

ABSTRACT

Genetic control of cell fate specification in *Caenorhabditis elegans* germline.

by

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The precise regulation of germ cell fates (sperm or oocyte) lies at the heart of reproduction and fertility. The nematode *C. elegans* hermaphrodites produce a discrete number of sperm during larval development and then switch to produce oocyte during adulthood. A number of positive (e.g., *fbf* genes) and negative (e.g., *gld-3*) regulators are important for this switch. Here, we found that aberrant activation of MPK-1 (an ERK homolog) by removal of both *fbf-1* and *lip-1* partially inhibits sperm-oocyte switch, resulting in Mog (masculinization of germline) sterility. The *fbf-1* gene encodes a conserved PUF (Pumilio and FBF) RNA-binding protein and the *lip-1* gene encodes an MPK-1/ERK phosphatase.

Notably, inhibition of MPK-1/ERK signaling by either genetic mutation or chemical inhibition reprograms the germ cell fate and thus helps in regaining the fertility. We also found that *fbf-1; lip-1* Mog sterility was enhanced by the depletion of G2/M cell cycle regulators, including CYB-3/Cyclin B, CDK-1/CDK1, and CDC-25.1/CDC25. Markedly, *cdc-25.1* mRNA is a direct target of FBF-1. These results suggest that FBF-1 and LIP-1 may promote sperm-oocyte switch by activating MPK-1/ERK signaling and G2/M cell cycle progression.

**Genetic control of cell fate *specification* in
Caenorhabditis elegans germline**

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By

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May, 2015

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**Dedicated to my family, friends and my mentor
and to the entire scientist fraternity.**

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- Srivalli Swathi Mamillapalli

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LIST OF SYMBOLS AND ABBREVIATIONS

PUF: PUMILIO/FBF domain-containing.
FBF: Fem-3 mRNA Binding Factor.
FBE: FBF Binding Element
LAG: Lin-12 and Glp-1
PUM: Pumilio
PBE: Pumilio Binding Element
LIP-1: Lateral-signal-Induced Phosphatase.
GLP-1: abnormal Germ Line Proliferation.
UNC: UNCoordinated.
MPK-1: Mitogen-activated Protein (MAP) Kinase.
MKP3: MAP Kinase Phosphatase 3.
ERK: Extracellular signal-regulated Kinase.
MOG: Masculinization of Germline.
FOG: Feminization of Germline.
IFE-1: Initiation Factor 4E (eIF4E) family.
eIF4E: Eukaryotic initiation factor.
CYB: Cyclin B
CDK: Cyclin Dependent Kinase
NGM: Nematode Growth Medium.
GSCs: Germline Stem Cells.
DTC: Distal Tip Cell.
IPTG: Isopropyl β -D-1-thiogalactopyranoside
DAPI: 4', 6-Diamidino-2-Phenylindole, Dihydrochloride.

PFA: Paraformaldehyde.

PTW/PBST: Phosphate Bovine Serum with Tween-20.

BSA: Bovine Serum Albumin.

EDTA: Ethylenediaminetetraacetic acid.

RISC: RNA Induced Silencing Complex.

SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis.

HRP Antibody system: Horse Radish Peroxidase Antibody system

Y3H: Yeast Three Hybridization

3-AT: 3- Amino Triazole

CHAPTER 1

Synopsis of *Caenorhabditis elegans* as a model system

INTRODUCTION

1. Stem cells and cell fate specification

Stem cells have an ability to self-renew and differentiate into a specific cell type. This balance between the two fates is crucial in the process of cellular homeostasis and normal tissue generation. Aberrant regulation of this balance is often associated with human disorders, including cancer. Therefore, understanding the various regulatory mechanisms controlling the stem cell and its cell fate specification is essential to develop novel diagnostic and therapeutic approaches towards the disorders in stem cell proliferation, differentiation and functioning in mammals.

2. *C. elegans* as a model system

The nematode *Caenorhabditis elegans* (*C. elegans*) is a distinguished organism whose genome is remarkably similar to the human genome (Kaletta and Hengartner, 2006). To date, it contributed conclusively in understanding regulation and progression of homologous processes in humans. *C. elegans* has an inherent capability for rapid and abundant proliferation in a laboratory environment. It undergoes a 3-day life cycle producing about 300-350 progeny from a single organism. This ability provides a significant advantage of determining the authenticity of the impact of various regulators that are being studied. In addition, the number of cells and the position of cells in a particular organism remain

constant through generations. This anatomical simplicity along with its physical transparency makes *C. elegans* an exceptionally suitable organism for phenotypic studies (Barriere and Felix, 2005). The characteristic of physical transparency also facilitates the usage of simple laboratory techniques like differential interference contrast microscopy to study the organism. *C. elegans* has another unique characteristic which enables it to be an appropriate organism to study homologous systems in higher animals. Induction of specific mutations in *C. elegans* is comparatively easier than in higher animals. The widely available and alterable *C. elegans* mutants thus became powerful tools in understanding various mechanisms and regulators of stem cells. An ideal model organism should be maintained and reused in the time of need. With the development of convenient methods for permanent storage of *C. elegans* stocks, the organism can be maintained for prolonged periods of time. Therefore, all these characteristics make *C. elegans* a simple, yet effective model system to study homologous cell systems of higher animals.

3. *C. elegans* life cycle

The average duration of reproductive life cycle in *C. elegans* is 3 days under optimal conditions. This generation time usually varies with temperature. It is 2.5 days at 25°C, 3.5 days at 20°C and about 5.5 days at 15°C (Klass, 1977). *C. elegans* passes through the stages of embryo and larval stages (L1, L2, L3 (or Dauer) and L4) before it becomes an adult (Figure 1). Embryogenesis (entry of the sperm till hatching of egg) in *C. elegans* takes about 14 hours of time. The organism takes 9 hours from the stage of egg to L1 stage (Figure 1). Usually the L1 stage larva takes 12 hours to reach the L2 stage (Figure 1). The gonad elongates and germ cells continue to enter meiosis during L3 and L4 stages. L4 progresses to adult and by about 72 hours from being hatched from an egg the organism will be ready to lay

eggs. However, in exceptional conditions like starvation - the organism enters into a stage called Dauer stage in its life cycle (Figure 1). The Dauer organism can directly progress to L4 and then to adult when provided with suitable conditions and food (Figure 1). In adults, germline transcends from mitotic zone to meiosis. Therefore, as the life cycle progresses from L1 to Adult stage – a typical germ cell enters into meiosis proximally to produce sperm, but remains in mitosis distally.

Markedly, *C. elegans* also undergoes molting as it progresses through its life cycle. It molts through four larval stages (L1-L4) before it becomes an adult. Molting (shedding of the old cuticle and synthesizing a new one) occurs at the end of each larval stage. This process allows the organism to maintain physical transparency.

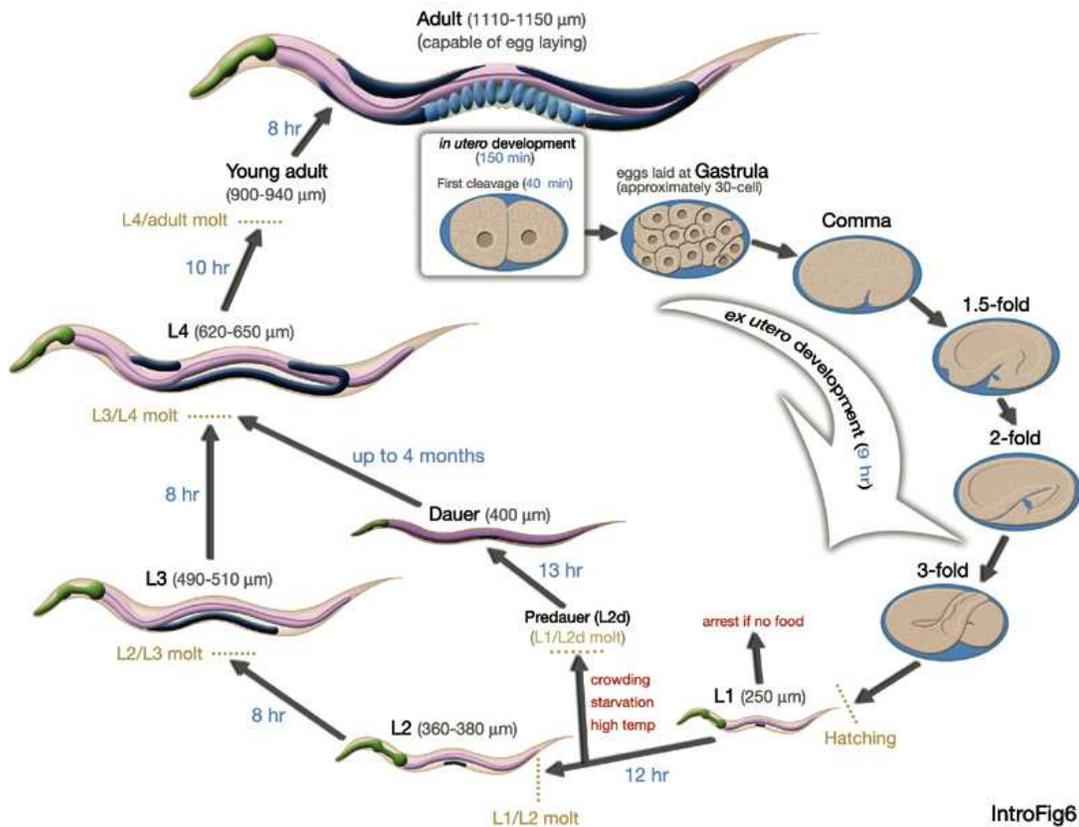


Figure 1. Review of life cycle of *Caenorhabditis elegans*

4. *C. elegans* germline development

The process of reproduction is different in the two sexes of *C. elegans*. The male worm can provide sperm for a cross-fertilization (but cannot bear the progeny), while the hermaphrodites are capable of self-fertilization and bearing progeny. Therefore, an adult hermaphrodite worm lays about 300 embryos by self-fertilization and about 1000 embryos by cross-fertilization in a lifetime. Moreover, the changes related to germ cell specification occur in relation with the life cycle progression. In a male worm, the spermatogenesis begins in the L4 larval stage and it continues throughout the lifetime. Whereas in a hermaphrodite, the spermatogenesis ceases and there is a switch to oogenesis in the adult stage and hence it can self-fertilize (Kimble and Crittenden, 2007).

5. *C. elegans* anatomy

In general, in both the sexes, the body of *C. elegans* is cylindrical and unsegmented.

Like any typical nematode, *C. elegans* possesses an outer tube and an inner tube with pseudocoelomic space in

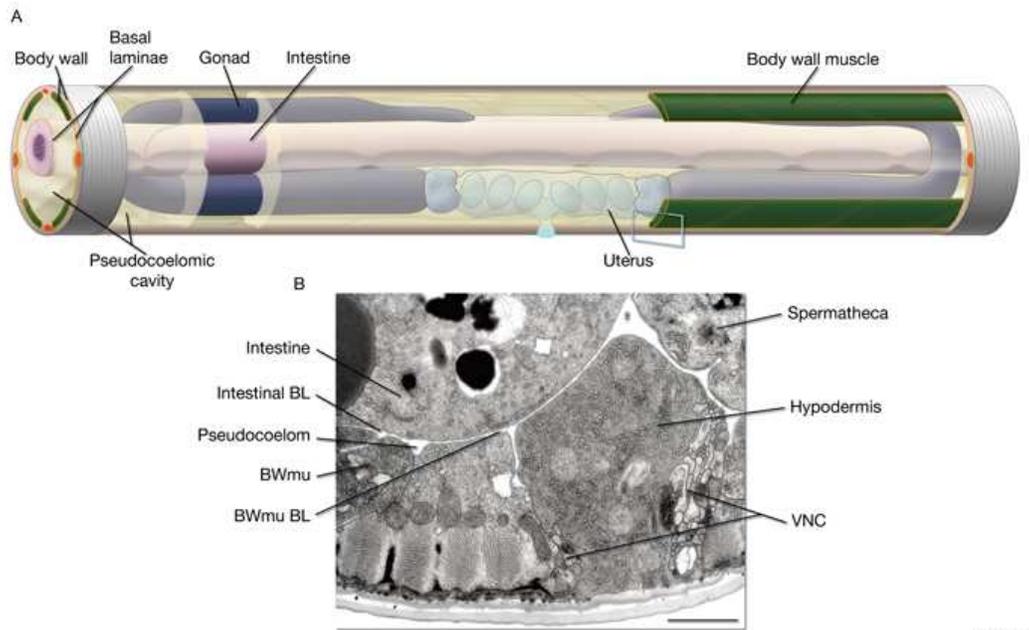


Figure 2. Schematic of cross-section of *C. elegans* worm

between (Figure 2). All of the tissues are under internal hydrostatic pressure, which is regulated by osmoregulatory system. The outer tube of *C. elegans* has cuticle

(which molts), hypodermis, excretory system, neurons and muscles. The inner tube comprises the pharynx, intestine and gonad (if the organism is an adult).

