Developmental Expression of FOG-1/CPEB Protein and Its Control in the *Caenorhabditis elegans* Hermaphrodite Germ Line

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The specification of a germ cell as sperm or oocyte and determination of cell number remain unsolved questions in developmental biology. This paper examines *Caenorhabditis elegans* FOG-1, a CPEB-related RNA-binding protein that controls the sperm fate. We find that abundant FOG-1 protein is observed transiently in germ cells just prior to their expression of an early sperm-differentiation marker. As the germline tissue elongates, abundant FOG-1 appears more and more distally as sperm become specified, but disappears when the germ line switches to oogenesis. This dynamic pattern is controlled by both globally acting and germline-specific sex-determining regulators. Importantly, the extent of FOG-1 expression corresponds roughly to sperm number in wild-type and mutants, altering sperm number. By contrast, three other key regulators of the sperm/oocyte decision do not similarly correspond to sperm number. We suggest that FOG-1 is precisely modulated in both time and space to specify sperm fate and control sperm number.

Key words: germ line; sex determination; cell number; RNA-binding protein

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INTRODUCTION

Animal germ lines produce either sperm or oocytes, depending on their sex. Many key regulators of the sperm/oocyte decision have been identified in *Caenorhabditis elegans* (Ellis and Schedl, 2005). Normally, this small nematode exists as either an XX self-fertilizing hermaphrodite or an XO male. The hermaphrodite germ line makes sperm first and then oocytes. Importantly, hermaphrodites make a reproducible number of sperm before switching to oogenesis. Therefore, the hermaphrodite germ line can be used to address mechanisms of sperm and oocyte specification as well as mechanisms that control cell number.

The adult hermaphrodite gonad is composed of two elongate "arms" of germline tissue, which develop in a similar manner during four larval stages (L1–L4; Fig. 1A). Proliferation occurs throughout development, with the total number of germ cells increasing from 2 in the newly hatched L1 larva to ~2,000 in adults (~1,000/arm). As the germ line elongates, it acquires polarity: germ cells remain proliferative at one end and begin differentiating at the other. Polarity is established and maintained by Notch signaling from the somatic distal tip cell (DTC; reviewed in Kimble and Crittenden, 2005). As germ cells divide and move away from the DTC, they enter the meiotic cell cycle and begin to differentiate (Fig. 1A). Of particular relevance to this work, germ cells are thought to be specified as sperm during the L3 and L4 stages of development in hermaphrodites, before the germ line switches into oogenesis (Barton and Kimble, 1990).

The sperm/oocyte decision is controlled by both globally acting and germline-specific regulators (Ellis and Schedl, 2005). Among the globally acting sex determination genes, *fem* and *tra-1* are most relevant to this work (Fig. 1B). Three *fem* genes direct male development, and *tra-1* specifies female development in all tissues. Molecularly, FEM-1 is an ankyrin repeat protein (Spence et al., 1990), FEM-2 is

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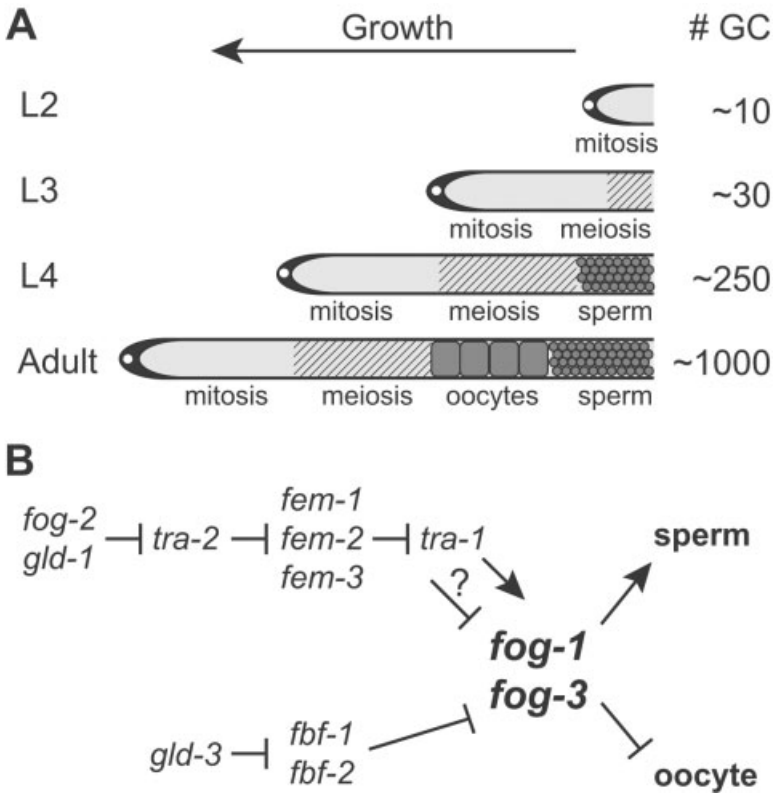


