After preabsorption of the anti-GFP antibodies with either acetone powder, the signal was reduced for all bands, including both the nonspecific and specific bands (data not shown). However, after several rounds of optimization with nonpreabsorbed antibody, a consistent strong signal was visible at the expected TRY-5::GFP size (Figure 4.2).

To ensure that samples are properly loaded so that an equal number of worms is loaded into each well, I sought to optimize a potential loading control. I tested an anti-α-tubulin antibody (DM1α, Sigma) that has been previously used as a *C. elegans* loading control. Using this antibody, a specific α-tubulin band can be seen near the expected size of 50 kDa (Figure 4.3, A. Snow). I found the level of protein observed did not correlate with the number of worms loaded. Optimization of this antibody is ongoing, after which the optimal conditions for stripping a blot probed with the anti-GFP antibody and then probing with the anti-α-tubulin antibody will be determined.

Protein expression

To test whether TRY-5 acts directly on sperm to signal activation, TRY-5 protein will be purified, and whether the purified protein is sufficient for sperm activation will be determined. TRY-5 is predicted to contain four disulfide bonds, based on conservation with other trypsin-like serine proteases. To increase the likelihood that TRY-5 would fold properly and be soluble, I expressed TRY-5 from several constructs consisting of TRY-5 fused to different tags in two different strains of *Escherichia coli*. I generated three constructs in expression vectors that expressed *try-5* cDNA: glutathione S-transferase fused to TRY-5 (GST-TRY-5), maltose binding protein fused to TRY-5 (Mal-TRY-5), and a 6X His tag fused to Trigger Factor (a chaperone) fused to TRY-5 (HisTF-TRY-5) (Table 4.1, Figure 4.4). I transformed each of the constructs into the standard

expression strain, BL21 (DE3), and a strain that contains two mutations that produce an environment that improves formation of disulfide bonds, Origami (DE3). Transformation of Origami (DE3) with Mal-TRY-5 did not occur successfully, and was subsequently not tested for expression of TRY-5.

To determine which constructs were most likely to express a high level of soluble protein, I induced expression in each construct-strain pair and looked for increased protein production by running a protein gel and staining with Coomassie blue. I found strong TRY-5 expression when using the GST-TRY-5 construct in BL21 cells only and the HisTF-TRY-5 construct in both BL21 and Origami cells (Figure 4.5A). I tested the solubility of the protein product in a buffer consisting of Tris, EDTA, salt, and glycerol, and found that TRY-5 produced from the HisTF-TRY-5 construct was highly soluble in this buffer (Figure 4.5B).

Discussion

At this point, not enough data has been collected to make any conclusions about the character of the TRY-5 protein. As observed by Western blot, TRY-5::GFP appears to be approximately the same size as that predicted based on cDNA sequence (Smith and Stanfield, 2011). This suggests that TRY-5 is not processed in any significant way, whether by cleaving or some other modification. However, any minor modifications would not be detected with the conditions used for the Western blot. In addition, due to nonspecific binding observed with all anti-GFP antibodies tested, cleavage products of TRY-5 may not be detectable. TRY-5 is the predicted length when expressed in bacteria, which suggests that the protein produced is stable in bacteria.

The level of TRY-5 protein in *swm-1* mutant males has not been examined at this point. However, conditions have been identified that would allow for comparison of the amount of TRY-5 present in different genetic backgrounds. Since conditions allow for clear observation of full-length TRY-5::GFP protein levels, comparison of wild-type and *swm-1* mutants can be performed. To ensure that proper loading occurs, a loading control still needs to be developed, either by continued optimization of the anti-α-tubulin antibody or use of a different loading control.

Similarly, active TRY-5 protein has not been purified at this point, but some conditions that may produce protein have been identified. This includes expressing TRY-5 from the HisTF-TRY-5 construct in BL21 or Origami cells. This expression system produces a high level of TRY-5 protein that is also soluble. However, steps have not been taken yet to purify TRY-5. When expressed from the HisTF-TRY-5 construct, TRY-5 would be purified using a nickel column, and then the Trigger Factor tag removed using a protease that targets one of the three protease recognition sites. TRY-5 would be further purified either by removing the tag and protease using a nickel column, or by using high-performance liquid chromatography to separate the different proteins.

Materials and methods

Strains used

Worm strains were grown on NGM at 20°C and fed with OP50 (Brenner, 1974). Strains used were (described in Smith and Stanfield, 2011): *jnSi62[Ptry-5::TRY-5::GFP] I; try-5(tm3813) him-5(e1490) V, jnSi62[Ptry-5::TRY-5::GFP] I; swm-1(me87) try-5(tm3813) him-5(e1490) V, jnSi92[Ptry-5::TRY-5::GFP] I; try-5(tm3813) him-5(e1490) V, jnSi93[Ptry-5::TRY-5::GFP] I; try-5(tm3813) him-5(e1490) V, mIs11[Pmyo-2::GFP,*

Ppes-10::GFP, gut::GFP] IV; him-5(e1490) V . Bacterial strains used for protein expression were BL21(DE3) and Origami(DE3) (Novagen).

