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MPK-1/ERK Regulatory Network Controls the Number of Sperm by Regulating Timing of Sperm-Oocyte Switch in *C. elegans* Germline

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Abstract

The precise regulation of germline sexual fate is crucial for animal fertility. In *C. elegans*, the production of either type of gamete, sperm or oocyte, becomes mutually exclusive beyond the larval stage. Hermaphrodites initially produce sperm and then switch to produce oocytes. This change of fate during germline development is tightly controlled by several regulators. In *C. elegans* hermaphrodites, FBF-1 and FBF-2 (>95% identical, members of the Pumilio RNA-binding protein family) proteins function redundantly to promote the sperm-oocyte switch. Here, we demonstrate that loss of LIP-1 (dual specificity phosphatase) in *fbf-1(ok91)* single mutants leads to excess sperm production due to a delayed sperm-oocyte switch. This phenotype was dramatically rescued by depletion of MPK-1 (an ERK homolog). In contrast, loss of LIP-1 in *fbf-2(q738)* single mutants leads to a premature sperm-oocyte switch and loss of sperm. Notably, *fbf-1 fbf-2; lip-1* triple mutants produce excess sperm. These results suggest that the MPK-1/ERK regulatory network, including FBF-1, FBF-2, and LIP-1, controls the number of sperm by regulating the timing of the sperm-oocyte switch in *C. elegans*.

1. Introduction

C. elegans has two sexes, hermaphrodites and males. The self-fertility of hermaphrodites is achieved by spermatogenesis at the L3 stage followed by a switch to oogenesis in late L4 or in young adult stage (the “sperm-oocyte switch”) (Fig. 1A). Males begin spermatogenesis in

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the L3 stage, and this continues through adulthood. In both hermaphrodites and males, sperm production requires *fog-1, fog-3* and the three *fem* genes [1] (see Fig. D). Mutations in any of these genes cause all germ cells to differentiate as oocytes, the Fog phenotype (for feminization of the germline) (Fig. 1B), and mutations in *tra* genes cause hermaphrodites to make sperm instead of oocytes, or the Mog phenotype (for masculinization of germline) (Fig. 1C) [1]. Moreover, the phosphorylation state of FOG-3 probably by MPK-1 (an ERK homolog) modulates the initiation and maintenance of the *C. elegans* sperm fate program [2]. Therefore, the precise regulation of the sperm-oocyte switch is critical for animal fertility.

Fig. 1D shows a simplified version of the germline sex determination pathway (reviewed in [1]). In this pathway, RNA-binding proteins such as FBF-1, FBF-2 (collectively known as FBF), PUF-8, and NOS-3 play important roles in controlling the sperm-oocyte switch. FBF-1, FBF-2, and PUF-8 belong to the PUF (Pumilio/FBF) family of RNA-binding proteins, whereas NOS-3 is one of three *C. elegans* Nanos homologs [3,4,5]. The FBF proteins bind specifically to regulatory elements of the *gld-1* [6], *fem-3* [3], *fog-1* [7], and *fog-3* mRNAs [7,8], to inhibit their mRNA translation. NOS-3 also participates in the sperm-oocyte switch through its physical interaction with FBF, forming a regulatory complex that controls *fem*-3 mRNA [4]. FBF-1 and PUF-8 affect FOG-2 abundance to promote the sperm-oocyte switch in the germline [5].

In this study, we demonstrate that the MPK-1/ERK regulatory network controls the timing of the sperm-oocyte switch in the *C. elegans* germline. Specifically, we found that mutant worms lacking both *fbf-1* and *lip-1* (dual specificity phosphatase; MPK-1/ERK inhibito) genes produced excess sperm by an MPK-1/ERK-mediated delayed sperm-oocyte switch. In contrast, mutant worms lacking both *fbf-2* and *lip-1* genes produced less sperm due to a precocious sperm-oocyte switch. Notably, mutant worms lacking the *fbf-1*, *fbf-2*, and *lip-1* genes continuously produced sperm, strongly suggesting that this triple mutation completely abrogated the sperm-oocyte switch. These results suggest an intricate interplay between FBF-1, FBF-2, and LIP-1 proteins to control the timing of the sperm-oocyte switch through MPK-1 activity during larval development. Importantly, these regulators are broadly conserved, suggesting that a similar molecular mechanism may be observed in other organisms, including humans.