The gastrointestinal system comprises of a feeding tube, the pharynx, which connects to the intestine that runs along the body length which then leads to the rectum and finally ends in an opening, the anus.

The reproductive system is unique in *C. elegans*. It has two sexes – male and the self-fertilizing hermaphrodite (Figures 3 and 4). In general, the reproductive system consists of a somatic gonad, the germ line and the egg-laying apparatus but the two sexes are phenotypically different. The differences can be observed with contrast microscopy as the organism is transparent. The male organism has a single armed gonad in its reproductive system while the hermaphrodite has two gonadal arms. In a hermaphrodite organism, the two bilaterally symmetric U-shaped gonad arms are connected to a central uterus through the spermatheca.

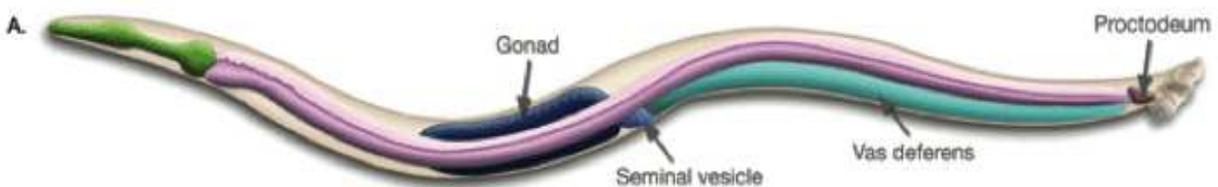


Figure 3. Anatomy of *C. elegans* hermaphrodite. Schematic drawing of anatomical structures, left lateral side

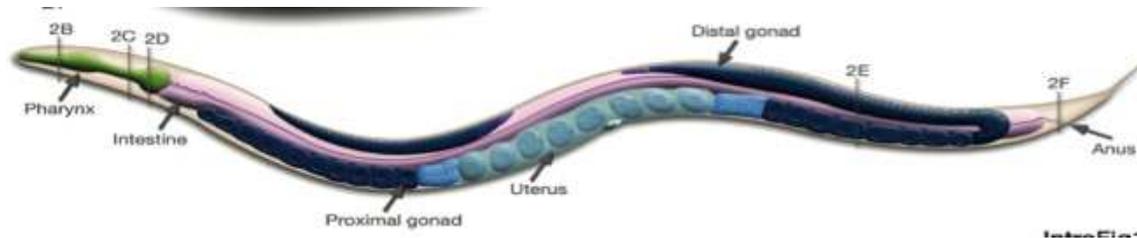


Figure 4. Anatomy of *C. elegans* male. Schematic drawing of anatomical structures, left lateral side

The germ line within the distal gonad arms is syncytial with germline nuclei surrounding a central cytoplasmic core. More proximally, germ cells pass sequentially through the mitotic, meiotic prophase and diakinesis stages. As they pass through the bend of the gonad arm (oviduct), oocytes enlarge, detach from the syncytium, and mature as they move more proximally. The oocytes are fertilized by the sperm in spermatheca. The resulting diploid zygotes are stored in the uterus and are laid outside through the vulva, which protrudes at the ventral midline. In the anatomy of a male worm, the shape of the posterior half of the worm is different and appears more tapered. The most distinguishing feature of a male worm is that it possesses a male copulatory apparatus in the posterior part of the body.

6. *C. elegans* somatic sex determination

The somatic sex specification of the worm is determined by chromosomes. *C. elegans* has five pairs of autosomes and one pair of allosomes. The allosome pair is XX for hermaphrodite and XO for male. Hermaphrodites are females that make sperms during larval stage and produce oocytes as adults. XO males make sperms throughout their lifetime. Hermaphrodite germline develop bi-directionally

whereas male gonad develops in a single direction. Only 0.1% of the total population is males through self-fertilization. However, almost 50% of the progeny are males after cross-fertilization as they do not inherit the X chromosome from their fathers in this case.

7. Review of *C. elegans* germline sex determination

The germ cells pass sequentially through the mitotic, meiotic prophase and diakinesis stages in the gonad. In a male worm, the spermatogenesis begins in the L4 larval stage and it continues throughout the lifetime. Whereas in *C. elegans* hermaphrodite self-fertility is achieved by the spermatogenesis in the L3 stage followed by a switch to oogenesis in the adulthood (Figure 5). Among the different RNA regulators controlling the sex differentiation in wild-type hermaphrodites *fbf-1*, *fbf-2*, *tra-1*, *tra-2*, *puf-8* and *daz-1* are important RNA binding proteins that promote oocyte production and the regulators *fog-1*, *fog-2*, *fog-3*, *fem-1*, *fem-2*, *fem-3*, *gld-1* and *gld-3* enhance sperm production (Kimble and Crittenden, 2007). An overview of germ cell fate specification is shown in the (Figure 6).

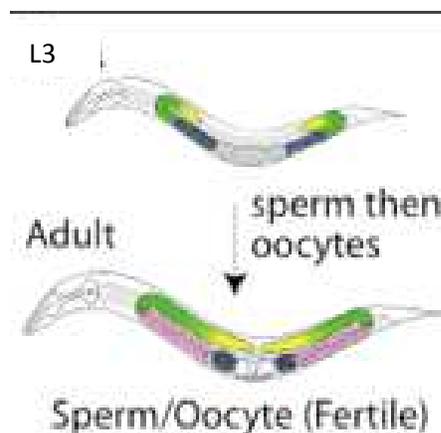


Figure 5. Schematic development of gametes in hermaphrodite gonad. Male gametes (sperm) develop in L4 stage and continue to adult stage. Oocytes develop later.

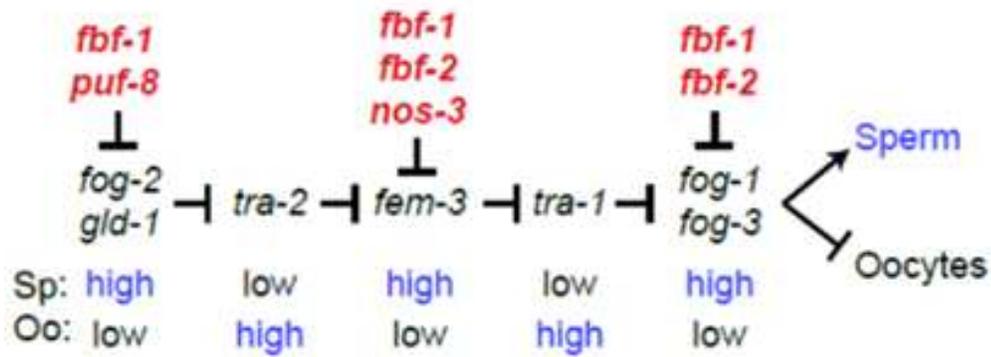


Figure 6. Overview of Simplified germ cell fate

Interestingly, the sperm promoting RNA regulators and oocyte fate promoters antagonize each other. (Barton and Kimble, 1990; Doniach and Hodgkin, 1984; Ellis and Kimble, 1995; Hodgkin, 1986). Mutations in any of sperm promoter genes cause all germ cells to differentiate as oocytes, called Fog (feminization of the germline). *tra* genes act as negative regulators of *fog-1* and *fog-3*. Mutations in *tra* genes cause hermaphrodite to make sperm instead of oocytes, called Mog (for masculinization of germline) (Shen et al., 2005). FBF (Fem-3 binding factor) RNA-binding proteins also promote sperm-oocyte switching by inhibiting the expression of sperm-promoting gene mRNAs, including *fem-3*, *fog-1*, and *fog-3* (Gorrepati et al., 2013; Zhang et al., 1997)(Figure 7). Therefore, translational control of sperm-oocyte gene mRNAs is critical for *C. elegans* sexual fate decision.

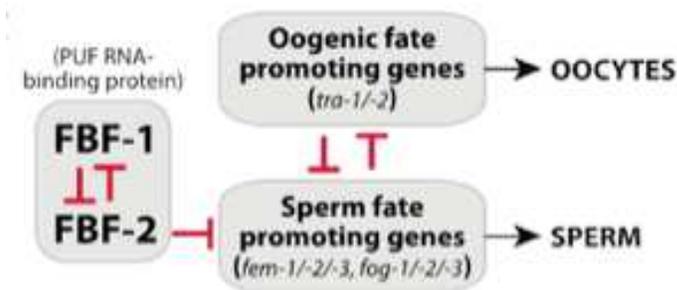


Figure 7. Diagram representing the mechanism of action of FBF. FBF-1 and FBF-2 (PUF RNA binding proteins) act redundantly to promote sperm-oocyte switch.

CHAPTER 2

REVIEW

Systemic mRNA selection mechanisms for germline stem cell homeostasis and its cell fate specification in *C. elegans*

ABSTRACT

Germline stem cells (GSCs) are the best understood adult stem cell types in *C. elegans*, and have provided an important system for studying of GSC and its cell fate *in vivo*. We here propose a novel mechanism that may control GSC homeostasis and germ cell fate specification through a systemic mRNA selection. This idea from a simple animal may provide insights into vertebrate stem cell and its cell fate specification broadly.

INTRODUCTION

C. elegans germline

Germline stem cells (GSCs) are characterized by their ability to both self-renew and generate gametes, sperm or eggs. In the adult gonads of many organisms, GSCs are maintained to replenish stocks of germ cells whose numbers are otherwise depleted by gamete production. GSCs are also responsible for transmitting genetic information across the generations. A self-renewal and differentiation of GSCs is called “GSC homeostasis”. GSC homeostasis is tightly regulated by a systemic regulatory network, which includes extrinsic cues and intrinsic regulation. Aberrant regulation of this network can either result in loss of specific germ cell type or excessive proliferation of GSCs, which are in turn associated with infertility or germline tumors respectively (Kobet et al., 2014) (Figure 8).

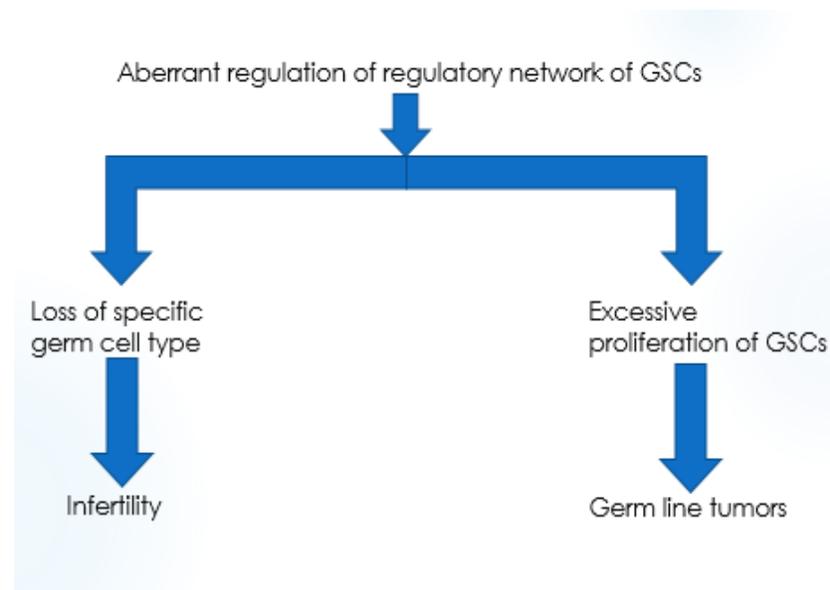


Figure 8 Schematic of effects of aberrancy in GSC regulatory network

The nematode *C. elegans* is a very attractive model organism that has greatly contributed to the understanding of germline development (Kimble and Crittenden, 2007). *C. elegans* either exists as a hermaphrodite or a male. Hermaphrodites produce a limited number of sperm during the early larval stage (L3-early L4) and switch to oogenesis in late larval stage (late L4).