Fig. 1. *Caenorhabditis elegans* germline development and sex determination. **A:** Basic features of hermaphrodite germline development, shown using schematics of one gonadal arm. Growth is directional (arrow points from proximal to distal). The black cell at the growing end is the somatic distal tip cell; germline tissue proliferates and acquires polarity during larval development. L1–L4, first to fourth larval stage. In L1 (not shown) and L2, all germ cells are in the mitotic cell cycle; in L3, proximal germ cells enter the meiotic cell cycle (hatched); in L4, spermatogenesis produces approximately 140 mature sperm (circles) per arm; in adults, mature sperm are stored in spermatheca (not shown), while oocytes are produced more distally (large squares). #GC, total number of germ cells per arm. **B:** Genetic pathway of germline sex determination. The *fog-1* and *fog-3* genes encode terminal regulators (both are required for sperm specification in the absence of upstream regulators). Bar, negative regulation; →, positive regulation. Chen and Ellis (2000) showed that TRA-1 acts directly upstream of the *fog-3* gene, and suggested that TRA-1 may also act upstream of *fog-1*. The *fbf-1* and *puf-8* genes act upstream of *fog-2* (Bachorik and Kimble, 2005), but are not shown.

a phosphatase (Pilgrim et al., 1995; Chin-Sang and Spence, 1996), and FEM-3 is novel (Ahringer et al., 1992). TRA-1 is a zinc finger transcription factor and the single *C. elegans* homolog of vertebrate GLI (Zarkower and Hodgkin, 1992).

Germline-specific regulators are also crucial to the sperm/oocyte decision (Fig. 1B). The *fog-1* and *fog-3* genes promote spermatogenesis in both sexes and have been proposed to act as terminal regulators of that fate decision (Barton and Kimble, 1990; Ellis and Kimble, 1995; Fig. 1B). FOG-1 is a homolog of CPEB, the cytoplasmic polyadenylation element-binding protein (Luitjens et al., 2000; Jin et al., 2001a), and FOG-3 is a Tob family protein with an uncertain molecular function (Chen and Ellis, 2000). *fog-1* expression is controlled posttranscriptionally by FBF-1 and FBF-2 (Thompson et al., 2005). FBF-1 and FBF-2 are two nearly identical RNA-binding proteins of the PUF family (PUF, for Pumilio and FBF; Wickens et al., 2002). FBF-1 and FBF-2, which are collectively known as FBF, function redundantly to maintain germline stem cells and to promote the hermaphrodite switch from spermatogenesis to oogenesis (Zhang et al., 1997; Crittenden et al., 2002; Lamont et al., 2004). Indeed, FBF represses FOG-1 expression and does so both directly and indirectly: FBF targets include *fog-1*, *gld-1*, and *fem-3* mRNAs (Zhang et al., 1997; Crittenden et al., 2002; Thompson et al., 2005). Therefore, FBF represses a battery of sperm-promoting regulators, consistent with its biological role of driving the switch from spermatogenesis to oogenesis. In addition, the *fog-2* gene is required for the onset of spermatogenesis in hermaphrodites, but does not affect males (Schedl and Kimble, 1988). FOG-2 is an F-box protein and may be involved in regulating protein stability (Clifford et al., 2000). The *gld-1* gene is required for hermaphrodite, but not male, spermatogenesis (Francis et al., 1995). GLD-1 belongs to the STAR/GSG family of RNA-binding proteins (Jones et al., 1996), and it represses translation of *tra-2* mRNA (Jan et al., 1999). Recently, two PUF RNA-binding proteins, *fbf-1* and *puf-8*, were placed upstream of *fog-2* in the regulatory pathway controlling the sperm/oocyte decision (Bachorik and Kimble, 2005).