2. Materials and Methods

2.1. C. elegans culture and strains

All strains used in this study were hermaphrodites, and were maintained at 20°C unless otherwise noted [9]. We used the wild-type Bristol strain N2 as well as the following mutants and balancers. *LG I: fog-1(q250)* [10]. Balancer: *hT2[qIs48]; LG II: fbf-1(ok91)* [6]*, fbf-2 (q738)* [11]. Balancer: *mnIn[mIs14 dpy-10(e128)]; LG III: mpk-1(ga111)* [12]; *LG IV: lip-1(zh15)* [13].

2.2. Germline immunohistochemistry

For immunohistochemistry, dissected gonads were fixed with 3% formaldehyde in 0.1 M K_2HPO_4 (pH 7.2) for 10 min at room temperature followed by 100% cold methanol for 5 min at −20°C [14]. After blocking for 30 min with 0.5% BSA in 1x 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 antibodies. MAPK-YT (Sigma, 1:200), SP56 (a gift from Dr. Samuel Ward, 1:400), and RME-2 (a gift from Dr. Barth Grant, 1:200) were used for this study. DAPI staining followed standard methods.

2.3. RNAi experiment

RNAi experiments were performed by feeding bacteria expressing double-stranded RNAs corresponding to the gene of interest [15,16]. RNAi bacteria were from *C. elegans* ORF-RNAi library (Open Biosystems).

2.4. U0126 treatments

Small molecule inhibitoin (U0126) of MEK1/2 was performed using a slightly modified method of the protocol previously described [17]. Briefly, *C. elegans* worms were synchronized by the alkaline hypochlorite method and arrested in M9 media at the first larval (L1) stage. L1 larvae were then plated onto NGM agar plates containing a mixture of 100 μM U0126 and OP50 *E. coli*, and grown at 20°C for 3 days. Fertility was scored under a dissection microscope and/or by germline immunohistochemistry.

2.5. Data analysis

Statistical significance was analyzed using the two-tailed student's t-test and ANOVA. The error bars reflect respective standard deviation values.

3. Results and Discussion

3.1 fbf-1; lip-1 mutants produce excess sperm with incomplete penetrance

In the *C. elegans* germline, FBF-1 and FBF-2 redundantly promote the sperm-oocyte switch at late L4 or young adult stages [6]. Therefore, most *fbf-1(ok91)* and *fbf-2(q738)* loss-of-function mutants produce both sperm and oocytes, and they are self-fertile [6]. However, *fbf-1(ok91) fbf-2(q738)* double mutants produce excess sperm without switching to oogenesis, resulting in a Mog phenotype [6]. We previously reported that MPK-1/ERK is required to promote sperm fate in the *C. elegans* germline [2,17]. Thus, we first tested whether MPK-1 activation by loss of LIP-1 in *fbf-1(ok91)* single mutants could affect the sperm-oocyte switch. To this end, the germline phenotypes of *fbf-1(ok91)* single, *lip-1(zh15)* single, and *fbf-1(ok91); lip-1(zh15)* double mutants were determined under a dissecting microscope. As previously reported, most *fbf-1(ok91)* and *lip-1(zh15)* adult hermaphrodites were self-fertile like wild-type hermaphrodites [11,18,19] (Fig. 2A–2D). However, *fbf-1(ok91); lip-1(zh15)* double mutants were sterile and produced excess sperm (ExSp) with incomplete penetrance (Fig. 2A and 2E). To measure sperm number, we stained adult *fbf-1; lip-1* mutant germlines (1 day after L4) with anti-SP56 (sperm-specific marker) antibody [20] and DAPI (a DNA marker), and we counted the number of mature haploid

