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A study of a novel self-fertility mechanism in the hermaphroditic nematode Rhabditis sp. SB347

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A study of a novel self-fertility mechanism
in the hermaphroditic nematode *Rhabditis* sp. SB347

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Thesis presented to the Undergraduate Faculty of The College of William & Mary in Candidacy for the Degree of Bachelor of Science

Biology Department

College of William and Mary
May, 2017
A thesis submitted in partial fulfillment of the requirements for the degree of Bachelor of Science in Biology

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Abstract

Germ cell development and gametogenesis are essential for the continuity of future generations in most eukaryotic organisms, including humans. One well-established system for studying the complex mechanisms of gametogenesis is the nematode species, *Caenorhabditis elegans*. The fact that *C. elegans* hermaphrodite germlines undergo different cellular development at the same life stage in a linear progression makes *C. elegans* a model system for the study of the conversion of uncommitted germ cells into either oocytes or sperm. *C. elegans* hermaphrodite produces spermatocytes during the final larval stage and then makes a one-time switch over to oogenesis as the worm enters adulthood. Recently our lab made the surprising discovery that hermaphrodites of another nematode species, *Rhabditis* sp. SB347, evolved an alternative mechanism for achieving self-fertility.

In Chapter one, we describe that the germline in *R*. sp. SB347 hermaphrodites is capable of producing both sperm and oocytes from the final larval stage throughout adulthood. Along the length of SB347 hermaphrodite germline, we found clusters of distinct “mystery cells” that divide mitotically outside of stem cell. These “mystery cells” serve as spermatocyte progenitors and share features of spermatogonial cells that are key components of sperm production in other organisms, including *Drosophila*, mice and humans. Our finding is significant for the understanding of germ cell development because it reveals a completely new reproductive characteristic that is not present in *C. elegans* but in other model organisms.

In Chapter two, we look at further characterization of spermatogonial cells using a key molecular player known as *fem-3* binding factor (FBF). Previously described to regulate both mitosis/meiosis switch and oocyte/sperm determination in *Caenorhabditis elegans*, FBF belongs to PUF (Pumilio and FBF) protein family and shares a conserved role of germline stem cell regulation. We report the presence of FBF in both the distal germline and the spermatogonia, and unexpectedly, in late maturing oocytes. Our results highlight SB347 spermatogonial cells as an intermediate stage of partially committed spermatocyte progenitors that remain features of germline stem cells.

Through this study of the first reported case of spermatogonial cells in the phylum nematode, we hope to extend our knowledge of germline stem cell development to decipher features of stem cell differentiation and provide more insights with broader, medical implications.
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Acknowledgements

I would like to thank Dr. Diane C. Shakes for mentorship in the past three years. She has given me invaluable insights to train me as a cell biology scientist. With her help, I have not only developed critical scientific thinking skills but also learned to conduct my own project as a researcher. I would like to especially thank her for her patience in revising my writings and providing advices in scientific presentations. I also want to thank her for giving me chances to go to scientific conferences and broaden my view of science community.

I would like to thank Caitlin McCaig and Maureen Farrell for their partnership in this work. Thank Maureen for training me to conduct research experiments when I first entered the lab. Thank Caitlin for the collaboration both in experiments and in writing processes. I would also thank Pablo Ordóñez and Brian Hur, who also contribute to this research project.

I would also like to thank everyone in our lab who have provided feedbacks to this project and have given mutual support in this project. Thank everyone outside the lab who have given suggestions for the future directions of the project.

Finally, I would like to thank DeFontes Family Scholarship for supporting my 2016 summer research, and thank National Science Foundation for the grant support (IOS 1122101) to Diane C. Shakes.

Finally, we thank K. Kiontke and D. Fitch for sharing unpublished data on the molecular phylogeny of the *Rhabditis* nematodes and David Greenstein for anti-MSP antibodies. We thank Pires-daSilva for sharing SB347 gene sequencing information.
Dedications

This thesis is dedicated to my parents, who have provided strong supports in my science career, and to my grandfather, who have shaped my determination to pursue my future path in science field.
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Introduction to the nematode germline

Most multicellular organisms contain populations of special cells called stem cells. Stem cells serve to maintain homeostasis within organisms, replenishing tissues whose cells are continuously lost due to tissue injuries with newly differentiating cells, thus maintaining a balance between cell proliferation and cell death (Oatley and Brinster, 2012). Most stem cell populations give rise to body cells in a variety of self-renewing organs, including bone marrow, gut, hair follicle and heart (Christensen et al. 2012; Muller-Sieburg et al. 1986; Spangrude 1988; Till and McCulloch 1961, Barker et al. 2007; Cheng and Leblond 1974; Marshman et al. 2002; Sangiorgi and Capecchi 2008; Costarelis 1990; Morris and Potten 1999; Hsieh et al. 2007).

However one particular type of stem cell contributes not to tissue renewal, but to the development of the next generation. That special type of is called a germline stem cell.

Germline stem cells (GSCs) are essential for the survival of future generations. In GSC lineages, developmental defects result in infertility. It has been estimated that at least 10% of human couples worldwide struggle with infertility issues (Matzuk and Lamb, 2008; Oatley and Brinster, 2012). Understanding the mechanisms underlying regulation of GSC self-renewal and differentiation is important in multiple biomedical fields, including tissue-regeneration and repair. Increasing evidence also suggests that defects in stem cell development contribute to directly to oncogenesis (Reya et al,
2001; reviewed in Spradling et al., 2011). Therefore, insights from studying how GSCs develop into differentiated gametes have important medical implications for both germline stem cell and general stem cell research.

The bacterial feeding nematode *Caenorhabditis elegans* serves as a wonderful model organism for cell biology research. It is easy to culture in the laboratory environment, self-fertile worms produce large numbers of offspring within a short period of time (~2 to 3 days), and the transparency of both the body and eggshell enables scientists to directly observe cell development under the microscope (Corsi and Chalfie, 2015). Studies on *C. elegans* have led to numerous, revolutionary discoveries including programmed cell death (Miura et al., 1993), RNA interference (RNAi) (Fire and Montgomery 1998), and microRNAs (Lee et al. 1993). *C. elegans* researchers were also key leaders in genomic sequencing and bioinformatics, as *C. elegans* was the first multi-cellular organism to have its full genome sequenced (Consortium CES 1998; Hodgkin 2005).

*C. elegans* has two sexual modes: males and self-fertile hermaphrodites. *C. elegans* hermaphrodites have exactly 959 somatic cells. There are also about 2,000 germ cells continued within the two gonad arms comprising a large portion of the worm’s total body mass (Wood 1988). The highly ordered *C. elegans* gonads have played a particularly important role in our understanding of GSC development (Figure 1). At the distal region of the gonad, GSCs proliferate mitotically. As germ cells progress to the proximal
region, they become either sperm or oocyte fated and enter into meiotic divisions before finally differentiating into fully mature gametes (Kimble and White, 1981). Since cells along the length of the germline are in specific developmental stages, this linear progression of gamete development makes the *C. elegans* germline an ideal model for the understanding of GSC development (Hubbard and Greenstein 2005).

Most nematode biologists study the model organism *C. elegans*. However, the larger phylum Nematoda is well known for its species richness (Sommer et al. 2005), and thus the analysis of GSC development in other nematode species promises to be highly informative. The goal of this study is to investigate GSCs in another closely related nematode, known as *Rhabditis* sp. SB347. *R*. sp. SB347 differs from *C. elegans* in that it contains not two but three sexual modes: hermaphrodite, male, and female (Félix, 2004). In chapter one, we report our discovery that *R*. sp. SB347 hermaphrodites employ a distinct strategy to achieve self-fertility. Furthermore, their mechanism of sperm production includes an intermediate stage of mitotically proliferating spermatogonial cells. In chapter two, we continue to characterize the features of their GSCs and spermatogonial cells using a stage-specific protein marker known as *fem*-3 binding factor (FBF). Since FBF protein has a conserved role of regulating GSC maintenance and differentiation across a variety of organisms, its protein alignments and expression are studied in *R*. sp. SB347. By the end of this study, we hope to provide more insights in comparing
different self-fertility strategies and the key features underlying the germ cell development in *R.* sp. SB347, with a broader implication of GSC regulation both within and outside of the phylum Nematoda.

**Figure 1:** *Caenorhabditis elegans* hermaphrodite with two gonad arms within which germ cells are arranged in a linear progression. A) *C. elegans* hermaphrodite with two gonad arms (also referred as two germlines). B) Two gonad arms within *C. elegans* hermaphrodite. Germ cells develop in a linear progression within each of the two gonad arms. From distal to proximal: distal tip cell, mitotic cells, meiotic cells, and maturing gametes.
Chapter 1. Germ cell cysts and simultaneous sperm and oocyte production in a hermaphroditic nematode

Contributions:

Rehain-Bell made the first observation in her 2012 B. S. thesis of the unexpected cells “of unknown function” that are characterized here. Farrell initially led these studies and reported that these cells were hermaphrodite-specific clusters that produce sperm in her M. S. thesis in 2015. Lin and McCaig partnered in collecting the majority of the data reported here unless otherwise noted. McCaig analyzed the quantitative data and carried out the experiments characterizing actin patterns, while Lin carried out the experiments testing collagenase treatment and collected data from closely related species. Lin and McCaig co-wrote the first drafts of the manuscript, and then McCaig edited the manuscript with thorough input from Dr. Shakes. Rehain-Bell and Farrell have also provided helpful suggestions for the manuscript text.
Introduction

Most animal species are dioecious (male/female). However, when mates are scarce, selective pressures can favor the evolution of self-fertile hermaphrodites (Ghiselin 1969; Clark 1978; Jarne & Charlesworth 1993). Within the phylum Nematoda, evolutionary transitions from outcrossing males and females to self-fertile hermaphrodites have occurred in multiple, separate lineages (Kiontke et al. 2004; Kiontke & Fitch 2005; Denver et al. 2011). Hermaphroditic nematodes can be found across the phylum in both parasitic and free-living species (Castro 1996; Criscione et al. 2005; Kiontke & Fitch 2005; Kanzaki et al. 2013).