Western blots

Adult virgin males (unless otherwise indicated) were picked into 2 µL of water. Eight µL of 1.25X SDS loading buffer (62.5 mM Tris-HCl pH 6.8, 125 mM DTT, 2.5% SDS, 12.5% Glycerol, 9.375 M Urea, Bromophenol Blue) were added. While optimizing detection of α-tubulin, protease inhibitors (Complete ULTRA, EDTA-free, Roche) were added to the loading buffer. The mixture was incubated at 98°C for 5 min, and then frozen on dry ice 1 hr. Samples were then either used immediately or frozen at -80°C. Samples were thawed, β-mercaptoethanol was added to 10 mM, and the sample was incubated at 98°C for 5 min. The samples were then briefly centrifuged and loaded on a 10% resolving SDS-PAGE gel. The samples were transferred to a nitrocellulose membrane (Hybond ECL, GE Healthcare) using a semi-dry blotter (BioRad) run at 15 V for 45 min. Transfer was confirmed by Ponceau S stain (Amresco). The membrane was then blocked using Blotto (5% dry milk added to TBST (20 mM Tris pH 7.4, 500 mM NaCl, 0.05% Tween-20)). Probing was performed 1-3 hr at room temperature or overnight at 4°C using primary antibody, the membrane was rinsed with TBST and washed six times for 5 min each with TBST, stained with secondary for 1 hr, and the washes were repeated. Then the membrane was incubate with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher). Membranes were exposed to X-ray film (Amersham Hyperfilm ECL, GE Healthcare) and the film was developed. Antibodies used were: anti-GFP: Living Colors GFP Monoclonal Antibody, Clontech (Catalog number: 632375), anti-α-tubulin: Monoclonal Anti-α-Tubulin antibody produced in

mouse, Sigma-Aldrich (Catalog number: T6199), and Blotting Grade Affinity Purified

Goat Anti-Mouse IgG (H + L) Horseradish Conjugate, Bio-Rad (Catalog number: 170-

6516).

Cloning *try-5* cDNA into expression vectors

To express TRY-5, I cloned *try-5* cDNA without the predicted signal sequence

(the first 24 amino acids of the sequence were removed) into the various vectors using

standard cloning techniques (see Table 4.1 for details) (Sambrook et al., 1989).

Constructs were transformed into BL21 (DE3) and Origami (DE3) cells. Correct

transformation was confirmed by purifying each construct from each strain and using

restriction digest to ensure that no large-scale modifications had occurred.

Protein expression

Single colonies of bacteria with the TRY-5 constructs were grown in 1.5 mL LB

starting cultures that were grown overnight at 37°C. Subsequently, the cultures were

diluted to an OD600 of approximately 0.2 in 2-3 mL LB and allowed to grow to an

OD600 of 0.5. 0.5 mM IPTG was added to the GST-TRY-5 and Mal-TRY-5 cultures,

and the cells allowed to express for 5 hr at 37°C. For the HisTF-TRY-5 construct, once

an OD600 of 0.5 was reached, the cells were placed in a shaking water bath set at 16°C

for 30 min before adding 0.5 mM IPTG and the cells were then allowed to express TRY-

5 for 24 hr at 16°C. After the expression period, a 0.5 mL sample was taken, centrifuged,

and the supernatant was removed and saved. The remainder of the culture was also

centrifuged and the supernatant discarded. To determine expression levels, the 0.5 mL

sample pellet was diluted in 0.1*OD600 µL of 2X SDS buffer (200 mM Tris pH 6.8, 4%

SDS, 10% β-mercaptoethanol, 30% glycerol, 8 M urea) and 15 µL was run on an SDS-PAGE gel. To ensure that cells were not releasing TRY-5 into the media, the media was also run on the gel. In cases in which expression was seen, the remaining pellet was resuspended in Buffer A (50 mM Tris-HCl pH 8.0, 0.5 mM EDTA, 50 mM NaCl, 5% glycerol) and sonicated using a sonication cup (10 min, 50% duty cycle, power level 0.7). The resulting solution was centrifuged 10 min, 4°C and the supernatant removed and saved. The pellet was resuspended in Buffer A (pH 8.0) plus 1% Triton X-100 (Sigma) and incubated on ice for 10 min, then centrifuged 10 min at 4°C, and the supernatant removed and saved.
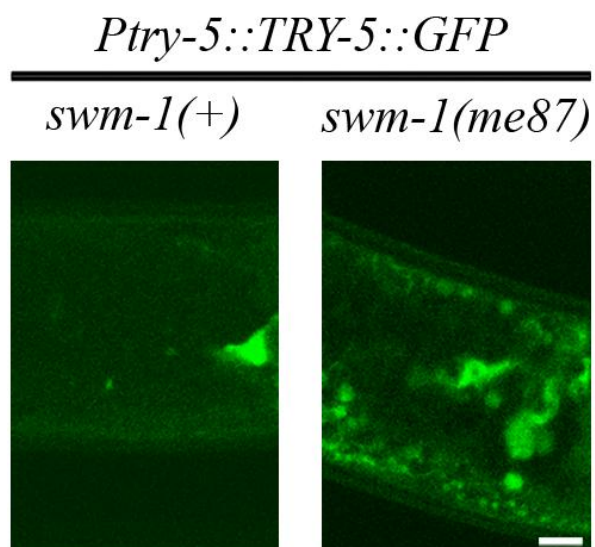
Acknowledgments