sperm within a SP56-positive region (see Fig. 3B). Wild-type, *fbf-1(ok91)*, and *lip-1(zh15)* hermaphrodites (1 day-old adult) possessed about 130 (range: 106–164), 103 (range: 81–103), and 293 (range: 185–385 [21]) sperm per gonadal arm (Fig. 2G). However, adult *fbf-1; lip-1* double mutants produced excess sperm (533, range: 123–866) (Fig. 2G). This result suggests that FBF-1 and LIP-1 proteins redundantly promote the sperm-oocyte switch to produce normal sperm numbers during hermaphrodite larval development.

3.2. fbf-2; lip-1 mutants produce less sperm

We also examined the germline phenotype of *fbf-2(q738); lip-1(zh15)* double mutants. As previously reported, most *fbf-2(q738)* single mutants produced both sperm and oocytes, and were fertile (Fig. 2A). However, some *fbf-2(q738); lip-1(zh15)* double mutants produced less sperm (LeSp), and then showed a Fog-like phenotype after the depletion of the small number of sperm as adults (Fig. 2A and 2F). We also counted the number of mature haploid sperm within a SP56-positive region of adult *fbf-2(q738); lip-1(zh15)* mutant germlines. Intriguingly, *fbf-2(q738); lip-1(zh15)* double mutants exhibited diminished sperm production (56, range: 0–100) probably due to a premature sperm-oocyte switch (Fig. 2G). This result indicates that FBF-2 and LIP-1 proteins redundantly inhibit an accelerate sperm-oocyte switch to keep sperm production within the physiological range during hermaphrodite larval development. Taken together, these results suggest that a regulatory mechanism involving FBF-1, FBF-2, and LIP-1 may be necessary for efficient sperm production by modulating sperm-oocyte switch timing.

3.3. MPK-1/ERK dependence

We previously reported that FBF-1 and LIP-1 proteins inhibit MPK-1/ERK activity at the post-transcriptional and post-translational levels, respectively [22] (Fig. 3A). We thus tested whether the ExSp phenotype observed in *fbf-1(ok91); lip-1(zh15)* mutants is dependent on MPK-1/ERK activity. To this end, we depleted the *mpk-1* mRNA by RNAi from L1 staged *fbf-1(ok91); lip-1(zh15)* mutants at 20°C. Three days later (1 day-old adults), their germline phenotypes were determined by staining dissected gonads with anti-SP56 and anti-RME-2 antibodies. 57% of *fbf-1(ok91); lip-1(zh15)* ExSp sterile germlines stained strongly for SP56 (Fig. 3B). Notably, *mpk-1(RNAi)* dramatically rescued the ExSp sterile phenotype of *fbf-1(ok91); lip-1(zh15)* hermaphrodite mutant germlines (Fig. 3C and 3D). To confirm this result, we employed a temperature-sensitive (ts) *mpk-1(ga111),* loss-of-function mutant (henceforth called *mpk-1(ga111ts)*). The *mpk-1(ga111ts)* mutants are fertile at permissive temperature (20°C), but show a sterile PAC (pachytene arrest) phenotype caused by reduced MPK-1 activity in the germline, at restrictive temperature (25°C) [23]. Notably, *mpk-1(ga111ts)* mutation also fully rescued the ExSp sterile phenotype of *fbf-1(ok91); lip-1(zh15)* mutant germlines and restored fertility even at 20°C (Fig. 3C and 3D). These results suggest that active MPK-1 in f*bf-1(ok91); lip-1(zh15)* distal germlines is required to maintain continuous sperm.

