ABSTRACT Matthew A. Gaddy. PUF-8 and MPK-1: Genetic and Chemical Control of Spermatocyte Dedifferentiation in Caenorhabditis elegans (Under the direction of Dr. Myon-Hee Lee). Department of Internal Medicine, May 2021.

Stem cells face a number of major fate decision during their development: the decision to self-renew or differentiate, and then whether to remain differentiated or dedifferentiate, as occurs in some oncogenesis. A regulatory network controlling these decisions is vital to the development of all multicellular organisms, including humans. Aberrant regulation can result in either loss of specific cell type or uncontrolled cell proliferation, leading to tumors. However, our understanding of how differentiated cell can be reverted to an undifferentiated state remains far more limited.

Using the nematode *C. elegans* germline as a model system, we previously reported that PUF-8 (a PUF RNA-binding protein) and LIP-1 (a dual-specificity phosphatase) inhibit the formation of germline tumors via repressing the dedifferentiation of spermatocytes into mitotic cells (termed "spermatocyte dedifferentiation") at least in part by inhibiting MPK-1 (an ERK MAPK homolog) activation. To gain insight into the molecular competence for spermatocyte dedifferentiation, we compared the germline phenotypes between two competent mutants – *puf-8(q725); lip-1(zh15)* with a high MPK-1 activity and *puf-8(q725); fem-3(q20gf)* with a low MPK-1 activity. *puf-8(q725); lip-1(zh15)* mutants developed germline tumors more aggressively than *puf-8(q725); fem-3(q20gf)* mutants at 25°C with aging. This result suggests that MPK-1 activation is critical to induce the formation of germline tumors via spermatocyte dedifferentiation. This idea was confirmed by treatment of *puf-8(q725); fem-3(q20gf)* mutant worms with Resveratrol, which stimulates MPK-1 activation. Our results show that 100 mM RSV significantly induced the formation of germline tumors via spermatocyte dedifferentiation at 25°C with aging. Therefore, we conclude that MPK-1 activation is required to promote the

formation of germline tumors via spermatocyte dedifferentiation in the absence of PUF-8. Since PUF-8 and MPK-1 are broadly conserved, we therefore suggest that similar molecular mechanisms may control dedifferentiation-mediated tumorigenesis in other organisms, including humans.

PUF-8 and MPK-1: Genetic and Chemical Control of Spermatocyte Dedifferentiation in *Caenorhabditis elegans*

A Thesis

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Master of Science

By:

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INTRODUCTION

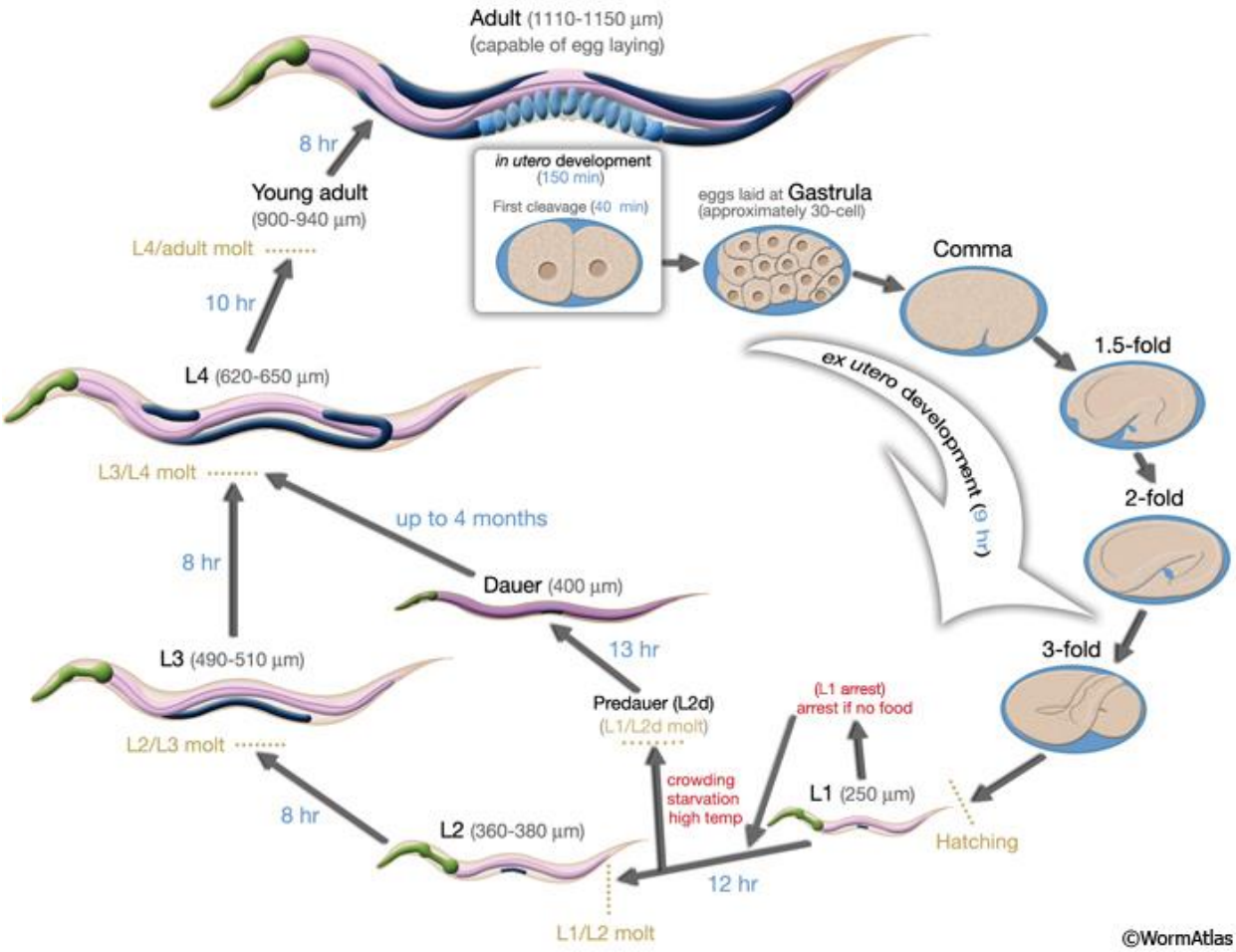
Background

1. Caenorhabditis elegans (C. elegans) as a model organism

The nematode *C. elegans* is a multicellular organism that has become a popular model for biological and basic medical research. It has also been widely used as a model system to explore fundamental questions in multiple aspects of biology, including evolution, development, cell fate specification, stem cell regulation, tumorigenesis, and aging.

C. elegans is a free-living, non-parasitic, soil nematode which measures approximately 1 mm long. In laboratory, they are grown in large numbers on NGM (nematode growth medium) agar plates feeding on OP50 *Escherichia coli* bacteria. The average lifespan of the worm is approximately two-three weeks. *C. elegans* molts through 4 larval stages (L1, L2, L3, and L4) before it becomes the adult (~3 days after L1 at 20°C). It has two sexes: male and hermaphrodite. In both males and hermaphrodites, spermatogenesis begins in the L4 larval stage, which continues throughout lifetime in a male. Whereas in a hermaphrodite, the spermatogenesis ceases, and switches to oogenesis in the late L4 stage and they are hence self-fertile. An adult hermaphrodite worm lays about 300 eggs by self-fertilization and about 1,000 eggs by cross-fertilization in a lifetime. A brief review of the lifecycle of the worm is shown in Fig. 1.

The worm is well suited for phenotypic studies also due to the transparency of the worm. The body of the worm has 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 anatomy of the worm – both male and hermaphrodite is shown in Fig. 2. The generation time of the worm varies with temperature due to its slow growth rate at low temperature and



faster growth rate at higher temperature. The generation time is approximately 2.5 days at 25°C and 3 days at 20°C.

Figure 1. Life cycle of *C. elegans* at 22°C. Cited from WormAtlas (https://www.wormatlas.org/hermaphrodite/introduction/mainframe.htm)

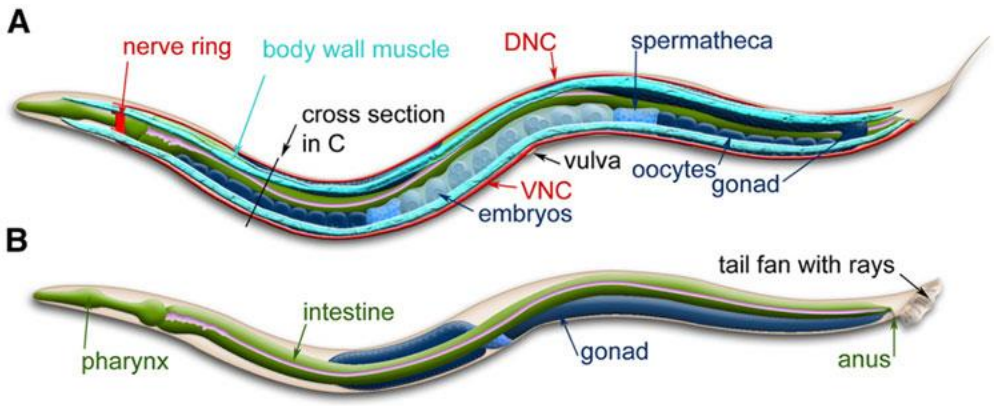


Figure 2. *C. elegans* as a model system. Major anatomical features of a hermaphrodite (A) and male (B). Cited from https://andor.oxinst.com/learning/view/article/advantages-of-using-caenorhabditis-elegans-as-a-model-organism.