Figure 4.1. TRY-5 expression appears to be increased in *swm-1* mutant males. *jnSi62[Ptry-5::TRY-5::GFP]; try-5(tm3813)* males were examined for levels of GFP within the male. Within the seminal vesicle, *swm-1(me87)* males had additional TRY-5::GFP when compared to *swm-1(+)* males. Figure from (Smith and Stanfield, 2011).
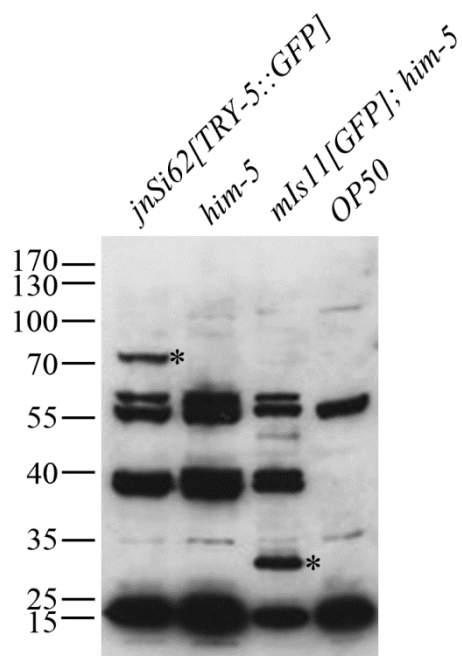
Figure 4.2. Western blot of TRY-5::GFP expressing worms. Worms were probed for GFP expression. In each lane 50 adult males were loaded, except for the *mIs11[GFP]* lane, in which 15 adult male worms were loaded. TRY-5::GFP has a predicted size of 66.5 kDa. GFP expressed in *mIs11* worms is predicted to be 27 kDa. Asterisks indicate the predicted specific band based on absence from lanes not expressing GFP. 1:10000 anti-GFP (Clontech) was used as the primary and 1:2000 chicken anti-mouse HRP (BioRad) was used for the secondary.
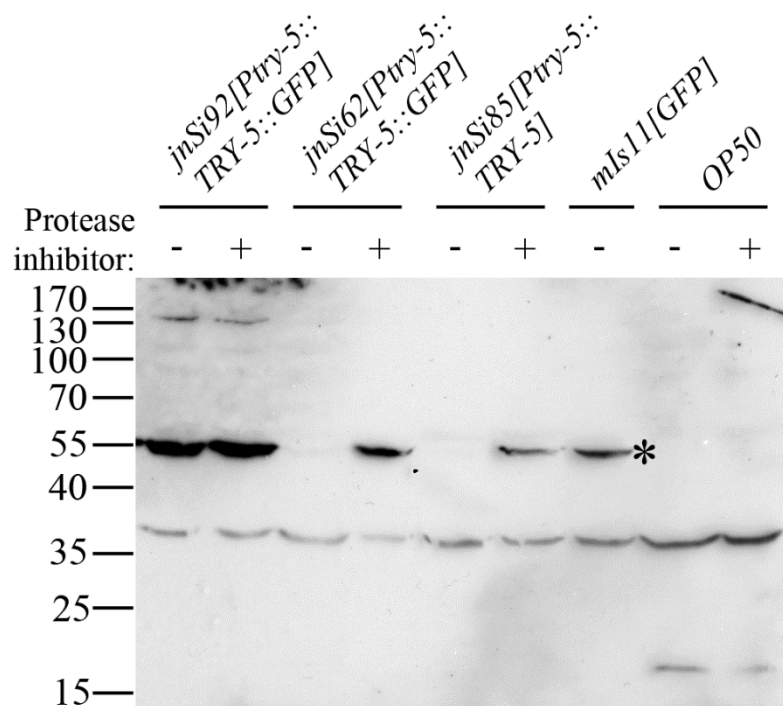
Figure 4.3. Western blot of α-tubulin. In each lane 50 adult males were loaded, except for the *mIs11[GFP]* lane, in which 15 worms were loaded. Protease inhibitors were added prior to lysis, as indicated. α-tubulin has a predicted size of 50 kDa. 1:500 anti-α-tubulin antibody (DM1α, Sigma) was used for the primary and 1:1000 chicken anti-mouse HRP (BioRad) was used for the secondary.
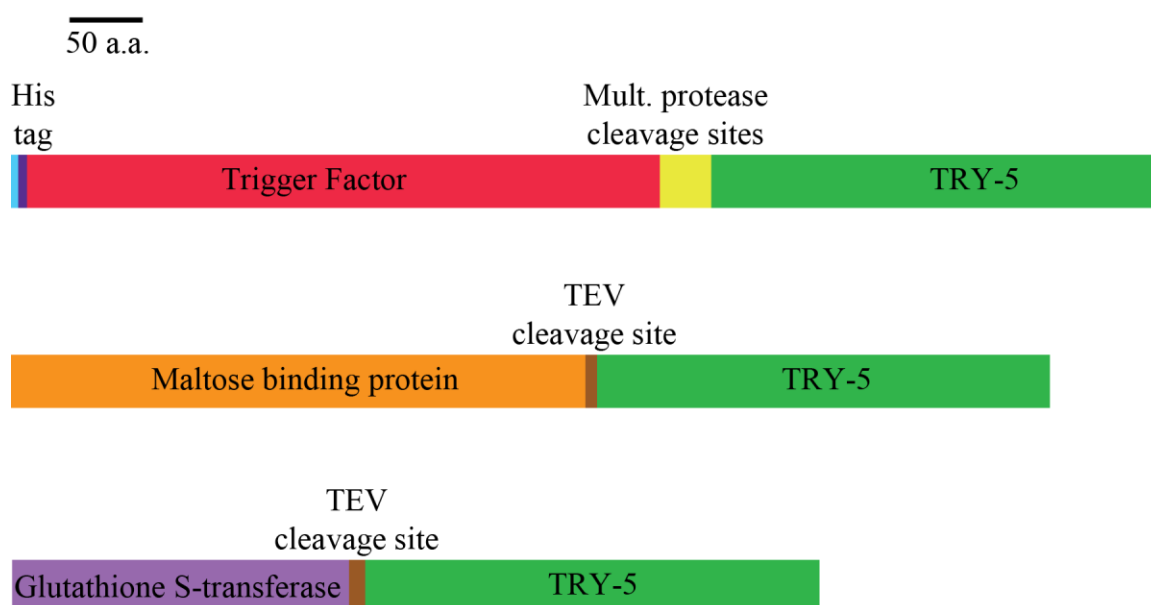