3.4. The distribution of activated MPK-1 in fbf-1(ok91); lip-1(zh15) and fbf-2(q738); lip-1(zh15) hermaphrodite germlines

In a wild-type hermaphrodite germline, activated (phosphorylated) MPK-1 is detected at high levels in the 2 to 3 most proximal oocytes (data not shown) and at moderate levels in

the proximal half of pachytene germ cells [24] (Fig. 3E). In contrast, in a wild-type male germline, activated MPK-1 is accumulated in the proximal mitotic region and transition zone [24] (Fig. 3F). LIP-1 phosphatase inhibits MPK-1 signaling to control germ cell proliferation and G(2)/M meiotic arrest in the wild-type germ line [18,19]. To ask whether the loss of LIP-1 controls germline sexual fate by altering the distribution of activated MPK-1 in *fbf-1(ok91)* and *fbf-2(q738)* mutant germlines, we stained dissected gonads with a monoclonal antibody that specifically recognizes the dual phosphorylation (YT) moieties of the active MPK-1 [22,24]. Notably, phosphorylated MPK-1 was detected in the proximal half of the transition zone and also very early in pachytene cells in *fbf-1(ok91); lip-1(zh15)* mutant germlines (Fig, 3G). This was also seen in the wild-type male germline (Fig. 3F). Conversely, in most *fbf-2(q738); lip-1(zh15)* germlines, phosphorylated MPK-1 was detected in the proximal pachytene cells (Fig. 3H), as seen in wild-type hermaphrodite germlines (Fig. 3E). These results suggest that the localization of activated MPK-1 in the transition zone may be correlated with sperm-oocyte switch and sperm production.

3.5. All fbf-1(ok91) fbf-2(q738); lip-1(lip-1) mutants display a Mog phenotype

FBF-1 and FBF-2 function redundantly to promote the sperm-oocyte switch, but how *lip-1(zh15)* mutation promotes opposite germline phenotypes in *fbf-1(ok91)* and *fbf-2(q738)* mutants remains elusive. A possible scenario is that activated MPK-1 may selectively influence the function of FBF-2. Consistent with this conjecture, we found that mammalian ERK phosphorylated FBF-2, but not FBF-1 (K. Friend unpublished result, personal communication). We thus hypothesized that MPK-1 may partially abrogate the function of FBF-2 in the *fbf-1(ok91); lip-1(zh15)* mutant germlines, leading to excess sperm production (see Fig. 4C and 4D). However, in an *fbf-2(q738); lip-1(zh15)* mutant germline, excess FBF-1 may still promote a premature sperm-oocyte switch, resulting in compromised sperm production (see Fig. 4E). To evaluate the selective effect of activated MPK-1 on FBF-2, we removed *fbf-1* gene in *fbf-2(q738); lip-1(zh15)* double mutants, generating an *fbf-1(ok91) fbf-2(q738); lip-1(zh15)* triple mutant. The germline phenotype of this triple mutant was determined by staining dissected gonads with anti-RME-2 and anti-SP56 antibodies. The results showed that all triple mutants had a complete Mog phenotype (negative for RME-2 and positive for SP56) and produced excess sperm (>500 sperm per gonadal arm; n=10). Next, to test if the Mog phenotype of *fbf-1(ok91) fbf-2(q738); lip-1(zh15)* triple hermaphrodite mutants depends on MPK-1, we depleted MPK-1 activity by either *mpk-1(RNAi)* or U0126 treatment on the *fbf-1(ok91) fbf-2(q738); lip-1(zh15)* mutants. Intriguingly, the Mog phenotype was not rescued either by *mpk-1(RNAi)* or U0126 treatment (Fig. 3D). While it remains unclear how MPK-1 inactivation fails to rescue the Mog phenotype in *fbf-1(ok91) fbf-2(q738); lip-1(zh15)* mutants, it is plausible that this mutant may lack cells responsive to MPK-1 inactivation-mediated sperm-oocyte switch. It was previously reported that sperm-oocyte fate decision occurs in cells undergoing transition from mitosis to meiosis [25]. However, the *fbf-1(ok91) fbf-2(q738); lip-1(zh15)* triple mutants lack mitotic and early meiotic cells as all mitotic germ cells were differentiated into sperm. Understanding this mechanism is beyond the scope of this work but will be an important challenge for future work.

