Dose-dependent control of proliferation and sperm specification by FOG-1/CPEB

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Summary

RNA-binding proteins control germline development in metazoans. This work focuses on control of the *C. elegans* germline by two RNA-binding proteins: FOG-1, a CPEB homolog; and FBF, a PUF family member. Previous studies have shown that FOG-1 specifies the sperm fate and that FBF promotes proliferation. Here, we report that FOG-1 also promotes proliferation. Whereas *fbf-1 fbf-2* double mutants make ~120 germ cells, *fog-1; fbf-1 fbf-2* triple mutants make only ~10 germ cells. The triple mutant germline divides normally until early L2, when germ cells prematurely enter meiosis and begin oogenesis. Importantly, *fog-1*/+; *fbf-1 fbf-2* animals make more germ cells than *fbf-1 fbf-2* double mutants, demonstrating that one dose of wild-type *fog-1* promotes proliferation more effectively than two doses – at least in the absence of FBF. FOG-1 protein is barely detectable in proliferating germ cells, but abundant in germ cells destined for spermatogenesis. Based on *fog-1* dose effects, together with the gradient of FOG-1 protein abundance, we suggest that low FOG-1 promotes proliferation and high FOG-1 specifies spermatogenesis. FBF binds specifically to regulatory elements in the *fog-1* 3′UTR, and FOG-1 increases in animals lacking FBF. Therefore, FBF represses *fog-1* expression. We suggest that FBF promotes continued proliferation, at least in part, by maintaining FOG-1 at a low level appropriate for proliferation. The dose-dependent control of proliferation and cell fate by FOG-1 has striking parallels with *Xenopus* CPEB, suggesting a conserved mechanism in animal development.

Key words: *C. elegans*, Germline, FBF, FOG-1, CPEB, Sex determination, Mitosis, Meiosis, Sperm, Oocyte

Introduction

Post-transcriptional regulators control both animal development and physiology (Richter, 1999; Wickens et al., 2002). In both invertebrates and vertebrates, two families of RNA-binding proteins control key events in germline development. The PUF family (for Pumilio and FBF) maintains germline stem cells in *C. elegans* and *Drosophila* (Crittenden et al., 2002; Forbes and Lehmann, 1998; Lin and Spradling, 1997) (reviewed by Wickens et al., 2002), and the CPEB family (for cytoplasmic polyadenylation element binding protein) controls progression through meiosis in *C. elegans*, *Drosophila*, *Xenopus* and mouse (Hake and Richter, 1994; Huynh and St Johnston, 2000; Luitjens et al., 2000) (reviewed by Mendez and Richter, 2001) (Tay and Richter, 2001). This work explores the relationship between these two families of RNA regulators during germline development in the nematode *C. elegans*.

The *C. elegans* germline provides a simple model for analyzing molecular and genetic mechanisms that coordinate growth and differentiation. During the first two stages of larval development (L1 and L2), germ cells actively proliferate; during the next larval stage (L3), distal germ cells continue proliferation, while proximal germ cells enter the meiotic cell cycle; during L4 and adulthood, germlines maintain proliferating cells at the distal end while continuously producing sperm or oocytes at the proximal end (see Kimble and Crittenden at http://dev.wormbook.org/). Four major regulatory pathways control growth and differentiation of the germline. Notch signaling promotes proliferation throughout development (Kimble and Simpson, 1997); an RNA regulatory network controls both mitosis/meiosis and sperm/oocyte decisions (Crittenden et al., 2003); the sex determination pathway controls the sperm/oocyte decision (see Ellis and Schedl at http://dev.wormbook.org/); and MAP kinase controls progression through meiosis and oocyte maturation (Church et al., 1995; Miller et al., 2001). Many components of these four regulatory systems are homologous to vertebrate regulators of growth and differentiation (e.g. GLP-1/Notch, FBF/Pumilio, TRA-1/GLI and MPK-1/MAP kinase). Therefore, understanding *C. elegans* germline development has direct implications for regulation of growth and differentiation in vertebrates.

The regulators most crucial for this work are FBF (for *fem-3* binding factor) and FOG-1 (for feminization of the germline). FBF is a collective term for two nearly identical proteins, FBF-1 and FBF-2, which belong to the PUF family of RNA-binding proteins (Wickens et al., 2002; Zamore et al., 1997; Zhang et al., 1997). Like other PUF proteins, FBF-1 and FBF-2 bind 3′UTR regulatory elements and repress target mRNA

expression (Bernstein et al., 2005; Crittenden et al., 2002; Eckmann et al., 2004; Lamont et al., 2004; Wickens et al., 2002; Zhang et al., 1997) (this work). FBF binds to regulatory elements called FBF binding elements (FBEs), for which a consensus sequence has been defined (Bernstein et al., 2005). In *fbf-1 fbf-2* double mutants, germline proliferation is normal until late L3 or early L4, but during L4 all germ cells enter meiosis and differentiate as sperm (Crittenden et al., 2002; Zhang et al., 1997). Therefore, FBF is required to maintain a population of germline stem cells in late larvae and adult animals, and to promote the switch from spermatogenesis to oogenesis in hermaphrodites.

FOG-1 belongs to the CPEB family of RNA regulatory proteins (Jin et al., 2001a; Luitjens et al., 2000). CPEB proteins in *Xenopus* bind U-rich elements, called CPEs (cytoplasmic polyadenylation elements), and thereby regulate both poly(A) tail length and translation of target mRNAs (Mendez and Richter, 2001). The *C. elegans* FOG-1 protein may also bind CPEs (Jin et al., 2001b). Before this study, FOG-1 was thought to have only one function in nematode development – specification of the sperm fate (Barton and Kimble, 1990). In the absence of *fog-1*, germ cells differentiate as oocytes rather than sperm. The sperm/oocyte choice is also controlled by *fog-3,* another germline regulator (Ellis and Kimble, 1995), as well as global sex-determining genes (e.g. Fem genes, *tra-1*) (see Ellis and Schedl at http://dev.wormbook.org/). The global sex-determining genes control *fog-1* and *fog-3* expression (Chen and Ellis, 2000; Jin et al., 2001a), and FOG-1/FOG-3 appear to be terminal regulators of sperm fate.

In this paper, we demonstrate that FOG-1 promotes early larval germline proliferation. Importantly, *fog-1* controls proliferation in a dose-dependent manner. The *fog-1* dose effects, together with FOG-1 immunocytochemistry, suggest that low FOG-1 promotes proliferation, whereas high FOG-1 promotes spermatogenesis. Three lines of evidence demonstrate that FBF represses *fog-1* expression, probably by binding directly to the *fog-1* 3′UTR. Similarly, FOG-3 and FEM-3 promote proliferation, and the *fog-3* 3′UTR also possesses an FBF-binding element. We suggest that FBF may coordinately repress sperm-specifying mRNAs to direct oogenesis and that it represses the *fog-1* mRNA to maintain FOG-1 at a low level appropriate for proliferation.

Materials and methods

Nematode methods

All strains maintained at 20°C unless specified (Brenner, 1974). Mutations and balancers are as follows. *LGI*: *fog-1(q219, q229, q250, q253, q272, q273, q325)* (Barton and Kimble, 1990; Jin et al., 2001b); *fog-3(q520)* (Chen et al., 2000; Ellis and Kimble, 1995). Balancers: *hT2[qIs48]; sep-1(e2406)* and *sys-1(q544)*. *LGII*: *fbf-1(ok91) fbf-2(q704)* (Crittenden et al., 2002). Balancer: *mnIn1[mIs14 dpy-10(e128)]*. *LGIII*: *glp-1(oz112 gf)* (Berry et al., 1997); *glp-1(q175 lf)* (Austin and Kimble, 1987); *unc-36(e251)*; *unc-32(e189)*. *LGIV*: *fem-3(e1996)* (Hodgkin, 1986). Balancer: *gon-3(e2548)*. RNAi was carried out using standard methods (Fire et al., 1998; Timmons and Fire, 1998). For RNAi into *glp-1(gf)* mutants, dsRNA (1 mg/ml) was injected into L4 *glp-1(gf) unc-32/ unc-36 glp-1(lf)* hermaphrodites.

In situ methods

To generate FOG-1 antibodies, rats were injected with keyhole-limpet-hemocyanin-coupled peptides corresponding to amino acids 2-22 of the long FOG-1 isoform (Genemed Synthesis). Extruded germlines were freeze-cracked, fixed with 1% paraformaldehyde and permeabilized with PBS + 0.5% BSA + 0.1% Triton X100; staining was carried out using affinity-purified α-FOG-1 antibodies at a concentration of 1:5 by standard methods (Crittenden and Kimble, 1998). Larvae were fixed as described by Finney and Ruvkun (Finney and Ruvkun, 1990). To stain with rabbit α-RME-2 (Grant and Hirsh, 1999), mouse SP56 (Ward et al., 1986) and rabbit α-PGL-1 (Kawasaki et al., 1998), larvae were freeze-cracked and fixed in –20°C methanol, followed by –20°C acetone (Crittenden and Kimble, 1998). 4′, 6-diamidino-2-phenylindole (DAPI) was included to visualize DNA. Epifluorescent images were captured with a Zeiss Axioskop equipped with a Hamamatsu digital CCD camera, and collected with Openlab 3.1.7. Confocal images were obtained on a Bio-Rad MR1024 confocal microscope and processed using Adobe Photoshop.

For mRNA in situ hybridization, adult male germlines were extruded and stained as described (Jones et al., 1996). Single-stranded probes were amplified from plasmid pJK1047, using primers BT35 (5′ TTACATCACGACGACGAGTTC 3′) and BT36 (5′ GGTACA-ATTCTCGGGAGTCCT 3′).