The functional relationships among the various regulators of the sperm/oocyte decision have been established by a combination of genetic and molecular assays (Ellis and Schedl, 2005; Zarkower, 2006; Fig. 1B). TRA-1 represses transcription of somatic target genes (reviewed in Zarkower, 2006) and appears to control *fog-1* and *fog-3* expression in the germ line. Both *fog-1* and *fog-3* promoters contain multiple TRA-1–binding sites (Chen and Ellis, 2000; Jin et al., 2001b), and Northern blot analyses show that FEM-1 and FEM-3 regulate the abundance of both *fog-1* and *fog-3* mRNAs, perhaps by regulating TRA-1 (Chen and Ellis, 2000; Jin et al., 2001b). Finally, TRA-1 binds the *fog-3* promoter in vitro, and these TRA-1 sites control activity of reporter transgenes in vivo (Chen and Ellis, 2000). However, the picture is not simple. Ellis and colleagues suggest that TRA-1 activates *fog-3* under certain circumstances and represses it under others (Chen and Ellis, 2000). The control of *fog-1* remains even more poorly understood.

In this work, we investigate expression of the FOG-1 protein and its control in the hermaphrodite germ line. Abundant FOG-1 is observed in a dynamic pattern from early L3 through the mid-L4 larval stage and disappears from spermatogenic precursors before expression of an early sperm-differentiation marker. This pattern is consistent with the role of FOG-1 in

sperm specification. FOG-1 expression is controlled by the regulatory network, as might be predicted from previous studies examining *fog-1* mRNA in a subset of mutants examined in this study. Importantly, TRA-1 represses FOG-1 expression, but FOG-3 does not. Furthermore, the extent of FOG-1 corresponds to sperm number, both in wild-type and mutants that change sperm number. Therefore, the regulatory network that controls *fog-1* expression precisely modulates its pattern in both time and space to control both sperm fate and sperm number.

RESULTS

FOG-1 Expression in Wild-Type Larvae

To investigate the *fog-1* gene and its role in sperm specification, we examined FOG-1 protein during hermaphrodite larval development. We focused on hermaphrodites, because we are interested in how hermaphrodite sperm number is controlled. We focused on larval development, because hermaphrodite sperm are made during the L4 stage, and because sperm specification occurs during L3 and L4 (Barton and Kimble, 1990).

In wild-type larvae, FOG-1 became "easily detectable" (well above background, see the Experimental Procedures section) in the second half of L2. In early L3 larvae, FOG-1 levels increased in the proximal region of most germ lines (95%, n = 36), and by late L3, FOG-1 staining was strong in that proximal region of all germ lines (Fig. 2A). FOG-1 remained abundant for approximately the first half of the L4 stage, although its location moved distally within the germ line (Fig. 2B; see below).

In L4 germ lines, we also examined SP56, the earliest known marker of sperm differentiation: SP56 antibodies recognize a minor sperm protein in primary, secondary, and mature spermatocytes (Ward et al., 1986). SP56 was first seen approximately 6 hr after the molt from L3 to L4 (3 hr, n = 17; 6 hr, n = 14; 10 hr, n = 19), where staining was limited to the proximalmost germ cells (Fig. 2C). As animals matured through the L4 stage, the SP56 domain expanded distally to include an increasing number of cells. Importantly, FOG-1 and SP56 were not present in the same cells at the same time. Instead, FOG-1 staining preceded SP56 staining in both time and space (Fig. 2B–D; data not shown). For example, the proximalmost germ cells expressed FOG-1 in late L3 and early L4 (Fig. 2A, right; data not shown); those same cells expressed SP56 in mid-L4 (6 hr), when the FOG-1 domain had moved distally (Fig. 2C,D; data not shown). We conclude that, in wild-type larvae, FOG-1 is expressed transiently in germ cells destined for spermatogenesis.