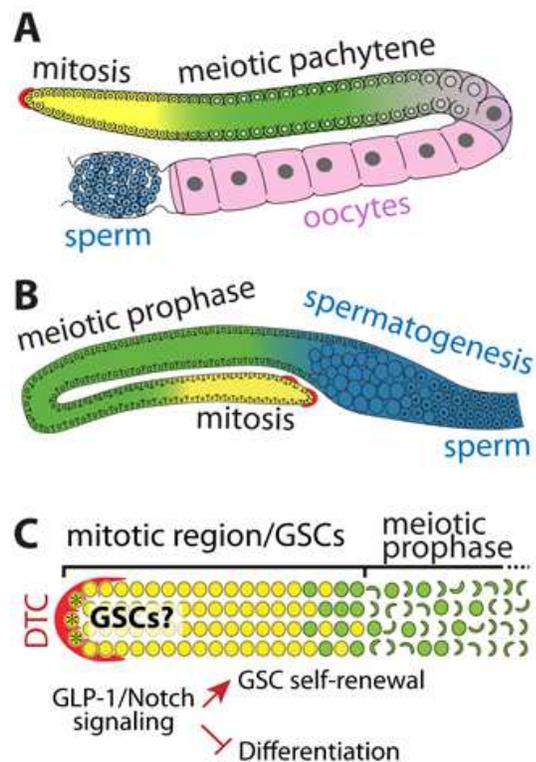


Figure 9 (A) Schematic of an adult *C. elegans* hermaphrodite gonad. Somatic DTC is located at the distal end. Cells at the distal end of the germline, including GSCs, divide mitotically (yellow). As cells move proximally, they enter meiosis (green) and differentiate into either sperm (blue) or oocytes (pink). (B) Schematic of an adult *C. elegans* male gonad. Two somatic DTCs reside at the distal ends of the adult male gonad. In male germline, all GSCs are differentiated into sperm (blue). (C) A simplified model for GSC and its differentiation. GSC is able to self-renew and differentiate into either sperm or egg. GLP-1/Notch signaling promotes GSC self-renewal and proliferation by inhibiting its differentiation. *, inferred actual GSCs.

They produce both sperm and oocytes, and they are therefore self-fertile (Figure 9A). However, males produce sperm continuously without switching into oogenesis (Figure 9B). *C. elegans* germline is organized in a simple linear pattern that progresses from GSCs at the distal region to maturing gametes at the proximal region (Figure 9A and B). In the *C. elegans* gonad, a single mesenchymal cell, called the Distal Tip Cell (DTC), functions as a GSC niche (or called microenvironment), and enhances mitotic cell cycle (Byrd and Kimble, 2009; Kimble and Crittenden, 2007). (Figure 9C). Although specific individual GSCs have not yet been precisely defined in the *C. elegans*, several genetic and cellular analyses propose that GSCs are the cells, which are located in the distal mitotic region that is in direct contact with DTC (Kimble and Crittenden, 2007). As a GSC leaves its DTC niche, it enters meiotic cell cycle and is eventually differentiated into either a sperm or an oocyte. Notably, several shared regulators control both GSC homeostasis and germ cell fate in the distal germ line. It suggests that self-renewal/differentiation decision and sperm/oocyte decision might be closely linked (Morgan et al., 2013).

In this report, we propose a mechanism to explain the systemic control of GCS and its cell fate specification through a systemic mRNA selection. Markedly, most regulators, identified from *C. elegans* are highly conserved in multicellular organisms, including humans and have been implicated in stem cell control and cell fate specification. Therefore, *C. elegans* may provide a great opportunity to understand mechanisms underlying stem cell regulation and cell fate specification in other higher model systems, including humans.

OBJECTIVES

1. Analysis of transcriptional activation by GLP-1/Notch signaling in GSCs

In *C. elegans*, the Distal Tip Cell (DTC) functions as a GSC niche, and it enhances the mitotic cell cycle in germ cells through transduction via GLP-1 (one of *C. elegans* two Notch receptors) signaling pathway (Byrd and Kimble, 2009; Kimble and Crittenden, 2007; Kobet et al., 2014) (Figure. 10A). The GLP-1/Notch signaling pathway maintains the germ cells in undifferentiated state through the transcriptional activation of target genes (Austin and Kimble, 1989; Crittenden et al., 1997; Yochem and Greenwald, 1989)(Figure. 10B). The Notch signaling pathway and its core components in *C. elegans* are highly conserved: Notch ligand “LAG-2” is expressed in the DTC and its receptor “GLP-1” is expressed in the membrane of mitotically dividing germ cells (Crittenden et al., 2003; Kobet et al., 2014) (Figure 10A). In the absence of signaling or progression of meiotic cell cycle, the transcription factor “LAG-1” is associated with repressor complex to inhibit the expression of GLP-1/Notch target genes. With an activated signaling mechanism, an ADAM-family metalloprotease and γ -secretase cleaves the GLP-1/Notch receptor and its intracellular domain (NICD) translocate from membrane to the nucleus. In the nucleus, the NICD interacts with LAG-1 and LAG-3 (a homolog of mastermind transcriptional co-activator) to activate the expression of Notch target genes (Figure 10A). Therefore, identifying the direct GLP-1/Notch target genes which drive GSC self-renewal is crucial for understanding the molecular mechanisms for stem cell regulation and hyper Notch signaling pathway-mediated tumorigenesis. Recently, bioinformatics has identified 163 putative GLP-1/Notch target genes with all harboring clusters of at least four LAG-1 binding sites (LBSs) (Kershner et al., 2014). Among them, four genes are validated to date as bona fide GLP-1/Notch targets in *C. elegans* germline. Those

include FBF-2 (a family of PUF RNA-binding proteins) (Lamont et al., 2004), LIP-1 (a homolog of the dual-specificity phosphatase) (Lee et al., 2006), LST-1 (lateral signaling target) (Kershner et al., 2014), and SYGL-1 (synthetic Glp) (Kershner et al., 2014). These genes function redundantly to maintain GSCs in the *C. elegans*: While *fbf-2*, *lip-1*, *lst-1* and *sygl-1* single mutants appear normal, the phenotype of *lst-1 sygl-1* double mutant is very similar to *glp-1* loss-of-function mutant, which does not maintain GSCs (Kershner et al., 2014). Notably, *fbf-2; lip-1* double mutant displays a defect in germ cell fate specification, rather than in GSC maintenance (Mamillapalli SS et al., unpublished results). These results suggest that GLP-1/Notch signaling and its targets may regulate both GSC maintenance and germ cell fate specification in the *C. elegans* germline.

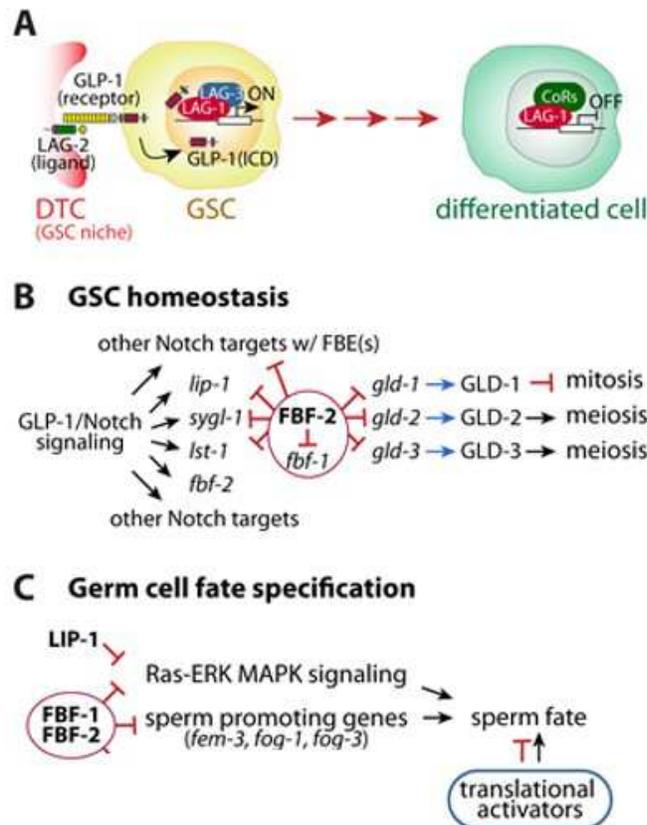


Figure 10. Depiction of systemic RNA selection mechanisms. (A) Transcriptional activation by GLP-1/Notch signaling. DTC functions a GSC niche. LAG-2, a Notch ligand is expressed in the membrane of DTC. Notch receptor, GLP-1, is expressed in the membrane of GSC and mitotically dividing germ cells. Upon Notch activation, GLP-1 intracellular domain (ICD) is trans-located from membrane to nucleus and forms a ternary complex with transcription activators, “LAG-1 and LAG-3” to activate the expression of target genes. Once GSC moves away from DTC, LAG-1 interacts with transcription corepressors (CoRs) to repress the expression of target genes. (B) GSC homeostasis. GLP-1/Notch signaling activates the expression of target genes, including FBF-2. FBF-2 acts as a regulatory hub for the proliferation and differentiation of GSCs. (C) Germ cell fate specification. FBF proteins inhibit the translation of the selected target mRNAs and translational activators (e.g., GLD-2, GLD-3, RNP-8, and IFE-1) promote the translation of the selected target mRNAs

2. Analysis of FBF-2, a regulatory hub for GSC homeostasis

FBF-2 is expressed in the GSC region (Lamont et al., 2004; Voronina et al., 2012). FBF-1 and FBF-2 (collectively known as FBF) are two nearly identical PUF RNA-binding proteins that regulate the switch from mitosis to meiosis in the *C. elegans* germline (Crittenden et al., 2002; Lamont et al., 2004). Being a translational repressor, FBF proteins specifically bind to element(s) in the 3'UTR (Untranslated Region) of target mRNAs (Kershner and Kimble, 2010; Kimble and Crittenden, 2007; Lamont et al., 2004; Lee et al., 2006; Lee et al., 2007a). FBF proteins inhibit mRNA stability and its translation by recruiting CCF-1 (Pop2P deadenylase homolog) or/and Argonaute protein (Friend et al., 2012; Goldstrohm et al., 2006). Kimble's group recently generated the list of several FBF targets, which includes about 1,350 mRNAs. The targets were established experimentally by using immunoprecipitation of FBF with associated mRNAs followed by microarray analysis (Kershner and Kimble, 2010). Interestingly, several FBF targets are also GLP-1/Notch targets (Kershner et al., 2014; Lee et al., 2006). It suggests that some GLP-1/Notch target genes are repressed by FBF in the GSC region. Why are mRNAs of GLP-1/Notch target genes also repressed by FBF-2?

One possible explanation is that FBF-2 maintains a balance between proliferation and differentiation (called GSC homeostasis) by the process of inhibition. Consistent with this idea, *fbf-2* single mutants are grossly normal, but their germlines have a larger mitotic region than normal (Lamont et al., 2004). In addition, FBF proteins repress the expression of target mRNAs, which normally promote differentiation of GSCs. The repressed target mRNAs may include GLD-1 (a KH-motif containing RNA-binding protein) (Crittenden et al., 2002), GLD-2 (a cytoplasmic poly (A) polymerase) (Millonigg et al., 2014), and GLD-3 (a bicaudal homolog) (Eckmann et al., 2002). These GLD proteins are critical for either promoting the differentiation of GSCs or inhibiting the proliferation of GSCs. Therefore, we propose that FBF proteins may act a regulatory hub to control GSC homeostasis.