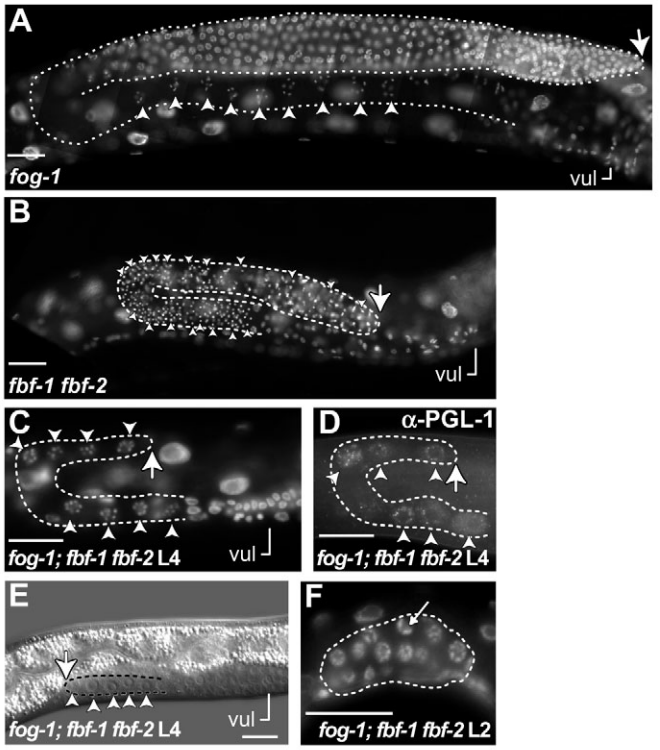
FBE analysis

A consensus FBE (Bernstein et al., 2005) was used to identify candidate sites, and three-hybrid assays were performed as described (Bernstein et al., 2002). DNA oligonucleotides containing predicted FBEs were cloned into pIIIA/MS2-2 vector. Gal4 activation domain fusion proteins with FBF-1 (amino acids 121-614), FBF-2 (amino acids 121-634) or PUF-5 (amino acids 1-553) were expressed from pACT2 plasmids in yeast strain YBZ-1. β-Galactosidase was quantified using the Beta-Glo system (Promega) as described by Hook et al. (Hook et al., 2005). For gel shifts, GST fused FBF-2 (amino acids 121-634) was purified as described (Bernstein et al., 2005) and combined with 100 fMol ^{32}P-end-labeled RNA oligoribonucleotides (IDT and Dharmacon). Shift conditions were identical to those described by Bernstein et al. (Bernstein et al., 2005), and binding constants were calculated as described in Hook et al. (Hook et al., 2005).

Results

A synthetic growth defect in *fog-1; fbf-1 fbf-2* germlines

Adult wild-type XX gonads have ~1000 germ cells in each of two U-shaped arms (Hirsh et al., 1976), and *fog-1* XX gonads have a similar number of germ cells (Fig. 1A). By contrast, *fbf-1 fbf-2* gonads have only ~60 germ cells in each arm (Fig. 1B) (Crittenden et al., 2002). We find that the *fbf* defect in germline proliferation is dramatically enhanced in *fog-1; fbf-1 fbf-2* triple mutants, which produce only about five germ cells/arm on average (n=21 arms, range, 1-10 germ cells) (Fig. 1C). This number was confirmed using α-PGL-1 antibodies to visualize germ cells (Kawasaki et al., 1998) (Fig. 1D) and by Nomarski microscopy (Fig. 1E). Therefore, germ cell number is reduced in *fog-1; fbf-1 fbf-2* triple mutants by ~100-fold compared with wild-type or *fog-1* single mutants, and by ~10-fold compared with *fbf-1 fbf-2* double mutants. We confirmed the synthetic defect using RNAi: *fog-1; fbf(RNAi)* and *fog-1(RNAi); fbf-1 fbf-2* germlines were both similar to *fog-1; fbf-1 fbf-2* triple mutant germlines (not shown). Therefore, the synthetic defect is not due to extraneous mutations on either the *fog-1* or *fbf-1 fbf-2* mutant chromosome. Fig. 1 shows the triple mutant with *fog-1(q250)*, a nonsense mutation and putative null (Jin et al., 2001b) (Table 1, Fig. 2A). We also tested other *fog-1* alleles for the synthetic defect using *fbf*



G Larval germline proliferation

Genotype	Average germ cell number				
	e L1	l L1	L2	L3	Adult
wild-type	2	8	14	42	>2000
fog-1	2	9	15	47	>2000
fbf-1 fbf-2	2	8	14	48	100
fog-1; fbf-1 fbf-2	2	8	11	11	10

Fig. 1. FBF and FOG-1 control larval germline proliferation. (A-C,F) DAPI-stained germlines. Broken lines indicate germlines; small arrowheads indicate sperm; large arrowheads indicate oocytes or oocyte-like cells; arrow in A-E indicates distal end; vul, vulva. *fog-1, fog-1(q250)*. (A) *fog-1* homozygote. Size of adult germline is normal, but only oocytes are made (Barton and Kimble, 1990). (B) *fbf-1 fbf-2* adult. Germline is reduced to ~60 germ cells/arm with only sperm (Crittenden et al., 2002). (C) *fog-1; fbf-1 fbf-2* triple mutant. Germline is reduced to ~10 germ cells (white arrowheads) and no sperm are made. (D) *fog-1; fbf-1 fbf-2* triple mutant, stained with antibodies to the germ cell marker PGL-1; few germ cells are present (white arrowheads). (E) *fog-1; fbf-1 fbf-2* Nomarski micrograph; few germ cells are present (white arrowheads). (F) L2 *fog-1; fbf-1 fbf-2*. Arrow indicates crescent-shaped nucleus typical of early meiosis. Scale bars: 10 μm. (G) Larval germline proliferation. eL1, early L1; lL1, late L1. $n \geqslant 4$ at all stages.

RNAi (Fig. 2A; Table 1). Three nonsense/frameshift mutations, *q219, q272* and *q273,* and one loss-of-function missense mutation, *fog-1(q229)* showed the synthetic defect, while two other alleles, *q253* and *q325,* did not (see next section).

To learn whether the small germ cell number in *fog-1; fbf-1 fbf-2* triple mutants reflects a defect in proliferation or survival, we examined germline proliferation during larval development. In both *fog-1* single mutants and *fbf-1 fbf-2* double mutants, germline proliferation appeared normal for the first three larval stages (L1-L3) (Fig. 1G) (Crittenden et al., 2002). The *fog-1; fbf-1 fbf-2* triple mutant had two primordial germ cells at hatching, which divided during L1 to generate eight germ cells by late L1. However, in L2 larvae, only ~10 total germ cells were present on average, and that number remained constant in L3s and L4s (Fig. 1G). No germ cells were observed outside the gonad; germ cells appeared healthy and no evidence of cell death was seen. Therefore, the defect appears specific for proliferation.

We next asked if the decreased proliferation was accompanied by early entry into meiosis. In wild-type germlines, crescent-shaped nuclei typical of early meiotic prophase are first seen in mid-L3 (Hansen et al., 2004a), and pachytene nuclei are seen a few hours later, just before the molt to L4 (Kimble and White, 1981); gametogenesis does not occur in wild-type germlines until L4. In *fog-1; fbf-1 fbf-2* triple mutants, germline nuclei appeared enlarged and granular during L2 by Nomarski microscopy; after DAPI-staining, crescent-shaped nuclei were observed in some L2 germlines (Fig. 1F). Typical pachytene nuclei were rarely seen, even in later germlines, but 12 univalents were present in some L4 germline nuclei (not shown). Furthermore, as described below, germ cells in the triple mutant were oogenic in L3, much earlier than gametogenesis begins in wild type. Therefore, triple mutant germ cells stop mitotic divisions and enter meiosis earlier than normal, although meiotic prophase does not progress normally. We conclude that FOG-1 can promote proliferation in the early larval germline.

Table 1. Summary of synergistic effects and epistasis

Depletion of *fog-1**	Depletion of *fbf-1 fbf-2*	Germline sex	Enhanced Glp defect
+	Mutant or RNAi	♂	N/A
fog-1(q250)	Mutant or RNAi	♀	Yes
fog-1(RNAi)	Mutant	♀	Yes
fog-1(q273)	RNAi	♀	Yes
fog-1(q219)	RNAi	♀	Yes
fog-1(272)	RNAi	♀	Yes
fog-1(q229)	RNAi	♀	Yes
fog-1(q253)	Mutant or RNAi	♀	No
fog-1(q325)	Mutant or RNAi	♀	No
fog-3(q520)	Mutant or RNAi	♀	Yes
fem-3 m+/z–	Mutant	♀	No
fem-3 m–/z–	Mutant	♀	Yes

**fog-1(q250, 273, q219, q325), fog-3(q520)* and *fem-3(e1996)* are all putative null alleles. *fog-1(q229, q253)* are loss-of-function missense mutations.
N/A, not applicable.

fog-1 dose affects germline proliferation

While constructing strains, we noticed that *fog-1/+; fbf-1 fbf-2* animals made more germ cells than the *fbf-1 fbf-2* double mutant (Fig. 2B,C). Indeed, *fbf-1 fbf-2* mutants made an average of 123 germ cells (n=18, range, 73-192), but *fog-1/+; fbf-1 fbf-2* mutants made an average of 557 germ cells (n=6, range, 261-795). Some *fog-1/+; fbf-1 fbf-2* mutants contained mitotically dividing germ cells into adulthood, which is not seen in *fbf-1 fbf-2* double mutants. The *fog-1/+; fbf-1 fbf-2* germlines made excess sperm and no oocytes (Fig. 2B,C), which is consistent with the presence of FOG-1 (which specifies sperm) and absence of FBF, which promotes oogenesis. All germ cells ultimately differentiated as sperm

(not shown). We conclude that one dose of wild-type *fog-1* is more effective in promoting germline proliferation than are two doses, at least in the absence of FBF. This finding supports the idea that a low level of FOG-1 promotes proliferation, whereas a high level of FOG-1 promotes spermatogenesis (see Discussion).