Figure 4.4. TRY-5 fusion proteins. Fusions that were made are: a 6X His tag::Trigger Factor::TRY-5 fusion (HisTF-TRY-5), a maltose binding protein::TRY-5 fusion (Mal-TRY-5), and a glutathione S-transferase::TRY-5 fusion (GST-TRY-5). The multiple protease cleavage sites region contains cleavage sites for HRV 3C protease, thrombin, and Factor Xa.
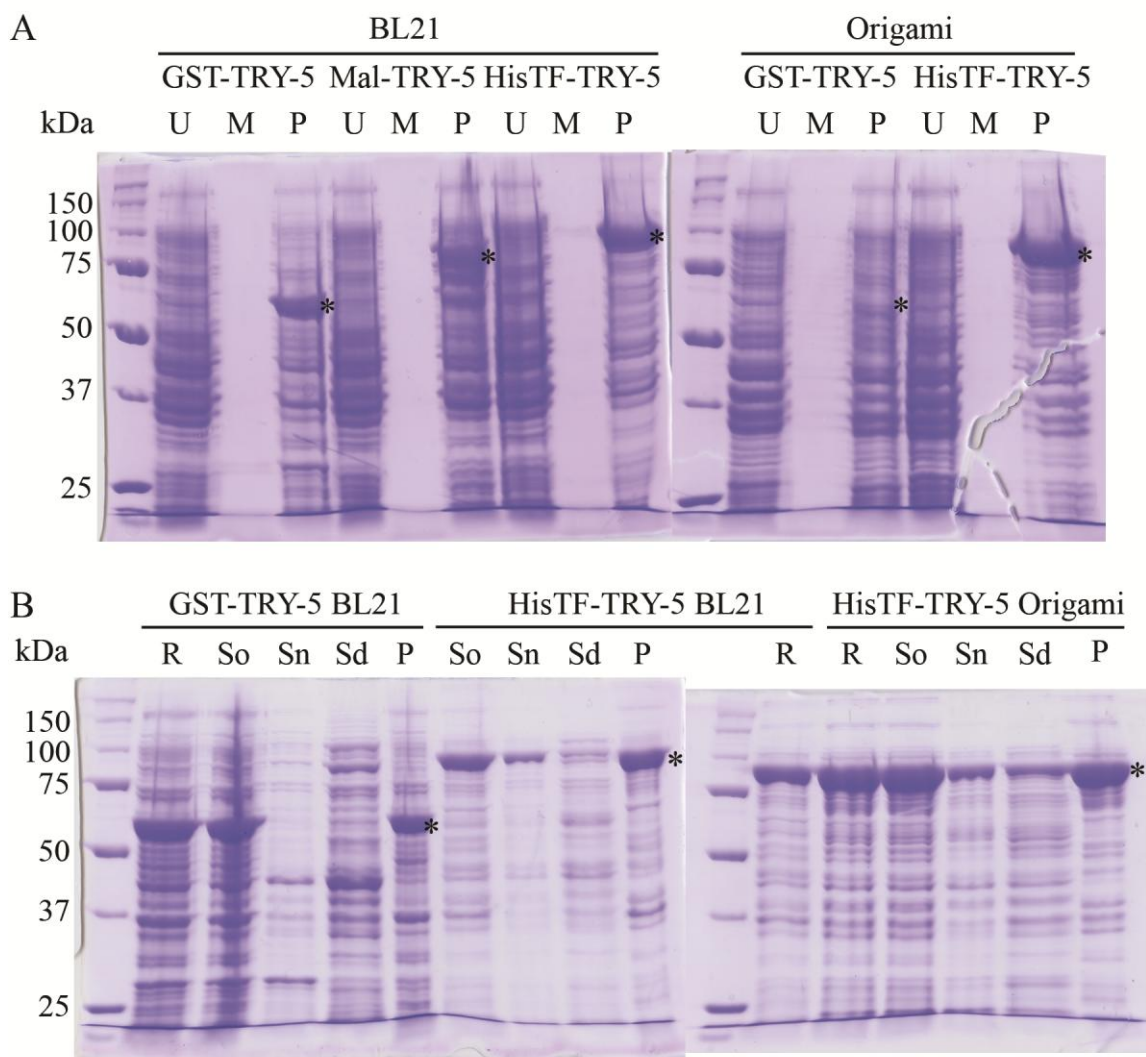
Figure 4.5. Expression and solubility of TRY-5 protein expressed in *E. coli*. A) Expression of TRY-5 in *E. coli*. TRY-5 was expressed in both BL21 and Origami cells in three different vectors. Protein was run on a 10% SDS-PAGE gel. Three samples were run for each condition: pelleted bacteria in which expression was not induced (U), media (M) and pellet (P) from bacteria that were induced with 0.5 mM IPTG. A similar amount of bacteria was run in each lane. Predicted band sizes are indicated with an asterisk. Predicted sizes are GST-TRY-5: 61.7 kDa, Mal-TRY-5: 80.8 kDa, HisTF-TRY-5: 86.8 kDa. B) Solubility of highly expressed TRY-5. Bacteria were sonicated to release expressed protein. Lanes are bacteria prior to sonication (R) and after sonication (So), the supernatant after centrifugation (Sn), supernatant after resuspending pellet in Buffer A plus 1% Triton X-100 and centrifuging (Sd), and the resultant pellet (P). Asterisks indicate the predicted band size. GST-TRY-5 did not appear soluble, while HisTF-TRY-5 did appear soluble.