3. Understanding the selective translational activation specifies germ cell fate

Once GSCs enter the meiotic cell cycle, dynamic changes occur in gene expression which program the germ cell fate. Normally, *C. elegans* hermaphrodites make sperm as larvae and oocytes as adult. This germ cell fate appears to be programmed in the early meiotic region (Morgan et al., 2013). In this region, sperm-promoting genes (e.g., *fem-3*, *fog-1*, and *fog-3*) are expressed in the fourth larval stage (L4) and are *dramatically decreased* when germ cell fate is switched to oogenesis in young adult stage (Arur et al., 2011; Lee et al., 2011b; Thompson et al., 2005). Notably, FBF protein promotes sperm-oocyte switch by inhibiting the expression of sperm-promoting genes (Kershner et al., 2013; Kershner and Kimble, 2010) (Figure 2). Recently, we reported that *C. elegans* Ras-ERK MAPK signaling promotes sperm fate specification (Morgan et al., 2010a). One of Ras-ERK MAPK targets is FOG-3 (a homolog of TOB/BTG anti-proliferative proteins) (Lee et al., 2011b). *C. elegans* FOG-3 directs germ cells to adopt sperm fate at the expense of

oogenesis (Ellis and Kimble, 1995; Lee et al., 2011b). We reported that non-phosphorylated FOG-3 initiates the sperm fate program and phosphorylated FOG-3 maintains that program for continued sperm production typical of males (Lee et al., 2011b). Notably, FBF proteins inhibit the expression of both *mpk-1* (an ERK homolog) (Lee et al., 2007a) and *fog-3* mRNAs (Snow et al., 2012; Thompson et al., 2005). These findings suggest that FBF proteins selectively repress the expression of both sperm-promoting genes and MPK-1/ERK MAPK signaling pathway to program sperm fate. In addition, we also found that FBF promotes sperm fate by inhibiting cell cycle regulators (See Chapter 3 results section). Markedly, it was earlier observed that male germline appears to have faster cell cycle progression than female germline (Morgan et al., 2010b). This finding supports an idea that cell cycle regulators may play a role in germ cell fate specification in *C. elegans*. In addition to selective mRNA repression, RNA regulators can promote the translation of the selected mRNAs that are associated with germ cell fate specification. For example, GLD-2/GLD-3 poly(A) polymerase complex activate the stability or/and translation of sperm fate-promoting gene mRNAs (Kim et al., 2009). In contrast, GLD-2/RNP-8 (RRM-motif RNA-binding protein) complex activates oogenic fate-promoting gene mRNAs (Kim et al., 2009). Therefore, mutants lacking GLD-2 are defective in gametogenesis: defective spermatocytes occur proximally, but mature sperm are normally found with absence of oocyte-like cells. (Kadyk and Kimble, 1998). Consistent with the GLD-2-mediated translational role in gametogenesis, the translational control of mRNAs, mediated by *C. elegans* IFE-1 is required for gametogenesis (Henderson et al., 2009). In *C. elegans*, IFE-1 is one of five translational initiation factors and is expressed in germ granule (called P granule in *C. elegans*), which share components with the P bodies and stress granules in mammals (Henderson et al., 2009). Interestingly, a mutant lacking IFE-1 arrests germ cells in secondary

spermatocytes and shows a modest defect in oocyte development, resulting in completely sterility (Henderson et al., 2009). Moreover, MALDI-TOF mass spectrometry using an *ife-1* mutant revealed that IFE-1 is required for the efficient translation of germ cell fate-specific genes, such as GSP-3 (Glc7-like protein phosphatases) and MSPs (Major Sperm Protein) (Kawasaki et al., 2011). Therefore, these findings support that translational activation of the selective mRNAs may coordinate germ cell fate in the premeiotic region of *C. elegans* germline.

4. Interpret the aberrant translational regulation and abnormal germline development

C. elegans GSCs are established in the early larval stages and continuously maintain their population by controlling the balance between self-renewal and differentiation. Aberrant regulation of this balance is often associated with germline tumors and infertility (Kobet et al., 2014). Therefore, studying the regulatory pathways controlling the balance between these two states is critical to understand how the aberrant regulation of GSCs causes germline tumors and infertility. To date, although significant progression has made in transcriptional regulation of GSCs and cell fate, little is known about how translational control influences GSC and its cell fate in multicellular organisms.

In *C. elegans*, many translational regulators, including RNA-binding proteins have been identified genetically. For example, FBF (FBF-1 and FBF-2) and GLD (GLD-1, GLD-2, GLD-3, and GLD-4) proteins are critical for GSC self-renewal and differentiation (Kimble and Crittenden, 2007). Mutants lacking FBF proteins do not maintain GSCs and all GSCs are differentiated into sperms

(Crittenden et al., 2002). This finding suggests that FBF proteins are required for GSC maintenance and oogenic fate specification. Once GSCs enter meiotic cell cycle, GLD-1 protein represses mitotic cell cycle by inhibiting GLP-1/Notch signaling. It also represses oogenic fate by inhibiting the expression of mRNAs of sperm-promoting genes (e.g., *tra-2*) (Clifford et al., 2000). In parallel with the function of GLD-1, GLD-2 (a poly(A) polymerase) and GLD-3 (a bicaudal-C homolog) together promote the translation of target mRNAs (Kim et al., 2009). One of GLD-2/GLD-3 targets is *gld-1* (Suh et al., 2009). All GLD proteins promote the meiotic entry of GSCs at the translational level. Therefore, mutations in GLD genes develop germline tumors, undergoing uncontrolled germ cell proliferation. These translational regulators also control germ cell fate in the pre-meiotic germline. One of key translational regulators is GLD-2. GLD-2 and its partners (GLD-3 and RNP-8) in combination control the germ cell fate (sperm or oocyte) by the following mechanisms: GLD-2/GLD-3 complex drives the sperm fate and GLD-2/RNP-8 complex drives the oocyte fate (Kim et al., 2009). Notably, GLD-3 and RNP-8 antagonize each other in the sperm/oocyte decision (Kim et al., 2009). Moreover, GLD-3 binds FBF and thereby inhibits its repression of target mRNAs (Eckmann et al., 2002). How do GSC regulators also govern germ cell fate? Though not thoroughly investigated, we can suggest an estimated proposition that these regulators either inhibit or promote the translation of target mRNAs at the different region of germline. Furthermore, GSC regulators and their targets regulate each other. These reciprocal regulations form the spatial boundary of germ cell fate decision (mitosis/meiosis and sperm/oocyte). The disruption of this regulatory mechanism leads to GSC-loss, germline tumor, or abnormal germ cell fate, which in turn are associated with infertility.

Discussion

In this review, we describe a new mechanism for *C. elegans* GSC homeostasis and its cell fate specification through a systemic mRNA selection. In GSC region, GLP-1/Notch signaling activates the expression of target genes. FBF-2, one of *C. elegans* GLP-1/Notch targets, likely controls GSC homeostasis by inhibiting both the proliferation and differentiation of GSCs. Once GSCs enter premeiotic cell cycle, FBF-2 selectively represses its target mRNAs, associated with sperm fate specification. In addition, translational activators selectively activate mRNAs, associated with oogenic fate specification. These multistep mRNA selections lead germ cells to a designated cell fate and inhibit the abnormal development of germ cells. Interestingly, most regulators, involved in this mechanism are localized in *C. elegans* P-granules (analogous to germ granule in mammals) (Lamont et al., 2004; Updike and Strome, 2010; Voronina et al., 2012). *C. elegans* P-granules are highly enriched for RNA and RNA-binding proteins and are key centers for specialized translational control (Sengupta and Boag, 2012; Updike and Strome, 2010). Importantly, these *C. elegans* RNA regulators, involved in this mechanism are highly conserved in other multicellular organisms, including humans. In addition, the function of the PUF RNA-binding proteins is conserved throughout many species in evolution (Wickens et al., 2002). Mammalian PUF proteins (e.g., PUM1 and PUM2) can bind to the PBE (Pumilio binding element) in the 3'UTR of the target mRNAs. Importantly, several PUF target mRNAs among *C. elegans*, *Drosophila*, and humans have been broadly conserved (Kershner and Kimble, 2010). Furthermore, mammalian PUM2 is expressed in embryonic stem cells (Moore et al., 2003), hematopoietic stem cells

(Spasov and Jurecic, 2003), and germ cells (Moore et al., 2003). It is suggested that PUM2 remarkably influences these stem cells. Therefore, we propose that the systemic activation/repression of mRNA pool may be a conserved mechanism that broadly influences both stem cell homeostasis and cell fate specification. Future directions include the possible role of microRNAs (miRNAs) in the regulation of GSC and its cell fate. The miRNAs are highly conserved in all eukaryotes and are involved in numerous cellular processes. Markedly, miRNAs mechanically bind to the 3'UTR of target mRNAs, and usually repress their translation. Therefore, the identification and functions of conserved miRNAs in GSCs and their cell fate specification still remains as major challenges.

CHAPTER 3

A systemic control of cell fate in *C. elegans* germline

ABSTRACT

The adjunct effect of various regulators on germ cell fate specification appeals further insight into their mechanisms of action and their collegial interactions. The *C. elegans* germline provides a remarkably simple yet effective model system to investigate the uncharted realms in germ cell fate specification. Among the different mRNA regulators, our study focused on FBF-1 which is a PUF RNA-binding protein. By meticulous screening, we elucidated the interaction of LIP-1 (an MPK-1/ERK phosphatase) with *fbf-1* and its subsequent influence on germ cell fate specification. Next, we found an increase in Mog (masculinization of germline) sterility in a genetic background lacking both *fbf-1* and *lip-1* and that MPK-1 (ERK homolog) activation inhibits the sperm-oocyte switch, thus promoting sperm fate. The partially penetrant Mog phenotype in *fbf-1;lip-1* mutant raised our suspicions about involvement of other regulators of germ cell fate specification in this genetic background. Here, we found that Mog sterility is enhanced by depletion of CYB-3/Cyclin B, CDK-1/CDK1, and CDC-25.1/CDC25 (G2/M phase cell cycle regulators) in *fbf-1;lip-1* genetic setting. Notably, an FBE (FBF Binding Element) is found on *cdc-25.1*. This study advocates an idea that both MPK-1/ERK signaling activation and interactions with G2/M phase cell cycle regulators influence the actions of FBF-1 and LIP-1 in promoting sperm-oocyte switch during germ cell fate specification.

INTRODUCTION

The germline of the nematode *C. elegans* provides a tractable system for studying sperm-oocyte decision. Sperms develop earlier (L3 stage) in *C. elegans* hermaphrodite followed by production of oocytes in adult stage (Figure 5). Further investigation is essential to understand about various factors regulating three *fog* genes and the three *fem* genes (Barton and Kimble (1990); (Doniach and Hodgkin, 1984); (Ellis and Kimble, 1995);(Hodgkin, 1986) (Figure 6) that are required for spermatogenesis. Among the various regulators influencing these genes, this project concentrates on a specific regulator called FBF. FBF is the collective term for FBF-1 and FBF-2, two nearly identical PUF (Pumilio and FBF) RNA-binding proteins that are largely redundant for germline stem cell maintenance and sperm-oocyte decision (Crittenden et al., 2002; Zhang et al., 1997). Earlier studies have elucidated that most *fbf-1(ok91)* and *fbf-2(q704)* single mutants produce both normal sperm and oocytes, and they are therefore self-fertile (Crittenden et al., 2002; Lamont et al., 2004). However, most *fbf-1 fbf-2* double mutants produce only sperm without switching to oogenesis (Zhang et al., 1997) (Figure 11) and fail to maintain germ line stem cells after L4 stage. Therefore, a single mutant called *fbf-1* is chosen to further the investigation. In order to understand the diverse actions of *fbf-1* on germ cell fate specification, we meticulously screened for other regulators which interact with FBF to influence germ cell fate specification. The aberrant phenotype observed in specific genetic background (*fbf-1;lip-1*) during our study, ignited the idea that systemic regulators (like cell cycle regulators) may also influence sperm fate specification.