In Caenorhabditis elegans, studies of the developmental and molecular underpinnings of nematode gametogenesis have contributed significantly to our understanding of both germ cell biology and fundamental mechanisms in cell and developmental biology (Corsi et al. 2015). Anatomically, the C. elegans hermaphrodite gonad is organized as a linear tube with germline stem cells (GSCs) at the distal end and differentiated, meiotic gametes at the proximal end (Figure 2A). A single somatic cell at the distal end (the distal tip cell, DTC) serves as the stem cell niche and maintains a zone of ~200 mitotic, undifferentiated germ cells (Kimble & White 1981; Hansen & Wilson-Berry 2004; Crittenden et al. 2006; Fox & Schedl 2015). As germ cells move beyond the niche, they both enter meiotic prophase and begin differentiating into either spermatocytes or oocytes (Figure 2A-B) (Lui & Colaiácovo 2013). C. elegans
hermaphrodites are somatic females which achieve self-fertility by producing sperm as larvae, storing their sperm in a pouch-like structure called the spermatheca, and then producing exclusively oocytes as adults (Figure 2A). As individual oocytes mature, they pass through the spermatheca in an assembly-line fashion where they are fertilized by the stored sperm (Figure 2A) (Ward & Carrell 1979; L'Hernault 2006).

This same pattern of “sperm first, then oocytes” gamete production has been observed in self-fertile hermaphrodites of the Caenorhabditis and Pristionchus genera (Rudel et al. 2005; Sommer 2005). Yet even within the same genus, C. elegans and C. briggsae hermaphrodites convergently evolved distinct molecular changes to support sperm production in an otherwise female body (Nayak et al. 2005; Hill et al. 2006; Hill & Haag 2009; Guo et al. 2009; Liu et al. 2012). Alternative patterns for achieving hermaphrodite self-fertility have been reported; for example, hermaphrodites in the parasitic species Rhabdias ranae, simultaneously produce both oocytes and sperm within a discrete zone of the ovatessis (Runey et al. 1978). However, current studies are too limited to assess whether the sequential hermaphroditism strategy adopted by Caenorhabditis and Pristionchus predominates within the larger phylum.

Rhabditis sp. SB347 is a free-living, trioecious (male/ female/ hermaphrodite) nematode from the same Rhabditidae family as C. elegans (Félix 2004; Kiontke & Fitch 2005), but its reproductive biology differs in
several ways (Félix 2004; Shakes et al. 2011; Chaudhuri et al. 2011; Chaudhuri et al. 2015). First, whereas *C. elegans* is androdioecious (male/hermaphrodite) (Maupas 1901; Corsi et al. 2015), *R*. sp. SB347 is trioecious (male/female/hermaphrodite), with XX animals developing into either females or hermaphrodites (Félix 2004). In *R*. sp SB347, hermaphroditism is specifically linked to passage through a stress-resistant and dispersive larval morph, referred to as “dauer” in free-living nematodes and “infective juvenile” in parasitic species (Félix 2004; Chaudhuri et al. 2011). Additionally, *R*. sp. SB347 exhibits non-Mendelian sex ratios (Shakes et al. 2011; Chaudhuri et al. 2015). More specifically, self-fertilizing hermaphrodites produce an unexpectedly high number of male offspring (~10%) (Félix 2004)(M. Farrell, unpublished), while males sire exclusively XX feminine progeny due to a modified spermatogenesis program in which only the X-bearing sperm are functional (Shakes et al. 2011) (DS, in press).

Given the many differences between the reproductive biology of *R*. sp. SB347 and *C. elegans*, we investigated whether *R*. sp. SB347 might also have evolved a distinct developmental solution to achieving self-fertility. Here we describe the unexpected discovery of germ cell clusters within the gonads of *R*. sp. SB347 hermaphrodites that enable the continuous and simultaneous production of sperm and oocytes in both larvae and adults. Characterization of these germ cell clusters revealed them as mitotically proliferative spermatogonia within germ cell cysts. Such cells are a feature of sperm
production in most other taxa but have not been previously reported in a nematode, including \textit{C. elegans}. Given this apparent novelty, we also examined near relatives of \textit{R.} sp. SB347. Other trioecious \textit{Rhabditis} species shared the \textit{R.} sp. SB347 pattern of hermaphrodite sperm production, whereas males of \textit{Rhabditis axei} and hermaphrodites of the more distant \textit{Oscheius tipulae} exhibited the \textit{Caenorhabditis} patterns. These data indicate that \textit{R.} sp. SB347 evolved heretofore undescribed mechanisms for both nematode sperm production and achieving hermaphrodite self-fertility, distinct from those of the model nematode \textit{C. elegans}.

\textbf{Results}

\textit{Rhabditis} sp. SB347 hermaphrodite gonads contain distinctive cell clusters that have no \textit{C. elegans} analog

To identify potential differences in germ cell patterning between \textit{R.} sp. SB347 and \textit{C. elegans} hermaphrodites, we compared isolated gonads co-labelled with DAPI and anti-\(\alpha\)-tubulin antibodies. Surprisingly, \textit{R.} sp. SB347 gonads contained isolated cell clusters positioned along the line of developing oocytes (Figure 2B - cc1-3). These cell clusters were distinct from somatic sheath cells in both their gonadal distribution ("s" in Figure 2B,C,E) and their chromatin morphology (Figure 2D). The cell clusters were specific to \textit{R.} sp. SB347 hermaphrodites: they were almost always in the gonad arms of
presumed hermaphrodites (576/596 scored) but never in those of females (0/24 scored) (Figure 2E). Analogous clusters were not present in *C. elegans* hermaphrodite gonads (Figure 2C).

**Cell clusters in *R. sp.* SB347 hermaphrodite gonads enable the production of sperm in oocyte-producing adults**

Because the cell clusters were specific to *R. sp.* SB347 hermaphrodites and not females, we hypothesized that they might function in hermaphrodite sperm production. To test if they were spermatocytes, hermaphrodite gonads were co-labeled with antibodies against α-tubulin and the major sperm protein (MSP). MSP is first detectable within late spermatocytes, where it localizes to discrete structures called fibrous bodies (FBs). MSP remains in this pattern until it disperses cytoplasmically in spermatids and partitions specifically to the pseudopods of crawling spermatozoa (Figure 3A) (Ward & Klass 1982; Shakes et al. 2011). For these studies, a total of 1,428 discrete clusters from 518 hermaphrodite gonad arms were scored. Cells with microtubule spindles were scored as undergoing division (mitotic or meiotic). MSP was used as a stage-specific marker of spermatocytes (Smith 2006; Chu & Shakes 2013).

In *R. sp.* SB347 adult hermaphrodites, MSP was detected not only in crawling spermatozoa (Figure 3B-viii), but also in some of the most proximal cell clusters (Figure 3Bv-vii; 2C). Within these MSP-positive (MSP+) cell clusters MSP was organized in the FB pattern of spermatocytes (Figure
3Bv-vi). 43% of these MSP+ clusters (95/222) also exhibited microtubule spindles, characteristic of meiotically dividing spermatocytes (Figure 3Bv-vi; 2C). The proximal region of many gonads also contained clusters of haploid spermatids and DNA-containing residual bodies, the immediate products of spermatocyte meiosis in R. sp. SB347 (Figure 3A, 2B – vii) (Shakes et al. 2011)(DS, in press). Thus, in striking contrast to the sequential “sperm first, then oocytes” pattern of gamete production in C. elegans hermaphrodites, adult R. sp. SB347 hermaphrodites simultaneously produce both sperm and oocytes within a single gonad.

Within these same gonads, numerous MSP-negative (MSP-) cell clusters were scored as either dividing (10% or 120/1206) (Figure 3B- iii; Figure 3C) or not dividing (90% or 1086/1206) (Figure 3Bii, iv) (Figure 3C). Since these dividing cells lacked MSP, we concluded that they could not be meiotically dividing spermatocytes, and must therefore be mitotically dividing. Dividing MSP- cell clusters were observed along the length of the gonad, with some adjacent to the most mature oocytes and thus physically distant from the stem cell niche (the DTC) (Figure 3C). Therefore, we hypothesized that these clusters were developmental intermediates between undifferentiated GSCs and late-stage MSP+ spermatocytes.

**MSP-negative clusters exhibit characteristics of spermatogonial cysts**

Although MSP-negative, mitotically dividing germ cell clusters have not
been described in *C. elegans* or other nematodes studied to date (Morgan et al. 2010; Chu & Shakes 2013; Sommer 2005; Denver et al. 2011), sperm production in other animals, including *Drosophila* and vertebrates, typically includes cysts of interconnected, mitotically dividing cells called spermatogonia. Spermatogonia, distinct from GSCs, undergo a limited number of mitotic divisions that expand the germ cell pool before they differentiate into spermatocytes — a process known as transient amplification (Reviewed in (White-Cooper & Bausek 2010; Kimble 2011; Spradling et al. 2011). Given this broader context, we hypothesized that the MSP- clusters observed in *R. sp.* SB347 hermaphrodite gonads were a nematode form of spermatogonial cysts.

To test this hypothesis, the MSP- cells were assessed for three key characteristics of spermatogonia: (1) transient amplification, (2) proliferation independent of the stem cell niche, and (3) presence of cytoplasmic bridges between cells. First, to determine if the cells undergo transient amplification that increases germ cell numbers, we plotted the relationship between cluster location and the number of cells per cluster. This illustrated a trend where cells per cluster increased as the developing clusters shifted proximally (Figure 7). Second, consistent with spermatogonia having the capacity to proliferate independently of signals from the stem cell niche (Kiger et al. 2001; Tulina & Matunis 2001), mitotically dividing MSP- clusters were observed distant from the DTC (Figure 3B-C).