2. C. elegans germline and its development.

Development of the *C. elegans* germline progresses by many of the same steps that are typical of other animal germlines (Kimble and Crittenden, 2005). *C. elegans* germline is organized in a simple linear fashion that progresses from germline stem cells (GSCs) at one end to maturing gametes at the other (Fig. 3A). Germ cells progress from GSCs at the distal end, through meiotic prophase as they move proximally to become differentiated gametes at the proximal end (Fig. 3A).

C. elegans germline development is tightly regulated by conserved external signaling pathways, including GLP-1/Notch and MPK-1/ERK (Fig. 3A) as well as intrinsic regulators, including gene expression regulators and cell cycle regulators (Kimble and Crittenden, 2007). The Notch signaling pathway and its core components in *C. elegans* are highly conserved. The *C. elegans* has two Notch receptors, GLP-1 and LIN-12, which mediate cell-cell interaction during development (Greenwald, 2005). Specifically, GLP-1/Notch signaling in the *C. elegans* germline is critical for germline stem cell maintenance and continued mitotic division (Kimble and Crittenden, 2007) (Fig. 3B-3D). LIN-12/Notch signaling in the *C. elegans* somatic cells specifies vulva cell fate during early larval stages (Greenwald, 2005). When LAG-2 (GLP-1/Notch ligand) is expressed in DTCs (distal tip cells, functions as GSC niche) (Fig. 3B-3D) (Henderson et al., 1994) and interacts with the GLP-1/Notch receptor, proteolytic cleavage of the GLP-1/Notch receptor follows (Fig. 3B-3D). GLP-1/Notch intracellular domain (NICD) is then trans-located from the membrane into the nucleus (Fig. 3B-3D). In the nucleus, the NICD forms a tertiary complex with LAG-1/CSL DNA binding protein and LAG-3/SEL-8/Mastermind transcription co-activator to activate the expression of target genes: *lst-1* (Nanos-like zinc finger domain-containing protein) (Kershner et al., 2014; Yoo et al., 2004), and *sygl-1* (Novel protein) (Kershner et al., 2014) (Fig. 3B-3D). Importantly, IST-1 and SYGL-1 work together with FBF-1

and FBF-2 to maintain GSC fate (Fig. 3B-3D). Therefore, loss of GLP-1/Notch signaling in germ-line causes a severe proliferation defect during early meiotic entry, resulting in no GSC maintenance and sterility (Austin and Kimble, 1989), while constitutive activation of this signaling promotes proliferation of GSCs and their progenitor cells as well as inhibits entry into meiosis, resulting in germline tumors and sterility (Berry et al., 1997).

C. elegans MPK-1/ERK signaling controls multiple developmental events, including meiotic cycle progression, oocyte activation, sperm fate specification, spermatogenesis, physiological apoptosis, axon guidance, and vulva development (Lee et al., 2006; Lee et al., 2007a; Lee et al., 2007b; Morgan et al., 2010; Sundaram, 2006). Dr. Lee's lab has previously reported that MPK-1/ERK signaling is required for the formation of germline tumors via dedifferentiation in the absence of PUF-8.

In addition to external signal pathways, a battery of RNA regulators including PUF RNA-binding proteins play critical roles in GSC maintenance, differentiation, cell fate specification, and cell fate reprogramming (see #3 below).

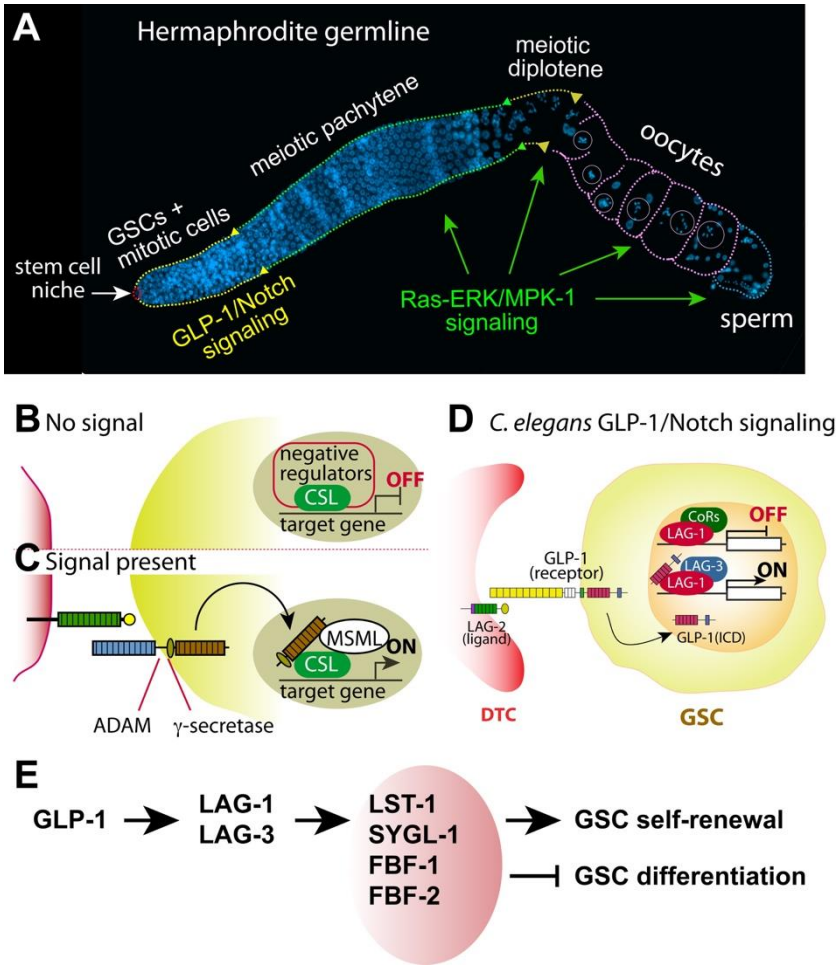


Figure 3. *C. elegans* germline. The hermaphrodite has two gonadal tubes. They produce both sperm and oocytes and are therefore self-fertile. (B) A dissected adult hermaphrodite germline stained with DAPI. In the distal end, somatic gonadal cell acts as a GSC niche that is essential for GSC maintenance. In the distal mitotic region, GLP-1/Notch signaling maintains GSC self-renewal and promotes mitotic cell cycle of progenitor cells. Once mitotic cells enter meiotic cell cycle, Ras-ERK MAPK signaling promotes meiotic germ cell progression, pachytene exit, oocyte maturation, and sperm fate specification. (B-D) *C. elegans* GLP-1/Notch signaling pathway. The DTC (distal tip cell, GSC niche) expresses GLP-1/Notch ligands (e.g., LAG-2) and employs GLP-1/Notch signaling to promote continued mitotic divisions. (E) GLP-1/Notch signaling pathway activates the expression of *lst-1* and *sygl-1* genes in the distal mitotic

cells. LST-1 and SYGL-1 work together with FBF-1 and FBF-2 to promote GSC self-renewal and repress GSC differentiation.

3. PUF RNA-Binding Proteins

RNA-binding proteins (RBPs) are proteins that bind to either single stranded or double stranded RNA and play a role in the post-transcriptional control of RNAs, such as mRNA stabilization, localization, splicing, polyadenylation, and translation (Hentze et al., 2018). A family of these RBPs that are heavily conserved among most eukaryotic organisms are PUF proteins (Wickens et al., 2002) (Fig. 4A). PUF proteins are conserved RBPs that maintain GSCs in worms and flies (Fig. 4B) and have also been implicated in this role in mammals (Lee et al., 2019; Mak et al., 2016; Moore et al., 2003; Naudin et al., 2017; Shigunov et al., 2012; Xu et al., 2007; Zhang et al., 2017). PUF proteins bind specifically to PUF binding elements (PBE: UGUAnAUA) within the 3' untranslated region (3'UTR) of their direct target mRNAs to repress their translation and stability (Datla et al., 2014; Wang and Voronina, 2020; Wickens et al., 2002) (Fig. 4C). PUF proteins also have diverse roles depending on the organism that they are found in. In *Drosophila melanogaster*, Pumilio is required for embryonic development through the regulation of *Hunchback* (important for the establishment of an anterior-posterior gradient) (Murata and Wharton, 1995). This very specific function in *Drosophila* is then contrasted with the yeast PUF protein Mpt5 which is a broad RNA regulator in *Saccharomyces cerevisiae* in which it binds to more than 1,000 RNA targets (Porter et al., 2015).