Control of FOG-1 Expression by Global Regulators

The onset and disappearance of FOG-1 is likely to be controlled by regulators identified genetically as critical for sex determination and the sperm/oocyte decision. We first examined control by the global sex determination pathway, focusing on the *fem* and *tra-1* genes, which reside near or at the end of the regulatory pathway (Ellis and Schedl, 2005; Zarkower, 2006). FOG-1 was absent from germ lines of *fem-1*, *fem-2*, and *fem-3* loss-of-function mutants at all stages examined (Fig. 3B,G). Therefore, the *fem* genes are required for FOG-1 expression. In *tra-1* null mutants, FOG-1 was not detectable in L1 larvae immediately after hatching, but could be observed in mid-L1 larvae, one full stage earlier than wild-type (Fig. 3G). FOG-1 expression then continued from L2 through the L4 stage (Fig. 3C,G; data not shown). In *tra-1* L4s and adults, FOG-1 staining was variable: in a total of 16 L4 germ lines, FOG-1 could be absent (1 of 16), barely detectable (2 of 16), moderate (5 of 16), or intense (8 of 16); of 19 adult germ lines, FOG-1 also could be absent (7 of 19), barely detectable (6 of 19), moderate (3 of 19), or intense (3 of 19). This variability in FOG-1 staining corresponds to the variability in germline phenotypes typical of these later stages (Hodgkin, 1987; Schedl et al., 1989).

We next examined FOG-1 in *tra-1; fem-1* double mutant germ lines. These mutants were somatically male, but their germ lines were variable. Some *tra-1;fem-1* XX germ lines made sperm and others made only oocytes; some expressed the SP56 early spermatogenic marker, while others expressed only the RME-2 oogenic markers, and did so prematurely. In adults, FOG-1 staining was also variable, but it did not correspond to the presence of SP56 or RME-2 (Fig. 3G; data not shown). More importantly, during larval development, all *tra-1; fem-1* double mutants expressed FOG-1 (Fig. 3D,G). Therefore, FOG-1 must be expressed in larval germ lines that go on to make only oocytes. This result supports the idea that TRA-1 represses FOG-1 expression, which has been suggested by others (Chen and Ellis, 2000), and also shows that FOG-1 is not sufficient for sperm specification.

Control of FOG-1 by Germline-Specific Regulators

We also examined FOG-1 in mutants defective for germline-specific regulators of sex determination. The *fog-2* gene is required for the onset of hermaphrodite spermatogenesis (Schedl and Kimble, 1988). Consistent with that role, FOG-1 protein was not detected in 89% of *fog-2* XX gonads at any time of development (n = 32; Fig. 3E,G); the remaining 11% showed weak staining that never acquired the intensity typical of prespermatogenic nuclei and was only found later than normal, in mid or late L4 larvae. Therefore, FOG-2 controls the onset of hermaphrodite spermatogenesis, at least in part, by promoting FOG-1 expression in young larvae. That regulation is probably indirect, as FOG-2 acts early in the germline sex determination pathway (Fig. 1B).

The *fog-3* gene is essential for specification of the sperm fate in both sexes (Ellis and Kimble, 1995). Furthermore, *fog-3* acts genetically in the same epistasis level as the *fem* genes and *fog-1* (Ellis and Kimble, 1995). In *fog-3* mutants, FOG-1 protein was expressed in a wild-type pattern (Fig. 3F,G). From this result, we draw two conclusions. First, FOG-3 does not affect *fog-1* expression. Second, FOG-1 is not sufficient for sperm specification. Instead, FOG-1 is likely to control the sperm fate, either in parallel to FOG-3 or by controlling FOG-3.