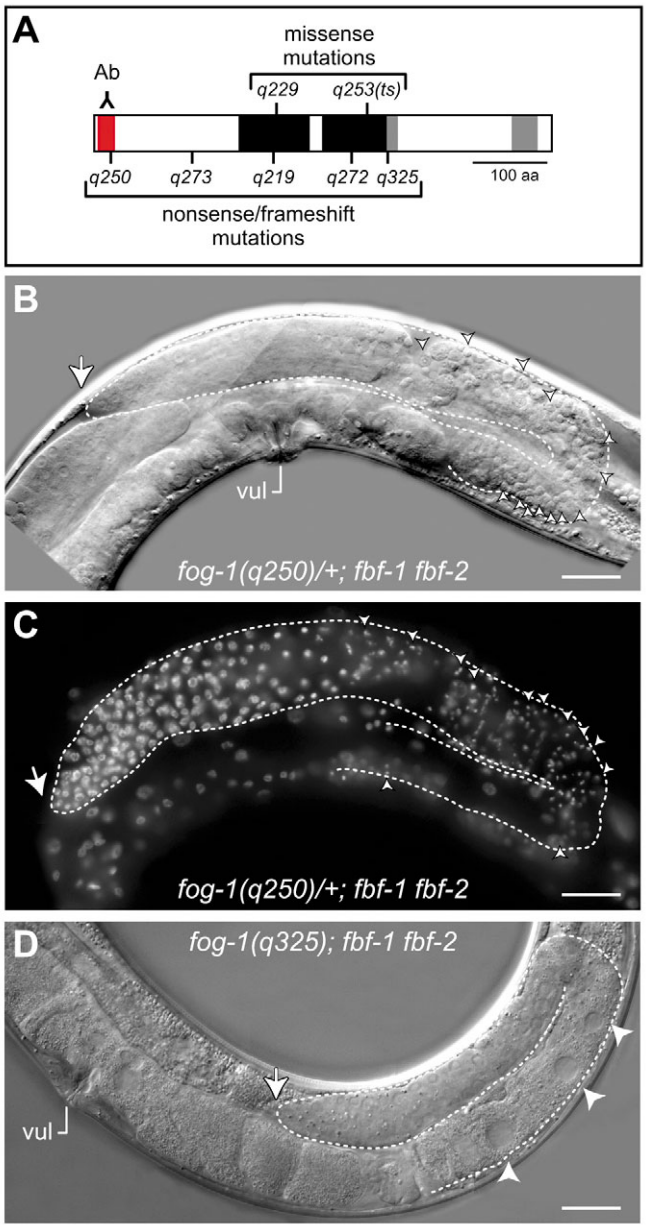


Fig. 2. FOG-1 dose affects germline proliferation. (A) The FOG-1 protein. Red indicates region used to generate FOG-1 antibodies (Ab, inverted Y); black boxes indicate RRM motifs; gray boxes indicate C/H Zn-finger motif. Sites of *fog-1* mutations are marked and bracketed by class of molecular lesion. (B-D) Broken lines indicate germlines; small arrowheads indicate sperm; large arrowheads indicate oocytes or oocyte-like cells; arrow indicates distal end; vul indicates vulva. (B) *fog-1(q250)/+; fbf-1 fbf-2* Nomarski micrograph. *fog-1/+; fbf-1 fbf-2* animals generate more germ cells than *fbf-1 fbf-2* animals; only sperm are made. (C) *fog-1(q250)/+; fbf-1 fbf-2*, DAPI stained. (D) *fog-1(q325); fbf-1 fbf-2* Nomarski micrograph. *fog-1(q325); fbf-1 fbf-2* animals generate more germ cells than *fbf-1 fbf-2* animals, but *fog-1(q325)* homozygotes only make oocytes. Scale bars: 10 μm.

Two *fog-1* mutants (*q325* and *q253*) did not have synthetically small germlines after *fbf* RNAi, although their germlines were fully feminized (Table 1). We confirmed this lack of synergy in triple mutants (Fig. 2D; not shown). The *fog-1(q325); fbf-1 fbf-2* mutants made more germ cells than *fbf-1 fbf-2* mutants: *fog-1(q325); fbf-1 fbf-2* triple mutants possessed an average of 206 germ cells per arm (n=7, range, 110-264), and mitotic divisions were detected in some adult germlines. As *fbf-1 fbf-2* double mutants only make ~60 germ cells per arm (~120 total germ cells), the *fog-1(q325)* allele partially suppressed its proliferation defect. This suppression was abolished by *fog-1* RNAi, suggesting that *fog-1(q325)* possesses residual *fog-1* activity. In *fog-1(q253 ts); fbf-1 fbf-2* triple mutants, germ cell numbers had to be counted at 25°C, a temperature at which *fbf-1 fbf-2* homozygotes make more cells than at lower temperatures (190 germ cells, n=5). The triple mutant made an average of 110 germ cells (n=7), a minor reduction compared with the double; however, germ cell number was further reduced after *fog-1* RNAi, again suggesting residual FOG-1 in the triple. A simple explanation is that these two non-null *fog-1* alleles made enough functional FOG-1 to support proliferation, but not enough to drive spermatogenesis. We conclude that the *fog-1* effect on germline proliferation is separable from its effect on germline feminization.

FOG-1 and FBF function downstream of Notch and upstream of *gld/nos*

We next investigated the relationship of *fog-1* and *fbf* with other regulators of germline proliferation. One key regulatory pathway is Notch signaling: in mutants lacking the GLP-1/Notch receptor, only one or two germ cell divisions occur before entry into meiosis (Austin and Kimble, 1987). To determine whether *fog-1* and *fbf* act downstream of GLP-1/Notch signaling, we employed a *glp-1* gain-of-function (gf) mutant that renders the germline tumorous. All *glp-1(gf)* homozygotes have a tumorous germline, but *glp-1(gf)/glp-1(lf)* heterozygotes can produce a few progeny before becoming tumorous (Berry et al., 1997). We co-injected *fog-1* and *fbf* dsRNAs into L4 *glp-1(gf)/glp-1(lf)* hermaphrodites. Among their progeny, 11 out of 19 *glp-1(gf)* homozygotes had small germlines with oocyte-like cells extending to the distal end (Fig. 3A). By contrast, neither *fog-1* RNAi (n=14) nor *fbf* RNAi (n=35) alone affected the *glp-1(gf)* tumors (Fig. 3B,C). Therefore, *fog-1* and *fbf* are likely to function downstream of Notch signaling (Fig. 3D). We conclude that Notch stimulation of germline proliferation is abolished when both *fbf* and *fog-1* are depleted. This finding suggests that Notch signaling promotes proliferation by controlling FBF and FOG-1. The *fbf-2* gene appears to be a direct target of Notch signaling (Lamont et al., 2004), but Notch targets that impinge on FOG-1 activity are unknown.

FBF promotes proliferation in the late larval germline, at least in part, by repressing mRNAs in each of two meiosis-promoting branches (see Fig. 3D) (Crittenden et al., 2002; Eckmann et al., 2004). To determine where *fbf* and *fog-1* act in relation to *gld/nos* regulators, we depleted *fog-1* by RNAi in quadruple mutants that lack the two Fbf genes and one gene from each meiosis-promoting branch. All *fbf-1 fbf-2 gld-3*

nos-3; fog-1(RNAi) and *fbf-1 fbf-2; gld-1 gld-2; fog-1(RNAi)* homozygotes had tumorous germlines, while control *fbf-1 fbf-2; fog-1(RNAi)* animals phenocopied the *fog-1; fbf-1 fbf-2* mutant (n>15 for each genotype). To confirm this result, we performed the reciprocal experiment, depleting *gld-1* and *gld-2* by RNAi in *fog-1; fbf-1 fbf-2* animals. Again, *fog-1; fbf-1 fbf-2; gld-1(RNAi) gld-2(RNAi)* animals were tumorous. To test if removal of *nos-3* alone might suppress the *fog-1; fbf-1 fbf-2* proliferation defect, we depleted *nos-3* by RNAi in *fog-1; fbf-1 fbf-2* mutants. *fog-1; fbf-1 fbf-2*; *nos-3(RNAi)* animals maintained a mitotic region and generated only oocytes (n=17). We conclude that *fog-1* and *fbf* act upstream of *gld/nos* genes to promote proliferation (Fig. 3D).