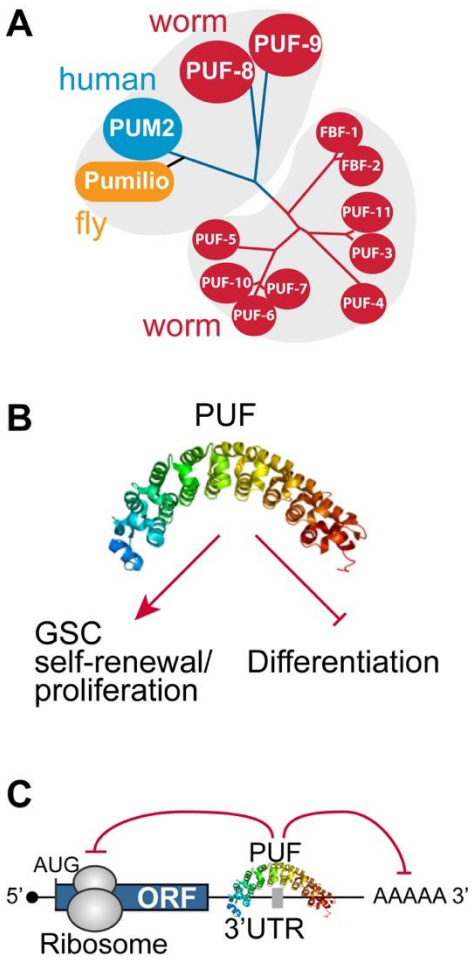


Figure 4. PUF protein family and their function. (A) PUF proteins are conserved from worms to humans. (B) PUF proteins are critical for GSC maintenance in worms and flies. (C) PUF inhibits the translation of mRNA by interacting with a regulatory element (called PUF binding element) on target mRNA 3'UTR and represses both polyadenylation and translational elongation.

3. C. elegans PUF proteins: PUF-8

PUF proteins control various physiological processes such as stem cell maintenance, cell fate specification, and cell fate reprogramming by interacting with the 3′ untranslated region (UTR) of specific mRNAs to repress the mRNA translation or stability (Wickens et al., 2002).

C. elegans have 11 PUF proteins that recognize a family of related sequence motifs in the target mRNAs (Fig. 4A), yet individual PUF proteins have clearly distinct biological functions (Wang and Voronina, 2020). FBF-1 and FBF-2 are required for GSC maintenance (Crittenden et al., 2002) and sperm/oocyte decision (Zhang et al., 1997).

PUF-8 (mostly like the Drosophila and human PUFs) protein controls multiple cellular processes, including GSC maintenance, proliferation, differentiation, sperm-oocyte decision, and dedifferentiation, depending on the genetic context (Datla et al., 2014). PUF-8 and its genetic partners regulate germline development at different places and time (Fig. 5).

- PUF-8 and MEX-3 (KH-type RNA-binding protein) are required for GSC maintenance in the early germline development (Ariz et al., 2009).
- PUF-8 also inhibits mitotic germ cell proliferation possibly by repressing GLP-1/Notch signaling (Racher and Hansen, 2012).
- PUF-8 and FBF-1 promote oocyte fate at the expense of sperm fate (Bachorik and Kimble, 2005)
- PUF-8 and LIP-1 prevent spermatocyte dedifferentiation by inhibiting MPK-1 signaling (Cha et al., 2012)

  PUF-8 and its target GLD-2 prevent spermatocyte dedifferentiation by repressing MPK-1 and activating GLD-1 (Park et al., 2020).

The GFP reporter-based expressional analysis showed that PUF-8 was first detected in the descendants of the primordial germ cells in the early L1 larva and its expression pattern continued through L2 and L3 stages (Ariz et al., 2009). In adult hermaphrodite germlines, PUF-8 was strongly expressed in the distal mitotic germ cells and gradually decreased as germ cells enter meiotic cell cycle (Ariz et al., 2009) (Fig. 6A and 6B) . However, in adult male germline, PUF-8 was expressed in distal mitotic germ cells, spermatocytes, and sperm (Fig. 6C and 6D).

These findings indicate that PUF-8 is critical for germline homeostasis – balancing the GSC self-renewal/differentiation, sperm/oocyte, and differentiation/dedifferentiation fates possibly by repressing its target mRNAs.

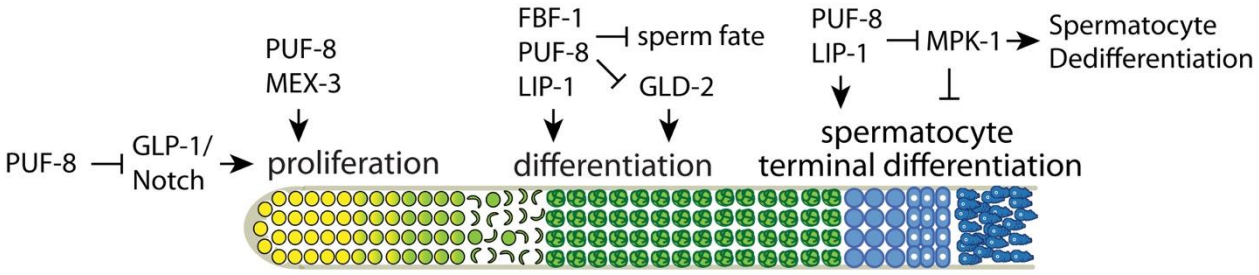


Figure 5. Multiple functions of PUF-8 proteins, depending on the genetic context. See text in details.

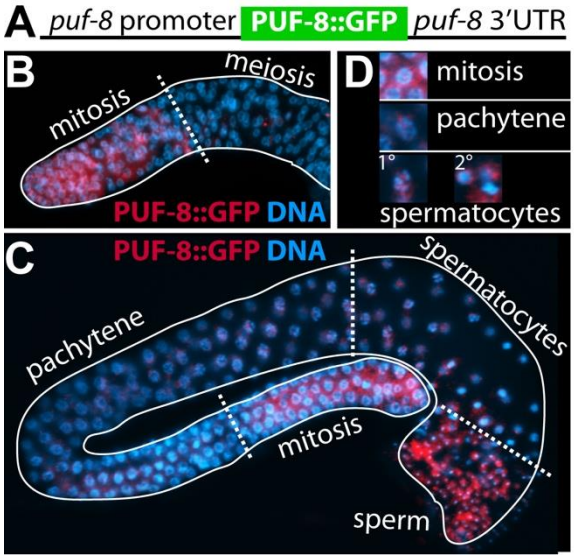


Figure 6. Expression of PUF-8::GFP in *C. elegans* germline. (A) *puf-8::GFP* transgene. (B & C) GFP expression in hermaphrodite mitotic region (B) and male germline (C). (D) Magnified images of mitotic, pachytene, and spermatocytes.

4. C. elegans Sex Determination Pathway

In *C. elegans*, terminal regulators of the sex determination pathway are *fog-1* and *fog-3* in which they are essential for sperm specification (Ellis, 2008) (Fig. 7). These terminal regulators are negatively controlled by an upstream sex determination gene, *tra-1*, which represses *fog-1/3*, and promotes oocyte production (Ellis, 2008). Directly upstream of the gene *tra-1* are the genes *fem-1/2/3*, which control *tra-1* repression via ubiquitin-mediated proteolysis (Starostina et al., 2007). This ubiquitin-mediated proteolysis is accomplished by *fem-1/2/3* participating in a complex known as CBC (which is comprised of CUL2, Elongin B, and Elongin C) to promote *tra-1* degradation (Starostina et al., 2007). Loss of any of the three *fem* genes leads to increased *tra-1a* levels indicating that all three FEM proteins are required to negatively regulate TRA-1 in *C. elegans* (Starostina et al., 2007). Interestingly, *fem-3(lf)* mutants only produce oocytes and *fem-3(gf)* mutants only produce sperm (Zanetti et al., 2012).

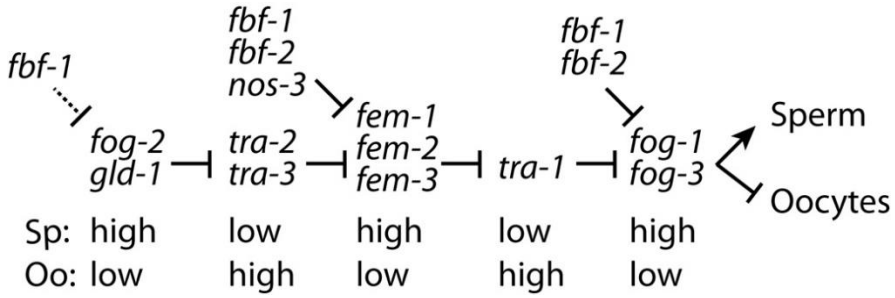


Figure 7. *C. elegans* germline sex determination pathway. Simplified version of the hermaphrodite germline sex determination pathway. Low and high refer to levels of above gene activation.