Finally, we examined three mu-

tant strains that produce sperm continuously and never switch to oogenesis: *fem-3(gf)* (Barton et al., 1987), *fbf-1 puf-8* (Bachorik and Kimble, 2005), and *gld-1(Mog)* mutants (Francis et al., 1995). In all three strains, FOG-1 persisted abnormally into adulthood (Fig. 3G). Given that *fog-1* is epistatic to *fem-3(gf)* (Barton and Kimble, 1990) and also to *fbf-1 puf-8* (Bachorik and Kimble, 2005), the simplest explanation is that the switch from spermatogenesis to oogenesis is effected, at least in part, by down-regulating the production of FOG-1 in late larvae.

FOG-1 and Sperm Number

FOG-1 appears to mark germ cells in the process of sperm specification, and its expression is controlled by both global and germline-specific regulators (Figs. 2, 3). Therefore, we asked if the spatial and temporal extent of FOG-1 expression might relate to sperm number in hermaphrodites. To investigate that idea, we examined FOG-1 in mutants making either fewer or more sperm than wild-type (Fig. 4A). For this study, we selected *gld-1* heterozygotes (*gld-1/hT2[qIs48]*, referred to as *gld-1/Bal*), which make fewer sperm than normal (Francis et al., 1995), and *fbf-1* homozygotes, which make more sperm than normal (Crittenden et al., 2002). Both *gld-1* heterozygotes and *fbf-1* homozygotes are healthy self-fertile animals with a germ line that is morphologically normal (except for sperm number). We first confirmed that the mutants made the reported number of sperm (Fig. 4A). We then examined the extent of FOG-1 in each of them. The first appearance of FOG-1 protein was de-