Third, we determined whether cells within the MSP- clusters were
interconnected by cytoplasmic bridges. To first test whether the cells were being held together solely by extracellular matrix, isolated gonads were treated with collagenase. Under these conditions, the clusters dissociated from the oocytes, but cells within clusters remained connected (Figure 4A). Next, since spermatogonia in other organisms are linked by actin-rich cytoplasmic bridges (reviewed by (Greenbaum et al. 2011), hermaphrodite gonads were labeled with F-actin-specific rhodamine-phalloidin. Within clusters, phalloidin labeled both the cortical microfilaments (Figure 4B-arrowhead) of individual cells as well as actin bridges between cells (Figure 4B-arrows). In contrast, the GSCs (Figure 8) and each developing oocyte (Figure 4B) were individually connected to a thin, shared rachis. The cell clusters were not attached to this rachis (Figure 4B).

If spermatogonia are connected by cytoplasmic bridges, cells within spermatogonial cysts should be synchronized in both their developmental and cell cycle timing (Guo & Zheng 2004). Cells within individual clusters were either uniformly MSP- or exhibited equivalent MSP+ patterns (Figure 3B), suggesting that they were developing synchronously. Cell cycle synchrony was tested in two ways. If cells within clusters were synchronously dividing, the number of cells within clusters should double after each division (illustrated in Figure 4C). As predicted, most MSP- clusters were scored with 1, 2, 4, or 8 cells (Figure 4C). In a more direct test of cell cycle synchrony, gonads were labelled with the monoclonal antibody MPM-2 that binds to the phosphorylated
target proteins of M-phase cyclin-dependent kinases (Davis et al. 1983; Golden et al. 2000). Within the distal mitotic zone, MPM-2 labeled individual M-phase putative GSCs (Figure 4D- GSCs). In contrast, within cell clusters, MPM-2 either labeled all of the cells (Figure 4D- cc3) or, more typically, none of the cells (Figure 4D- cc1, cc2, cc4). This pattern suggests that cells within clusters were synchronously either in M phase or interphase. Consistent with previous descriptions of C. elegans mitotic cells (Crittenden et al. 2006; Maciejowski et al. 2006), MPM-2 labeling of comparable C. elegans hermaphrodite gonads was restricted to individual cells in the mitotic zone (Figure 4E).

Because the cell clusters in R. sp. SB347 hermaphrodite gonads exhibited these four key features of spermatogonial cysts — amplification of germ cell number, mitotic proliferation independent of the stem cell niche, cytoplasmic bridges, and developmental and cell cycle synchrony — we concluded that they are a nematode version of spermatogonial cysts.

**Spermatogonial cysts are also a feature of sperm production in *Rhabditis sp. SB347* males**

To determine whether sperm production in R. sp. SB347 males likewise included spermatogonia-based germ cell expansion, male gonads were also co-labelled with DAPI and MPM-2 antibody (Figure 4F). In the distal region of male gonads were two DTCs and a line of putative GSCs (Figure 4F-GSCs)
whose chromatin morphology was similar to putative GSCs in hermaphrodite gonads (Figure 3B-i). As in hermaphrodites, some male gonads included singlets or pairs of MPM-2+ GSCs (data not shown). More significantly, large clusters of MPM-2+ cells were also observed far away from the DTCs. MPM-2+ cells immediately adjacent to the haploid sperm were presumed to be meiotic M-phase spermatocytes (Figure 4F-meiotic), while those within the less proximal clusters (Figure 4F-spermatogonia) were synchronously in mitotic M-phase and appeared to be the male equivalent of hermaphrodite spermatogonia (Figure 4D-cc3).

*R. sp. SB347* hermaphrodites continuously simultaneously produce spermatocytes and oocytes production at all ages

In *C. elegans*, the “sperm first then oocytes” pattern of gamete production (Ward & Carrell 1979) can be observed by comparing the gonads of hermaphrodites in their last larval (L4) stage with those that are transitioning to adulthood (Figure 2A). Gonads from *C. elegans* hermaphrodite L4 larvae contained a line of maturing and meiotically dividing MSP+ spermatocytes that give rise to haploid spermatids (Figure 5A) (Ward & Klass 1982), whereas hermaphrodites making the one-time switch to oogenesis during the L4-to-adult molt contain only a few remaining spermatocytes followed by developing oocytes (Figure 5B).
To determine whether the *R.* sp. SB347 pattern of simultaneously producing sperm and oocytes begins during early gamete production, we also examined the gonads of L4 larval hermaphrodites. Even as L4 larvae, hermaphrodite gonads contained both spermatogonial cysts and developing oocytes (Figure 5C), indicating that simultaneous production of oocytes and sperm is not preceded by an extended period of only sperm production. Notably, though, the most proximal (most mature) germ cells were invariably a cyst of MSP- spermatogonia or MSP+ spermatocytes. Relative to *C. elegans*, the development of haploid spermatids was delayed, because only 25.4% (16/63) of the L4 gonads contained MSP+ spermatocytes and haploid sperm were never (0/63) observed.

To determine whether *R.* sp. SB347 hermaphrodites continue to produce both spermatocytes and oocytes as they age, we compared germ cell patterns at different ages (Figure 5D). Hermaphrodites maintained an average of 2-3 MSP+/−-clusters per gonad arm (Figure 5D). Moreover, most (128/144) of the gonad arms in the most elderly (60-72 hours post-adult-molt) still contained both developing oocytes and spermatogonia (Figure 5E). Therefore, *R.* sp. SB347 hermaphrodite sperm and oocyte production is not only simultaneous but also continuous.

Hermaphrodites in closely related species employ similar reproductive strategies.
To our knowledge, this is the first report in nematodes of either continuous, simultaneous hermaphroditism or spermatogonia. To begin to address how this particular reproductive strategy might have evolved, we examined patterns of gamete production in nematode species closely related to *R.* sp. SB347. The phylogenetic relationships of these species are depicted in Figure 6A (Kiontke and Fitch 2005; Kiontke and Fitch, unpublished 20S rRNA data). Two closely related trioecious *Rhabditis* species *Rhabditis* sp. JU1782 and *Rhabditis* sp. JU1783 exhibited a pattern of gamete production similar to that of *R.* sp. SB347 (Figure 6B-D). Adult hermaphrodite gonads of both species contained both spermatogonia and developing oocytes (Figure 6B), while adult females contained only oocytes (Figure 6C). Furthermore, L4 hermaphrodite gonads of both species also contained both spermatogonia and oocytes (Figure 6D).

To determine if a dioecious (male/female) species within the *Rhabditis* genus might also utilize spermatogonia, we labeled male gonads from the closest known dioecious relative, *Rhabditis axei*, with DAPI and MPM-2 antibody (Figure 6E). MPM-2 labelled single GSCs in the distal mitotic zone, yet no clusters of MPM-2+ mitotic cells were observed.

Outside the *Rhabditis* genus, we also investigated whether hermaphrodites of the androdioecious (male/ hermaphrodite) species *Oscheius tipulae* would exhibit simultaneous hermaphroditism. However, much like *C. elegans*, *O. tipulae* hermaphrodites produced sperm as L4 larvae
before making a one-time switch to oocyte production during the L4 to adult molt (Figure 6F).

Discussion

We discovered two novel features of gamete production in the nematode *Rhabditis* sp. SB347. First, the hermaphrodites simultaneously produce both sperm and oocytes throughout their reproductive lifetimes. Simultaneous and continuous production of sperm and oocytes within a single gonad had been reported in the parasitic nematode *R. ranae* (Runey et al. 1978), but never in a lab cultivatable nematode. Second, both *R. sp. SB347* hermaphrodites and males amplify sperm production via spermatogonial cysts. Spermatogonanial cysts have not been previously described in a nematode, yet these *R. sp. SB347* cysts share key features with those in other taxa (i.e. flies and mice): their cells divide mitotically to increase germ cell numbers, they proliferate without signals from the stem cell niche, they are linked by cytoplasmic bridges, and, within individual cysts, they develop and divide in synchrony.

**Distinct features of *R. sp. SB347* germ cell development**

Both *C. elegans* and *R. sp. SB347* hermaphrodites achieve self-fertility by producing sperm and oocytes within a single gonad, yet the two species evolved distinct solutions for producing two types of gametes from what we
hypothesize to be a common pool of GSCs. In both cases, the GSCs themselves are linked through physical connections to a shared cytoplasmic rachis. In *C. elegans*, as germ cells move out of the niche, exit mitosis, and begin to differentiate, both the developing spermatocytes and oocytes maintain their linkage to the rachis (Gumienny et al. 1999; Amini et al. 2014; Lints & Hall 2009). The one-time switch from producing spermatocytes to producing oocytes is achieved through regulatory changes (reviewed in Ellis & Schedl 2007; Zanetti & Puoti 2013). In *R. sp. SB347*, as germ cells move out of the niche, they follow two distinct paths. Developing oocytes exit mitosis and begin to differentiate while maintaining their linkage to a thin, shared rachis. The route towards sperm production differs in two ways: cells leaving the niche continue to divide mitotically and detach from the rachis. As they divide, they develop distinct cytoplasmic connections to their daughter cells, forming discrete spermatogonial cysts. Mitotic exit and meiotic entry occur only much later as the cyst reaches the proximal end of the germline.

In other organisms, mitotically dividing gametogonial cysts are a standard part of sperm and oocyte production and help to expand the germ cell pool (reviewed in Greenbaum et al. 2011; Kimble 2011; Spradling et al. 2011). In *R. sp. SB347*, this feature is specific to sperm production. In *C. elegans*, germ cell cysts are notably absent, although the mitotic zone contains some cells with a limited capacity to proliferate independent of the niche (Fox & Schedl 2015; Cinquin et al. 2010). However, the coordinated commitment of
C. elegans germ cells to both meiosis and differentiation as they leave the niche (reviewed in (Kimble & Crittenden 2007; Hansen & Schedl 2013) makes it difficult to study these two events in isolation. During R. sp. SB347 spermatogenesis, but not oogenesis, these two events are separated in time and space. Key unanswered questions include how R. sp. SB347 spermatogonia maintain a proliferative state despite their distance from the niche and whether they can be experimentally manipulated to de-differentiate into GSC-like cells, as can occur in Drosophila and mouse (Sheng et al. 2009; Nakagawa et al. 2010).