An important gene that plays a role in sperm fate in *C. elegans* is the gene *fem-3*, which along with *fem-1/2*, work together to repress downstream sex determination genes (Fig. 7). Interestingly, *fem-3 (q20)* gain-of-function (gf) mutants [*fem-3(q20gf)*] only produce sperm at

restrictive temperatures of 25°C. To gain insight into the molecular competence for spermatocyte dedifferentiation, we have generated a *puf-8(q725); fem-3(q20gf)* double mutants. Notably, this mutant produced only sperm without switching to oogenesis (called Mog (masculinization of germline) phenotype) at both permissive (15-20°C) and restrictive temperatures (25°C) (see Fig. 8).

Specific Aims

1. Analyze the germline phenotype of *puf-8(q725); fem-3(q20gf)* mutants.
2. Compare spermatocyte dedifferentiation competence using *puf-8(q725); lip-1(zh15)* and *puf-8(q725); fem-3(q20gf)* mutants.
3. Test the hypothesis that resveratrol (an MPK-1 activator) may induce the formation of germline tumors via spermatocyte dedifferentiation in *puf-8(q725); fem-3(q20gf)* mutants.
4. Test the potential function of mitochondria in the formation of germline tumors via spermatocyte dedifferentiation in *puf-8(q725); fem-3(q20gf)* mutants.

MATERIALS AND METHODS

1. C. elegans culture.

Worms are cultured in the laboratory on 60 mm Nematode Growth Media (NGM) agar plates seeded with OP50 *Escherichia coli* (*E. coli*) bacteria as a food source at permissive temperature (20°C) or restrictive temperature (25°C) as per (Brenner, 1974).

1) Inoculate OP50 *E. coli* bacteria colony in 100 mL LB media.
2) Culture at 37°C with shaking for overnight.
3) Apply approximately 0.5 ml of OP50 *E. coli* bacteria liquid culture to 60 mm NGM plates using pipet.
4) Allow the OP50 *E. coli* bacteria lawn to grow overnight at room temperature.
5) Transfer worms to NGM plates with OP50 *E. coli* bacteria and culture at 20°C.

2. Making culture media and plates

2.1. Nematode Growth Media (NGM)

For 1 L of NGM agar media, mix 3 g NaCl, 2.5 g Bacto Peptone, 17 g agar, 1 mL 1 M $CaCl_2$, 1 mL 1M $MgSO_4$, 25 mL Potassium phosphate (pH 6.0), and H_2O to a final volume of 971 mL, and autoclave. After autoclaving and cooling to ~55°C, add 1 mL of 5 mg/mL Cholesterol in 95% Ethanol (EtOH). Pour NGM media to 6 mm Petri dishes manually or using a pouring system.

2.2. Luria Bertani (LB) for OP50 E. coli bacteria culture

For 1 L of LB media, mix 10 g Bacto Tryptone, 5 g Bacto Yeast, 10 g NaCl, and H_2O to a final volume of 1 L, and autoclave.

2.3. M9 buffer

For 1 L of M9 buffer, mix 5 g NaCl, 6 g Na_2HPO_4, 3 g KH_2PO_4, and H_2O to a final volume of 999 mL, and autoclave. After autoclaving, add 1 mL of autoclaved 1 M $MgSO_4$.

2.4. Bleaching solution

For 1 L of bleaching solution, mix 245.9 mL of 1 N Sodium hydroxide, 196.7 mL of Sodium hypochloride (household bleach) and 557.4 mL of autoclaved H_2O in a sterilized bottle.

2.5. RNAi plates

After making normal NGM media and autoclaving, add 1 ml of ampicillin (100 mg/mL) and 1 ml of 0.5 M IPTG when autoclaved NGM media are cooled down to 55°C and pour 96-well deep-well plates or 35 mm Petri dishes manually or using a pouring system.

3. C. elegans strains (see Table 1)

C. elegans strains were provided by CGC or Dr. Kimble's lab or generated by Dr. Lee's lab using a standard genetic method.

Table 1. Strain information

Strain	**Major Germline Phenotype**	**Source**	**References**
N2	Normal (fertile)	CGC	(Brenner, 1974)

puf-8(q725)	Normal (fertile)at 20°C, but 9% germline tumors via dedifferentiation (sterile) at 25°C	Dr. Kimble's lab	(Bachorik and Kimble, 2005; Cha et al., 2012)
lip-1(zh15)	Partially sterile with small oocytes	CGC	(Hajnal and Berset, 2002)
puf-8(q725); lip-1(zh15)	100 % Mog at 20°C; 100% germline tumors via dedifferentiation (sterile) at 25°C	Dr. Kimble's lab Dr. Lee's lab	(Cha et al., 2012; Morgan et al., 2010)
fem-3(q20) gain-of-function (gf)	Normal (fertile) at 20°C 100% Mog sterile at 25°C	Dr. Kimble's lab	(Barton et al., 1987)
puf-8; fem-3(q20gf)	Mog sterile at 15°C, 20°C, and 25°C. Germline tumors during aging at 25°C	Dr. Lee's lab	Unpublished
puf-8(q725); fem-3(q20gf); (tn1541[GFP::tev::s::lin-41])	Tn154 partially rescue *puf-8; fem-3(q20gf)* Mog phenotype at 20°C	Dr. Lee's lab	Unpublished

5. Generation of *puf-8(q725)/mln1[mIs14 dpy-10(e128)]; fem-3(q20gf)/fem-3(q20gf)* double mutants

Adult *puf-8(q725)* homozygote mutant males (3 ea) were mated with adult *fem-3(q20)* homozygote hermaphrodite mutants (5 ea) at 20°C. Male progeny (predicted genotype: *puf-8(q725)/+; fem-3(q20gf)/+*) were mated with dumpy homozygote hermaphrodite mutants with *mln1[mIs14 dpy-10(e128)]* GFP balancer chromosome. Non-dumpy GFP (in pharynx) hermaphrodites were selected and singled out. *puf-8(q725)/mln1[mIs14 dpy-10(e128)]; fem-3(q20gf)/+* progeny were identified by PCR analysis and phenotype analysis at 25°C (~25% F1 progeny exhibited Mog phenotype. Finally, *puf-8(q725)/mln1[mIs14 dpy-10(e128)]; fem-*

3(q20gf)/fem-3(q20gf) double mutants were identified by PCR and phenotype analysis in the next generation.

6. Generation of puf-8(q725)/mln1[mIs14 dpy-10(e128)]; fem-3(q20gf)/fem-3(q20gf); (tn1541[GFP::tev::s::lin-41]) mutant strain.

Adult *puf-8(q725)/mln1[mIs14 dpy-10(e128)]; fem-3(q20gf)/fem-3(q20gf)* homozygote mutant males (3 ea) were mated with adult *(tn1541[GFP::tev::s::lin-41])* homozygote hermaphrodite alleles (5 ea) at 20°C. Male progeny (predicted genotype: *puf-8(q725)/+; fem-3(q20gf)/+; (tn1541[GFP::tev::s::lin-41]/+*) were mated with dumpy homozygote hermaphrodite mutants with a *mln1[mIs14 dpy-10(e128)]* GFP balancer chromosome. Non-dumpy GFP (in pharynx) hermaphrodites were selected and singled out. *puf-8(q725)/mln1[mIs14 dpy-10(e128)]; fem-3(q20gf)/+; (tn1541[GFP::tev::s::lin-41]/+* progeny were identified by PCR analysis, oocyte GFP expression, phenotype analysis at 25°C (~25% F1 progeny exhibited Mog phenotype. Finally, *puf-8(q725)/mln1[mIs14 dpy-10(e128)]; fem-3(q20gf)/fem-3(q20gf); (tn1541[GFP::tev::s::lin-41]/(tn1541[GFP::tev::s::lin-41]* strains were identified by PCR, oocyte GFP expression, and phenotype analysis in the next generation.

7. RNA interference (RNAi)

RNAi experiments were performed by feeding bacteria expressing double strand RNAs corresponding to the gene of interest (Kamath et al., 2001)). Briefly, synchronized L1 staged worms were plated onto RNAi plates and incubated at 25 °C. Germline phenotypes 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 (Lee et al., 2007a; Morgan et al., 2010). Other RNAi bacteria were from *C. elegans* RNAi feeding library (Source Bioscience LifeSciences) and *C. elegans* ORF-RNAi library (Open Biosystems).

8. Germline Immunochemistry

For antibody staining, dissected gonads were fixed in 3% paraformaldehyde with 100 mM K_2HPO_4 (pH 7.2) for 20 min at room temperature followed by 100% cold methanol for 5 min at -20 °C. After blocking for 30 min with 0.5% BSA in 1 × PBS (+ 0.1% Tween 20), fixed gonads were incubated for 1.5 h at room temperature with primary antibodies followed by 1 h at room temperature with secondary antibodies. Anti-MSP (a marker for sperm, purchased from the DSHB (Cat#: 4A5) and anti-HIM-3 (a marker for meiotic cells: NOVUS Cat#:53470002). DAPI staining followed standard methods.