3.6. MPK-1 may act upstream of FOG-1 and FOG-3

The *fog-1* (a CPEB homolog) and *fog-3* (a TOB/BTG homolog) genes are terminal regulators essential for sperm specification. We first tested whether MPK-1 activation in *fbf-1(ok91); lip-1(zh15)* mutants could force sperm production in a *fog-1(q785)* loss-of-function background. All *fog-1(q785)* mutants display a complete Fog phenotype and are exclusively oocyte-producing, sterile worms [10]. Our results showed that loss of both FBF-1 and LIP-1 failed to produce sperm in a *fog-1(q785)* mutant background (Fig. 4A and 4B). Independently, we depleted the expression of *fog-3* by RNAi in wild-type(N2) and *fbf-1(ok91); lip-1(zh15)* mutants. Expectedly, *fog-3(RNAi)* significantly induced Fog phenotype in *fbf-1(ok91); lip-1(zh15)* germlines (Fig. 4A). These results suggest that activated MPK-1 in *fbf-1(ok91); lip-1(zh15)* mutant germlines may act upstream of FOG-1 and FOG-3.

3.7. Proposed working models

Here, we demonstrate that the MPK-1/ERK regulatory network controls sexual fate in *C. elegans* hermaphrodite germlines (Fig. 4C). The significance of our findings is twofold: 1) the MPK-1/ERK regulatory network, including FBF-1, FBF-2, and LIP-1 proteins, controls normal sperm number probably by regulating the timing of the sperm-oocyte switch (Fig. 4C), 2) although FBF-1 and FBF-2 are nearly identical proteins, their role in the sperm-oocyte switch is not interchangeable. A likely scenario is that MPK-1 may selectively inhibit the function of FBF-2 by phosphorylation (Fig. 4C). Specifically, in wild-type hermaphrodite germlines, FBF-1, FBF-2, and LIP-1 proteins inhibit MPK-1 activation and other sperm-promoting genes (e.g., *fem-3*, *fog-1*, and *fog-3*) to expedite the sperm-oocyte switch. However, in *fbf-1(ok91); lip-1(zh15)* hermaphrodite germlines, activated MPK-1 may stimulate excess sperm production, probably through the simultaneous activation of FOGs and inhibition of FBF-2 (Fig. 4D). In *fbf-2(q738); lip-1(zh15)* hermaphrodite germlines, however, excess FBF-1 protein may continue to inhibit *mpk-1* expression and consequently delay the sperm-oocyte switch (Fig. 4E). Also, additional removal of the *fbf-1* gene in *fbf-2(q738); lip-1(zh15)* double mutants promoted a Mog phenotype with complete penetrance (Fig. 4F). Since examined regulators are highly conserved and have been identified in all mammalian germlines, a similar control mechanism of sperm production likely exists in other organisms, including humans.

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Abbreviations

FBF	Fem-3 Binding Factor
LIP-1	Lateral signaling-Induced Phosphatase-1
MPK-1	Mitogen activated Protein Kinase-1

DAPI	4′,6-diamidino-2-phenylindole
CPEB	Cytoplasmic Polyadenylation Element Binding
TOB/BTG	Transducer of Erb2/B-cell Translocation Gene

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Highlights

- FBF-1 and LIP-1 repress excess sperm production by inhibiting MPK-1 activation.
- FBF-2 and LIP-1 promote sperm production by delaying the sperm-oocyte switch.
- MPK-1/ERK controls sperm number by regulating the timing of the sperm-oocyte switch.