FBF and FOG-1 in germline sex determination

The *fog-1* germline makes only oocytes (Fig. 1A) (Barton and Kimble, 1990), but *fbf-1 fbf-2* germlines make only sperm (Fig. 1B) (Crittenden et al., 2002; Zhang et al., 1997). In *fog-1; fbf-1 fbf-2* triple mutants, germ cells appeared oocyte-like. By Nomarski, these cells were larger than mitotic germ cells and somewhat granular, but not as large as typical oocytes (Fig. 2A). We therefore used antibodies to assess gamete differentiation. Specifically, we used the RME-2 yolk protein receptor as the oocyte marker (Grant and Hirsh, 1999) and SP56 as the sperm marker (Ward et al., 1986). Germ cells in *fbf-1 fbf-2* double mutants stained with the sperm marker (Fig. 4A), but not the oocyte marker (Fig. 4B); by contrast, *fog-1; fbf-1 fbf-2* germ cells failed to express the sperm marker (Fig. 4C), but expressed the oocyte marker (Fig. 4D). Consistent with an early entry into meiosis, germ cells began to express the oocyte marker in L3 germlines and continued to express it in L4 germlines. We conclude that *fog-1; fbf-1 fbf-2* germ cells differentiate as oocytes and that *fbf* acts upstream of *fog-1* in the sex determination pathway. A simple interpretation is that FBF represses *fog-1* expression to promote the hermaphrodite switch from spermatogenesis to oogenesis (Fig. 4E).

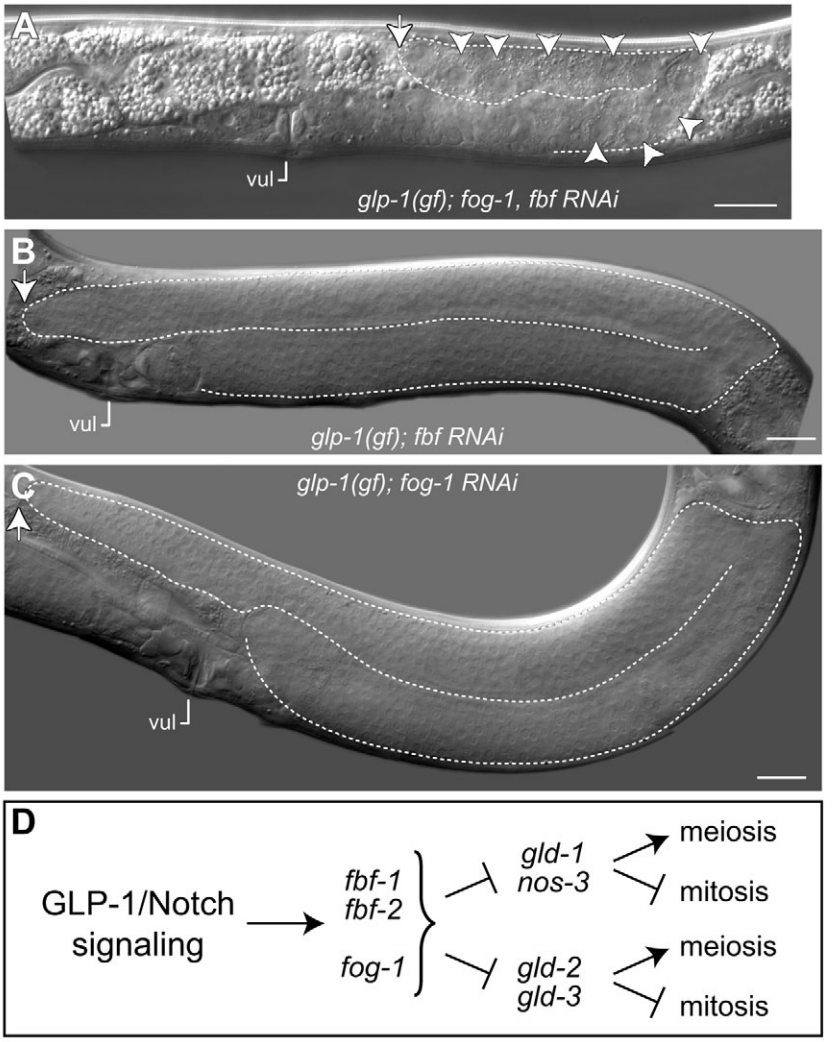


Fig. 3. FBF and FOG-1 act downstream of Notch signaling. (A-C) Large arrowheads, oocytes or oocyte-like cells; arrow indicates distal end; vul, vulva. (A) *glp-1(gf); fog-1(RNAi); fbf(RNAi)* germlines possess small granular oocyte-like cells extending to distal end (arrow). (B) *glp-1(gf); fbf(RNAi)* germlines are tumorous. (C) *glp-1(gf); fog-1(RNAi)* germlines are tumorous. Scale bars: 10 μm. (D) Model for FBF and FOG-1 in germline proliferation pathway.

Control of germline proliferation by other sex-determining regulators

The *fog-1* gene is not the only sex-determining gene required for specification of the sperm fate; in addition, *fem-1*, *fem-2*, *fem-3* and *fog-3* are all crucial (see Ellis and Schedl at http://dev.wormbook.org/). To determine whether other Fem/Fog genes also affect germline proliferation redundantly with *fbf*, we focused on *fog-3* and *fem-3*. The *fog-1* and *fog-3* genes have much in common: both are germline-specific, essential for the sperm fate, controlled by *fem* genes, repressed by TRA-1 and act at the end of the sex-determination pathway (Barton and Kimble, 1990; Chen and Ellis, 2000; Ellis and Kimble, 1995; Jin et al., 2001a; Luitjens et al., 2000). We found that *fog-3; fbf-1 fbf-2* triple mutants made ~10-fold fewer germ cells than *fbf-1 fbf-2* (approximately seven germ cells/arm; n=31). Therefore, *fog-3,* like *fog-1,* controls early larval germline proliferation redundantly with *fbf*.

We also analyzed *fbf-1 fbf-2; fem-3* triple mutants. The *fem-3* gene has a strong maternal effect (Barton et al., 1987; Hodgkin, 1986), so we examined mutants derived from *fem-3* homozygous parents. Such progeny are called *fem-3(m–z–),* because they possess no maternal (m) or zygotic (z) *fem-3*. The *fbf-1 fbf-2; fem-3(m–z–)* germline was indistinguishable from that of *fog-1; fbf-1 fbf-2* (Table 1; not shown). By contrast, *fbf-1 fbf-2; fem-3(m+z–)* animals (which retain maternal, but lack zygotic, *fem-3*) had more germ cells than *fbf-1 fbf-2* double mutants and were similar in size to *fog-1/+; fbf-1 fbf-2* germlines. Mitotic divisions were observed in some *fbf-1 fbf-2; fem-3(m+z–)* adult germlines, and only oocytes were made. Therefore, maternal *fem-3* is sufficient to achieve some germline proliferation, but

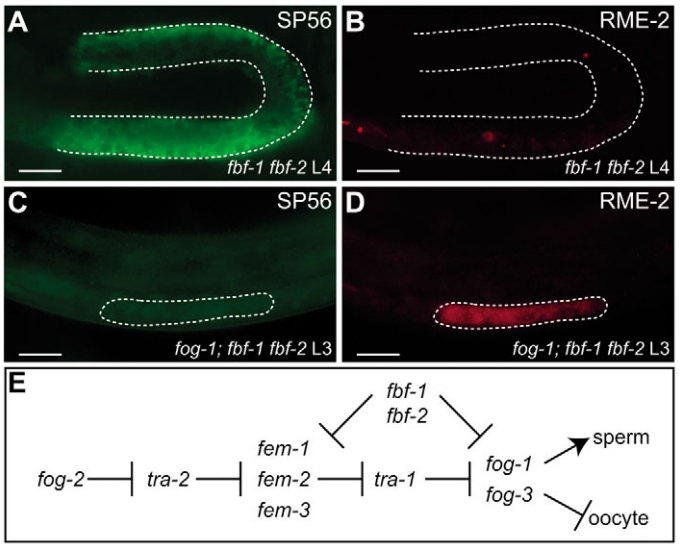


Fig. 4. FBF acts upstream of *fog-1* in the sperm/oocyte decision. (A-D) Broken lines indicate germlines. Green indicates sperm-specific SP56 marker; red indicates oocyte-specific RME-2 marker. Genotypes, stages and markers are indicated in micrographs. (A,C) *fbf-1 fbf-2* mutants express sperm marker, but not oocyte marker. (B,D) *fog-1; fbf-1 fbf-2* mutants do not express sperm, but express oocyte marker. Scale bars: 10 μm. (E) Model for FBF repression of multiple genes in germline sex determination pathway.

not sufficient for specification of sperm. Importantly, germline proliferation in *fbf-1 fbf-2; fem-3(m+z–)* is dependent on *fog-1*; it is abolished if FOG-1 is depleted by RNAi. Thus, *fem-3* effects on proliferation may be explained by absence of FOG-1 in *fem-3(m–z–); fbf-1 fbf-2* germlines and low FOG-1 in *fem-3(m+z–); fbf-1 fbf-2* germlines. We suggest that the sex-determining pathway influences germline proliferation by controlling FOG-1 and FOG-3.

FBF binds the *fog-1* and *fog-3* 3′UTRs

The synthetic proliferation defect shows that *fbf* and *fog-1* are redundant at some level. However, *fog-1* is epistatic to *fbf* in sex determination, suggesting that *fog-1* mRNA may also be a target of FBF repression. Therefore, we examined the *fog-1* 3′UTR for FBF binding elements (FBEs). Three putative FBEs were identified using the consensus UGURHHAUW (where H is A, C or U and W is A or U) (Fig. 5A,B) (Bernstein et al., 2005). FBF-1 and FBF-2 interacted specifically with each of these FBEs in the yeast three-hybrid system (Fig. 5C,D), but did not interact with mutant RNAs in which the core UGU was replaced with ACA (Fig. 5B,D). Another *C. elegans* PUF protein, PUF-5, failed to interact with the FBEs (Fig. 5D). We confirmed that the FBF/FBE interactions were direct, specific and high affinity, using a gel electromobility shift assay. Purified recombinant FBF-2 bound to ^{32}P-labeled synthetic RNAs containing each site, suggesting that the protein-RNA interaction was direct (Fig. 5C). This interaction was not observed in RNAs carrying a UGU to ACA substitution in the core element, suggesting the interaction is specific. Apparent binding constants were determined for the interaction of FBF-2 with each FBE and found to be consistent with known targets of FBF regulation (see below).