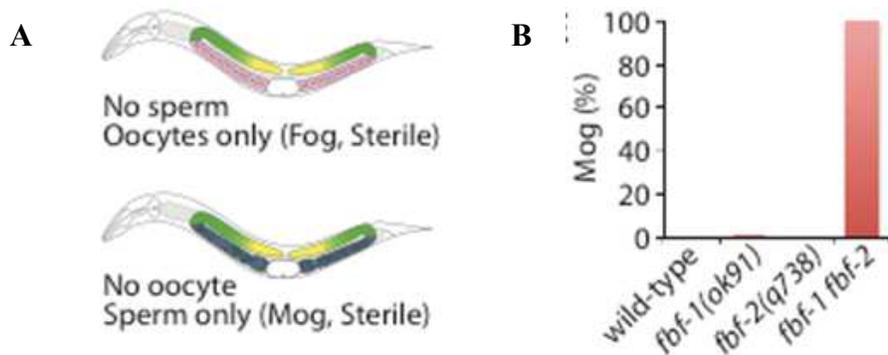


Figure 11. (A) Schematic of two types of sterile organisms. (B) Bar graph representing Mog phenotype *fbf-1* single mutant and *fbf-2* single mutant produce both sperm and oocyte. *fbf-1*: *fbf-2* double mutant promotes sperm fate at the expense of oocytes.

Therefore, understanding the mechanisms and interactions of various key sexual fate regulators is critical for modulating and investigating the *C. elegans* sexual fate decision and associated germ line disorders like infertility.

MATERIALS AND METHODS:

C. elegans culture

All strains were maintained at 20 °C as described (Brenner, 1974), unless noted otherwise. We used the wild-type Bristol strain N2 and the mutants: *C. elegans fbf-1 (ok91)*, *fbf-2 (q704)*, *lip-1(zh15)*, *mpk-1 (gal11)*. *C. elegans* worms were grown on NGM plates covered with *Escherichia coli* OP50 cells. To harvest embryos, the gravid worms at mixed stages were lysed in 10 volumes of a 1% NaOCl and 0.5 M NaOH solution for 5 min at room temperature. After multiple washes of embryos in M9 buffer, *C. elegans* embryos were harvested by centrifugation at 2000 rpm for 2 min.

Gonad staining

For antibody staining, dissected gonads were fixed in 3% paraformaldehyde with 100 mM K_2HPO_4 (pH 7.2) for 10–60 min at room temperature followed by 100% cold methanol for 5 min at $-20\text{ }^\circ\text{C}$ (Lee et al., 2007b). After blocking for 1 h with 0.5% BSA in $1 \times$ PBS (+ 0.1% Tween 20), fixed gonads were incubated for 2 h at room temperature with primary antibodies followed by 1 h at room temperature with secondary antibody. SP56 (sperm marker—a gift from S Ward) were used as primary antibodies. DAPI staining followed standard methods.

Embryo Isolation (to generate synchronized worm populations):

The plate with a high density of embryos is washed with PTW or M9 solution and the worms with embryos are collected into a micro tube, where they are exposed to the bleaching solution until the adult worm bodies are dissociated (this is further facilitated by frequent vortexing) while the embryos are still intact due to a tough protective coating. The sample is then spun down to remove the supernatant and subsequently washed thrice with M9 solution. In the last washing step, the embryos are synchronized overnight in M9 solution where the L1 stage worms are arrested. They are then plated.

- Bleaching solution protocol: 3.75 ml of 1M sodium chloride and 3 ml bleach (Clorox) are added to 8.25 ml autoclaved distilled water to make the bleaching solution.

RNA interference

RNAi experiments were performed by feeding bacteria expressing double strand RNAs corresponding to the gene of interest (Kamath et al., 2001). Briefly, about fifty L1 larvae were plated onto RNAi plates and allowed to grow at 20 °C for 2 days. Germline phenotypes of F1 progeny were determined by staining dissected gonads with specific markers and DAPI. For *mpk-1b* isoform-specific RNAi, the unique region (exon 1; 1–240 nt) of the *mpk-1b* gene was amplified by PCR from *C. elegans* genomic DNA and cloned into the pPD129.36 (L4440) vector containing two convergent T7 polymerase promoters in opposite orientations separated by a multi-cloning site. Other RNAi bacteria were from *C. elegans* RNAi feeding library (Source Bioscience LifeSciences) and *C. elegans* ORF-RNAi library (Open Biosystems).

RNA interference Mechanism –

The phenomenon of RNAi was first discovered by (Fire et al., 1998) and since then it has become much easier to study the phenotype of the worm by gene inactivation through RNAi approach by knocking out the transcript levels of the gene. It is a post-transcriptional mechanism where the dsRNA is cleaved by the enzyme Dicer into small siRNAs (small interfering RNAs). These siRNAs together with the RISC (RNA induced silencing complex) bind to the mRNAs in the cell by base-pairing. This bound mRNA is then degraded by an enzyme within RISC (Gao et al., 2006). The various methods to trigger RNAi is by injecting the adult worms with dsRNA (Fire et al., 1998), by soaking them in dsRNA solution (Tabara et al.,

1998) by feeding them with a strain of *E.coli* bacteria engineered to produce large amounts of the specific dsRNA (Timmons and Fire, 1998), or by the transgenesis mechanism (Tavernarakis et al., 2000). We used the RNAi of *mpk-1*, *fbf-1*, *lip-1*, *cyb-3*, *cdk-1* and *cdc-25.1* to study the role of MPK-1/ERK MAPK signaling in dedifferentiation and effect of cell cycle regulators in development and germ cell fate specification.

U0126 treatments

Small-molecule inhibitor (U0126) of MEK was performed using a slightly modified method of the protocol previously described (Morgan et al., 2010a). Briefly, *fbf-1*; *lip-1* double mutants were synchronized by the alkaline hypochlorite method and arrested in M9 media at the first larval or L1 stage. L1 larvae were then plated onto NGM plates containing mixture of 100 μ M U0126 and OP50 *E. coli*, and grown at 25 °C for 68 h, corresponding to day one of adult life. Fertility was observed using a dissecting microscope and germline phenotypes were determined by staining dissected gonads with DAPI.

Western blots

Blots were prepared by standard procedures. Protein samples were separated on 4%–20% gradient gels (Cambrex), and the blot was probed with 1:20,000 rabbit polyclonal anti-ERK-1/2 antibody (Sc94; Santa Cruz Biotechnology), followed by washing and incubation with 1:10,000 HRP-anti-Rabbit (Jackson ImmunoResearch). Blots were re-blocked and re-probed with 1:10,000 Mouse monoclonal anti- α -tubulin (Sigma-Aldrich) and 1:10,000 HRP-conjugated anti-mouse (Jackson ImmunoResearch).

Yeast three-hybrid assay and 3-AT assay

Three-hybrid assays were performed as described (Bernstein et al., 2002). Levels of 3-aminotriazole (3-AT) resistance were determined by assaying multiple transformants at 12 different concentrations of 3-AT, up to 11 mM. For b-galactosidase assays, cells were grown in selective media to an OD600 of 1.0 and mixed with an equal volume of b-Glo (Promega) reagent. Luminescence was measured after 1 h. Gel shift assays were performed as described (Hook et al., 2005)

Yeast three-hybridization assay mechanism

Yeast three hybridization assay elucidates the Protein-RNA-Protein interaction, if any present (Figure 12). Three-hybrid assays were performed as described (Bernstein et al., 2002). FBF proteins fused with the Gal4 activation domain were expressed from pACT2 plasmid. N-terminal truncations were expressed for FBF-1 (amino acids 121–614). DNA oligonucleotides were designed to express various RNA sequences and cloned into the XmaI and SphI sites of pIIIA/MS2-2. Assays were performed in the yeast strain YBZ-1 (Bernstein et al., 2002; Hook et al., 2005).

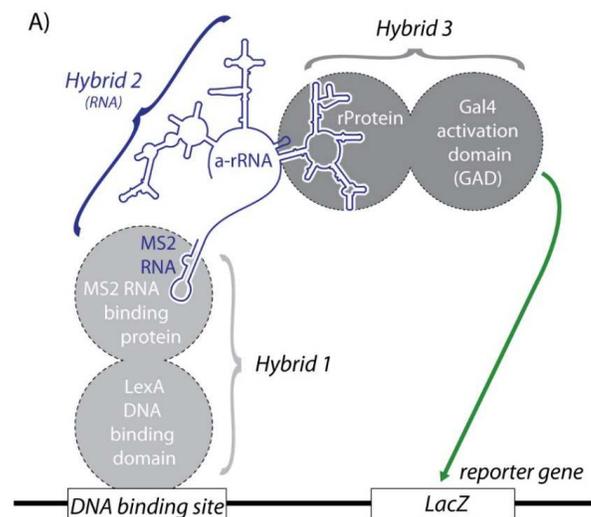


Figure 12 Schematic of mechanism of Yeast three hybridization assay – Interaction between protein-RNA-protein

3-AT assay mechanism:

3-Aminotriazole (3-AT) acts by competitively inhibiting the *HIS3* gene product, His3p. Cells containing more His3p can survive at higher concentrations of 3-AT (Figure 13). Thus, the level of resistance to 3-AT monitors the strength of an RNA–protein interaction. Selective medium plates that lack Histidine with increasing amounts of 3-AT (0, 2, 4, 6, 8 and 10 mM respectively) are prepared. Similar plates lacking leucine and Uracil

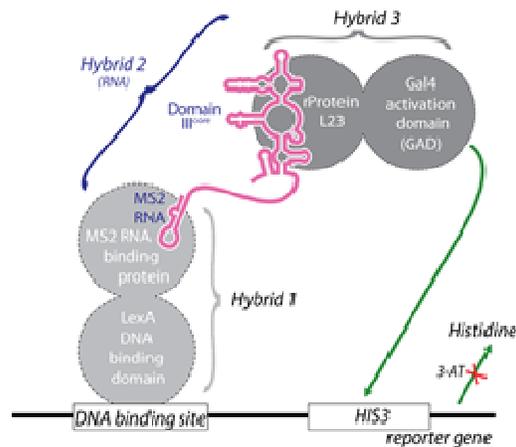


Figure 13 Schematic of mechanism of 3-AT assay – Competitive inhibition of Histidine by 3-AT

are also prepared. The specific RNA and protein-encoding plasmids are transformed into a three-hybrid strain and are plated on SD-Leu-Ura medium and are incubated for 2 days at 30 °C. Average-sized colonies are picked up and streak for single colonies on –Leu –Ura and –His plates. They are further incubated at 30 °C for 3–5 days. Growth is observed by the presence of individual colonies at the respective 3-AT concentration (Hook et al., 2005).

Supporting material for the methods

1. NGM Agar Plates for *C. elegans* culture: To make 1 liter of NGM Agar solution, about 3 grams of sodium chloride, 2.5 grams of peptone, 17 grams of agar, 1 ml of cholesterol (5 mg/ml in ethanol), 1 ml of 1M calcium chloride (29.4

grams of calcium chloride dihydrate in 200 ml autoclaved distilled water), 1 ml of 1M magnesium sulfate (49.2 grams of magnesium sulfate heptahydrate in 200 ml autoclaved distilled water) and 25 ml of 1M Potassium phosphate buffer (pH 6.0) are added to a large conical flask and the final volume is made up to 1 liter with distilled water and then autoclaved and plated. OP50 *E. coli* bacteria and HT115 are respectively inoculated in 2XYT buffer is spread on the NGM agar plates to feed *C. elegans*.

2. 2XYT media: About 10 grams of bacto tryptone, 5 grams of bacto yeast extract and 5 grams of sodium chloride are added to a large conical flask and the final volume is made up to 500 ml with distilled water and then autoclaved and stored at 4°C after cooling. It serves as a medium good for the growth of *E. coli* bacteria.

3. M9 Buffer: About 3 grams of potassium monobasic phosphate, 6 grams of sodium dibasic phosphate and 5 grams of sodium chloride are dissolved in 1 liter distilled water and autoclaved. After autoclaving, 1 ml of 1M magnesium sulfate is added. It is added after autoclaving to avoid precipitation.