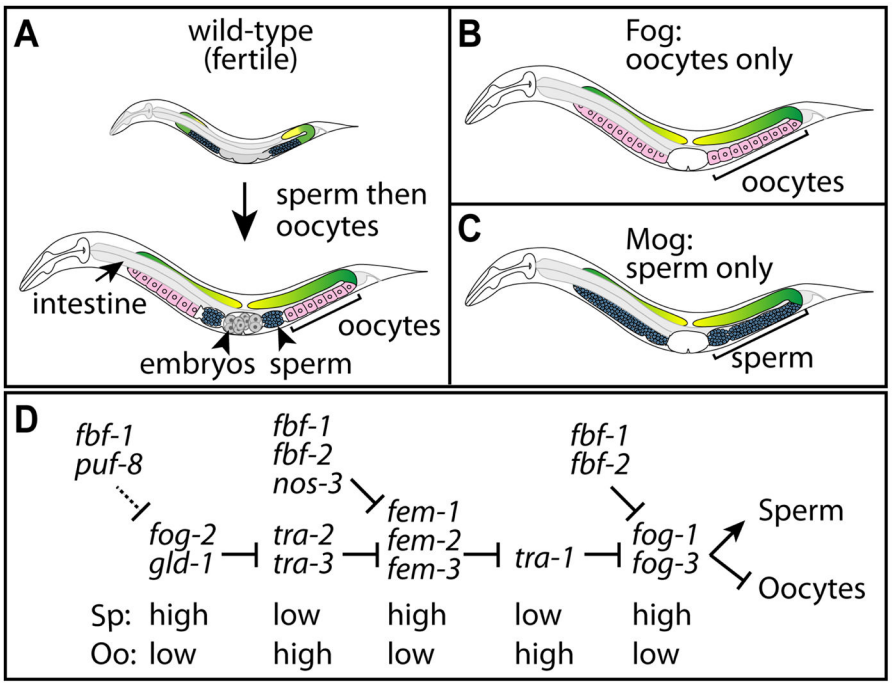


Fig. 1.

C. elegans sexual fate decision and its regulators

(A–C) Schematic germline phenotypes caused by aberrant sperm/oocyte switch. (A) Wild-type hermaphrodite, (B) Fog (for feminization of the germ line), (C) Mog (for masculinization of germline). In each cartoon, anterior is left, dorsal is up. The gonad consists of two arms that share a central uterus. Sperm (dark blue); Oocytes (light pink). (D) Simplified version of the hermaphrodite germline sex determination pathway. Low and high refer to levels of above gene activities.

A

Strain (20°C)	Gametogenesis (%) Sp+Oo	ExSp	Fog	n
wild-type (N2)	100	10	0	100
fbf-1(ok91)	99	1	0	100
lip-1(zh15)	100	0	0	100
fbf-1(ok91); lip-1(zh15)	43	57	0	101
fbf-2(q738)	98	0	2	100
fbf-2(q738); lip-1(zh15)[a]	52	0	48	100