9. 5-Ethynyl-2'-deoxyuridine (EdU) labeling

To label mitotically cycling cells, worms were incubated with rocking in 0.2 mL M9 buffer (3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, 1 mL 1M $MgSO_4$, H_2O to 1 L) containing 0.1% Tween 20 and 1 mM EdU for 30 min at 20°C. Gonads were dissected and fixed in 3% paraformaldehyde/0.1M K_2HPO_4 (pH 7.2) solution for 20 min, followed by −20°C methanol fixation for 10 min. Fixed gonads were blocked in 1× PBST/0.5% BSA solution for 30 min at 20°C. EdU labeling was performed using the Click-iT EdU Alexa Fluor 488 Imaging Kit (Invitrogen, CA, United States, #C10337), according to the manufacturer's instructions. For co-

staining with antibodies, EdU-labeled gonads were incubated in the primary antibodies after washing for three times, and subsequently in the secondary antibodies as described above.

10. Resveratrol (RSV) treatment

RSV (Sigma, St. Louis, MO, USA; Cat# R5010) was dissolved in ethanol (EtOH) to stock concentrations of 100 mM. RSV was directly added to the NGM media before pouring the solution into petri dishes. The worms were transferred to the EtOH- or RSV-containing NGM agar plates. All worms tested were transferred to fresh plates every 2 days and their germline phenotypes were determined by staining dissected gonads with cell type-specific antibodies or EdU-labeling kit.

RESULTS

1. puf-8 depletion enhances fem-3(q20gf) Mog phenotype

To test whether excess sperm production might enhance the formation of germline tumors at 25°C, we have generated *puf-8; fem-3(q20)* gain-of-function (gf) mutants using a standard genetic method (see Material and Methods). Most *fem-3(q20gf)* mutants produce both sperm and oocytes, and they are self-fertile at permissive temperature (15°C-20°C), but they produce sperm continuously throughout adulthood (Masculinization of germline (Mog) phenotype) at restrictive temperature (25°C). To confirm this phenotype, we have generated a *puf-8(q725); fem-3(q20gf); lin-41(tn1541[GFP::tev::s::lin-41])* mutant using a standard genetic method (see Materials and Methods). *lin-41(tn1541[GFP::tev::s::lin-41])* allele was used to visualize oocytes (Fig. 8A-8C).

This phenotype was determined by staining dissected gonads with anti-MSP (a marker for sperm fate cells) and DAPI (a marker for DNA). Wild-type worms produced sperm (MSP-positive) and oocyte (GFP::LIN-41-positive) at 15, 20, and 25°C (Fig. 8C). *puf-8(q725)* single mutants produced both sperm and oocytes at permissive temperature (15-20°C), but they exhibited Mog (10%) and germline tumor (9%) phenotypes at 25°C (Fig. 8C). *fem-3(q20gf)* allele is a temperature-sensitive, gain-of-function mutant (Barton et al., 1987). ~ 1%, ~12%, and 100% of *fem-3(q20gf)* mutants showed Mog phenotype at 15°C, 20°C, and 25°C, respectively (Fig. 8C). Notably, most *puf-8(q725); fem-3(q20gf)* hermaphrodite mutants produced only sperm (MSP-positive, GFP::LIN-41-negative) without switching to oogenesis even at 15°C-20°C (Fig. 8C). This result suggests that *puf-8(q725)* depletion enhances *fem-3(q20gf)* Mog phenotype.

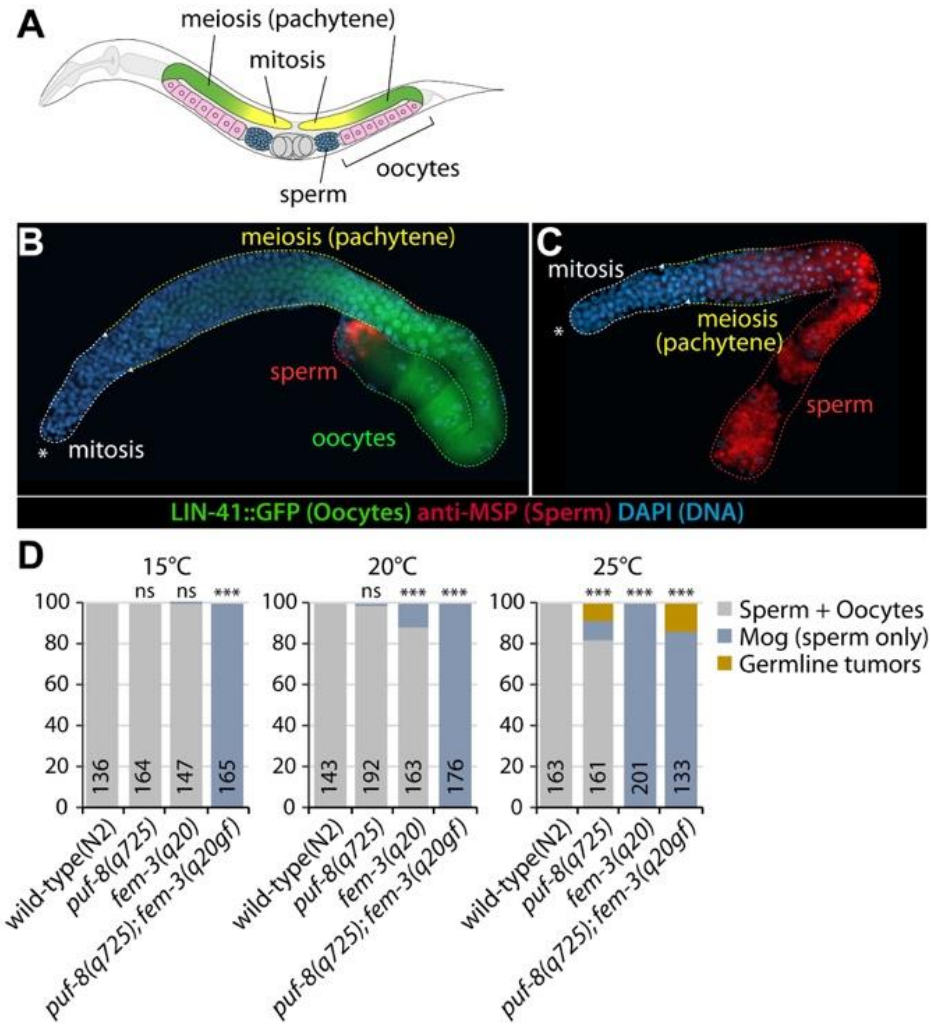


Figure 8. Phenotype analysis of *puf-8(q725); fem-3(q20gf)* germlines (A) Schematic of adult *C. elegans* hermaphrodite gonads. (B-C) Expression of LIN-41::GFP (oocyte marker) and MSP (sperm marker) in wild-type and *puf-8; fem-3(q20gf)* mutant germlines. (D) Germline phenotypes were determined by staining dissected gonads with anti-MSP antibody and DAPI.

2. MPK-1 dependence of puf-8; fem-3(q20gf) Mog phenotype

C. elegans mpk-1 gene encodes two major transcripts - *mpk-1a* and *mpk-1b*, which produce MPK-1A and MPK-1B proteins, respectively. The *mpk-1a* mRNA is contained entirely within *mpk-1b*, but *mpk-1b* harbors a unique exon (Lee et al., 2007a). Previous studies showed that *mpk-1a* isoform is predominantly expressed in somatic cells, but *mpk-1b* isoform is abundantly expressed in germ cells (Lee et al., 2007a; Lee et al., 2007b). We previously reported that *puf-8(q725); lip-1(zh15)* Mog phenotype depends on MPK-1 activity (Morgan et al., 2010). Depletion of germline *mpk-1b* isoform by RNAi dramatically rescued *puf-8(q725); lip-1(zh15)* Mog sterility (Morgan et al., 2010). To test whether *puf-8(q725); fem-3(q20gf)* Mog sterility is also dependent on MPK-1 activity, we depleted the expression of germline *mpk-1b* isoform by RNAi in *puf-8(q725); fem-3(q20gf)* mutants and determined their germline phenotype by staining dissected gonads with anti-MSP antibody and DAPI. Notably, *puf-8(q725); fem-3(q20gf)* Mog phenotypes were partially suppressed by *mpk-1b(RNAi)* (Fig. 9). This result indicates that the *puf-8(q725); fem-3(q20gf)* Mog phenotype is largely independent of the MPK-1 activity.

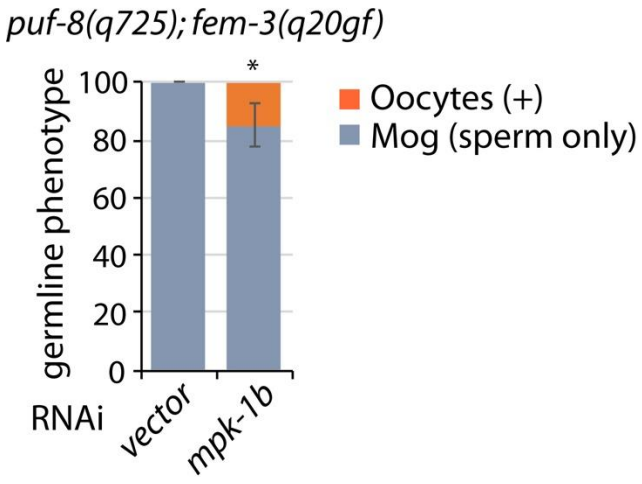


Figure 9. MPK-1 dependence of *puf-8(q725); fem-3(q20gf)* Mog phenotype. *puf-8(q725); fem-3(q20gf)* Mog phenotype was partially suppressed by *mpk-1b(RNAi).* The germline phenotype was determined by staining dissected gonads with anti-MSP antibodies.