Spermatogonial cysts in R. sp. SB347 share the hallmark features of those in other organisms. Within individual cysts, the R. sp. SB347 spermatogonia were interconnected by actin bridges, similar to the actin-rich cytoplasmic bridges that result from incomplete cytokinesis after gametogonial divisions in flies and mice (reviewed in (Greenbaum et al. 2011)). Likely due to these interconnections, cells within individual cysts exhibit developmental and cell cycle synchrony. The same is true in flies and mice (Guo & Zheng 2004). The discovery of both cyst and rachis based interconnections within R. sp. SB347 hermaphrodites begs the question: what is the functional difference between these two structural organizations? While both structures join individual cells with interconnecting cytoplasm, the extent that signals pass between cells through these connections may differ. In C. elegans, molecules injected into the rachis diffuse through the germline, yet cells maintain cell
cycle independence (Crittenden et al. 2006; Maciejowski et al. 2006) and recent studies suggest that diffusion blockers slow diffusion between sub-regions of the germline (Cinquin et al. 2015). The structure and properties of the thin R. sp. SB347 rachis or how the spermatogonia might detach and initiate their distinct division patterns have yet to be fully investigated.

**Evolution of *Rhabditis* sp. SB347 hermaphrodite self-fertility**

It is possible, that within the *Rhabditis* clade, spermatogonial cysts may have originated with the evolution of simultaneous hermaphroditism since we observed them in three trioecious *Rhabditis* species, but not in the dioecious near relative *Rhabditis axeii*. Certainly, the spermatogonial cysts enable simultaneous hermaphroditism in that they allow sperm-fated mitotic germ cells to develop independently from adjacent developing meiotic oocytes. Since the *R*. sp. SB347 clade is currently represented by only a few species, it is yet unclear whether the three species examined here arose from a common trioecious ancestor with these features or through independent acquisitions of convergently-evolved hermaphroditism.

Why might *R*. sp. SB347 male sperm production employ spermatogonial cysts while *R. axeii* male sperm production does not? One possible explanation is that *R*. sp. SB347 sperm production utilizes spermatogonial mitotic divisions to compensate for having smaller pool of GSCs in the distal mitotic zone. Conversely, *C. elegans*, *O. tipulae*, and *R. axeii*
sperm production may not need spermatogonial cysts because their gonads contain much larger GSC pools. Fox & Schedl (2015) proposed a similar explanation for the lack of either gametogonial cysts or non-cyst forming, transient amplifying cells in C. elegans gonads, hypothesizing that the larger stem cell pool confers C. elegans a greater adaptability in rapidly producing gametes when environmental conditions are favorable.

Because the diversity of nematode reproductive strategies is poorly studied (Pires-daSilva 2007; Denver et al. 2011), it is possible that continuous simultaneous hermaphroditism and spermatogonial cysts are not limited to the Rhabditis species examined here. Simultaneous hermaphroditism has been described in a parasitic lungworm nematode R. ranae (Runey et al. 1978), and may be common in parasitic forms. The linkage of hermaphrodite self-fertility in R. sp. SB347 to passage through a behaviorally dispersive dauer phase (Félix 2004; Chaudhuri et al. 2011) combined with the observation that males mate primarily with females rather than hermaphrodites (Chaudhuri et al. 2015) suggest that hermaphrodite outcrossing is rare; a condition that would favor the continuous production of sperm to maintain self-fertility. More nematodes should be examined to determine if these features are limited to Rhabditis or are perhaps more common within the phylum Nematoda. Outside of Nematoda, examples of simultaneous hermaphroditism have been documented in diverse phyla (Ghiselin 1969; Clark 1978). However, the ease of studying simultaneous hermaphroditism in the linearly organized gonad of a
free-living and lab-cultivatable nematode reveals *R*. sp. SB347 as a promising system for studying the cellular and molecular mechanisms required to simultaneously produce both sperm and oocytes within a single ovate testis.

**Materials and Methods**

**Strains**

All strains were cultured at room temperature using standard methods (Lambie, 1994). The *Rhabditis axei* strain (DF5006) and *Caenorhabditis elegans* strain Bristol N2 were obtained from the *Caenorhabditis* Genetics Center. The other *Rhabditis* strains were gifts from André Pires-daSilva. *R*. sp. SB347 was originally isolated from a dead deer tick in Connecticut by Walter Sudhaus, *R*. sp. JU1782 from a rotting stem in Ivry, France by Marie-Ann Félix, and *R*. sp. JU1783 from a star fruit in La Réunion by Melissa Doma. *Oscheius tipulae* was a gift from and isolated by Theresa Grana in Virginia.

To isolate hermaphrodites of the trioecious *Rhabditis* species (SB347, JU1782, 1783), dauers were isolated and matured into adult hermaphrodites. Dauers and L3 females are similar in appearance, so human error likely occasionally resulted in females in hermaphrodite samples. *Rhabditis* females were isolated by picking early L3 feminine progeny from early (first 12 hours) broods of selfing hermaphrodites, allowing them to mature in the absence of males, and
monitoring for a lack of embryos.

**Immunocytochemistry and microscopy**

Hermaphrodite and male gonads were dissected in 6 µL of 1% levamisol in Edgar’s buffer (Boyd et al. 1996) on ColorFrost Plus slides (Fisher Scientific) coated with poly-L-lysine (Sigma Aldrich Co.). Anti-tubulin and anti-MSP labeling was done as previously described (Shakes et al. 2009) at 1:20,000 anti-MSP (G3197 polyclonal, gift from David Greenstein; (Kosinski et al. 2005)), 1:100 (0.025 mg/mL) FITC-conjugated anti-α-tubulin DM1A (Sigma). MPM-2 (EMD Millipore) labeling was done as described in (Kulkarni et al. 2012) but at a 1:200 dilution (0.005 mg/mL). Secondary antibodies were the same as described in (Kulkarni et al. 2012). Slides were mounted with Fluoro-Gel II (Electron Microscopic Science) containing 6-diamidino-2-phenylindole (DAPI).

For actin staining, slides with dissected gonads were fixed for 15 minutes in 4% fresh paraformaldehyde (Fisher) in 1x PBS. Samples were quenched in 1M glycine (Fisher) in PBS (Fisher) for at least 5 minutes, permeabilized in 0.1% Triton-X-100 (Fisher) in PBS for 1 minute, then dip washed in 1xPBS. Slides were incubated with rhodamine-conjugated phalloidin (Molecular Probes) diluted 1:100 in 1xPBS for 15 minutes in the dark before washing three times in PBS for 5 minutes each. Slides were mounted as above.
All images were acquired under epifluorescence using an Olympus BX60 microscope equipped with a QImaging EXi Aqua cooled CCD camera with an Olympus PlanApo 40x or 60x objective lens and IPLab software. Images were minimally processed to enhance contrast with IPLab software or Adobe Photoshop.

**Collagenase Treatment**

Hermaphrodite gonads were dissected as above and subsequently treated in a final concentration of 1% collagenase (Worthington Biochemical) in Edgar’s buffer (Boyd et al. 1996) for 15 minutes at room temperature before freeze-cracking and fixation in -20°C methanol.

**Cell cluster feature scoring**

Data shown in Figures 2C, S1, 3C, 4D are from a series of experiments where a total of 1,428 discrete clusters from 518 hermaphrodite gonad arms were scored. For each experiment, microtubule presence, MSP expression, the number of cells within each cluster, and the clusters’ locations in the gonad were scored. Number of cells within clusters was counted according to DAPI-stained nuclei at all focal planes. Cluster location was scored according to the adjacent oocyte’s distance from the spermatheca, where the oocyte nearest the spermatheca is -1 and then counting backwards (-2, -3, etc.) distally. When a cluster resided between oocytes, the cluster was scored at the
more proximal oocyte location.

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
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<tr>
<td>Germline stem cells (GSCs)</td>
<td>Undifferentiated, self-renewing stem cells that are maintained by a stem cell niche (the distal tip cell (DTC))</td>
</tr>
<tr>
<td>Spermatogonia</td>
<td>A proliferative, developmental intermediate between GSCs and spermatocytes. Exist in germ cell cysts.</td>
</tr>
<tr>
<td>Gametocytes</td>
<td>Germ cells that have committed to the meiotic program and are differentiating or have differentiated. Either spermatocytes or oocytes.</td>
</tr>
<tr>
<td>Cell cluster</td>
<td>An isolated group of cells physically associated together.</td>
</tr>
<tr>
<td>Germ cell cyst</td>
<td>A cluster of germ cells that are interconnected by cytoplasmic bridges.</td>
</tr>
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Table 1. Terms used.
Figure 2. Organization of *Caenorhabditis elegans* and *Rhabditis* sp. SB347 oocyte-producing gonads. A) Schematic of germ cell development in *C. elegans* hermaphrodites. Description in text. One half of whole gonad (one of the two gonad arms) is shown. Spth= spermatheca. B-E) Isolated gonad arms labeled with DAPI (blue) and anti-α-tubulin antibody (green). *= location of distal tip cell (DTC). Spth= spermatheca. som= somatic sheath cell. Mt= distal mitotic region. cc= cell cluster. B) *R. sp.* SB347 adult hermaphrodite gonad. C) *C. elegans* adult hermaphrodite gonad. D) Full-size DAPI images of cell clusters from (B) and somatic sheath cell nuclei from (C), (D), and (E). E) *R. sp.* SB347 female gonad. All scale bars = 20 µm.
Figure 3. MSP and microtubule patterns during *Rhabditis* sp. SB347 hermaphrodite spermatogenesis. A) Top: schematic of MSP localization during *Rhabditis* sp. SB347 spermatogenesis. Blue = DNA. Red = MSP. FBs =
fibrous bodies. Below: examples of spermatocytes and sperm from R. sp. SB347 males labeled with DAPI (blue), anti-α-tubulin (green), and anti-MSP (red) antibodies. Scale bars = 5 µm. Description in text. B) Isolated R. sp. SB347 hermaphrodite gonads stained as in (A). Scale bars = 20 µm. Arrows and roman numerals indicate regions corresponding to full-size images below, where scale bars = 5 µm. Locations of clusters are scored according to the adjacent oocyte, starting counting backwards from -1 at the most proximal oocyte, as shown. * = DTC location. C) Histogram of each cluster type at scored locations. Locations were scored by the adjacent oocytes, as in (B). All 1,428 scored clusters are represented, but the outer distal 5% of clusters’ data are shaded gray because very few hermaphrodite gonads contained such large numbers of oocytes.