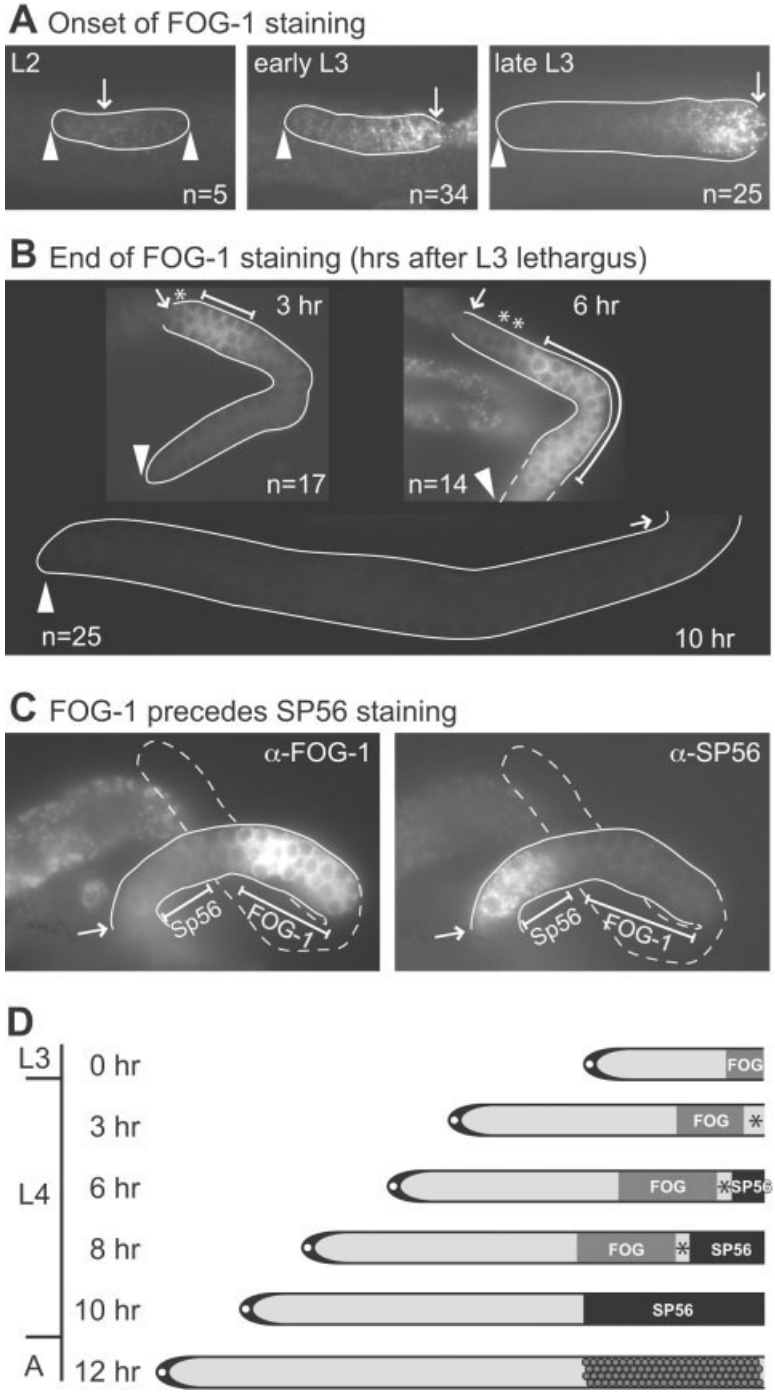


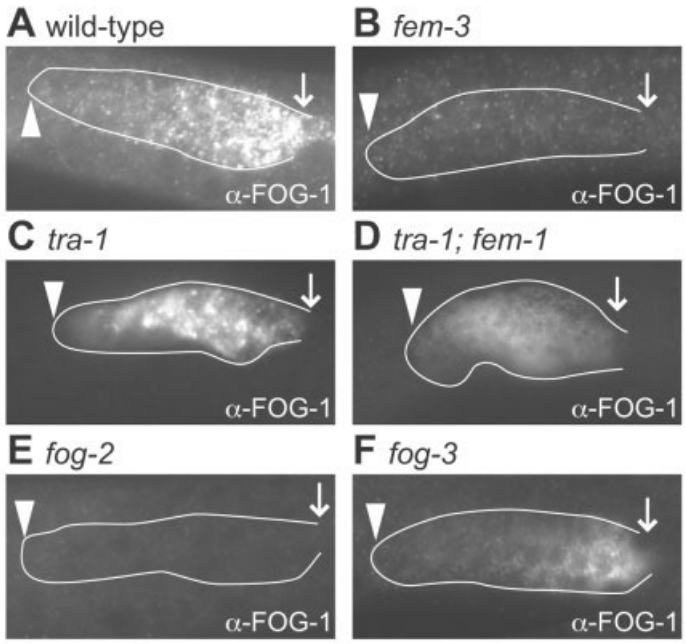
Fig. 2. FOG-1 is expressed at the right time and in the right place to specify the sperm fate. A–C: Solid line, gonad boundary; arrowhead, distal end; arrow, proximal-most germ cells. **A:** Onset of "easily detectable" FOG-1 expression in wild-type hermaphrodites. Left, L2: FOG-1 is not abundant; middle, early L3: FOG-1 is abundant in proximal-most germ cells, but not in distal germ cells; right, late L3: FOG-1 remains strong in proximal-most germ cells, but disappears from distal germ cells. L2 shows complete developing gonad, which does not yet have discrete arms. By early L3, arms are distinct; each L3 image shows only a single gonadal arm. **B:** End of FOG-1 expression in wild-type hermaphrodites. Extent of FOG-1 staining is marked by a bar. Top left, 3 hr into L4: FOG-1 staining is limited to the proximal germ line, but the few proximal-most germ cells (asterisk) no longer express FOG-1; top right, 6 hr into L4: FOG-1–expressing germ cells are found more distally as the germ line extends; below, 10 hr into L4: FOG-1 is no longer seen. **C:** Comparison of FOG-1 and SP56 staining. L4 germ line costained with FOG-1 (left) and SP56 (right), an early marker of sperm differentiation (Ward et al., 1986). **D:** FOG-1 and SP56 expression during L4 stage. Diagrams similar to Figure 1A: proximal on right and distal growth toward left. Asterisk, the proximal-most germ cells do not express FOG-1, but they do express SP56. (At L3 lethargus, the proximal-most germ cells express FOG-1 [Fig. 3A, right], whereas at later time points those same proximal-most cells express SP56 and begin spermatogenesis [Fig. 3C, right].) Larval stages defined in Figure 1.