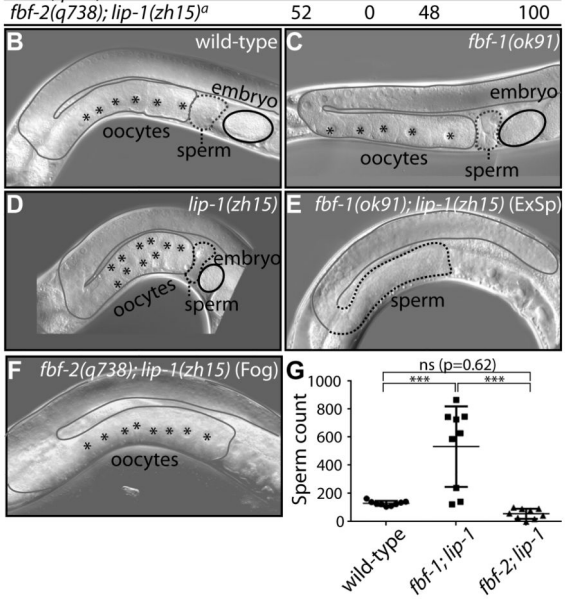
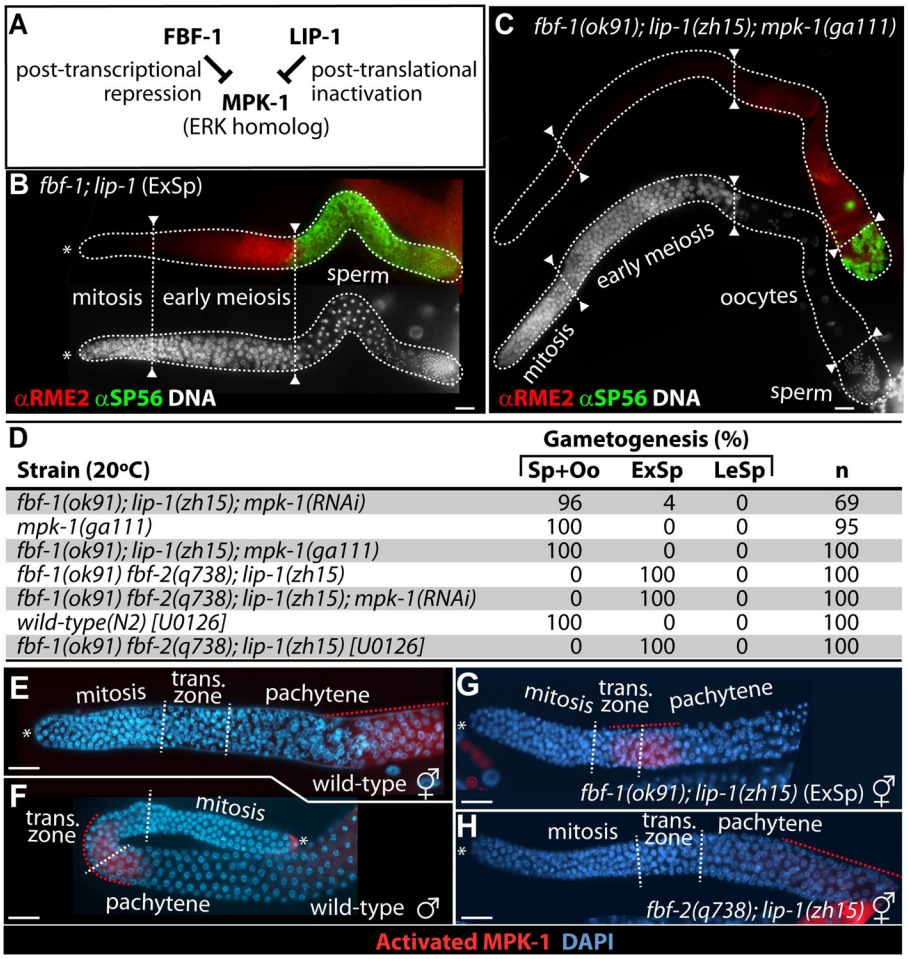


Fig. 2.

Germline phenotypes of *fbf-1(ok91); lip-1(zh15)* and *fbf-2(q738); lip-1(zh15)* mutants.(A) Germline phenotypes of adult hermaphrodites. Sp+Oo, making both sperm and oocytes; ExSp, excess sperm production (>500 sperm per gonadal arm); LeSp, less sperm production (<100 sperm per gonadal arm). Germline phenotype was scored 2 days after L4. (B–E) DIC pictures of adult worms (1 day after L4). Solid grey lines, boundary of gonad; broken lines, sperm; sold black circles, embryos; asterisks, oocyte nuclei. Scale bars: 10 um. (G) Sperm number in hermaphrodite worms. Statistical analysis was performed using ANOVA. n, number of gonadal arms scored. $p<0.0001$ (***). No statistically significant difference (ns).