4. Bacteria Stock: Bacteria are inoculated to 2XYT media and shaking incubated overnight at 37°C. The next day, 800 µl of this 2XYT buffer concentrated with bacteria is transferred to a stock tube (vial) to which 200 µl of 75% glycerol (should be 20% of the total volume) is added and the vial is then stored at -80°C.

5. *C. elegans* Stock: A non-contaminated, starved plate with plenty of L1 and L2 stage worms (in dauer stage) of the desired strain is washed with a little more than 500 µl of worm stock solution (S-medium) and is transferred to a stock tube (vial) to which 500 µl of worm freezing solution is added to make 1 ml of the worm

stock frozen at -80°C. A portion of the frozen stock can be test thawed a week later to ensure the viability of worms.

6. DAPI Staining: The dissected gonads are first washed 1X with PTW/PBST, then fixed in 3% PFA for 10 minutes to 1 hour (time of fixation is based on the germline phenotype – tumorous or non-tumorous) at room temperature, then washed 3X with PTW/PBST and then incubated in cold methanol at - 20°C for 10 min. (Time period of incubation in cold methanol can range over a period of 10 minutes to 1 month). After that, the sample is spun down and washed with PTW. Later, DAPI solution is added to the sample and incubated at room temperature for 10 minutes to 1 hour. Finally, the DAPI stained germlines (stains DNA) are mounted on 2% agarose pads and analyzed for the required phenotype under Nomarski microscope.

Solutions Used in the Staining Processes are

- DAPI working solution: 100 µl of 10X PBS and 20 µl of DAPI in water (stock solution) are added to 880 µl of autoclaved distilled water, vortexed and stored at 4°C.
- 1X PTW/PBST: 1 ml of Tween-20 (0.1%) is added to 100 ml of 10X PBS and the final volume is made up to 1 liter with distilled water.
- Levamisole/Tetramisole working solution (25 mM): About 60 mg of levamisole hydrochloride powder is dissolved in 10 ml of autoclaved distilled water and stored in 1 ml aliquots at -20°C.

- PTW+Levamisole solution: 1 ml of 25 mM levamisole solution is added to 100 ml of 1X PTW – used for paralyzing/immobilizing the worms to provide a better grip during dissection.
- 3% PFA: In a 50 ml conical tube, 5 ml of 1M potassium dibasic phosphate solution (pH 7.2),
- 9.375 ml of 16% PFA and 35.625 ml of autoclaved distilled water are mixed, vortexed and finally stored in 1 ml aliquots at 4°C.
- 2% Agar: 2 grams of fine quality agar is dissolved in 100 ml of autoclaved distilled water.
- Vector shield: Commercially available and is used as mounting medium with DAPI.

RESULTS

FBF-1 and LIP-1 partially repress oogenic fate specification

We found that *lip-1* mutation in either *fbf-1* or *fbf-2* alters sexual fate. Most *lip-1(zh15)* single mutants are self-fertile, albeit with small brood size (*lip-1*: ~130, n=8; wild-type: ~230, n=5). Notably, 34% of *fbf-1; lip-1* double mutants continued to produce excess sperm, without switching to oogenesis (Figure 14). 38% of *fbf-2; lip-1* (n=100) did not have sperm, but they had fertilized embryos in uterus, suggesting that FBF-2 and LIP-1 control the number of sperm production. Therefore, we concluded that FBF-1, FBF-2 and LIP-1 proteins control sexual

fates in *C. elegans*. In this proposal, we focus on the effect of FBF-1 and LIP-1 on germ cell fate specification in the *C. elegans* germline.

Genotype	Gametogenesis (%)		n
	Sp/Oo	ExSp	
wild-type	100	0	many
<i>fbf-1(ok91)</i>	99	1	many
<i>lip-1(zh15)</i>	100	0	180
<i>fbf-1; lip-1</i>	66	34	101

Table 1. Table showing that *fbf-1; lip-1* double mutant promotes sperm fate while *fbf-1* and *lip-1* single mutants show both sperm and oocyte.

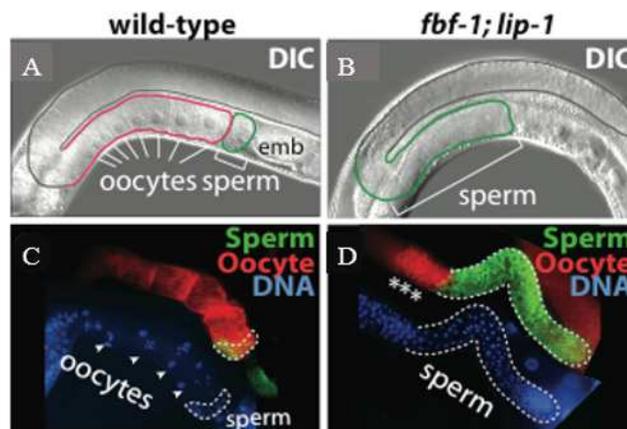


Figure 14. (A and B) DIC pictures showing that *fbf-1; lip-1* double mutant promotes sperm fate while *fbf-1* and *lip-1* single mutants show both sperm and oocyte. (C and D) Depiction of staining by sperm markers, oocyte makers and DAPI. Staining dissected gonads with sperm marker (SP56, green), oocyte marker (RME-2, Red), and DAPI (Blue).

***fbf-1; lip-1* Mog phenotype depends on MPK-1/ERK activity**

LIP-1 protein inhibits MPK-1 activation in somatic and germ cells (Berset et al., 2001; Hajnal and Berset, 2002; Lee et al., 2006). FBF-1 inhibits the translation

of *mpk-1* mRNA by interaction with its 3'UTR (Lee et al., 2007b). Therefore, we questioned whether *fbf-1; lip-1* defects are dependent upon activation of MPK-1 or its expression. The *mpk-1* gene encodes two major transcripts, *mpk-1a* and *mpk-1b*, which produce MPK-1A and MPK-1B proteins, respectively (Lee et al., 2007b)(Figure 15A). MPK-1A is abundantly expressed in the somatic cells and MPK-1B protein is predominantly expressed in the germline (Lee et al., 2007b). We first depleted the two MPK-1 isoforms or the MPK-1b isoform by RNAi in wild-type hermaphrodites and certified their specific-depletion by Western blot analysis (Figure 15B) (Cha et al., 2012). Interestingly, both *mpk-1ab* and *mpk-1b* RNAi were able to suppress *fbf-1; lip-1* Mog phenotype (Figure 15C). These results suggest that aberrant MPK-1/ERK activation promotes sperm fate specification in *fbf-1; lip-1* mutants.

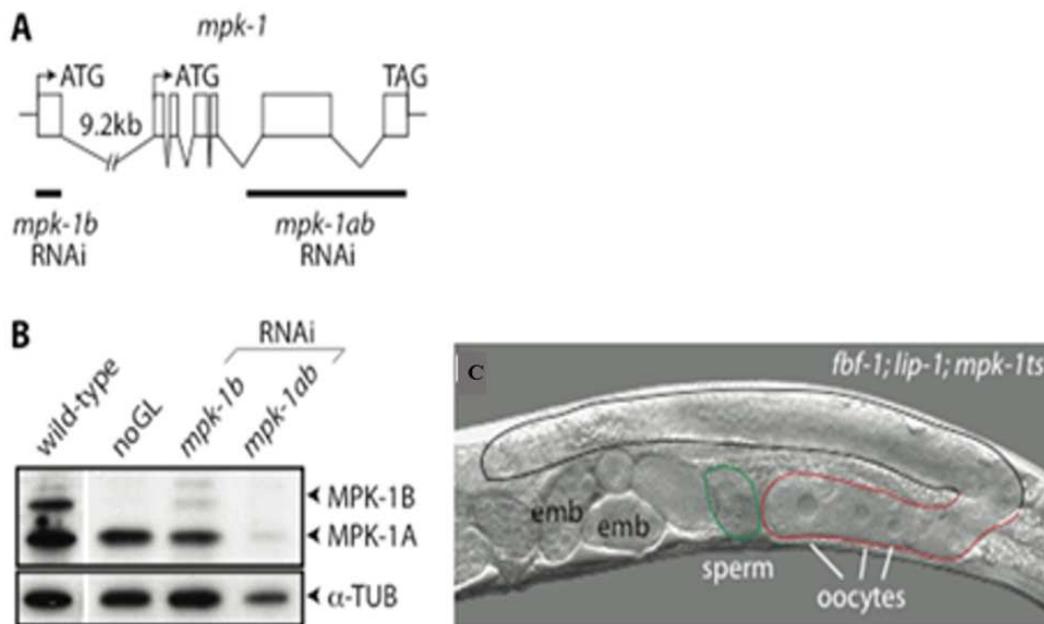


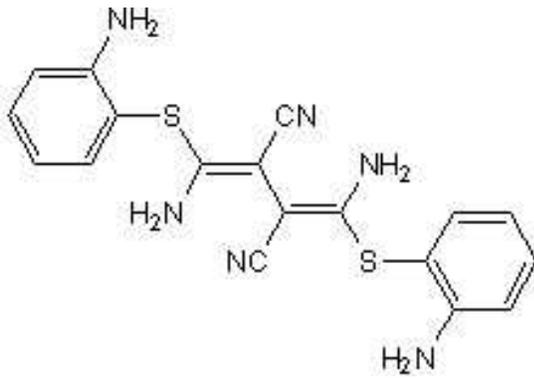
Figure 15. (A) Depiction of 2 variants of *mpk-1* (*mpk-1a* and *mpk-1b*). (B) Western blot analysis of specific deletion in *mpk-1* isoforms. (C) DIC picture of *fbf-1; lip-1; mpk-1* triple mutant.

Strain (*RNAi)	Gametogenesis (%)			n
	Sp/Oo	ExSp	Others	
<i>fbf-1; lip-1</i>	43	57	0	>100
<i>mpk-1ab*</i>	48	0	52	118
<i>mpk-1b*</i>	100	0	0	28
<i>mpk-1(ga111ts)</i>	100	0	0	>100
<i>fbf-1; lip-1; mpk-1ab*</i>	97	3	0	78
<i>fbf-1; lip-1; mpk-1b*</i>	68	32	0	59
<i>fbf-1; lip-1; mpk-1ts</i>	99	1	0	>100

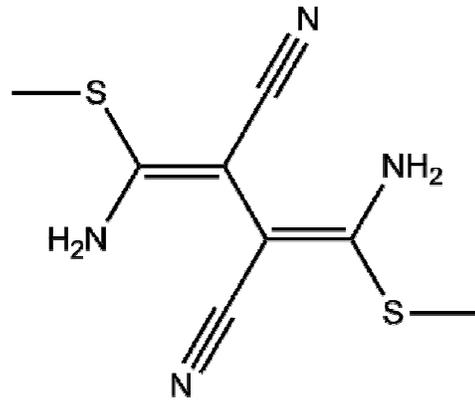
Table 2. *fbf-1; lip-1* double mutant shows increased Mog phenotype. RNAi of *mpk-1* in *fbf-1; lip-1* double mutant promotes oocytes and hermaphrodite phenotype, thus elucidating that *mpk-1* is essential for sperm fate specification.

Activated MPK-1/ERK continues to promote sperm fate

To investigate whether MPK-1/ERK activation (phosphorylation) is required for sperm fate specification, we treated *fbf-1; lip-1* double mutants with a small-molecule MEK inhibitor, U0126 (Chemical structure is depicted in Figure 16). Interestingly, U0126 sufficiently rescued *fbf-1; lip-1* Mog sterility (Figure 17). These results indicate that FBF-1 and LIP-1 inhibit sperm fate by inhibiting MPK-1/ERK activity. To rule out the off-target effects of U0126 as the mechanism of germ cell fate reprogramming, we tested an inactive but structurally similar analog, U0124 (Morgan et al., 2010a) (Figure 16). U0124 failed to rescue *fbf-1; lip-1* Mog sterility (Figure 17). These findings strongly suggest that activated MPK-1/ERK promotes sperm fate without switching into oocyte fate and Ras-ERK inhibitor acts as a chemical switch to reprogram germ cell fates in mutants.