The binding of FBF to two adjacent sites, *fog-1* FBE bc, was particularly robust (Fig. 5D,E). These binding sites are predicted to overlap, though their core elements are distinct. We assayed the FBF binding to an RNA carrying a UGU to ACA substitution in one or both FBEs (Fig. 5B). FBF bound the wild-type RNA strongly; the level of β-galactosidase activity in three-hybrid assays (Fig. 5D) and apparent binding constant in gel shifts (Fig. 5E) were similar to those reported for the strong interaction between FBF and an FBE in the *gld-1* 3′UTR (Bernstein et al., 2005). This wild-type *fog-1* FBE bc RNA yielded two complexes, one comparable in mobility with that obtained with the single FBE a (Fig. 5E, left), and the other migrating more slowly (Fig. 5E, center). This ‘supershift’ was reduced, although still detectable, when either FBE was mutated (Fig. 5E, center and right). We conclude that FBF binds specifically to all three FBEs in the *fog-1* 3′UTR, and that the overlap of two FBEs creates a particularly strong binding site.

We also examined other *fem* and *fog* mRNAs for putative FBEs (Fig. 5A,B). We found one FBE in the *fog-3* 3′UTR; both FBF-1 and FBF-2 interacted specifically with this element in the yeast three-hybrid system, and FBF-2 bound it specifically in vitro (Fig. 5D,E). We also found one FBE in the *fem-1* 3′UTR; both FBF-1 and FBF-2 interacted specifically with this element in yeast, but FBF-2 did not bind it in the gel retardation assay. Such a discrepancy is unusual (Bernstein et al., 2005). The *fem-2* 3′UTR carried two UGURxxAU sequences, but these did not conform to the more restricted UGURHHAUW consensus (Fig. 5D,E) and did not bind FBF (data not shown). The *fog-2* 3′UTR possessed no potential FBEs. We conclude that FBF binds FBEs in the *fog-1* and *fog-3* 3′UTRs, in addition to the *fem-3* FBE identified in previous work (Zhang et al., 1997).

FOG-1 expression and its regulation by FBF

The *fog-1* dose effects predicted that FOG-1 might be less abundant in proliferating germ cells and more abundant in cells destined for spermatogenesis. In addition, genetic epistasis and the identification of FBEs in the *fog-1* 3′UTR predicted that *fog-1* expression might be subject to FBF repression. To test these predictions, we raised rat polyclonal antibodies against the long isoform of FOG-1, which is the crucial isoform for *fog-1* function (Jin et al., 2001a).

In wild-type animals, FOG-1 protein was observed in the germline and was predominantly cytoplasmic (Fig. 6). In L2s, FOG-1 became detectable, but staining was faint (Fig. 6A). In L3s, the level of FOG-1 remained low distally in proliferating germ cells, but FOG-1 was abundant more proximally in germ cells that had entered meiosis and were destined for spermatogenesis (Fig. 6B). Temperature shift experiments with a *fog-1(ts)* allele showed that FOG-1 specifies spermatogenesis in L3 when germ cells enter meiosis (Barton and Kimble, 1990), consistent with the idea that the abundant FOG-1 in early meiotic germ cells is specifying the sperm fate. In adult male germlines, FOG-1 was spatially graded: FOG-1 was either not detected or barely visible in the distal half of the mitotic region, became detectable in the proximal half of the mitotic region where some germ cells have entered pre-meiotic S phase (Hansen et al., 2004a) (S. Crittenden, personal communication), intensified in the transition zone and remained high in distal

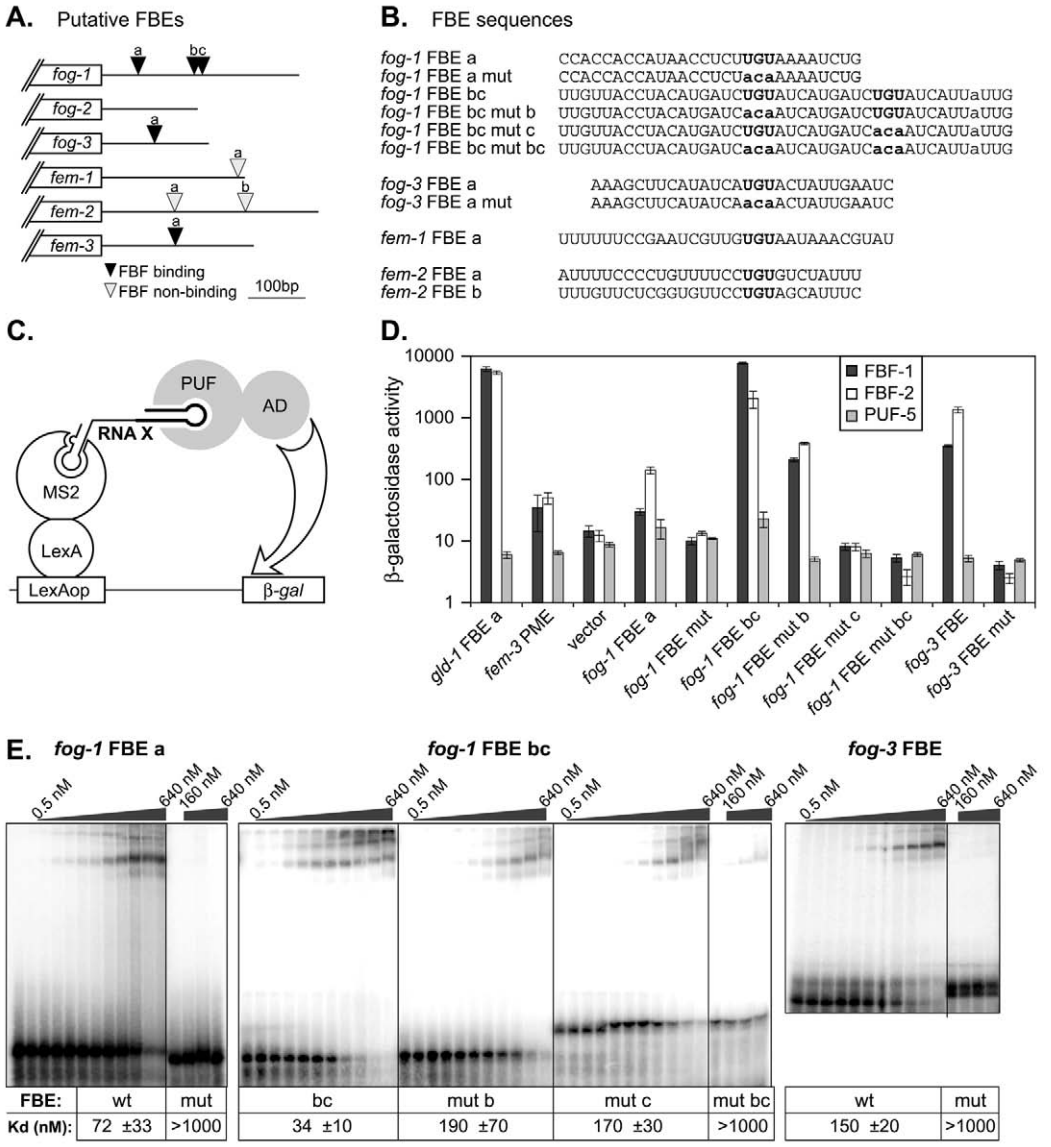


Fig. 5. FBF binds specifically to elements in *fog-1* and *fog-3* 3′UTRs. (A) Putative FBF binding elements (FBEs) in *fog* and *fem* 3′UTRs; black triangles, elements that bind in vitro; gray triangles, elements that do not bind in vitro. (B) Nucleotide sequence of predicted FBF binding elements. Sequences are aligned by conserved UGU motif (bold). Mutated nucleotides are lowercase. [UUUUU towards 3′ end of *fog-1* FBE bc RNA was disrupted to increase expression in three-hybrid system (indicated by 'a').] (C) Yeast three-hybrid assay. (D) Yeast three-hybrid results. Error bars represent standard deviation of at least four repetitions. *gld-1* FBEa, element in *gld-1* 3′UTR (Bernstein et al., 2005; Crittenden et al., 2002); *fem-3* PME, element in *fem-3* 3′UTR (Zhang et al., 1997), which is called *fem-3* FBEa in A. (E) FBF-2 binds FBEs in *fog-1* and *fog-3* 3′UTRs. Protein concentrations were 0.5 nM, 1 nM and 5 nM, then consecutively doubled to reach 640 nM. Apparent binding constants are shown below each shift.

pachytene germ cells; no FOG-1 was detected in more proximal pachytene cells (Fig. 6E). This adult male pattern of *fog-1* expression was confirmed by in situ hybridization using an antisense probe to detect *fog-1* mRNA (Fig. 6F); no RNA was seen with a sense probe (not shown). Germlines dissected from *fog-1(q250)* mutant males had no detectable FOG-1 protein (Fig. 6G), consistent with its being a null allele and demonstrating specificity of the antibody. In contrast to adult male germlines, no FOG-1 was detected in adult hermaphrodites (Fig. 6H). Therefore, FOG-1 expression is sexually dimorphic in adults. FOG-1 is graded in spermatogenic germlines: FOG-1 is low or undetectable in proliferating cells but abundant in cells entering the meiotic cell cycle and destined for spermatogenesis. By contrast, FOG-1 is not detected in oogenic germlines.