Table 4.1. Protein expression constructs and their construction.

Plasmid	Description	Length	Vector	Construction
GST-TRY-5	Glutathione S-transferase fusion with *try-5* cDNA	5906 bp	B337[1]	cDNA amplification primers
				Fwd: GGCCGGATCCAatgatgaattgtgcggtcg
				Rev: CCGGGCTCGAGGTaagcttgattaataaaattcacg
				Ligated the 918 bp BamHI-XhoI fragment from cDNA into the 4988 bp BamHI-XhoI fragment from B337
Mal-TRY-5	Maltose binding protein fusion with *try-5* cDNA	7511 bp	B332[1]	Ligated the 910 bp BamHI-HindIII fragment from GST-TRY-5 into the 6601 bp BamHI-HindIII fragment from B332
HisTF-TRY-5	6X His tag Trigger Factor fusion with *try-5* cDNA	6667 bp	pColdTF	Ligated the 910 bp BamHI-HindIII fragment from GST-TRY-5 into the 5757 bp BamHI-HindIII fragment from pColdTF

[1] Vectors were a gift of B. Leibold.

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CHAPTER 5

SUMMARY, FUTURE DIRECTIONS, AND CONCLUSIONS

Summary

Sperm activation in *C. elegans* has presented an opportunity to study a system for the rapid acquisition of motility in cells and mechanisms for regulation of sperm motility. *try-5* has been identified as necessary for sperm motility to occur in *swm-1* mutant males. Before this study, the activation signal in both the hermaphrodite sperm activation and male sperm activation pathways were unidentified. TRY-5 represents an activation signal in the male sperm activation pathway.

Efforts to identify the sperm activation pathway within males were made difficult by activation not occurring until after mating. In addition, the hermaphrodite sperm activation signal is likely able to activate male sperm, thus screening for infertile males would not have identified the signal. The identification of *swm-1* (Stanfield and Villeneuve, 2006) as necessary for the inhibition of sperm activation within males allowed for a simplified screen for activation signals in males, since sperm activation could be scored within virgin males. Such screens have identified *try-5* and other genes necessary for sperm activation.

In vitro studies have found that proteases can activate sperm, suggesting that such a mechanism might also activate sperm *in vivo*. This model was supported by the identification of *swm-1*, which encodes a protein with two trypsin inhibitor-like (TIL)

domains (Stanfield and Villeneuve, 2006). The role of proteases in sperm activation is further supported by the identification of *try-5* as necessary for the premature sperm activation seen in *swm-1* males. However, at this point, whether TRY-5 has protease activity and what the targets of TRY-5 are remains unknown. Genes necessary for protease-dependent sperm activation may have been identified in the *swm-1* suppression screen (A. Hansen, G. Stanfield, personal communication).

Protease signaling is conserved in multiple organisms. Protease signaling is required for blood clot formation after injury. Frequently, a pathway involves multiple proteases that amplify the signal. Protease cascades are involved in coagulation (reviewed in (Maas et al., 2011)) and recently have been found to function in *Drosophila* seminal fluid to signal multiple postmating female responses (LaFlamme et al., 2012). Protease cascades have been hypothesized to have roles in male fertility (Yousef and Diamandis, 2001); however, no evidence for such function exists. Protease cascades have not been identified in mammalian seminal fluid, but proteases have been found to function in regulating sperm motility. The most well characterized protein is prostate specific antigen (PSA). After mating, a seminal clot forms that restricts sperm movement. PSA dissolves the clot by cleaving the proteins that form it, allowing sperm to become motile again (Christensson et al., 1990). The cleavage products are also thought to have antimicrobial properties (Bourgeon et al., 2004). TRY-5 represents another protease signal that has a clear function in sperm activation. Sperm activation is a significant morphological change, and by using activation as an indication of signaling, TRY-5 may be useful for studying how proteases signal quick, irreversible responses and how the protease activity can be regulated.