D

Strain (20°C)	Gametogenesis (%) Sp+Oo	ExSp	LeSp	n
fbf-1(ok91); lip-1(zh15); mpk-1(RNAi)	96	4	0	69
mpk-1(ga111)	100	0	0	95
fbf-1(ok91); lip-1(zh15); mpk-1(ga111)	100	0	0	100
fbf-1(ok91) fbf-2(q738); lip-1(zh15)	0	100	0	100
fbf-1(ok91) fbf-2(q738); lip-1(zh15); mpk-1(RNAi)	0	100	0	100
wild-type(N2) [U0126]	100	0	0	100
fbf-1(ok91) fbf-2(q738); lip-1(zh15) [U0126]	0	100	0	100

Fig. 3.

MPK-1 activity in the distal germline is required for continued sperm production. (A) The inhibition of MPK-1 by FBF-1 and LIP-1 at post-transcriptional and post-translational levels [22]. (B and C) Immunohistochemistry using anti-RME2 (a marker for oocytes) and anti-SP56 (a marker for sperm) antibodies. Scale bars: 10 um. (D) Germline phenotypes of adult worms (1 day after L4). (E–H) Immunohistochemistry using anti-MAPK(YT) antibody that recognizes *C. elegans* activated MPK-1. Broken red lines indicate a region where activated MPK-1 proteins were detected. Asterisks (*), distal end. Scale bars: 10 um.

A

Strain (20°C)	Gametogenesis			n*
	Sp+Oo	ExSp	Fog	
fog-1(q785)	0%	0%	100%	50
fog-3(RNAi)	2%	0%	98%	111
fog-1(q785); fbf-1; lip-1	0%	0%	100%	43
fog-3(RNAi); fbf-1; lip-1	12%	0%	88%	33

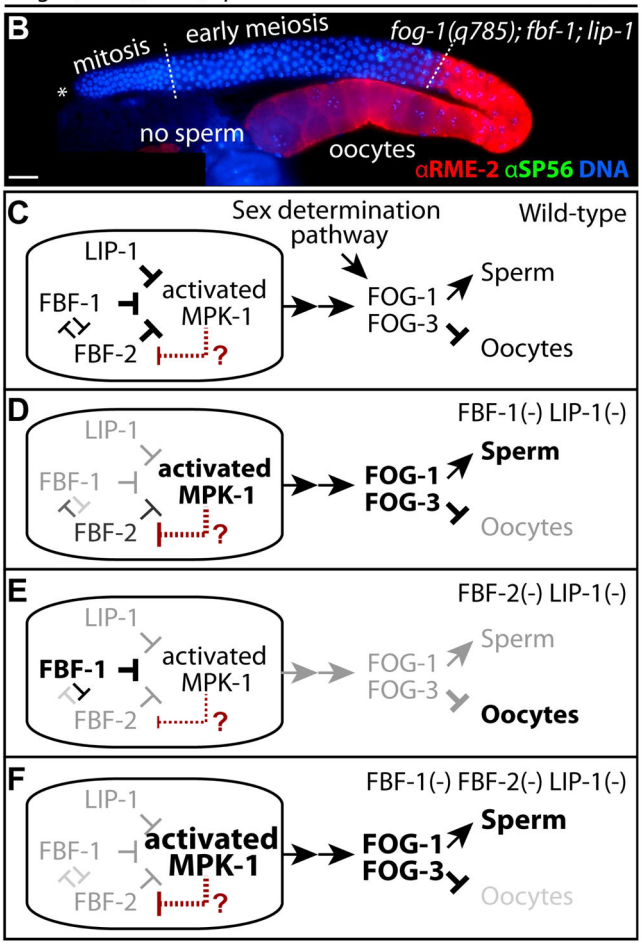


Fig. 4.

MPK-1 acts upstream of FOG-1 and FOG-3. (A) Germline phenotype of adult worms. n*, number of dissected gonad arms scored. (B) Immunohistochemistry using anti-RME2, anti-SP56, and DAPI. Scale bars: 10 um. (C–F) Proposed working models.