3. puf-8 functions upstream of fog-2 in germline sex determination pathway

It was previously reported that sex determination in *C. elegans* is controlled by PUF proteins such as PUF-8 and FBF-1 that act redundantly to switch the gonad from sperm fate to oocyte fate (Bachorik and Kimble, 2005). This redundancy can be seen with single mutant worms for *puf-8* and *fbf-1* resulting in a Mog phenotype < 3% whereas *puf-8(q725) fbf-1(ok91)* double mutants resulted in 100% Mog phenotype (Bachorik and Kimble, 2005). Previous epistasis analyses studies also show that *puf-8* and *fbf-1* are upstream of *fog-2*, a gene thought to be near the top of the germline sex determination pathway (Bachorik and Kimble, 2005). This is further confirmed with the abundance of FOG-2 increasing dramatically in *puf-8(q725) fbf-1(ok91)* double mutants which would suggest that either PUF-8 or FBF-1 actively represses FOG-2 under normal conditions (Bachorik and Kimble, 2005). Since FBF-1 represses the expression of *fem-3* mRNA (Zhang et al., 1997), we also performed epistasis experiments. Specifically, genes required for sperm production were depleted by RNAi in *puf-8(q725); fem-3(q20gf)* mutants. Briefly, L1-staged *puf-8; fem-3(q20gf); (tn1541[GFP::tev::s::lin-41])* mutants were cultured on RNAi plate seeded each RNAi bacteria for 3 days at 20°C. Their germline phenotypes were determined by observing the expression of GFP::LIN-41 (an oocyte marker) in the germlines under fluorescence microscopy. Our results show that *puf-8(q725); fem-3(q20gf)* Mog phenotypes were completely suppressed by the depletion of *fog-1*, *fog-2*, *fog-3*, or *fem-3*, but not vector (control) (Fig. 10A-10C). Interestingly, each RNAi dramatically rescued the Mog sterile phenotype and made them fertile. These results indicate that *puf-8* acts upstream of all germline sex determination genes and *puf-8(q725); fem-3(q20gf)* Mog phenotypes are likely due to increased levels in *fog-1*, *fog-2*, *fog-3*, or *fem-3* gene.

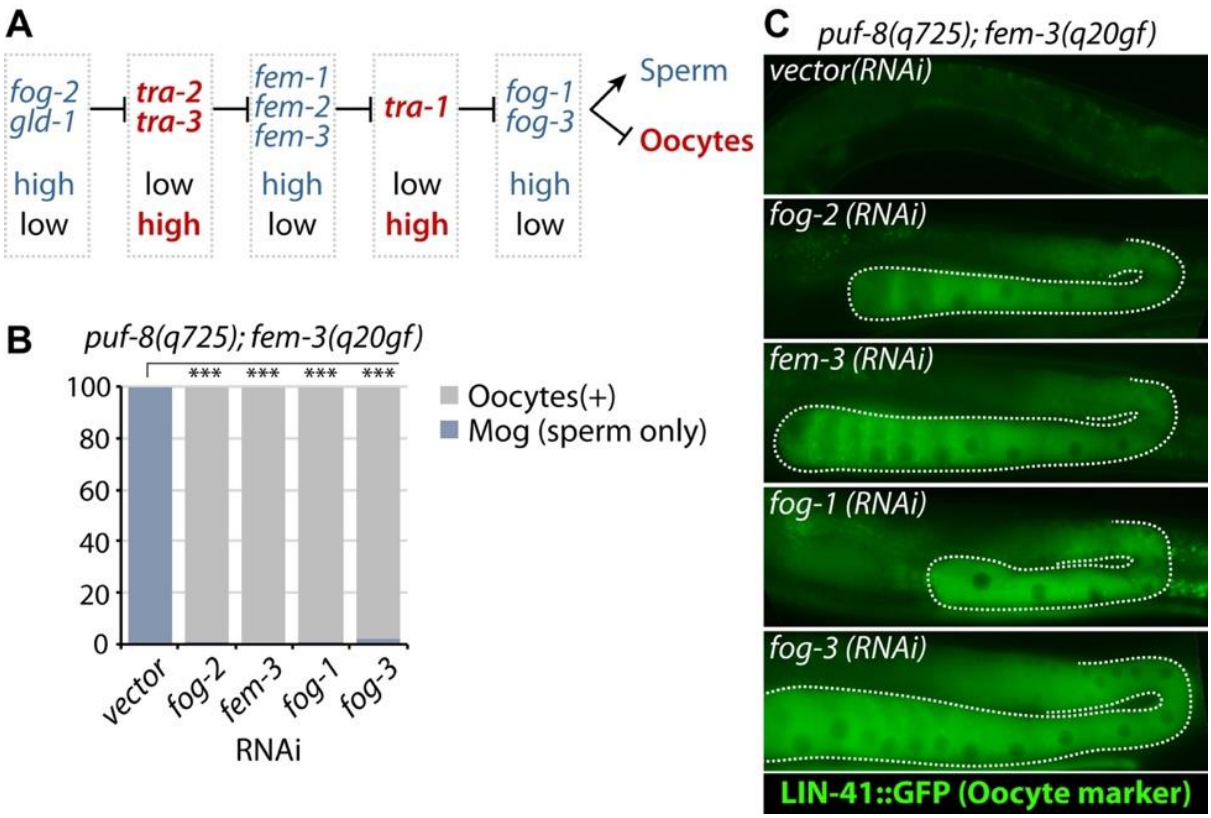


Figure 10. Epistatic analysis. (A) Simplified version of the germline sex determination pathway. Red genes promote oocyte fate and blue genes promote sperm fate. (B) Depletion of sperm-promoting genes by RNAi rescue *puf-8; fem-3(q20gf)* Mog sterility. (C) LIN-41(oocyte marker)::GFP expression.

4. Competence for spermatocyte dedifferentiation

We next tested the hypothesis that competence for spermatocyte dedifferentiation requires MPK-1 activation in the absence of PUF-8. To this end, we employed three mutant strains: *fem-3(q20gf)* mutant with wild-type *puf-8(+/+)* and *lip-1(+/+)* genes, *puf-8(q725); fem-3(q20gf)* with wild-type *lip-1(+/+)* gene (low MPK-1 activity), and *puf-8(q725); lip-1(zh15)* (high MPK-1 activity). All three mutants are all Mog at 25°C. To score the percentage of worms with germline tumors, synchronized L1-staged mutants were placed on NGM plates seeded with OP50 bacteria food at 25°C and their germline phenotypes were scored daily by staining dissected gonad with DAPI. As we expected, *fem-3(q20gf)* mutants never formed germline tumors at 25°C (Fig. 11). However, *puf-8(q725); lip-1(zh15)* (high MPK-1) mutants developed germline tumors more aggressively than *puf-8(q725); fem-3(q20gf)* (low MPK-1 activity) mutants (Fig. 11). This result indicates that MPK-1 activity is required to induce the formation of germline tumors via spermatocyte dedifferentiation in the absence of PUF-8 at 25°C.

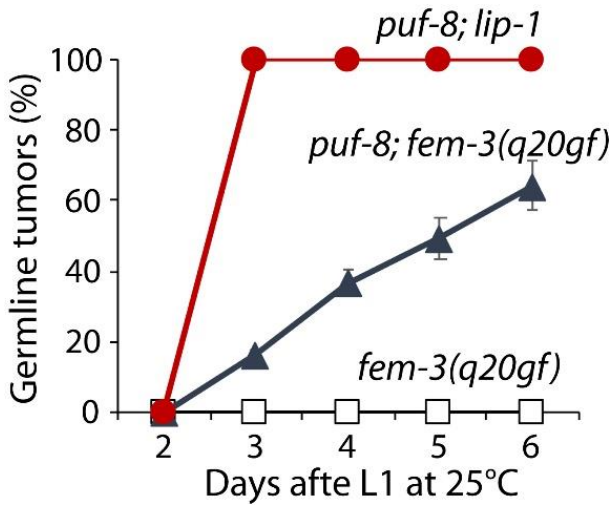


Figure 11. Germline tumors via spermatocyte dedifferentiation require *puf-8* depletion and MPK-1 activation. This result is the means of three independent experiments. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. ns, not statistically significant.