Chemical structure of U0126



Chemical structure of U0124

Figure 16 Depiction of chemical structure of U0126 and U0124

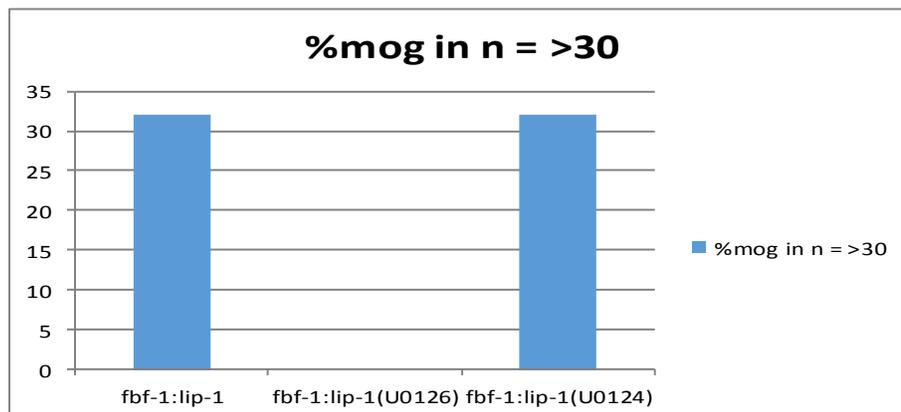


Figure 17. Bar chart depicting that Mog phenotype is rescued in *fbf-1; lip-1* when exposed to U0126 (MEK inhibitor) whereas Mog phenotype is not rescued in presence of the inactive MEK inhibitor (U0124)

G2/M cell cycle progression is required for oocyte fate specification

Cell cycle control is a central step in generating specific cells and tissue during development and maintains cellular homeostasis in adults. Moreover, the disruption of the cell cycle process can cause developmental abnormalities or cancer. To date, significant progress has been made, but little is known about the mechanism of how cell cycle regulators control specific developmental events. Previously, it was observed that PUF proteins inhibit cell cycle regulators in multi-cellular organisms, including worms, flies, and humans (Qiu et al., 2012). ERK MAPK signaling was also observed to regulate cell cycle progression, specifically the G1/S phase. (Chambard et al., 2007). Moreover, the cell cycle duration is observed to be different in the two sexes of *C. elegans*. It is faster in male worms than in hermaphrodites (Morgan et al 2010b). Therefore, we here aimed to identify cell cycle regulators that have an impact on germ cell fate specification. To this end, we initially used *fbf-1; lip-1* mutant because its Mog sterility phenotype shows partial penetrance (Figure 15C). Among the several cell cycle regulators shown in Figure 18, we first depleted cycline genes by feeding RNAi in *fbf-1 lip-1* mutants and then determined their germline phenotypes by staining dissected gonads with DAPI (DNA). Notably, the depletion of CYB-3 dramatically enhanced *fbf-1; lip-1* Mog sterility (Figure 20A). CYB-3 interacts with CDK-1 (Cyclin-Dependent

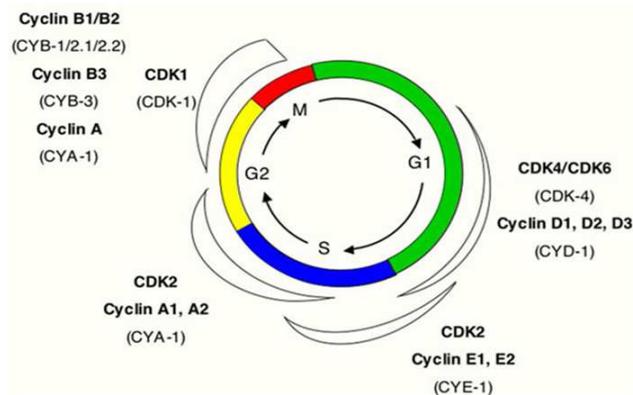


Figure 18 Cell cycle regulators and overview of cell cycle regulation

regulators control specific developmental events. Previously, it was observed that PUF proteins inhibit cell cycle regulators in multi-cellular organisms, including worms, flies, and humans (Qiu et al., 2012). ERK MAPK signaling was also observed to regulate cell cycle progression, specifically the G1/S phase. (Chambard et al., 2007). Moreover, the cell cycle duration is observed to be different in the two sexes of *C. elegans*. It is faster in male worms than in hermaphrodites (Morgan et al 2010b). Therefore, we here aimed to identify cell cycle regulators that have an impact on germ cell fate specification. To this end, we initially used *fbf-1; lip-1* mutant because its Mog sterility phenotype shows partial penetrance (Figure 15C). Among the several cell cycle regulators shown in Figure 18, we first depleted cycline genes by feeding RNAi in *fbf-1 lip-1* mutants and then determined their germline phenotypes by staining dissected gonads with DAPI (DNA). Notably, the depletion of CYB-3 dramatically enhanced *fbf-1; lip-1* Mog sterility (Figure 20A). CYB-3 interacts with CDK-1 (Cyclin-Dependent

Kinase 1) to drive G2/M cell cycle progression (Boxem et al 2006). CDK-1 has to be dephosphorylated to regulate G2/M cell cycle progression. To test whether CYB-3-mediated G2/M cell cycle progression is required for germ cell fate specification, we also performed *cdk-1* RNAi in *fbf-1; lip-1* mutants at 20°C. Consistent with the novel role of CYB-3 in germ cell fate specification, *cdk-1(RNAi)* also enhanced *fbf-1; lip-1* Mog sterility (Figure 20B). Also, another regulator called CDC-25.1 (CDC25 phosphatase) is necessary for activation of CYB-3/CDK-1 complex to activate G2/M cell cycle progression (Figure 19), but WEE-1.3 (Wee1 Kinase) inhibits G2/M cell cycle progression (Lamitina and L'Hernault, 2002). Next, we tested whether CDC-25.1

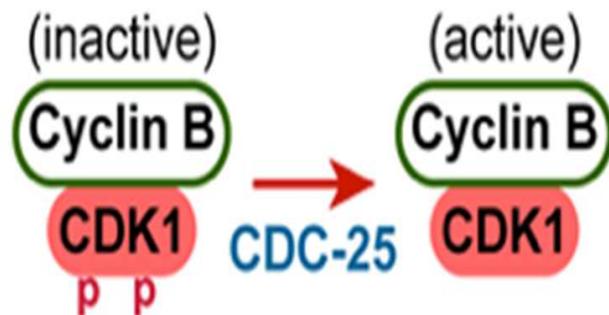


Figure 19 Diagrammatic representation of activation of Cyclin/CDK complex by CDC-25

and WEE-1.3 also control *fbf-1; lip-1* Mog sterility by RNAi. Interestingly, *cdc-25.1(RNAi)* also enhanced *fbf-1; lip-1* Mog sterility (Figure 20C) as seen in *cyb-3(RNAi)* (Figure 20A) and *cdk-1(RNAi)* (Figure 20B), but *wee-1.3(RNAi)* failed to enhance *fbf-1; lip-1* Mog sterility (Figure 20C). Finally, to ask whether CYB-3, CDK-1, and CDC-25.1 normally inhibit sperm fate, we depleted these genes by RNAi in wild-type and *eri-1 (mg366)* mutant, which are hypersensitive to RNAi (Kennedy et al., 2004). RNA of these genes did not affect germ cell fate (not shown). These results suggest that the inhibitory actions of CYB-3/CDK-1 and CDC-25.1 on sperm fate specification, strictly depends on genetic context.

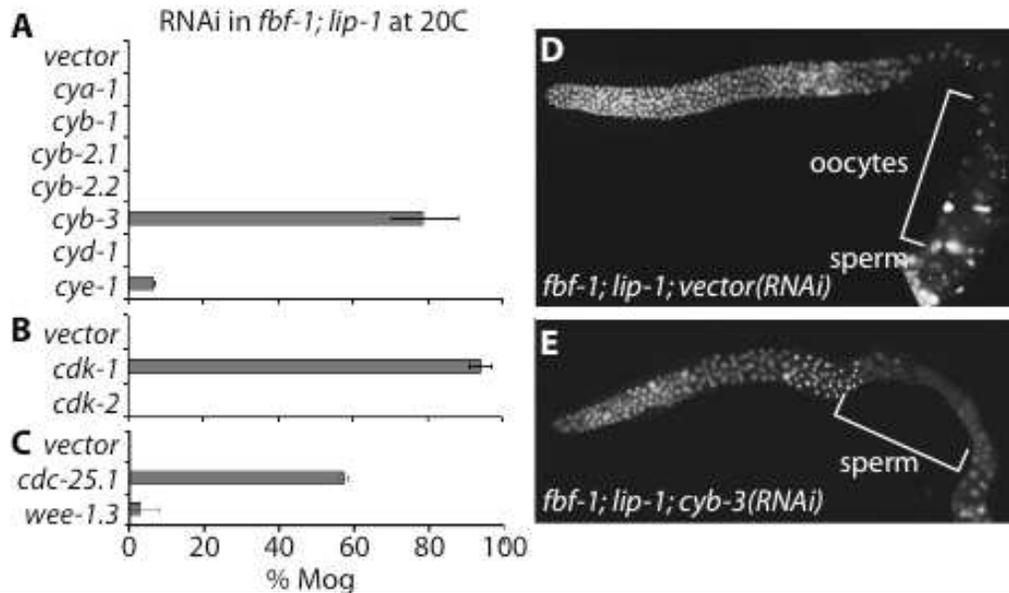


Figure 20 (A-C) Bar graph showing that the RNAi of cell cycle regulators (specifically *cyb-3*, *cdk-1* and *cdc-25.1*) promote Mog phenotype (sperm) in *fbf-1; lip-1* double mutant background. (D) Pictorial depiction of phenotype of hermaphroditism (sperm and oocyte) in *fbf-1; lip-1* double mutant. (E) Mog phenotype of *cyb-3(RNAi)* in *fbf-1; lip-1* double mutant, elucidating that cell cycle regulators like *cyb-3*, *cdc-1* and *cdc-25.1* influence cell fate specification.

FBF-1 may inhibit G2/M cell cycle progression

How do CYB-3/CDK-1 and CDC-25.1 inhibit sperm fate specification in *fbf-1; lip-1* mutant background? One possible idea is that FBF may regulate G2/M cell cycle regulators. FBF proteins inhibit translation by binding to FBF binding element (FBE: UGU(G/A)nnAU) within the 3'UTR of specific mRNAs (Bernstein et al., 2005). Therefore, we questioned whether any of the cell cycle regulator has FBF binding site. In collaboration with Dr Kimble's lab, we found that the *cdc-25.1* 3'UTR possesses one potential FBE that conforms to the sequence UGU (G/A) nnAU within the 3'UTR region (Figure 21A). To assess the binding of FBF to this potential *cdc-25.1* FBE, we used yeast three-hybrid assay. Yeast three-hybrid interaction was monitored by production of β -galactosidase from a *lacZ* reporter (Figure 21B). Our yeast three-hybrid assay showed that the *cdc-25.1* FBE

interacted with FBF-1 (Figure 21B). Furthermore, this interaction was specific: wild-type *cdc-25.1* FBE bound FBF-1, but not PUF-8, which is another *C. elegans* PUF proteins with a different binding specificity (Not shown). Moreover, this interaction was disrupted by mutation of the UGU in the consensus binding site (FBE mutation: UGU was changed to aca). Next, we measured the strength of interaction between FBF-1 protein and *cdc-25.1* FBE using a 3-AT (3-aminotriazole) assay (Figure 21C). Growth was monitored on media lacking Histidine and containing varying concentration of HIS-3 competitor 3-AT. Notably, yeast with FBF-1 and *cdc-25.1* FBE (wt) was able to grow on media containing 8 mM 3-AT. This result suggests that FBF-1 interacts strongly with *cdc-25.1* FBE in yeast system.