TRY-5 appeared to not only have activity in signaling male sperm activation, but also in signaling activation of *spe-8* mutant hermaphrodite sperm. Since the discovery of *spe-8*, two separate sperm activation pathways have been hypothesized: a male pathway and a hermaphrodite pathway. Hermaphrodite *spe-8* group mutants are infertile, since their sperm are not able to activate. *spe-8* group mutant males are fertile. In addition, these male retain the ability to activate *spe-8* group mutant hermaphrodite sperm. These results suggested that male worms likely contain an activator in their seminal fluid that is independent of the hermaphrodite sperm activator. *In vitro* activation studies showed that proteases were not able to fully activate the *spe-8* mutant sperm; instead, spikes formed. These spikes are likely not due to the conditions used, since *spe-8; swm-1* mutant males also contain spiky sperm. These results suggest that the *spe-8* group may be necessary even in male sperm activation. Formation of spikes may be the result of reduced activity. I found that TRY-5 is necessary for transactivation of *spe-8* mutant hermaphrodite sperm, which suggests that TRY-5 is either the sperm activation signal or upstream of the activation signal. I confirmed that TRY-5 is necessary for the activation of *spe-8* mutant sperm by examining *spe-8* group*; try-5* animals. I found that these worms were completely infertile, including after mating, which shows that all paths to sperm activation have been blocked. The differences between hermaphrodite and male *spe-8* sperm activation suggest that male and hermaphrodite sperm may contain different gene products. The only confirmed difference between male and hermaphrodite sperm is size. However, eight genes that may be male-sperm specific have been identified using microarray of males and hermaphrodites with and without germlines (Reinke et al.,

2004). Whether these genes indeed function only in the male has not been confirmed.

The differences in activation may only be due to extracellular signals.

To determine whether TRY-5 could be directly responsible for sperm activation, I

examined TRY-5::GFP fusion-expressing males. I found that only males express TRY-5

in the somatic gonad. The fusion protein in virgin adult males localized to the vas

deferens (a known seminal fluid contributor) and the valve region (a poorly understood

region that is thought to regulate the release of sperm). In a few cases, I found TRY-

5::GFP within the lumen of the seminal vesicle, surrounding sperm. In these cases sperm

tended to be activated. This observation suggests that TRY-5 could act as a signal for

sperm activation. This model is supported by *swm-1* mutants, in which TRY-5::GFP fills

the lumen, surrounding activated sperm.

To be directly responsible for signaling activation of *spe-8* mutant hermaphrodite

sperm, TRY-5 would have to be transferred. I do indeed observe transfer during mating.

TRY-5::GFP represents the first marker of seminal fluid transfer. I performed some

initial characterization of seminal fluid transfer and found that seminal fluid transfer

occurred in an ordered process. First, TRY-5 from the vas deferens was transferred,

which was followed by a brief pause, before valve TRY-5::GFP was transferred,

immediately followed by sperm transfer. Upon further examination, transfer appeared to

occur in small vesicles that are released from the valve region. Whether release of TRY-

5 from the vas deferens occurs in vesicles is unknown. Currently, the localization of

SWM-1 is unknown, thus the interactions that occur between SWM-1 and TRY-5 during

the storage and transfer of sperm remain unknown.

While investigating the localization of TRY-5::GFP, I attempted to rescue TRY-5 activity in specific tissues. I found that expression in neurons still resulted in sperm activation in *swm-1* mutant males. Proteases may be able to cleave proteins that seal the lumen of the somatic gonad, thus TRY-5 may be able to enter the somatic gonad when expressed anywhere in the worm. In some cases, worms that were overexpressing TRY-5 appeared very unhealthy, sometimes with tissues disconnecting from others (data not shown), supporting that TRY-5, when expressed outside of the somatic gonad, may cause tissue damage that allows for openings into the somatic gonad. Whether TRY-5 may damage hermaphrodite tissue after mating has not been determined. Hermaphrodites may or may not have reduced lifespan after mating. Van Voorhies (1992) found that lifespan was reduced in males, but not hermaphrodites, after mating. Gems and Riddle (1996) found that lifespan was reduced in mated hermaphrodites, and that this reduction was independent of production of progeny and reception of sperm from males. That result suggests that any lifespan reduction would be due to something within the seminal fluid. TRY-5 could be responsible for reduction of lifespan. If a protease is responsible for decreased lifespan, then hermaphrodites may have evolved a protective mechanism against damage.

These results have led to a model where TRY-5 is the seminal fluid male sperm activation signal. Before mating, TRY-5 is stored in the tissues of the somatic gonad, and any prematurely released TRY-5 is inhibited by SWM-1. During mating, TRY-5 is transferred in discrete steps. TRY-5 activates sperm, either during transfer or once in the hermaphrodite. If a male mates with a *spe-8* mutant hermaphrodite, the transferred TRY-5 is able to signal hermaphrodite sperm activation.

Despite acting as a sperm activation signal, *try-5* is not necessary for male fertility. If *try-5* is not necessary for fertility, then *try-5* must be maintained for some other reason. One possibility is that the hermaphrodite sperm activation mechanism evolved recently, and thus *try-5* has not been selected against. This hypothesis is unlikely since hermaphrodites evolved 20-50 million years ago (Cutter, 2008); selection against *try-5* should have been possible over such a time period. Alternatively, *try-5* has some other role that our experiments have not been able to detect. *try-5* may have subtle roles in ensuring fertility when mated to wild-type hermaphrodites. Two nonexclusive models for how *try-5* may improve male fertility are possible. TRY-5 protein may ensure timely male sperm activation. Also, TRY-5 may damage sperm present in the hermaphrodite improving competition. Experiments testing these models are ongoing.