In *fbf-1 fbf-2* mutants, FOG-1 levels were elevated compared with wild-type (compare Fig. 6A,C for L2 with Fig. 6B,D for L3). In both L2 and L3, FOG-1 was easily detectable or abundant in all *fbf-1 fbf-2* germ cells (Fig. 6C,D). Therefore, FBF repression is required for maintaining low FOG-1 in L2 germlines and for establishing the FOG-1 spatial gradient in L3 germlines.

To assess FOG-1 in germlines that possess proliferative cells throughout the entire tissue, we stained germlines that are tumorous in the presence and absence of FBF. The quantity of FOG-1 protein was low in *gld-3 nos-3* tumorous germlines (Fig. 6I), but consistently higher in *fbf-1 fbf-2 gld-3 nos-3* tumorous germlines (Fig. 6J). We conclude that the FBF represses *fog-1* expression in vivo and that FBF is required for establishing the temporal and spatial pattern of FOG-1 expression.

Discussion

In this work, we demonstrate that FOG-1, a member of the CPEB family of RNA-binding proteins, promotes proliferation in addition to its previously known role in sperm specification. Our results support the idea that FOG-1 activity is concentration dependent, with a low level promoting proliferation and a higher level directing the sperm fate. The

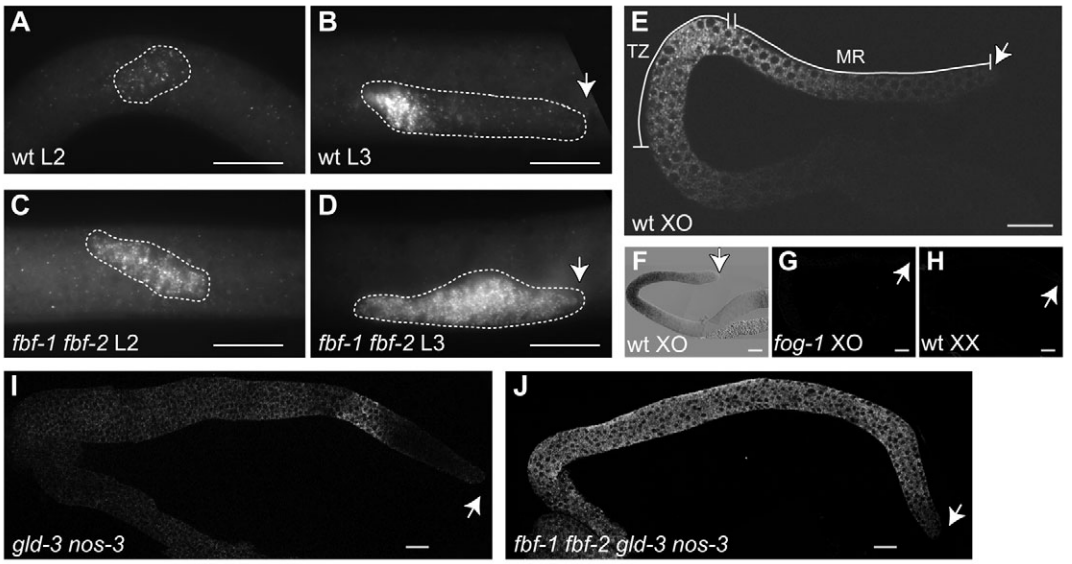


Fig. 6. FBF represses *fog-1* expression in vivo. (A-J) Arrow indicates distal end. Scale bars: 10 μm. (A-E,G-J) Animals or dissected germlines stained with α-FOG-1 antibodies. Broken lines indicate germlines in A-D. (A) Wild-type L2. FOG-1 is barely detectable. (B) Wild-type L3. FOG-1 is abundant in proximal germline where cells are destined to become sperm; FOG-1 is low in distal germline where cells remain in mitotic cell cycle. (C,D) L2 *fbf-1 fbf-2*. FOG-1 is more abundant than in wild type (compare with A) and its distribution is not graded. (D) L3 *fbf-1 fbf-2*. FOG-1 is more abundant than in wild type (compare with B) and its distribution is not graded. (E) Wild-type adult male germline. MR, mitotic region; TZ, transition zone. FOG-1 levels are low in mitotic region and become high as germ cells begin entry into meiosis in proximal part of mitotic region and transition zone. FOG-1 disappears in more mature stages of meiotic prophase. (F) Wild-type adult male germline hybridized with *fog-1* antisense probe. *fog-1* mRNA has same pattern as FOG-1 protein (compare with E). Sense probe has no detectable staining (not shown). (G) *fog-1(q250)* homozygous male germline. No FOG-1 protein is detectable. (H) Wild-type adult hermaphrodite germline. No FOG-1 protein is detectable. (I) Adult *gld-3 nos-3* tumorous germline. FOG-1 levels are low. (J) Adult *fbf-1 fbf-2 gld-3 nos-3* tumorous germline. FOG-1 levels increase in the absence of FBF (compare with I).

temporal and spatial pattern of FOG-1 protein expression is controlled, at least in part, by FBF, a member of the PUF family of RNA-binding proteins. The following discussion highlights these key points and presents parallels in other organisms.

FOG-1 promotes germline proliferation and spermatogenesis

FOG-1 is required for sperm specification (Barton and Kimble, 1990), whereas FBF is required for germline proliferation during late larval development and adulthood (Crittenden et al., 2002). We show that FOG-1 can also promote germline proliferation and that it does so redundantly with FBF. The ability of FOG-1 to control both proliferation and sperm specification provides a molecular link between these two biological processes. This regulatory link is not new. Many genes regulate both proliferation and sex determination in the *C. elegans* germline. Examples include FBF (Crittenden et al., 2002; Zhang et al., 1997), GLD-1 (Jan et al., 1999; Kadyk and Kimble, 1998), GLD-3 (Eckmann et al., 2004; Eckmann et al., 2002) and NOS-3 (Eckmann et al., 2004; Hansen et al., 2004b; Kraemer et al., 1999). We can now add FOG-1, FOG-3 and FEM-3 to this list.

A major challenge for the future is to understand how FOG-1 and FOG-3 control both proliferation and sperm specification. One intriguing possibility is that a central aspect of sperm specification is cell cycle control. Spermatogenic cells continue to divide rapidly, albeit by meiotic divisions, whereas a conserved aspect of oogenesis is cell cycle arrest and growth. The *fog-1* gene appears to specify sperm as germ cells enter meiosis, a conclusion based on temperature shifts (Barton and Kimble, 1990) and FOG-1 expression (this work, see below). We speculate that FOG-1 and FOG-3 may control cell cycle regulators to both control mitosis and specify sperm. We do not know if the FOG-1 regulation of proliferation effects a male mode of the mitotic cell cycle. One argument against this idea is that FOG-1 can promote mitosis in an oogenic germline (e.g. *fog-1(q325); fbf-1 fbf-2* or *fem-3(m+z–); fbf-1 fbf-2*). However, these mutant germlines are aberrant, and in wild-type germlines, FOG-1-dependent mitoses generate primary spermatocytes, both in early larval hermaphrodite development and in males.

FOG-1 levels controls distinct germline fates

One dose of wild-type *fog-1* promotes proliferation better than two doses, and FOG-1 is less abundant in proliferative cells and more abundant in cells entering the meiotic cell cycle and destined for spermatogenesis. These two lines of evidence lead us to propose that the level of FOG-1 may determine biological outcome. Fig. 7A depicts this idea, with a broken line indicating the threshold of FOG-1 activity, low FOG-1 promoting mitosis and high FOG-1 specifying sperm. The *fog-1* dose effects are only seen in a mutant background that lacks FBF, which normally represses FOG-1 levels. One simple view of those dose effects is provided in Fig. 7A. Briefly, two wild-type *fog-1* genes lead to a rapid accumulation of high FOG-1, the threshold is crossed and all germ cells are driven into spermatogenesis; one wild-type *fog-1* gene generates high FOG-1 levels more slowly, the threshold is crossed later and

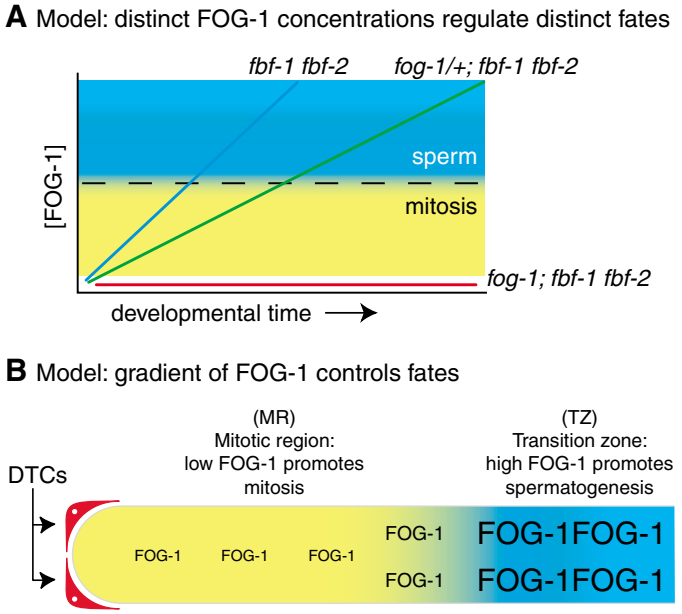


Fig. 7. Model for control of germline fate by FOG-1 abundance. (A) Threshold model for FOG-1 control of two germline fates. [FOG-1], hypothetical FOG-1 concentration; broken line, threshold of FOG-1 concentration; yellow, low FOG-1 promotes mitosis; blue, high FOG-1 that specifies spermatogenesis. *x*-axis, developmental time. In *fbf-1 fbf-2* double mutants (blue line), FOG-1 protein increases with time and ultimately promotes spermatogenesis in all germ cells. In *fog-1/+; fbf-1 fbf-2* animals (green line), FOG-1 levels are lower than in *fbf-1 fbf-2* double mutants, but they still increase with time; as a result, mitosis continues longer and more germ cells are generated before FOG-1 accumulates to a level that promotes the sperm fate. In *fog-1; fbf-1 fbf-2* triple mutants (red line), no FOG-1 is generated, few mitotic divisions occur (presumably under control of some other regulator), and no sperm are specified. The FOG-1 increase with time is depicted as linear for simplicity, but it may well increase non-linearly because of a combination of FBF repression and positive autoregulation (see text). (B) Model for FOG-1 spatial gradient in adult male germline. FOG-1 levels are depicted by font size. We suggest that low FOG-1 in the mitotic region promotes proliferation, whereas high FOG-1 in distal MR and transition zone specifies the sperm fate as germ cells enter meiosis.

more mitotic divisions occur; but the absence of wild-type *fog-1* results in few mitotic divisions and oogenesis. A more complex idea, which is perhaps more likely, is that the FOG-1 increase is not linear. Such a non-linear increase might result from the dual regulation of FOG-1 abundance by FBF repression (this work) and FOG-1 positive autoregulation (Jin et al., 2001b).