5. Resveratrol enhances the formation of germline tumors via spermatocyte dedifferentiation during aging.

Our previous study found that Resveratrol (RSV) maintains MPK-1 activity throughout the lifespan of *C. elegans* (Yoon et al., 2019). Importantly, RSV also activates ERK in human mesenchymal stem cells, depending on cell passage (Choi et al., 2019; Yoon et al., 2015). These findings led us to test whether RSV could induce the formation of germline tumors via spermatocyte dedifferentiation in *puf-8(q725); fem-3(q20gf)* mutant germlines. L1 staged *puf-8(q725); fem-3(q20gf)* mutant worms were cultured on NGM agar plates containing 100 μM RSV or 0.1% ethanol (EtOH) control at 25°C. Their germline phenotypes were analyzed daily by staining dissected gonads with EdU-labeling kit (a marker of mitotic cells) and DAPI. Notably, RSV significantly induced the formation of germline tumors probably by activating MPK-1 proteins (Fig. 12A). This result suggests that RSV (potential MPK-1 activator) can induce the formation of germline tumors via spermatocyte dedifferentiation *in vivo*.

Next, to test whether RSV-induced germline tumors rely on MPK-1 activity, we depleted the expression of *mpk-1* by RNAi from L1-staged *puf-8(q725); fem-3(q20gf)* mutants and their germline phenotypes were determined by staining dissected gonads with DAPI. While vector (RNAi) control did not suppress the formation of germline tumors via spermatocyte dedifferentiation, *mpk-1(RNAi)* significantly suppressed the formation of *puf-8(q725); fem-3(q20gf)* germline tumors until day 5 (Fig. 12B). On day 6, germline tumors were dramatically increased as the levels of vector (RNAi) control. We still do not know why, but suggest that a few of dedifferentiated cells might be actively divided on day 6 or another regulators might promote the formation of germline tumors via spermatocyte dedifferentiation in old *puf-8(q725); fem-3(q20gf)* mutants.

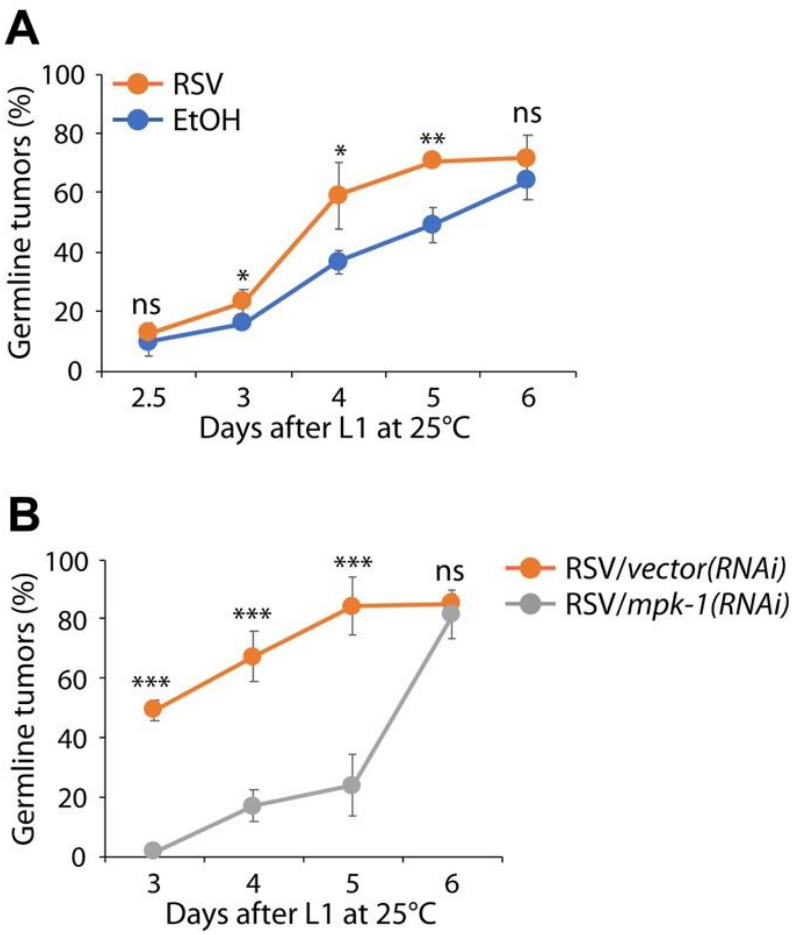


Figure 12. RSV induces the formation of germline tumors via spermatocyte dedifferentiation probably by activating MPK-1. (A) 100 μM RSV-treated *puf-8(q725); fem-3(q20gf)* mutants had more germline tumors than EtOH (control vehicle)-treated *puf-8(q725); fem-3(q20gf)* mutants. (B) RSV-induced *puf-8; fem-3(q20gf)* germline tumors are dependent on MPK-1 signaling.

6. Effects of bacterial foods on the formation of puf-8(q725); fem-3(q20gf) germline tumors.

OP50 is an *E. coli* strain conventionally used as food source for *C. elegans* maintenance. HT115 is an RNase III-deficient *E. coli* strain used for feeding RNAi in *C. elegans* (Asencio et al., 2003; Reinke et al., 2010). It was reported that HT115 *E. coli* strain serves as a higher metabolic energy source than OP50 strain (Chi et al., 2016). Our unpublished results showed that HT115 feeding significantly enhanced the formation of *puf-8(q725); lip-1(zh15)* germline tumors even at 20°C (Park et al., unpublished results). To test whether HT115 feeding also could enhance more germline tumors than OP50 feeding, synchronized L1-staged *puf-8(q725); fem-3(q20gf)* mutant worms were cultured on NGM agar plates seeded either OP50 or HT115 bacteria at 25°C. Their germline phenotypes were determined daily by staining dissected gonads with DAPI. Intriguingly, HT115-fed *puf-8(q725); fem-3(q20gf)* mutant worms formed significantly more germline tumors than OP50-fed *puf-8(q725); fem-3(q20gf)* mutant worms (Fig. 13A). Next, to test whether the RNase III-deficient contributes to the formation of *puf-8(q725); fem-3(q20gf)* germline tumors, *puf-8(q725); fem-3(q20gf)* mutants were cultured on NGM agar plates seeded with an RNase III-deficient OP50 *E. coli* strain (OP50/rnc-). Notably, the *puf-8(q725); fem-3(q20gf)* fed OP50/rnc- bacteria showed the similar percentage of germline tumors to *puf-8(q725); fem-3(q20gf)* fed HT115 bacteria (Fig. 13B). One possible reasoning for this is that there is a recycling of excess nucleotides by RNase III-deficiency that may provide an increased metabolic energy for the formation of germline tumors in *puf-8(q725); fem-3(q20gf)* mutant germlines. Another interesting reasoning would be an increase in the stabilization of higher mRNAs due to the RNase III-deficiency.

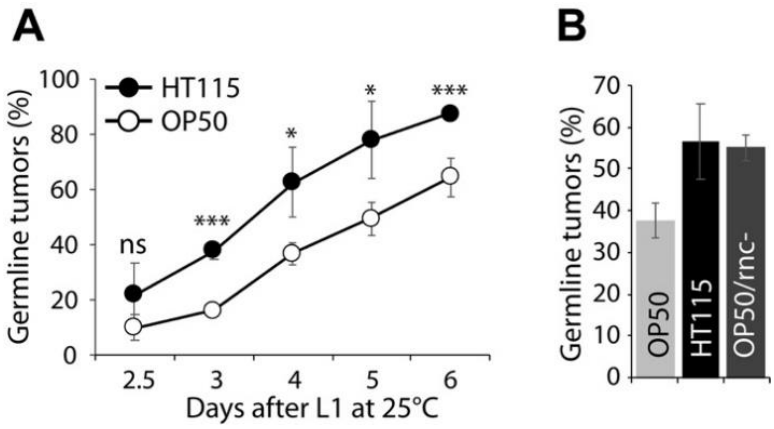


Figure 13. Different food diets affect the formation of germline tumors in *puf-8; fem-3(q20gf)* germlines. (A) The effect of bacteria food sources on the formation of germline tumors in *pur-8; fem-3(q20gf)* mutants. (B) An RNase III-deficiency contributes to the formation of germline tumors in the *puf-8(q725); fem-3(q20gf)* mutants.

7. Mitochondrial activity is required for the formation of germline tumors in puf-8; fem-3(q20gf) mutants.

Mitochondria serve as a signaling hub for metabolic pathways in the cell (Wanet et al., 2015). Recent evidence implicates additional functions of mitochondria in stem cell self-renewal and differentiation (Lisowski et al., 2018; Zhang et al., 2018). We here tested the hypothesis that changes in mitochondrial activity influences the formation of germline tumors in *puf-8(q725); fem-3(q20gf)* mutants. Two genes, *isp-1* (an iron sulfur protein of mitochondrial complex III) and *clk-1* (an ortholog of human COQ7 hydroxylase), are critical components of mitochondrial functions (Khan et al., 2013). The depletion of *isp-1* or *clk-1* results in low oxygen consumption, decreased sensitivity to ROS, and increased lifespan (Feng et al., 2001).