Figure 4. Physical and cell cycle characteristics of spermatogonial cysts in *Rhabditis* sp. SB347 gonads. A) Portion of R. sp. SB347 hermaphrodite gonad after collagenase treatment, labeled anti-α-tubulin antibody (green). Arrow points to cell cluster separated from oocytes. B) R. sp. SB347 hermaphrodite gonad stained with rhodamine-conjugated phalloidin labelled actin microfilaments (red). *=DTC. Box indicates cell cluster shown at full size.
to the right, where arrowhead points to cortical microfilaments and arrows point to actin bridging between cells. C) Illustration of predicted mitotic doubling pattern of spermatogonia is shown above histogram of frequencies of numbers of cells scored in MSP-negative clusters. D) Two *R. sp.* SB347 adult hermaphrodite gonads labeled with MPM-2 antibody (green). Arrowheads indicate regions shown at full size below. cc= cell cluster. *= DTC. E) Distal mitotic region of a *C. elegans* adult hermaphrodite gonad labeled with MPM-2 antibody (green). Arrows indicate MPM-2+ cells. F) *Rhabditis* sp. SB347 adult male gonad labeled with MPM-2 antibody (green). All gonads are stained with DAPI (blue). *=DTC. Scale bars = 5 \(\mu\)m (A), 20 \(\mu\)m (B, D-F).
Figure 5. Sequence of gamete production over hermaphrodites’ lifetimes in *C. elegans* and *R.* sp. SB347. A-C) Isolated gonads co-labeled with DAPI (blue) and antibodies against α–tubulin (green) and MSP (red). Arrows (1), (2), (3) indicate primary spermatocyte, meiotic spermatocyte, and sperm respectively. A) *C. elegans* L4 hermaphrodite B) *C. elegans* hermaphrodite during the L4-to-adult molt. C) *R.* sp. SB347 L4 hermaphrodite. D) Average number of (both MSP+ and MSP-) clusters per gonad arm scored at different
ages. Bars = standard error. E) Older R. sp. SB347 adult hermaphrodite (60 hours after adult molt) gonad stained as in (A-C). cc= cell clusters. Spth= spermatheca. All scale bars = 20 µm.
Figure 6. Pattern of gamete production in closely related nematodes. A)
Phylogenetic relationships between species examined and an outgroup *Pristionchus* ([Kiontke & Fitch 2005](#)); Kiontke & Fitch, 20s unpublished rRNA data). Left column indicates sexes in that species and right column indicates mode of hermaphroditism. Seq= sequential, making sperm in L4 and then oocytes as adults. Sim= sperm and oocytes simultaneously. B-D) Isolated *R.* sp. JU1782 (left) and *R.* sp. JU1783 (right) gonads labeled with DAPI (blue) and antibodies against α-tubulin (green) and MSP (red). B) Adult hermaphrodite gonads. C) Adult female gonads. D) L4 hermaphrodite gonads. E) *R.* axei adult male gonad labeled with MPM-2 antibody (green). In the top right corner are full-size images of the distal mitotic region. F) Left to right: L4, molt, and adult *O. tipulae* hermaphrodite gonads stained as in (B-D). cc = cell cluster. All gonads are stained with DAPI (blue). All scale bars = 20 micrometers.

**Figure 7.** Relationship between *R.* sp. SB347 hermaphrodite MSP-negative clusters’ location and the number of cells scored in that cluster.

Box-and-whisker representation of the number of cells scored in MSP-negative clusters found at each location. Location was scored by adjacent oocyte, as illustrated in Figure 3B. Dots = outliers outside of 1.5 × IQR.
Figure 8. The GSCs in *R. sp. SB347* hermaphrodite gonads develop along a thin rachis. Isolated *R. sp. SB347* hermaphrodite gonad stained with DAPI (blue) and rhodamine-conjugated phalloidin specific to actin microfilaments (red). *=DTC.
Chapter two: Characterizing the expression pattern of a mitotic protein marker in *R. sp. SB347* spermatogonial cells

**Introduction**

Most sexually reproducing organisms generate gametes from a population of germline stem cells (GSCs) that divide mitotically to give rise to both stem and non-stem cell daughters. The understanding of molecular mechanisms that govern the subsequent GSC growth and its differentiation into the non-stem cell are crucial to the unsolved questions in developmental biology, with broad medical implications (Crittenden et al. 2002).

One gene family, known as PUF (Pumilio and FBF), plays a critical role in developmental biology, in that members of the PUF protein family are key regulators of cell division and differentiation in both the germline and somatic gonad. PUF proteins are functionally conserved across a broad range of organisms, including fungi, plants and animals (Tam et al. 2010). Within animals, it is found in a wide range of species, including *Caenorhabditis elegans, Xenopus, Drosophila*, mice, and humans (Wickens et al. 2002; Salvetti et al. 2005; Xu et al. 2007; Zamore and Lehmann 2006). In *Drosophila* ovaries, the PUF family protein, Pumilio (Pum), is required for both the regulation of asymmetric GSC division and for the maintenance of GSCs (Forbes and Lehmann 1998; Lin and Spradling 1997). *pum* mRNA is also expressed during the earliest stages within the egg chamber. Its expression is
down regulated in subsequent stages and then up regulated again within the nurse cells during later stages of oocyte development (Macdonald 1992; Parisi and Lin 1999). In mice, the PUF homolog Pumilio1 (PUM1) suppresses p53-mediated apoptosis during spermatogenesis by inhibiting translation of mRNA-encoded activators of p53 (Chen et al. 2012). In female mice, PUM1 also controls the maturation of ovarian follicles (Mak et al. 2016). In humans, the PUF homolog PUM2 is found in the cytoplasm of early and late spermatocytes, in the cytoplasm of oocytes, and in the cytoplasm of primordial germ cells of fetal testis and ovaries (Moore et al., 2003). Therefore, the PUF protein family has conserved roles in regulating germ cell specification and development in diverse organisms (Extavour and Akam 2003).

Outside of the germline and somatic gonad, PUF family proteins are expressed in diverse stem cell populations. In humans, PUM2 is expressed in embryonic stem cells, although its exact function remains unclear (Moore et al., 2002). In mice, Pum1 and Pum2 transcripts are present in both fetal and adult hematopoietic stem cells (HSCs) (Spassov and Jurecic 2002).

PUF family proteins are also present in the model nematode organism C. elegans. Two PUF family genes in particular, fbf1 and fbf2, collectively known as fbf (fem-3 binding factor), are involved in regulating the development of germline stem cells (GSCs) (Zhang et al. 1997; Crittenden et al. 2002). As mentioned in previous chapter, the C. elegans germline provides a model for studying the conversion of uncommitted germ cells into either oocytes or
sperm because this process occurs in a linear progression. At the distal end, GSCs proliferate mitotically before the more proximal cells switch from mitosis to meiosis and commit to either the spermatocyte or oocyte fate (Reviewed in L’Hernault 2006; figure 1 in introduction). Recent studies indicate that the molecular regulation of these developmental processes is tightly controlled. In particular, regulation of the mitosis/meiosis switch is linked to sperm/oocyte decision (Kimble and Crittenden 2007). As GSCs located in the mitotic, distal region of the hermaphrodite germline shift proximally, they begin to exit from their undifferentiated stem cell state and begin expressing cell differentiation markers, including GLD-1. GLD-1 is an RNA-binding protein that promotes both gamete fate commitment and the mitosis/meiosis switch by inhibiting the translation of multiple transcripts that repress gamete differentiation, including the fbf.

FBF is an RNA-binding protein that binds to 3’ untranslated regions (UTR) of target mRNAs and represses expression of target proteins either by promoting RNA turnover or by repressing mRNA translation (Lee and Schedl, 2001; Wickens et al. 2002; Bachorik and Kimble 2005). In C. elegans, FBF is present in the GSC pool. Its levels gradually diminish as cells progress through the transition zone (TZ), where the cells first switch from mitosis to meiosis and differentiate into spermatocytes (Crittenden et al. 2002; Figure 9). FBF was first discovered as a regulator of C. elegans germline sex determination, promoting the hermaphroditic switch from spermatogenesis to oogenesis.
(Zhang et al. 1997). Knockdown of FBF expression by RNAi results in hermaphrodites producing only sperm in their germline (Zhang et al. 1997). Subsequent studies revealed that FBF not only regulates the sperm/oocyte decision, but also regulates the mitosis/meiosis switch through interacting with \textit{gld-1} mRNA activity (Crittenden et al. 2002; Suh et al. 2009). The dual function of \textit{C. elegans} FBF and its interaction with \textit{gld-1} activity suggests a key role in coordinating the mitosis/meiosis switch and gamete fate commitment.

The molecular conservation of PUF proteins across a variety of species allows us to study their homologs in other species. In this study, we examined PUF proteins in the \textit{rhabditid} nematode \textit{Rhabditis} sp. SB347. Our previous studies (see chapter 1) have shown that, in \textit{R.} sp. SB347, the hermaphrodites simultaneously undergo spermatogenesis and oogenesis throughout their reproductive lifespan. Spermatocyte progenitors develop within cysts, unlinked from either the oocytes or germline rachis, and divide mitotically as spermatogonial cells prior to becoming spermatocytes. Our findings raise the question: what molecular players regulate these spermatogonial cells? Because the Andre Pires daSilva lab (University of Warwick) recently obtained both genomic deep sequencing and transcriptome data for \textit{R.} sp. SB347 (personal communication), it is possible to identify the likely FBF homolog in \textit{R.} sp. SB347 within these databases and look at FBF expression in the germlines. This study is significant because, distinct from \textit{C. elegans} where germ cells undergo sperm/oocyte decision and mitosis/meiosis switch at the same time
via the co-regulation of FBF and GLD-1, *R.* sp. SB347 sperm-fated germ cells are able to continue dividing mitotically as cysts before initiating their meiotic divisions. Therefore through this study, we could obtain more insights about how FBF might function in a germline that includes mitotically dividing spermatogonial cells.