Fig. 7B diagrams the FOG-1 distribution in the wild-type adult male germline, and shows how the FOG-1 spatial gradient fits with the idea that distinct FOG-1 levels promote distinct fates. Briefly, low FOG-1 is present in the adult mitotic region, whereas a higher level is observed as germ cells enter meiosis. This spatial gradient is mimicked temporally during larval development. Therefore, although the idea that *fog-1* dose is important was formulated from results in *fbf-1 fbf-2* mutants, those ideas are supported by FOG-1 expression in wild-type germlines.

How might FOG-1 promote proliferation at a low level and spermatogenesis at a high level? One idea is that CPEB monomers and CPEB multimers affect translation of target mRNAs differently (Mendez et al., 2002). This idea derives from the observation that in *Xenopus*, Mos, which contains a single CPE site, is activated at a high CPEB concentration, whereas cyclin B1, which contains two CPE sites, is activated at a low CPEB concentration and repressed at a high concentration. Thus, Mendez et al. (Mendez et al., 2002) suggest that CPEB monomers activate target mRNAs, while CPEB multimers repress target mRNAs. According to this scenario, FOG-1 might activate a mitosis-promoting mRNA at a low concentration, but repress that same mRNA at a higher concentration. However, many possibilities exist. Indeed, FEM-3 and FOG-3 may also contribute to the gradient of activity specifying these two fates. Understanding the underlying molecular mechanisms by which FOG-1 controls germline fates will require the identification and characterization of specific FOG-1 target mRNAs.

Relationship between FBF and FOG-1

FBF promotes both proliferation and oogenesis, whereas FOG-1 promotes proliferation and spermatogenesis. How do these two regulators accomplish both common and antagonistic roles? We suggest that FBF and FOG-1 have partially redundant roles, but that FBF is also a repressor of *fog-1* expression. The partial redundancy is based in large part on the synthetic proliferation defect of the *fog-1; fbf-1 fbf-2* triple mutant. We do not yet understand this redundancy at a molecular level. PUF and CPEB family proteins bind distinct RNA sequences in vitro (Bernstein et al., 2005; Mendez and Richter, 2001; White et al., 2001), so it seems unlikely that FBF and FOG-1 control mitosis by binding the same regulatory element in vivo. One simple idea is that FOG-1 might activate mitosis-promoting mRNAs, while FBF represses meiosis-promoting mRNAs. Two known FBF target mRNAs, *gld-1* and *gld-3*, promote entry into meiosis (Crittenden et al., 2002; Eckmann et al., 2004; Kadyk and Kimble, 1998), but FOG-1 targets are unknown. Another possibility is that FBF and FOG-1 both repress the same key target. Consistent with this idea, *Xenopus* Pumilio and CPEB both repress cyclin B1 mRNA (Groisman et al., 2002; Nakahata et al., 2003). In *C. elegans, gld-1* might be a common target mRNA: a putative CPE is present in the *gld-1* 3′UTR, although it has not been confirmed as a FOG-1 binding site (B.E.T., unpublished). A third possibility is that FBF and FOG-1 control the same mRNA, but do so using antagonistic activities. For example, FBF repression and FOG-1 activation might cooperate to obtain the correct level of a dose-dependent regulator of mitosis. The identification of FOG-1 and FBF target mRNAs should clarify which of these three plausible possibilities are involved.

In addition to their redundancy, FBF represses *fog-1* expression. This repression is logical for the sperm/oocyte decision: FBF promotes oogenesis by repressing *fog-1*, which is required for spermatogenesis. We previously showed that FBF represses the *fem-3* mRNA (Zhang et al., 1997); now *fog-1* and *fog-3* are also likely targets. Therefore, FBF appears to regulate the switch from spermatogenesis to oogenesis by coordinately repressing several key regulators. Similarly, PUF3 in *S. cerevisiae* binds and may regulate more than 100 nuclear-encoded mRNAs with mitochondrial functions (Gerber et al.,

2004; Olivas and Parker, 2000). Therefore, PUF proteins are emerging as master regulators of developmental and cellular processes by regulating batteries of genes at a post-transcriptional level.

FBF repression of *fog-1* seems counterintuitive for proliferation. However, one possible explanation is that FBF repression maintains FOG-1 at an appropriately low level to promote proliferation. In wild-type animals, FBF levels decrease and FOG-1 levels increase as germ cells enter meiosis (Crittenden et al., 2002; Lamont et al., 2004) (this work). By contrast, in *fbf-1 fbf-2* mutants, FOG-1 levels increase throughout the germline, and all germ cells enter spermatogenesis. Therefore, FBF repression of *fog-1* appears to be a crucial mechanism by which FBF promotes mitosis. However, given the redundancy of FBF and FOG-1, we note that FBF must promote germline mitoses by other mechanisms as well (e.g. repression of *gld-1* and *gld-3*) (Crittenden et al., 2002; Eckmann et al., 2004).

CPEB homologs may control mitosis broadly in animal development

FOG-1 is the second CPEB known to control mitotic divisions. *Xenopus* CPEB is required for progression through the mitotic cell cycle (Groisman et al., 2000; Groisman et al., 2002) in addition to its well-known role in meiosis (reviewed by Mendez and Richter, 2001). Specifically, *Xenopus* CPEB promotes mitotic divisions during early embryogenesis (Groisman et al., 2000; Groisman et al., 2002). A striking parallel between *Xenopus* CPEB and *C. elegans* FOG-1 is that concentration is crucial in both cases. In *C. elegans*, low FOG-1 promotes mitosis, while high FOG-1 specifies sperm; in *Xenopus,* the amount of CPEB is reduced by regulated degradation, and that decrease is necessary to promote mitotic divisions (Mendez et al., 2002). At a high level, *Xenopus* CPEB regulates Mos RNA and progression through meiosis, but it cannot promote mitosis. Given the striking parallels between *Xenopus* CPEB and *C. elegans* FOG-1, we suggest that CPEB family members may control mitosis broadly during animal development.

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References

Austin, J. and Kimble, J. (1987). *glp-1* is required in the germ line for regulation of the decision between mitosis and meiosis in C. elegans. *Cell* **51**, 589-599.

Barton, M. K. and Kimble, J. (1990). *fog-1*, a regulatory gene required for specification of spermatogenesis in the germ line of *Caenorhabditis elegans*. *Genetics* **125**, 29-39.

Barton, M. K., Schedl, T. B. and Kimble, J. (1987). Gain-of-function mutations of *fem-3*, a sex-determination gene in *Caenorhabditis elegans*. *Genetics* **115**, 107-119.

Bernstein, D. S., Buter, N., Stumpf, C. and Wickens, M. (2002). Analyzing mRNA-protein complexes using a yeast three-hybrid system: methods and applications. *Methods* **26**, 123-141.

Bernstein, D., Hook, B., Hajarnavis, A., Opperman, L. and Wickens, M. (2005). Binding specificity and mRNA targets of a *C. elegans* PUF protein, FBF-1. *RNA* **11**, 447-458.

Berry, L. W., Westlund, B. and Schedl, T. (1997). Germ-line tumor formation caused by activation of *glp-1*, a *Caenorhabditis elegans* member of the *Notch* family of receptors. *Development* **124**, 925-936.

Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics* **77**, 71-94.

Chen, P.-J. and Ellis, R. E. (2000). TRA-1A regulates transcription of *fog-3*, which controls germ cell fate in *C. elegans*. *Development* **127**, 3119-3129.

Chen, P.-J., Singal, A., Kimble, J. and Ellis, R. E. (2000). A novel member of the Tob family of proteins controls sexual fate in *Caenorhabditis elegans* germ cells. *Dev. Biol.* **217**, 77-90.

Church, D. L., Guan, K.-L. and Lambie, E. J. (1995). Three genes of the MAP kinase cascade, *mek-2, mpk-1/sur-1* and *let-60 ras*, are required for meiotic cell cycle progression in *Caenorhabditis elegans*. *Development* **121**, 2525-2535.