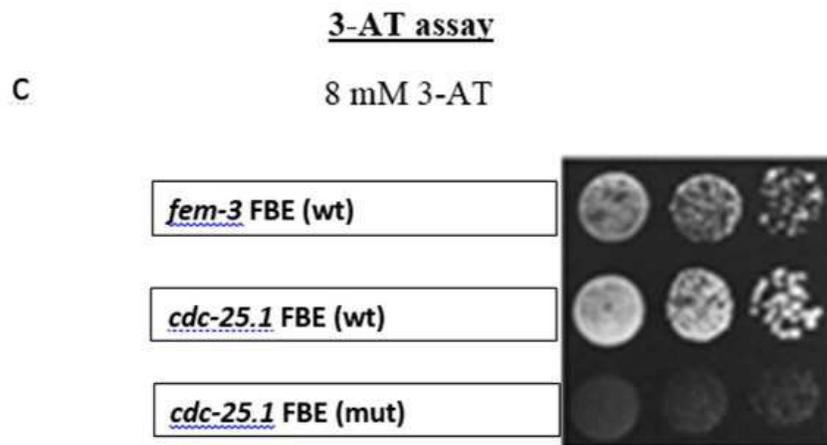
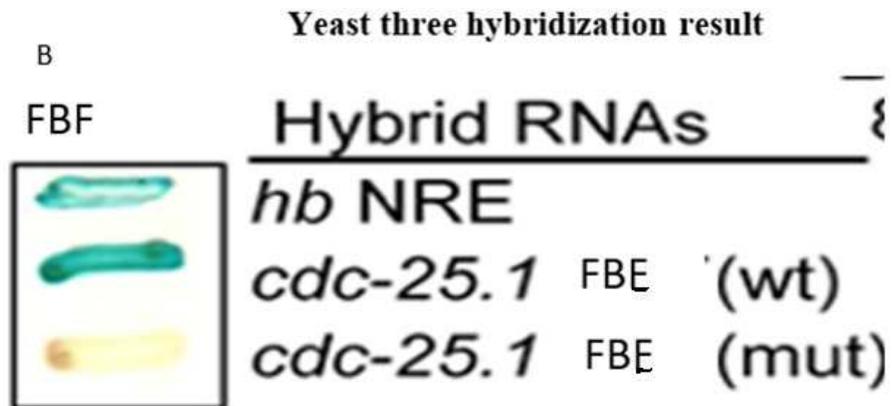


Figure 21. Depiction of results: FBF binds to FBE with *cdc-25.1* mRNA 3'UTR. (A) *cdc-25.1* 3'UTR possesses a putative FBE site. (B) Yeast three-hybrid assay. (C) 3-AT assay.

Proposed model for germ cell fate specification

We found that FBF-1, LIP-1 and CDC-25.1 promote oocyte fate by inhibiting sperm fate. How to explain the two-way regulation of FBF-1 on its repressing target, CDC-25.1 and also on sperm fate (Figure 22)?

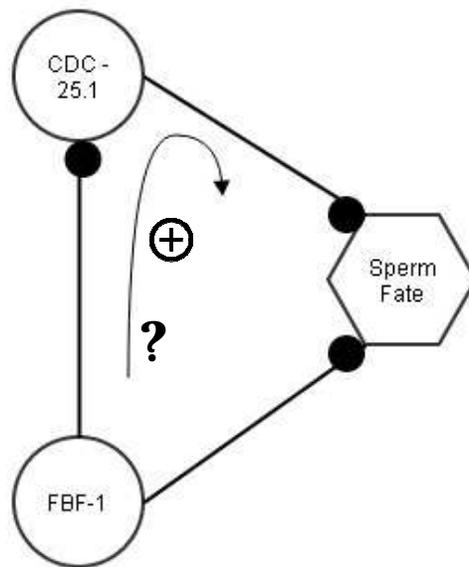


Figure 22 Pictorial representation of possible two-way regulation in germ cell fate specification. FBF-1 inhibits both CDC 25.1 and sperm fate. Question about possible dual regulation.

It can be possibly explained by a recently proposed novel regulatory mechanism, called dual-negative regulation (Datla et al., 2014). The concise explanation of this mechanism is - Regulator A and Regulator B individually inhibit the effector C. Regulator A also represses regulator B (Datla et al., 2014) (Figure 23A). But this repression in turn activates Effector C. Therefore, any single mutation does not affect effector C, but A and B double mutations activate the effector C. Consistent

with this idea, most *fbf-1*, *lip-1*, and *cdc-25.1* single mutation produce both sperm and oocytes (Figure 23B). However, *fbf-1; lip-1* double or *fbf-1; lip-1; cdc-25.1(RNAi)* animals displayed Mog sterile phenotype. Therefore, our findings and proposed model will provide a novel regulatory mechanism for stem cell and cell fate specification in other multicellular organisms, including humans.

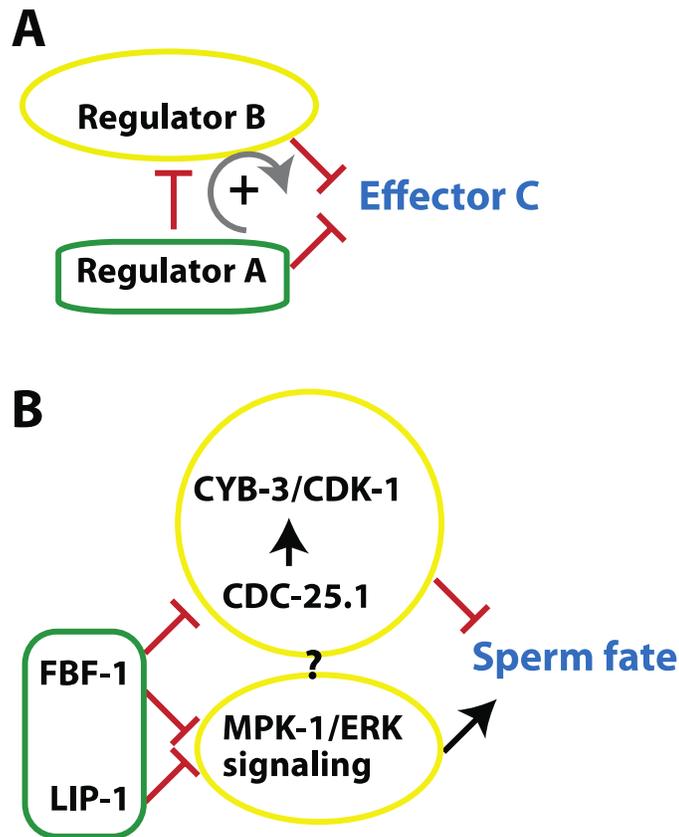


Figure 23. Diagrammatic representation of (A) Concept of Dual negative regulation. (B) Application of the dual negative regulation concept in *fbf-1; lip-1*

CONCLUSION AND FUTURE DIRECTIONS

Earlier studies showed that FBF-1 promotes oocyte fate by inhibiting the sperm fate regulators, such as FOG-1 and FOG-3 (Kimble and Crittenden, 2007). It was also established that LIP-1 (a dual specificity phosphatase) functions by dephosphorylating MPK-1 thus inhibiting MPK-1/ERK signaling pathways (Berset et al., 2001); (Hajnal and Berset, 2002); (Lee et al., 2006). By using *fbf-1; lip-1* mutants, we observed in our study that FBF-1 and LIP-1 redundantly inhibit sperm fate specification. But in our studies, we also observed that the *fbf-1; lip-1* double mutant showed only 34% Mog (masculinization of gamete) phenotype. Therefore, we next questioned the presence of other regulators influencing the action of FBF-1 in the germ cell fate specification. Also, in support of the investigation, previous studies elucidated that the ERK MAPK signaling regulates cell cycle progression, specifically G1/S. Earlier studies also showed few differences in the duration of cell cycle in different sexes. It was observed that the cell cycle duration is faster in male worms rather than in hermaphrodites (Morgan et al., 2010b).

Trying to connect the dots from the knowledge of previous studies and our research observations, we next questioned the role of cell cycle regulators in germ cell fate specification. We also questioned if there are any interactions between FBF-1 and cell cycle regulators. To this end, first, we used the method of RNAi interference and found that CYB-3 is a potential regulator for sperm-oocyte switch. Next, by means of Bioinformatics, yeast three hybridization and 3-AT assay we found that *cdc-25.1* mRNA has a putative FBE at 3'UTR region and that FBF-1 binds specifically and strongly to the 3'UTR region of *cdc-25.1* mRNA. This study demonstrates one of the control mechanisms of germ cell fate specification where

FBF-1 possibly interacts and inhibits CDC-25.1, which in turn promotes oocyte fate through CYB-3/CDK-2.

The function of the PUF RNA-binding proteins like FBF-1 is conserved throughout many species in evolution (Wickens et al., 2002). Mammalian PUF proteins (e.g., PUM1 and PUM2) can bind to the PBE (Pumilio binding element) in the 3'UTR of the target mRNAs. Importantly, several PUF target mRNAs among *C. elegans*, *Drosophila*, and humans have been broadly conserved (Kershner and Kimble, 2010).

Mechanism of germ cell fate specification in *C. elegans*:

Possible homologous mechanism in Human hematopoietic cell system:

Figure 24 (A)

Figure 24 (B)

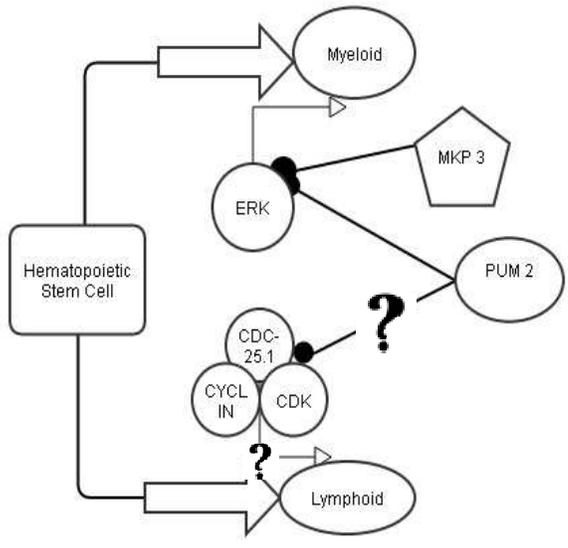
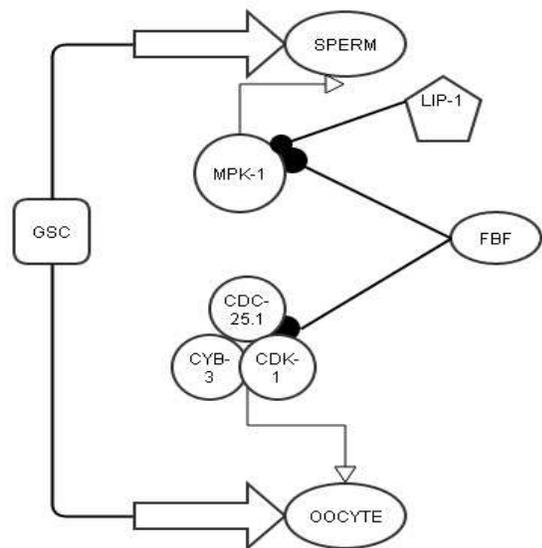


Figure 24 (A) Schematic of mechanism of germ cell fate specification in *C. elegans*. FBF inhibits both Cell cycle regulators (CYB-1, CDK-1 and CDC 25.1) and sperm fate via MPK-1 by the mechanism of dual negative regulation. (B) Depiction of possible application of similar mechanism in Human homologous systems like Human hematopoietic cell lineage differentiation. Further investigation is necessary in studying the interaction between PUM2 (FBF analog) and cell cycle regulators in Human system and effect of this interaction of human hematopoietic cell lineage specification.

Furthermore, mammalian PUM2 is expressed in embryonic stem cells (Moore et al., 2003), hematopoietic stem cells (Spasov and Jurecic, 2003), and germ cells (Moore et al., 2003). PUM2 is established to be a human homolog of FBF protein. Therefore, it warrants further study into possible presence of cell cycle control mechanisms on hematopoietic cell lineage specification (Figure 24 A and B).

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