To further understand the cellular features of spermatogonial cells in *Rhabditis* sp. SB347, we generated a polyclonal antibody against *R.* sp. SB347 FBF to determine whether it is restricted to the distal region of the germline or also expressed in the spermatogonial cells of *R.* sp. SB347 hermaphrodites and males. In this chapter, we will show that 1) there is potentially one FBF protein homolog in SB347; 2) SB347 FBF protein is expressed within the distal region of the germline, and its levels appear diminished as cells become oocyte-fated, suggesting a role in GSC maintenance; 3) FBF protein is present in the distal spermatogonial cell cysts in hermaphrodite germlines, and 4) FBF protein is expressed in late maturing oocytes.
Results

Generating an antibody against R. sp. SB347 FBF protein

To investigate if FBF is present in the R. sp. SB347 germline and in what pattern, we first identified the potential SB347 FBF homologous sequence. To do this, we aligned C. elegans FBF amino acid sequence to the translated sequences from the SB347 transcriptome (unpublished data collected by Sophie Tandonnet from Andre Pires da Silva lab, University of Warwick, by RNAseq). R. sp. SB347 protein sequences homologous to C. elegans FBF were identified by NCBI BLAST search tool. The resulting R. sp. SB347 mRNA sequences homologous to C. elegans FBF were kindly provided by Sophie Tandonnet. R. sp. SB347 fbf mRNA sequence was then translated in silico using expasy translate tool to obtain R. sp. SB347 FBF homolog sequence (Swiss Institute of Bioinformatics, http://web.expasy.org/translate/) (Artimo et al. 2012) (Figure 10A).

To check if the protein homolog was a member of PUF family and if the homolog was conserved to other nematode PUF proteins, a BLAST search was performed against the organism set “Nematoda”. Results indicated that the R. sp. SB347 sequence contains the conserved Pumilio domain (Figure 10B). The protein sequence has multiple PUF homolog alignments in other nematodes, including several C. elegans (Figure 10C). Another BLAST search against “C. elegans” was also performed (Figure 16). In both BLAST protein
alignments, protein homologs were considered a good match to SB347 FBF when the alignment has an amino acid identity >30%, and an E value <10e-3 (Dalal and Atri 2014). The resulting alignments showed C. elegans PUF-3/PUF-11, FBF-1/FBF-2 and PUF-5 as the top matches to the R. sp. SB347 sequence. Among these three other C. elegans PUF proteins, PUF-3 and PUF-11 together limit oocyte growth, while PUF-5 controls late steps of oogenesis (Hubstenberger et al. 2012). R. sp. SB347 FBF homolog was then aligned with the above five well-matched C. elegans PUF proteins using COBALT (Figure 10D), and also alone with C. elegans FBF-2 using pBLAST(Figure 10E).

To look at R. sp. SB347 FBF protein expression within the germline, we first designed antibodies against SB347 FBF homolog. The full SB347 FBF peptide sequence was given to Yenzym (South San Francisco, CA) to analyze peptide antigenicity, and a length of 18 amino acids was selected as an appropriate antigen (labeled with brown box, Figure 10D). The rabbit polyclonal antibodies used in the following experiments were produced and affinity purified by Yenzym.

**Testing anti-SB347 FBF antibody specificity using Western Blot**

To test whether the newly generated polyclonal anti-SB347 FBF antibody (referred below as anti-FBF antibody) binds specifically to the SB347 FBF homolog, we applied the antibody on a western blot. C. elegans him-8 strain
and R. sp. SB347 were used in the western blot, the former C. elegans sample being used to check cross-reactivity. The C. elegans him-8 is a strain that gives rise to high incidence of male offspring but otherwise serves as a wildtype control (Raymond et al. 1998). Meanwhile, the molecular weight of both C. elegans FBF and SB347 FBF were obtained by entering the full sequences into The Sequence Manipulation Suite (http://www.bioinformatics.org/sms/prot_mw.html). The predicted molecular weights (m.w.s) are 70.37kDa and 60.55kDa for C. elegans and R. sp. SB347 FBF, respectively. The resulting western blot probed with anti-FBF antibody showed one C. elegans protein band of approximately 61kDa (see method for m.w. estimation), implying possible antibody cross-reactivity with either C. elegans FBF or other C. elegans proteins (Figure 11A). R. sp. SB347 sample showed one expected protein band at approximately 60kDa (Figure 11B). Unexpectedly, R. sp. SB347 sample also showed one other protein bands with slightly different m.w. (approximately 56kDa). The nature of the other band is yet unable to be determined at this stage.

Testing FBF antibody specificity using immunocytochemistry

We then tested the validity of the antibody in immunofluorescence studies via two control methods. In the first method, R. sp. SB347 samples were treated with either only antibody buffer as a negative control or with anti-FBF antibody. As expected, samples treated with buffer only showed low levels of
uniform, non-specific background staining, while the slides treated with anti-FBF antibody in antibody buffer showed positive FBF signaling (Figure 12A-12B). In another method, competing peptides with lower binding specificity are added to anti-FBF antibody to block the antigen-binding sites and therefore to reduce the affinity of the antibody. Peptide-antibody complex is then applied to the samples (Figure 12C).

After determining that the anti-FBF antibody specifically binds to certain regions within the germline, we then tested the antibody under a variety of conditions to optimize antibody specificity (Figure 17A). When samples were either fixed overnight in -20°C methanol or in a quick fixation protocol (5 min -20°C methanol followed by 5 min -20°C acetone), we found that the quick fixation treatment followed by usual steps primary and secondary antibody incubations, gave better specificity and stronger fluorescence intensity under the epifluorescent microscope (Figure 17B-17C) (see method). However, one puzzle is that, with the quick fixation method, FBF localizes in the nucleolus of the oocytes, while with the overnight methanol fixation, FBF was shown to localize in the cytoplasm of the oocyte (Figure 17B-17C). We are not yet able to explain the difference in cell staining under different fixation methods.

**FBF levels are elevated both in the distal region of the germlines and in the mitotically dividing spermatogonial cells**

To ask where FBF may be functioning in the germline, we proposed two
possible models. One, based on the C. elegans patterns, is that FBF might be expressed specifically in the distal region of the germline, in accordance with its conserved role in GSC maintenance and potentially sperm/oocyte decision in hermaphrodite germline. Alternatively, FBF might be expressed in both the distal region of the germline and the SB347 spermatogonial cells to regulate GSC maintenance as well as the mitosis/meiosis switch.

We observed FBF expression in multiple regions along the germline. First, FBF is expressed at the distal region of all three sexual morphs (Figure 13A-13C). As cells progress to more proximal region, FBF expression diminishes, presumably as cells exit from GSC state and commit to oocyte fate in the same pattern as previously described in C. elegans adult hermaphrodites (Suh et al. 2009; Figure 9 and 13). Such an expression pattern is in parallel to its conserved function in GSC regulation.

Additionally, FBF stands positively expressed in the spermatogonia of both male and hermaphrodites (Figure 13B-13C). Within male germlines, FBF is expressed at the distal region, gradually diminishing its signal towards more proximal region presumably as cells are switching from mitosis to meiosis (Figure 13B). The germline shows a smooth transition from early GSCs with FBF-positive signal to the developing germ cells with FBF-negative signal, although FBF is very likely inactive towards the proximal regions where the cells have clearly entered spermatogenesis (Figure 13B, indicated by yellow dotted lines). Meanwhile in the germline of hermaphrodite, the most proximal
cell clusters, which can be either spermatogonia or spermatocytes, are occasionally FBF-negative (16/47) (Figure 13D). We hypothesize that the cells within these clusters have become spermatocytes.

We then arrive at whether FBF expression decreases within the distal region. First, immunostaining showed that FBF is not expressed in the somatic distal tip cell (indicated by arrows, Figure 14A). FBF is present in the cytoplasm but not the nucleus of the GSCs (Figure 14A-14B). GSCs closest to the distal tip have less FBF than more proximal GSCs. FBF levels then diminish in cells as they transition from stem cells to committing to a specific gamete fate (Figure 14).

These results suggest that SB347 FBF is expressed both in the GSC and in the intermediate mitotically dividing spermatogonia population during sperm production.

**Hermaphrodite and female germlines express FBF in late maturing oocytes**

One surprising result was the discovery of elevated FBF levels in the proximal SB347 oocytes (Figure 15). Initially, we observed that the two most proximal oocytes (often refer to as the -1 and -2 oocytes) have the strongest FBF expression (Figure 15A). Follow-up studies revealed that while most hermaphrodite gonads have high FBF levels in the most proximal -1 oocyte (30/42) (Figure 15A), in other gonads, the FBF labelling was lowered (8/42) or
absent (4/42) in the -1 oocyte (Figure 15B).

In *C. elegans*, changes in protein patterns in the most proximal oocytes are typically associated with oocyte maturation (Masui 1985; McCarter et al. 1999; Miller et al. 2001; Greenstein 2005). However, in contrast to *C. elegans* in which oocyte maturation is induced by the presence of sperm (McCarter et al. 1999; Miller et al. 2001; Greenstein 2005), *R.* sp. SB347 hermaphrodites seem to maintain their differences in FBF expression, despite that sperm is always present to the -1 oocyte. To determine if the variation in FBF levels within the -1 oocyte of *R.* sp. SB347 hermaphrodites might be related to the state of oocyte maturation, we examined female germlines. In these initial experiments, we did not exclusively study unmated females, but we did find the same variation on FBF expressions as we had observed in hermaphrodites (7C-7D), including the presence of both FBF-positive and FBF-negative -1 oocytes in mated females.