Crittenden, S. L. and Kimble, J. (1998). Immunofluorescence methods for *C. elegans*. In *Cells: A Laboratory Manual* (ed. D. Spector, R. Goldman and L. Leinwand), pp. 108.1-108.9. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Crittenden, S. L., Bernstein, D. S., Bachorik, J. L., Thompson, B. E., Gallegos, M., Petcherski, A. G., Moulder, G., Barstead, R., Wickens, M. and Kimble, J. (2002). A conserved RNA-binding protein controls germline stem cells in *Caenorhabditis elegans*. *Nature* **417**, 660-663.

Crittenden, S. L., Eckmann, C. R., Wang, L., Bernstein, D. S., Wickens, M. and Kimble, J. (2003). Regulation of the mitosis/meiosis decision in the *Caenorhabditis elegans* germline. *Philos. Trans. R. Soc. London Ser. B* **358**, 1359-1362.

Eckmann, C. R., Kraemer, B., Wickens, M. and Kimble, J. (2002). GLD-3, a Bicaudal-C homolog that inhibits FBF to control germline sex determination in *C. elegans*. *Dev. Cell* **3**, 697-710.

Eckmann, C. R., Crittenden, S. L., Suh, N. and Kimble, J. (2004). GLD-3 and control of the mitosis/meiosis decision in the germline of *Caenorhabditis elegans*. *Genetics* **168**, 147-160.

Ellis, R. E. and Kimble, J. (1995). The *fog-3* gene and regulation of cell fate in the germ line of *Caenorhabditis elegans*. *Genetics* **139**, 561-577.

Finney, M. and Ruvkun, G. (1990). The *unc-86* gene product couples cell lineage and cell identity in C. elegans. *Cell* **63**, 895-905.

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E. and Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391**, 806-811.

Forbes, A. and Lehmann, R. (1998). Nanos and Pumilio have critical roles in the development and function of *Drosophila* germline stem cells. *Development* **125**, 679-690.

Gerber, A. P., Herschlag, D. and Brown, P. O. (2004). Extensive association of functionally and cytotopically related mRNAs with Puf family RNA-binding proteins in yeast. *PLoS Biol.* **2**, E79.

Grant, B. and Hirsh, D. (1999). Receptor-mediated endocytosis in the *Caenorhabditis elegans* oocyte. *Mol. Biol. Cell* **10**, 4311-4326.

Groisman, I., Huang, Y. S., Mendez, R., Cao, Q., Theurkauf, W. and Richter, J. D. (2000). CPEB, maskin, and cyclin B1 mRNA at the mitotic apparatus: implications for local translational control of cell division. *Cell* **103**, 435-447.

Groisman, I., Jung, M. Y., Sarkissian, M., Cao, Q. and Richter, J. D. (2002). Translational control of the embryonic cell cycle. *Cell* **109**, 473-483.

Hake, L. E. and Richter, J. D. (1994). CPEB is a specificity factor that mediates cytoplasmic polyadenylation during Xenopus oocyte maturation. *Cell* **79**, 617-627.

Hansen, D., Hubbard, E. J. A. and Schedl, T. (2004a). Multi-pathway control of the proliferation versus meiotic development decision in the *Caenorhabditis elegans* germline. *Dev. Biol.* **268**, 342-357.

Hansen, D., Wilson-Berry, L., Dang, T. and Schedl, T. (2004b). Control of the proliferation versus meiotic development decision in the *C. elegans* germline through regulation of GLD-1 protein accumulation. *Development* **131**, 93-104.

Hirsh, D., Oppenheim, D. and Klass, M. (1976). Development of the reproductive system of *Caenorhabditis elegans*. *Dev. Biol.* **49**, 200-219.

Hodgkin, J. (1986). Sex determination in the nematode *C. elegans:* analysis of *tra-3* suppressors and characterization of *fem* genes. *Genetics* **114**, 15-52.

Hook, B., Bernstein, D., Zhang, B. and Wickens, M. (2005). RNA-protein

interactions in the yeast three-hybrid system: affinity, sensitivity, and enhanced library screening. *RNA* **11**, 227-233.

Huynh, J.-R. and St Johnston, D. (2000). The role of BicD, Egl, Orb and the microtubules in the restriction of meiosis to the *Drosophila* oocyte. *Development* **127**, 2785-2794.

Jan, E., Motzny, C. K., Graves, L. E. and Goodwin, E. B. (1999). The STAR protein, GLD-1, is a translational regulator of sexual identity in *Caenorhabditis elegans*. *EMBO J.* **18**, 258-269.

Jin, S.-W., Kimble, J. and Ellis, R. E. (2001a). Regulation of cell fate in *Caenorhabditis elegans* by a novel cytoplasmic polyadenylation element binding protein. *Dev. Biol.* **229**, 537-553.

Jin, S. W., Arno, N., Cohen, A., Shah, A., Xu, Q., Chen, N. and Ellis, R. E. (2001b). In *Caenorhabditis elegans*, the RNA-binding domains of the cytoplasmic polyadenylation element binding protein FOG-1 are needed to regulate germ cell fates. *Genetics* **159**, 1617-1630.

Jones, A. R., Francis, R. and Schedl, T. (1996). GLD-1, a cytoplasmic protein essential for oocyte differentiation, shows stage-and sex-specific expression during *Caenorhabditis elegans* germline development. *Dev. Biol.* **180**, 165-183.

Kadyk, L. C. and Kimble, J. (1998). Genetic regulation of entry into meiosis in *Caenorhabditis elegans*. *Development* **125**, 1803-1813.

Kawasaki, I., Shim, Y.-H., Kirchner, J., Kaminker, J., Wood, W. B. and Strome, S. (1998). PGL-1, a predicted RNA-binding component of germ granules, is essential for fertility in *C. elegans*. *Cell* **94**, 635-645.

Kimble, J. and Simpson, P. (1997). The LIN-12/Notch signaling pathway and its regulation. *Annu. Rev. Cell Dev. Biol.* **13**, 333-361.

Kimble, J. E. and White, J. G. (1981). On the control of germ cell development in *Caenorhabditis elegans*. *Dev. Biol.* **81**, 208-219.

Kraemer, B., Crittenden, S., Gallegos, M., Moulder, G., Barstead, R., Kimble, J. and Wickens, M. (1999). NANOS-3 and FBF proteins physically interact to control the sperm-oocyte switch in *Caenorhabditis elegans*. *Curr. Biol.* **9**, 1009-1018.

Lamont, L. B., Crittenden, S. L., Bernstein, D., Wickens, M. and Kimble, J. (2004). FBF-1 and FBF-2 regulate the size of the mitotic region in the *C. elegans* germline. *Dev. Cell* **7**, 697-707.

Lin, H. and Spradling, A. C. (1997). A novel group of *pumilio* mutations affects the asymmetric division of germline stem cells in the *Drosophila* ovary. *Development* **124**, 2463-2476.

Luitjens, C., Gallegos, M., Kraemer, B., Kimble, J. and Wickens, M. (2000). CPEB proteins control two key steps in spermatogenesis in *C. elegans*. *Genes Dev.* **14**, 2596-2609.

Mendez, R. and Richter, J. D. (2001). Translational control by CPEB: a means to the end. *Nat. Rev. Mol. Cell Biol.* **2**, 521-529.

Mendez, R., Barnard, D. and Richter, J. D. (2002). Differential mRNA translation and meiotic progression require Cdc2-mediated CPEB destruction. *EMBO J.* **21**, 1833-1844.

Miller, M. A., Nguyen, V. Q., Lee, M. H., Kosinski, M., Schedl, T., Caprioli, R. M. and Greenstein, D. (2001). A sperm cytoskeletal protein that signals oocyte meiotic maturation and ovulation. *Science* **291**, 2144-2147.

Nakahata, S., Kotani, T., Mita, K., Kawasaki, T., Katsu, Y., Nagahama, Y. and Yamashita, M. (2003). Involvement of *Xenopus* Pumilio in the translational regulation that is specific to cyclin B1 mRNA during oocyte maturation. *Mech. Dev.* **120**, 865-880.

Olivas, W. and Parker, R. (2000). The Puf3 protein is a transcript-specific regulator of mRNA degradation in yeast. *EMBO J.* **19**, 6602-6611.

Richter, J. D. (1999). Cytoplasmic polyadenylation in development and beyond. *Microbiol. Mol. Biol. Rev.* **63**, 446-456.

Tay, J. and Richter, J. D. (2001). Germ cell differentiation and synaptonemal complex formation are disrupted in CPEB knockout mice. *Dev. Cell* **1**, 201-213.

Timmons, L. and Fire, A. (1998). Specific interference by ingested dsRNA. *Nature* **395**, 854.

Ward, S., Roberts, T. M., Strome, S., Pavalko, F. M. and Hogan, E. (1986). Monoclonal antibodies that recognize a polypeptide antigenic determinant shared by multiple *Caenorhabditis elegans* sperm-specific proteins. *J. Cell Biol.* **102**, 1778-1786.

White, E. K., Moore-Jarrett, T. and Ruley, H. E. (2001). PUM2, a novel murine Puf protein, and its consensus RNA-binding site. *RNA* **7**, 1855-1866.

Wickens, M., Bernstein, D. S., Kimble, J. and Parker, R. (2002). A PUF family portrait: 3′UTR regulation as a way of life. *Trends Genet.* **18**, 150-157.

Zamore, P. D., Williamson, J. R. and Lehmann, R. (1997). The Pumilio protein binds RNA through a conserved domain that defines a new class of RNA-binding proteins. *RNA* **3**, 1421-1433.

Zhang, B., Gallegos, M., Puoti, A., Durkin, E., Fields, S., Kimble, J. and Wickens, M. P. (1997). A conserved RNA-binding protein that regulates sexual fates in the *C. elegans* hermaphrodite germ line. *Nature* **390**, 477-484.