Furthermore, evidence for a function of proteases in sperm activation has been found in *Ascaris suum*. A serine protease inhibitor, As_SRP-1, was identified that is necessary for maintenance of sperm activation. As_SRP-1 binds to a serine protease, which was named As_TRY-5. A partially purified male gonad extract that contained As_TRY-5 was capable of sperm activation, except when As_SRP-1 was added, which suggests that in *Ascaris* a protease is also responsible for sperm activation. These results suggest that protease activation of sperm is conserved in nematodes.

Future work

I have primarily examined how *try-5* functions in sperm activation using mutations in the *try-5* gene and transgenes expressing *try-5*. My results support a model in which the TRY-5 protein signals sperm activation; however, whether this interaction is direct has not been tested. To show whether TRY-5 directly signals sperm activation

requires biochemical techniques. To determine whether TRY-5 directly acts on sperm to signal activation, purified TRY-5 can be added to sperm *in vitro*. If sperm activate in the presence of TRY-5, then it likely acts directly on sperm. An activation curve testing the level of activation with different concentrations of TRY-5 would indicate whether TRY-5 could activate under physiological conditions. If TRY-5 does not signal activation, then TRY-5 may act indirectly to activate sperm. For instance, TRY-5 may signal through a protease cascade or further upstream in the process of sperm activation. Alternatively, TRY-5 may be necessary for the secretion of the activator. In either case, the function of TRY-5 would need to be confirmed, including testing if TRY-5 is a protease. Such a function could be shown by testing a library of fluorogenic peptides for cleavage. Sequence specificity could be determined using this library. The specificity of TRY-5 could give clues in identifying the likely target of TRY-5 function, by searching for sperm-expressed, membrane-bound proteins that have the specificity sequence in that protein's extracellular domain.

The sperm components that receive the activation signal have yet to be identified; however, identification of candidates is ongoing, based on genes identified in *swm-1* suppressor screens that are necessary for the premature sperm activation phenotype. If TRY-5 does signal sperm activation directly, then these candidates should be cleaved in a *swm-1* mutant background. Furthermore, suppression of this cleavage should occur in a *swm-1 try-5* mutant background. In addition, direct activation of sperm by TRY-5 would allow testing of cleavage in isolated sperm *in vitro*. While additional extracellular signaling components may exist upstream of SWM-1 and TRY-5, identifying a direct interaction between TRY-5 and a sperm membrane component that signals activation

would suggest that TRY-5 is the direct sperm activator. Thus, all proteins that function between TRY-5 and the receptor would have been identified, except for redundant signals. Redundant signals likely are not present since mutations in the *spe-8* group and *try-5* eliminate all forms of activation.

Sperm activate *in vitro* when exposed to a mixture of proteases called Pronase (Shakes and Ward, 1989); however, the activated sperm are not capable of fertilization. Two models exist for how this ability is lost. Pronase might cleave proteins that are necessary for fertilization that normally would not be cleaved by TRY-5. Certain proteins necessary for fertilization are only on the surface of sperm after activation (Xu and Sternberg, 2003). These and other proteins necessary for sperm competition may be cleaved by both Pronase and TRY-5. In the first case, Pronase contains multiple proteases, thus it likely cleaves many different proteins, and Pronase may cause damage to sperm that TRY-5 while the endogenous protease signal would not. Alternatively, TRY-5 or the protease signal may cleave the same proteins. This translocation may occur as a result of the membranous organelles fusing to the sperm surface during activation. Such a model is supported by the translocation of SPE-41 during sperm activation (Xu and Sternberg, 2003). In such a case, TRY-5 may be exposed to sperm during a short period after which it diffuses to a low concentration, is removed from the hermaphrodite, or is degraded. This short period could be sufficient to activate sperm, and the protease could be removed prior to exposure of the fertilization components. I have observed that as males terminate mating, some TRY-5 frequently leaves the uterus as the male removes its spicules. This model would be supported if sperm activated *in vitro* by TRY-5 were not capable of fertilization unless exposure occurred for a brief

period of time. Much TRY-5 remains in the uterus, thus for this model to be likely TRY-5 would have to be quickly degraded or inhibited. TRY-5 may represent a possible mechanism for sperm competition. Sperm already activated in the hermaphrodite could be damaged by the transfer of TRY-5. Sperm being transferred from the male would be undergoing the initial steps of activation, so might not be damaged and would be able to outcompete the pre-existing sperm.

TRY-5::GFP represents the first fluorescent marker of seminal fluid transfer in worms. Being the first marker, the discovery of TRY-5 allows for many additional experiments that were not previously possible. For instance, using this marker, identification of additional seminal fluid components could be aided by using TRY-5 as a control for successful mating and transfer of seminal fluid. Identification of other seminal fluid proteins may be possible using mass spectroscopy based techniques. One possibility would be to mark males using the ^{15}N isotope, mate those males to hermaphrodites, and compare proteins in mated hermaphrodites to unmated hermaphrodites (Findlay et al., 2008). In this case, TRY-5 would serve as a control to ensure that seminal fluid proteins were detected.