**Discussion**

In this study, we generated a new antibody specific to the SB347 FBF homolog, and used this antibody to analyze the location of FBF expression within the germline. We first obtained the homologous FBF sequence in SB347 and BLAST searched the protein alignment with other *Caenorhabditis elegans* PUF proteins. Our results showed that *C. elegans* PUF-3, PUF-11, FBF-1, FBF-2 and PUF-5 have significant alignment results with *R.* sp. SB347
homolog, with PUF-3 and PUF-11 having even higher alignment scores than FBF-1/-2, implying that the SB347 sequence might have alternative functions as other *C. elegans* PUF proteins. We then ran a western blot and found that the anti-FBF antibody binds to two proteins in SB347 adult samples with different molecular weights. While one of the bands matched the m.w. of SB347 FBF homolog (~61kDa), the other band was of unknown origin. Our western blot also showed one protein band of approximately 61kDa in *C. elegans* sample, not matching to the *C. elegans* FBF m.w. (70.37kDa) but more closely to the *C. elegans* PUF-5 m.w. (62.58kDa). In our following immunocytochemistry experiment, SB347 FBF was found present in both the distal region of the germlines and the spermatogonia, and was present unexpectedly in late maturing oocytes in both female and hermaphrodite germlines.

**Is the SB347 protein homolog an FBF or a PUF protein homolog?**

Although the original SB347 protein sequence was identified through aligning the entire SB347 amino acid sequences with *C. elegans* FBF, the resulting SB347 sequence gives higher alignment scores with *C. elegans* PUF-3/11 than *C. elegans* FBF-1/2 proteins. In addition, the anti-FBF antibody was generated against a small portion of amino acid sequence that also has high alignment score with other *C. elegans* PUF proteins (Figure 10D). Together with anti-FBF antibody binding to two proteins of different m.w.s in
the western blot, these results have two implications. One is that the SB347 FBF sequence has been either post-transcriptionally or post-translationally modified in different ways, potentially performing different functions at different locations. Previous studies have suggested that in *C. elegans*, other PUF proteins, PUF-3/11 and PUF-5/6/7, combine with Ras-MAPK signaling to control oocyte differentiation (Hubstenberger et al. 2002). If the same FBF protein binds to both mitotically dividing germ cells and maturing oocytes within oocytes, then the SB347 FBF homolog may also function as *C. elegans* PUF-3, PUF-11 or PUF-5 as being suggested by their protein alignments, to regulate oocyte maturation. Alternatively, the anti-FBF antibody is potentially binding to two proteins, one FBF homolog and the other PUF protein (potentially functional homologs of PUF-3, PUF-11 or PUF-5) with the same binding domain. Since the alignment scores are high in the antigenic peptide sequence among the five *C. elegans* PUF homologs, anti-FBF antibody may in fact bind to other PUF protein homolog that has the same m.w. as indicated on the western blot. However, the number of PUF homologs varies in different species, and we are unsure of the total number of PUF proteins in *R. sp.* SB347. The nature of the anti-FBF antibody and the protein needs further investigation.

**Does SB347 FBF antibody cross-react with a *C. elegans* protein?**

Our western blot results showed that the anti-FBF antibody binds with one
protein in *C. elegans*, however the estimated m.w. of the band was 61kDa, which is not similar to *C. elegans* FBF (70.37kDa). Meanwhile, the m.w. is 56.95 kDa for *C. elegans* PUF-3, 57.11 kDa for PUF-11, and 62.58 kDa for PUF-5, and therefore PUF-5 might be the binding protein according to the similarity with the protein band’s m.w. The result indicates that while anti-FBF antibody potentially cross-reacts with *C. elegans*, the antibody perhaps binds to another protein rather than *C. elegans* FBF. However, since the m.w.s of the protein bands were only estimates, follow-up experiments are needed to confirm our western blot result.

Is the localization pattern of the SB347 FBF homolog consistent with it controlling both the mitosis/meiosis switch and sperm/oocyte determination?

As described previously, *C. elegans* FBF regulates both mitosis/meiosis switch and sperm/oocyte determination. In our experiments, in *R. sp.* SB347, FBF was present both in the distal region and within the spermatogonia, which is in agreement with its conserved role of repressing the mitosis/meiosis switch. However, since the distal GSCs are smaller in size, FBF might be more concentrated in less cytoplasmic content than in the proximal oocytes. Further investigation is required to confirm that the distal region has higher FBF expression than the developing oocytes through analyzing the quantity of FBF expression in both the distal region and the developing oocytes.
Additionally, whether SB347 FBF homolog also controls sperm/oocyte switch needs further investigation. The characteristics of SB347 FBF can be further understood if we combine our localization studies with additional functional studies, such as gene silencing, ectopic expression, and gene knockout. Such an understanding of SB347 FBF function is important because different from C. elegans hermaphrodites in which the germline produces one gamete type at one life stage, R. sp. SB347 hermaphrodite germlines are capable of alternating in between sperm and oocyte production and maintaining two independent stem cell niche throughout the entire lifespan. If the role of this SB347 FBF homolog in sperm/oocyte switch can be elucidated based on both localization studies and functional studies, the homolog can be of great importance in understanding the mechanisms of germ cell specification in a germline that keeps two distinct mitotic cell populations, and potentially in providing insights within and outside of germline lineage studies.

Does the SB347 protein homolog play a role in oocyte maturation and what are the potential factors influencing the expression pattern?

Aside from its presence in spermatogonia and GSCs, the protein is present unexpectedly in late maturing oocytes in both female and hermaphrodite germlines. Although we are unsure of the nature of the protein, we can at least suggest the likelihood that this PUF protein (regardless of whether it is FBF) is involved in oogenesis, which has been indicated in other
species (Macdonald, 1992; Parisi and Lin, 1999; Moore et al., 2003). In *Drosophila* ovary, FBF protein homolog is found in the nurse cells in the later stage of egg chambers (Macdonald 1992; Parisi and Lin 1999). Additional studies have also shown its presence in the cytoplasm of human oocytes, although the exact function is unknown (Moore et al. 2003).

We also showed that, in most cases, FBF signal increases as oocytes approach the proximal region (30/42). Alternatively, FBF signal is either diminished (8/42) or completely negative at the -1 oocyte (4/42). Such a difference in the expression level in the -1 oocytes is presumably related to specific cellular stages during oocyte development. One possibility is the potential influence of sperm presence in the FBF expression of the -1 oocyte. Previous studies have shown that, in *C. elegans*, sperm promote oocyte meiotic maturation and ovulation through binding the major sperm protein MSP on the oocyte surface facilitating oocytes to exit from pre-meiotic prophase I (Masui 1985; McCarter et al. 1999; Miller et al. 2001; Greenstein 2005), although other studies have shown that some parthenogenic nematode species trigger oocyte-to-embryo transition in the absence of sperm (Heger et al. 2010). Whether or not SB347 sperm can have the similar regulation over oocyte development as *C. elegans* does remains to be investigated.

What might the FBF expression pattern tell us about features of spermatogonial cells?
Our studies provide an alternative model for the study of spermatogonial cells. Both *C. elegans* and *R. sp. SB347* germlines express FBF at the distal region to regulate GSC maintenance. However, different from *C. elegans*, which does not express FBF as cells progress through the germline, *R. sp. SB347* germline spermatogonial cells maintain FBF expression. Therefore, spermatogonial cells are partially committed to sperm fate but retain certain features of GSCs.

However, at this stage, we have limited knowledge about FBF expression pattern along the germline. We have not yet fully quantified FBF expression level within different cell populations, nor do we know whether FBF protein maintains its activity to inhibit translation of the target mRNAs in the spermatogonia. At this point, we propose that anti-FBF antibody is binding to either one or two proteins which function in mitosis/meiosis maintenance and potentially oocyte maturation. The nature of the antibody-binding proteins and the function of these proteins need further investigation.

**Future Directions**

Future experiment will look at whether *C. elegans* germlines cross-reacts with *R. sp. SB347* anti-FBF antibody using immunocytochemistry. Because the western blot probed with anti-FBF showed a protein band with a m.w. (~61kDa) different from *C. elegans* FBF (70.33kDa) but similar to *C. elegans* PUF-5 protein (62.58kDa), future experiments will first repeat the western blot and
confirm our calculation of the m.w. estimate, and then will perform immunocytochemistry probing *C. elegans* hermaphrodites with anti-FBF antibody to investigate the cross-reactivity of the protein.

Future experiment will look at whether the two SB347 protein bands on the western blot result are the same protein but modified post-translationally. One technique to use is immunoprecipitation, followed by the western blot to determine whether m.w. difference between the two bands is similar to any molecular groups that can be added post-translationally to the protein. In addition, one could check the possibility of post-translational modification (PTM), in particular, phosphorylation. To see whether the same protein might be phosphorylated to show up on the western blot with a slightly heavier m.w., future experiment will apply phosphatase to remove potential phosphate group on the target protein and see whether one protein band might disappear on the western blot.

To investigate the FBF-negative clusters at the proximal region of the germline, one future study is to co-label germlines with both FBF antibodies and antibodies against spermatocyte markers, including major sperm protein (MSP) and SPE-44 (Kulkarni et al. 2012). By looking at the expressions of both FBF and the spermatocyte marker within the proximal clusters, we could determine whether FBF is only expressed in the spermatogonial cells and not in spermatocytes.

To understand whether SB347 FBF functions in sperm/oocyte switch,
another future direction is to knockdown FBF function in SB347 via RNA interference and assess the resulting phenotype. Such an experiment will provide direct information on the function of SB347 FBF homolog in oocyte/sperm switch, which would be indicated by any absence of either sperm or oocyte production.

Because the levels of FBF expression have not been fully examined, future experiments will look at FBF localization using confocal microscopy and quantify the level of FBF expression through 3D imaging. The forthcoming results will help understand whether FBF expression level changes within the spermatogonial cells, what are the differences in the expression level between the distal region and the spermatogonia, and also potentially whether FBF is actively involved in inhibiting meiotic entry in spermatogonial cells.