RNAi of *isp-1* or *clk-1* began at L1 stage of *puf-8(q725); fem-3(q20gf)* mutant worms at 25°C and their germline phenotypes were determined 5 day later by staining dissected gonads with DAPI. Notably, RNAi of *isp-1* or *clk-1* significantly suppressed the formation of germline in the *puf-8(q725); fem-3(q20gf)* mutant worms (Fig. 14). These results indicate mitochondrial activity is required for the formation of germline tumors in the *puf-8(q725); fem-3(q20gf)* mutants. These experiments will be repeated at least two more times.

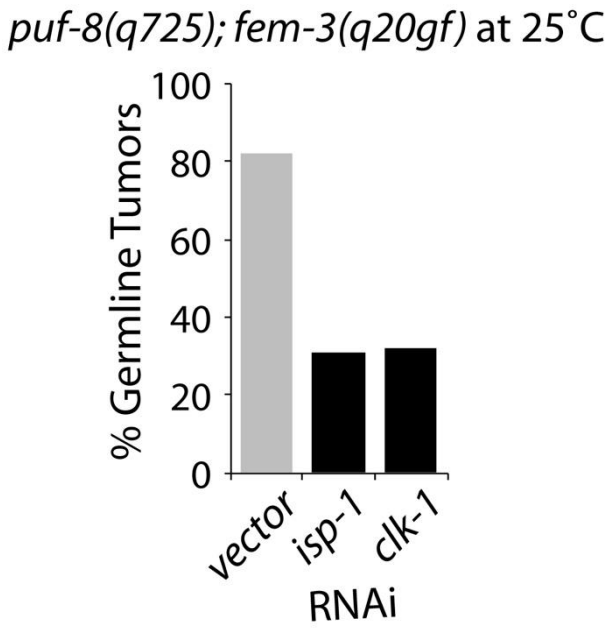


Figure 14. Mitochondrial activity is likely required for the formation of germline tumors in the *puf-8(q725); fem-3(q20gf)* mutant worms. RNAi of *isp-1* or *clk-1* significantly suppressed the formation of *puf-8(q725); fem-3(q20gf)* germline tumors at 25°C. Germline phenotypes were determined by staining dissected gonads with DAPI.

DISCUSSION

Cellular dedifferentiation counteracts the decline of stem cells during aging but has also been implicated in the formation of tumor-initiating cells (TICs) (Bansal and Banerjee, 2009). Thus, a comprehensive examination of what causes stem cells to differentiate into desired cell types, and how committed cells return to undifferentiated cells (i.e., TICs), is a central question in stem cell biology, regenerative medicine, and tumorigenesis (Daley, 2009). This cellular dedifferentiation can take many forms depending on the specific organism and specific tissue type. In zebrafish (Danio rerio), cellular dedifferentiation occurs in a controlled environment in which cardiomyocytes partially dedifferentiate to repopulate lost ventricular tissue (Jopling C. et al., 2010). In Drosophila melanogaster, it has been shown that differentiating germ cells can revert into functional stem cells both in second instar larval ovaries and in adult fruit flies (Kai and Spradling, 2004). Drosophila have also been shown to induce dedifferentiation in spermatogonial cells as there is considerable plasticity due to Jak-STAT signaling (Brawley and Matunis, 2004). Reduction of stem cell division in Drosophila has been shown due to an accumulation of germline stem cells with misoriented centrosomes that increases as the flies age (Cheng et al., 2008). In Mus musculus, dedifferentiated basal-like cells that originated from luminal airway cells can function as stem cells in the repopulation of damaged airway epithelia (Tata et al., 2013). Dedifferentiation in the mouse model has also been shown in the intestine where tumorigenesis is initiated due to increased Wnt-activation allowing for polyp formation to occur (Schwitalla et al., 2013). In each of these different examples of dedifferentiation occurring, different signaling pathways are reverting differentiated cells back into either stem cell-like or TICs.

In *C. elegans*, dedifferentiation in the germline can occur in both spermatogenic (Park et al., 2020) and oogenic (Jones and Schedl, 1995) germlines. In a mutant lacking *gld-1* (a KH-motif containing RNA-binding protein), germ cells destined for oogenesis early in the meiotic cell fate return to a mitotic cell cycle, which results in germline tumors (Jones and Schedl, 1995). In a double mutant lacking *puf-8* and *lip-1*, spermatocytes do not undergo normal meiotic division, but instead return to mitosis, resulting in germline tumors. Notably, the formation of germline tumors via dedifferentiation in both oogenic and spermatogenic germlines were inhibited by the depletion of *mpk-1*. This result indicates that MPK-1 activity may be critical for the formation of germline tumors via dedifferentiation. Consistently, mammalian Ras-ERK MAPK signaling has similarly been implicated in cellular dedifferentiation of Sertoli cells [18], myoblasts [19], and islet cells [20]. In addition to MPK-1, my studies using *puf-8(q725); fem-3(q20gf)* and *fem-3(q20gf)* mutants demonstrated that spermatocyte dedifferentiation also requires *puf-8* depletion. Dr. Lee's lab also examined the germline phenotypes of other PUF mutants in the absence of LIP-1 such as *fbf-1(ok91) ; lip-1(zh15)*, *fbf-2(q704); lip-1(zh15),* and *puf-9(ok1136); lip-1(zh15)*. None of them formed germline tumors, indicating that PUF-8 has a special function in inhibiting spermatocyte dedifferentiation. Therefore, the identification and characterization of PUF-8 target genes, involved in spermatocyte dedifferentiation will be critical to understand the mechanism of spermatocyte dedifferentiation.

Mitochondria constitute the center of heterotrophic aerobic life, processing and generating key metabolites that feed into pathways leading to growth, division, and signaling (Aon & Camara, 2015). One of these key metabolites created by mitochondria is ATP which is

created through respiration and oxidative phosphorylation (Hsu et al., 2016). For cells to differentiate such as mesenchymal stem cells, an upregulation of both mitochondrial biogenesis and aerobic metabolism must occur (Hsu et al., 2016). This upregulation of oxidative phosphorylation has also been shown in hepatic cells where increased mitochondrial fission also occurred (Wanet et al., 2014). However, in this same study on hepatic differentiation, dedifferentiation of cells found a reduction of several mitochondrial proteins involved in oxidative phosphorylation as well fusion of mitochondria which demonstrated opposite changes in mitochondrial morphology between differentiating and dedifferentiating cells (Wanet et al., 2014). In my study, two proteins required for *C. elegans* electron transport chain to properly work, *isp-1* and *clk-1*, were knocked down using RNAi. Previous studies have shown that loss of *isp-1* resulted in the rate of development being severely affected (Suthammarak W, Morgan P.G, & Sedensky M.M., 2010). However, unlike the previous study by Wanet et al., we found a decreased amount of dedifferentiation once mitochondrial function was downregulated. One molecule not mentioned is reactive oxygen species or (ROS) which are created when electrons leak out of the electron transport chain (Hsu et al., 2016). These species can inflict various oxidative damage upon macromolecules (nucleic acids, lipids, and proteins) (Hekimi S, Wang Y, & Noe A., 2016). ROS most importantly have been shown to affect the activation of MAPK, like ERK1/2 (Zhang & Jope, 1999). Although our model shows a reduction in dedifferentiation, there is a possibility that this alternative could have an effect in dedifferentiation in other systems.

What this tells us is that in this specific genetic context, loss of the RNA binding protein PUF-8 combined with the gain-of-function mutation in *fem-3* (q20gf), means that *fem-3* (q20gf) will increase the number of cells going through spermatogenesis. At the same time, *puf-8* being knocked out in the worm would lead to an increase in these cells to dedifferentiate and form

germline tumors. While this finding paints a nice picture of germline dedifferentiation and tumorigenesis it is by no means the complete picture of what is going on here. During the testing of germline tumors with resveratrol and subsequent MPK-1 RNAi, an unexpected finding was that worms fed a diet of OP50 had a lower percentage of tumor formation than those fed the RNAi diet of HT115. We proposed that a possible reasoning for this is that the HT115 *E. coli* provided a greater metabolic energy source due to recycling of excess nucleotides by the bacteria being RNase III-deficient. Due to this increase in energy boosting the formation of germline tumors, we decided to look at the mitochondria's role namely by knocking out specific genes in the electron transport chain *isp-1* and *clk-1*. Both genes are critical to mitochondrial function with *clk-1* (an ortholog of human COQ7 hydroxylase) needed for synthesis of ubiquinone and *isp-1* (an iron sulfur protein of mitochondrial complex III). RNAi of both genes resulted in a dramatic decrease in tumor formation which we believe is due to the impairment of the electron transport chain. However, it is important to note that the role of the gene *isp-1* in complex III deals with cytochrome c which is implicated in both apoptosis and energy production (Feng et al., 2001).

Altogether, findings from this project will not only elucidate the fundamental mechanisms of the differentiation/dedifferentiation decision *in vivo*, but will also provide a novel working platform for the identification of therapeutic targets for dedifferentiation-mediated cellular regeneration and tumorigenesis.

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