While aspects of how seminal fluid transfer is accomplished have been studied using TRY-5::GFP, many other aspects of seminal fluid transfer are not understood. For example, mating is coupled to secretion, such that secretion occurs shortly before sperm transfer. This process is not understood at several levels, including what cells and molecules are involved. One possibility is that neurons coordinate the secretion and sperm release processes, and molecules involved in synaptic vesicle fusion to the membrane could be involved in the process of secretion. Better characterization of this

process would also aid in analysis of secretion. In my experiments, I was limited by the resolution of the microscope I was using and the light necessary for fluorescence interfering with mating. Improving on the techniques used to monitor mating and using improved microscopy techniques may allow for higher resolution images of mating and shorter exposure times, increasing time resolution. Better imaging could allow for observation of events that lead to secretion.

An interesting phenotype was observed with *jn21* males. Some *swm-1(me66) try-5(jn21)* males contained activated sperm, which suggests that *jn21* is a hypomorphic allele of *try-5*. However, transactivation was completely eliminated. Such a result suggests that functional TRY-5 is produced, but secretion may not occur as in the wild type. By generating a TRY-5::GFP construct with this mutation, the localization of the mutant protein could be determined. A differing localization during mating could suggest the function of the secretion signal. The mRNA produced by this mutant could also indicate where translation begins and whether alternate splicing occurs. Alternate splicing is a possibility, since the next translational start site is after the cysteine that is mutated in the *jn2* allele. Since *jn2* appears to be a functional null, this cysteine might be critical for proper protein folding.

In some strains of *C. elegans*, a plug is deposited over the vulva during mating. The plug is formed by the protein PLG-1 and is thought to decrease the likelihood that the hermaphrodite mates with other males (Palopoli et al., 2008). Study of TRY-5 function may reveal additional functions for plugging. PLG-1 is likely secreted from the vas deferens, from a subset of the same cells that TRY-5 is expressed in. Whether TRY-5 and PLG-1 are transferred simultaneously is unknown. If TRY-5 is transferred with

PLG-1, then that pool of TRY-5 may be trapped in the plug or may help with its formation. Additionally, the vas deferens pool of TRY-5 is transferred prior to sperm. If PLG-1 is transferred simultaneously, then sperm must pass through the forming plug. Transfer in such a way would ensure quick plug formation behind sperm. If a plug does form quickly, then TRY-5 dynamics in the hermaphrodite may be changed by the plug, such as increasing the time TRY-5 is in the hermaphrodite. In my movies without plugging I saw that a significant amount of TRY-5 was pulled out of the hermaphrodite as the male removed his spicules. A plug may cause TRY-5 to remain in the hermaphrodite, allowing extra time for sperm activation.

Despite the function of TRY-5 in sperm activation, *try-5* was not required for male fertility in matings to *spe-8* mutant hermaphrodites. A function that is selectable would be necessary for maintenance of *try-5*. One possibility is that *try-5* is necessary for competition again hermaphrodite sperm. *try-5* mutant males would have lower fertility or a lower percentage of cross progeny when mated to wild-type hermaphrodites. To reach a further understanding of how *try-5* functions, examining related *Caenorhabditis* species that have *try-5* may reveal a more significant role in fertility, especially in gonochoristic species. In male-female species, *try-5* may act as the only signal for activation or may have roles in male-male competition. Thus, mutants or *try-5* knockdown in the animals would reduce male fertility, resulting in a strong selection for males that express *try-5*.

Conclusions

I have shown that a protease is a signal for sperm activation in *C. elegans*. Protease signaling in worm fertility has been hypothesized since the discovery that

Pronase activates sperm *in vitro*. TRY-5 likely is that protease signal. In addition, TRY-5 represents that first marker of seminal fluid transfer. Previously, a seminal fluid component, PLG-1, had been identified, but transfer was not directly observed (Palopoli et al., 2008). Thus, TRY-5 allows for studying of the process of seminal fluid transfer to better characterize mating.

This study has led to a model of sperm activation in which TRY-5 is expressed and stored in the somatic gonad, specifically the valve region and the vas deferens. Any secreted TRY-5 is inhibited by SWM-1. During mating TRY-5 is released from the somatic gonad and transferred with sperm to the hermaphrodite. During transfer or shortly after entry into the hermaphrodite, the male sperm activates. TRY-5 is also transferred into the hermaphrodite, since *spe-8* mutant hermaphrodite sperm activate after mating. This process of sperm activation is partially redundant with the hermaphrodite activation pathway, which remains poorly understood. Intracellular sperm activation signals have been found, including the *spe-8* group, which is necessary for hermaphrodite sperm activation to occur. However, *spe-8* sperm are competent for activation, since males are able to transactivate sperm. Males may be partially dependent on the *spe-8* group, since males are less able to transactivate hermaphrodite with stronger mutant alleles of *spe-8*.

Proteases have been shown to have important roles in signaling. One example is with blood clotting. In clotting, a protease-activated receptor, similar to G-protein coupled receptors, is responsible for receiving the protease signal (reviewed in (Ossovskaya and Bunnett, 2004)). The downstream signal of protease activation in

nematode sperm remains unknown, but identification of a different class of receptor may suggest other mechanisms for protease signaling.

Proteases also have roles in human fertility, and many different proteases are found in seminal fluid. The function of many of the proteases remains unidentified, with the exception of PSA. Further characterization of the male sperm activation pathway in nematodes may suggest parallels that exist between nematode and human sperm activation, which could also suggest ways to regulate protease activity in human seminal fluid. Such an understanding may aid in treatment of male infertility.

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