To compare oocyte maturation and FBF expression, future studies will assess the transitions during oocyte maturation stage through a few factors: chromosomal morphology of oocytes, the presence of sperm, and the expression of oocyte maturation markers such as mitogen-activated protein kinase (MAPK).

Chromosomal compactness is indicative of different stages of oocyte development and can be compared with levels of FBF expression in the -1 oocyte. In C. elegans, the maturing oocytes go through an arresting stage in pre-meiotic prophase (Reviewed by Masui and Clarke, 1979; Masui, 2001), where chromosome remains diffused within the nucleus. The chromosome
condenses as the oocyte enters diplotene and then diakinesis. Supposedly if *R.* sp. SB347 oocytes have similarly stage-specific chromosomal patterning, *R.* sp. SB347 -1 oocytes with corresponding chromosome morphologies might have distinct levels of FBF expression. Future oocyte scoring to look at the relationship between the chromosomal morphology and FBF signal can provide more insights to the potential role of FBF in *R.* sp. SB347 oocyte development.

To investigate whether the presence of sperm influences FBF expression in the -1 oocyte, another future experiment is to compare the germlines of mated vs. unmated female in relation to their FBF expression patterns. We suspect that as oocytes in unmated females arrest at one specific cell cycle stage and wait for sperm, they are not being stimulated to mature. Therefore the -1 oocytes will more frequently contain higher FBF expression than in mated females. Meanwhile, future studies will use oocyte maturation markers, for instance mitogen-activated protein kinase MAPK (Miller et al. 2001), to co-label the -1 oocytes with FBF and study the correlation between the maturation stage and the level of FBF.

**Materials and Methods**

**Strains**
All isolated strains were described in the method section in chapter one.

Bioinformatics and antibody manufacture for SB347 FBF

R. sp. SB347 transcriptome data were acquired by Sophie Tandonnet of André Pires daSilva lab, University of Warwick, by RNAseq. Homologous sequences were identified by BLAST searching for C. elegans protein sequences in R. sp. SB347 in silico translated sequences (BLASTx search function). The resulting mRNA sequences were kindly provided by Sophie Tandonnet. The sequences were translated in silico using xpasy translate tool (choosing the longest translated peptide) (Swiss Institute of Bioinformatics, http://web.expasy.org/translate/) (Artimo et al. 2012) and subsequently BLAST searched with C. elegans protein sequences. For FBF alignments from multiple nematode species, NCBI’s COBALT (https://www.ncbi.nlm.nih.gov/tools/cobalt/re_cobalt.cgi) (Papadopoulos & Agarwala 2007) was used directly from the BLAST search results. The translated protein sequences of R. sp. SB347 FBF homologs were aligned with the corresponding C. elegans protein using Geneious version 9.0.2 (Kearse et al. 2012). The text file of the pairwise alignments were then edited to highlight the conserved domains spanning the residues indicated by CDD Conserved Domains Database) search (Marchler-Bauer et al. 2009) on each protein sequence. To design antibodies of FBF, the full peptide sequences were given to Yenzym (South San Francisco, CA), who analyzed the peptides for
antigenicity. The antibodies were ordered from and affinity purified by Yenzym. Two rabbits were immunized per peptide. Experiments here were from the rabbit that produced polyclonal antibodies that showed more specific binding in immunohistochemistry analysis (see also McCaitg Thesis, May 2017).

**SDS-PAGE and Western Blotting**

SDS-PAGE and western blot are performed by Caitlin McCaig and are described in details in McCaig’s thesis, May, 2017.

The m.w. of each protein band was approximated relative to the two nearest molecular weight standards of the standard proteins (Biorad Precision Plus Protein Dual Color cat # 1610374).

**Immunocytochemistry and microscopy**

For FBF staining, different incubation conditions were tried to first obtain high specificity (Figure 17A) and the conditions with the best specificity result were obtained. To prepare FBF staining immunocytochemistry, slides with dissected gonads were freeze-cracked with liquid nitrogen, immediately fixed for 5 minutes in -20°C methanol, followed by 5 minutes in -20°C acetone. Samples were washed with three consecutive 5-minute washes in 1xPBS followed by a 15-minute incubation in blocking solution (PBS+0.5% BSA and 0.1% Tween 20). Samples were then incubated with 1:10 diluted goat serum for 10 minutes. Anti-FBF labeling was done at 1:50 anti-FBF (Yenzym),
incubated overnight at 4°C. Secondary antibodies were the same as described in Kulkarni et al. (2012). Slides were mounted with DAPI as described in chapter one.

For FBF peptide competition treatment, FBF peptide was first aliquotted to 25mg/ml. The peptide stock was diluted with Ab buffer with a ratio of 1:260. Equal volume of 1:1 FBF glycerol stock was added to the diluted peptide solution. The FBF-peptide solution was placed to rock for 30 minutes on ice, followed by 15 minutes of centrifuge at 10,000RPM, at 4°C (Rockland Peptide Competition Assay Protocol). The FBF-peptide solution was applied to the dissected samples as primary antibody.

Cell cluster feature scoring

The scoring system is described in chapter one. To score FBF-positive and FBF-negative clusters, FBF intensity level is measured in Adobe Photoshop CS6 using “level adjustment” to compare FBF level of the proximal cluster to the distal FBF-positive clusters.

Figure 9: C. elegans FBF expression pattern along male/L4 hermaphrodite germlines. FBF expression is high at the distal end, but gradually diminishes as cells become sperm-fated and enter into meiosis. Yellow = FBF-positive germline stem cell
Figure 10: R. sp. SB347 FBF homolog bioinformatics. A) R. sp. SB347 FBF sequence translated from nucleotides using BLASTx. B) R. sp. SB347 FBF sequence with conserved *pumilio* domain (indicated in green box). C) A list of proteins that align with SB347 FBF protein with highest sequencing. Red box indicates high alignment result with *C. elegans* protein (see also figure 16). D) R. sp. SB347 FBF protein alignments with five *C. elegans* proteins that have a low E value and high % amino acid identity. Red colors: high alignment score. Blue colors: fair alignment score. Grey: low alignment score. From top to bottom: SB347 FBF homolog (query_238256), *C. elegans* PUF-3 (NP_502606.1), *C. elegans* PUF-11 (NP_741425.1), *C. elegans* FBF-2 (NP_495216.1); *C. elegans* FBF-1 (NP_495216.1), and *C. elegans* PUF-5 (NP_495814.1). Brown box indicates the peptide sequence selected by Yenzym Company as the sequence with high antigenicity for generating FBF antibody. E) Protein alignments between SB347 FBF homolog and *C. elegans* FBF-2. E-Value is indicated in red box.
Figure 11: SDS-PAGE Western blot result with FBF antibody done by lab partner Caitlin McCaig. A) Western blot result with C. elegans and R. sp. SB347 samples. Number 100, 150, and 200 indicate the number of worms in each sample. Mix indicates that the sample contains worms with a mix of sexual modes. Red box is the sample enlarged on (B). B) Western blot with sample of 200 R. sp. SB347 hermaphrodites probed with FBF antibody where gel was run longer than in (A).

Figure 12: Comparison of SB347 germlines under control treatments. All three germlines are dissected within the same experiment, and all images are taken with the same exposure time. A) SB347 germline labelled with anti-FBF antibody. B) SB347 germline immunolabelled in the same experiment and with the same conditions as A, but without primary antibody. C) Competing peptide control. Immunolabelling of SB347
germline in the presence of competing peptides against which the anti-FBF antibody was generated. Scale bar = 20 µm.

Figure 13: R. sp. SB347 female, male, and hermaphrodite germlines. A) R. sp. SB347 female germline. B) R. sp. SB347 male germline. Yellow dotted lines indicate the unclear region where FBF is positive but cells have clearly entered spermatogenesis due to their smaller chromatin size. C) R. sp. SB347 hermaphrodite germline. #1 and 2 indicate distal and proximal spermatogonial cysts, which are both
FBF-positive. D) R. sp. SB347 hermaphrodite germline. #1 and 2 indicate distal and proximal clusters. The distal cluster is FBF-positive while the proximal cluster is FBF-negative. Yellow line = distal region of the germline (determined by condensed chromosome morphology). White line = differentiating gametes that have entered meiotic division. White arrows label cell clusters. Scale bar = 20 µm.

Figure 14: A closer look at the distal region of the germlines. A) Distal region of R. sp. SB347 female germline. B) Distal region of R. sp. SB347 hermaphrodite germline. Yellow line = distal region of the germline. Arrowhead = distal tip cell. Scale bar = 20 µm.
Figure 15: Comparison of FBF expression in the -1 oocytes between hermaphrodites and females. A) R. sp. SB347 hermaphrodite germline with FBF-positive -1 oocyte. B) Hermaphrodite germline with FBF-negative -1 oocyte. C) R. sp. SB347 female germline with FBF-positive -1 oocyte. D) R. sp. SB347 female germline with FBF-negative -1 oocyte. White line = differentiating oocyte gametes that have entered meiotic division. Mt = mitotic zone. Scale bar = 20 µm.

Figure 16: pBLAST search result of SB347 FBF homolog to C. elegans proteins. Alignments with >30% amino acid identity and <10e-3 E value are considered significant. Red box indicates the top five C. elegans PUF homologs with the most significant alignments: PUF-3, PUF-11, FBF-2, FBF-1, and PUF-5 (in order of alignment significance).
Figure 17: Optimization anti-FBF antibody specificity using different treatment conditions. A) Table of a list of tests on different treatment conditions in incubating anti-FBF antibody. The optimal protocol for immunocytochemistry is determined and highlighted in red box. B-C) Comparisons of antibody specificity under overnight methanol fixation treatment (B) or quick fixation treatment (C). B) R. sp. SB347 germline with anti-FBF antibody going into the cytoplasm of maturing oocytes. C) R. sp. SB347 germline
with anti-FBF antibody going into the nucleolus of maturing oocytes.
References