layed in *gld-1*/*Bal* heterozygotes, but not in *fbf-1* homozygotes (Fig. 4B,C). In addition, the disappearance of FOG-1 occurred earlier than wild-type in *gld-1*/*Bal* heterozygotes, but later in *fbf-1* homozygotes (Fig. 4C; data not shown). By contrast, the larval pattern of expression of other sperm/oocyte regulators (e.g., FBF-2, and GLD-1) was not altered in either *gld-1*/*Bal* heterozygotes or *fbf-1* homozygotes (data not shown). Therefore, FOG-1 stands out as the only regulator identified to date whose extent of expression correlates with sperm number.

DISCUSSION

FOG-1 and Sperm Specification

Previous studies suggested that the *fog-1* gene is essential for sperm specification: in both XX and XO *fog-1* null mutants, germ cells that normally would differentiate as sperm are sexually transformed into oocytes (Barton and Kimble, 1990). Previous work also suggested that *fog-1* functions during mid-larval development for sperm specification (Barton and Kimble, 1990) and that FOG-1 protein is present in L3 larvae but absent from young adult hermaphrodites (Thompson et al., 2005). In this work, we have investigated the production of abundant FOG-1 protein during hermaphrodite development and its control. Temporally, FOG-1 is expressed from late L2 through the first half of L4, with maximal expression in mid-L3 through mid-L4. Spatially, abundant FOG-1 is first seen in the proximal germ line, in the same germ cells that will later differentiate as sperm. The domain of FOG-1 staining moves distally as the germ line grows and elongates distally. Importantly, germ cells first express abundant FOG-1 and later express SP56, an early marker of sperm differentiation. Therefore, FOG-1 appears to mark germ cells destined for spermatogenesis. One simple idea might have been that the presence of FOG-1 was sufficient for sperm specification. However, FOG-1 is expressed in mutants that produce only oocytes (see below).



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Genotype	GL	L1	L2	L3	L4	A
wild-type	sp/oo	–	–/+	+	+/–	–
fem-1	oo	–	–	–	–	–
fem-2	oo	–	–	–	–	–
fem-3	oo	–	–	–	–	–
tra-1	sp/–	–/+	+	+	vab	vab
tra-1; fem-1	oo	–	+	+	+	vab
tra-1; fem-1	sp	–	+	+	+	vab
tra-1; fem-1	sp/oo	–	+	+	+	vab
fog-2	oo	–	–	–	–	–
fog-3	oo	–	–/+	+	+/–	–
fem-3(gf)	sp	–	–/+	+	+	+
fbf-1 puf-8	sp	–/+	+	+	+	+
gld-1(Mog)	sp	–	–/+	+	+	+

Fig. 3. Regulation of FOG-1 expression. A–F: FOG-1 in wild-type or mutant L3 larvae; one gonadal arm shown. Conventions as in Figure 2A–C. **A:** Wild-type; **B:** *fem-3(e1996)*; **C:** *tra-1(e1834)*; **D:** *tra-1(e1099);fem-1(hc17)*; **E:** *fog-2(q71)*; **F:** *fog-3(q520)*. **G:** Summary of FOG-1 expression during development in wild-type and sex determination mutants. GL, germ line makes sperm only (sp), oocytes only (oo), or both sperm and oocytes (sp/oo). A, adult. Note that *tra-1;fem-1* double mutants sometimes made only oocytes (oo), sometimes only sperm (sp), and sometimes both (sp/oo); however, the larval FOG-1 pattern did not vary. −, no FOG-1 in any germ lines (see below for one exception); +, FOG-1 easily detectable in all germ lines; −/+, FOG-1 absent from first part of stage, but present later in same stage; +/−, FOG-1 present in first part of stage, but absent later in same stage; vab, variable. In *fog-2* mutants, weak FOG-1 staining was seen in some L4s (4/32), but none had abundant FOG-1 and most had no FOG-1; we, therefore, recorded *fog-2* mutants as "−." The number of germ lines (n) scored for each mutant and each stage are as follows: *fem-1*: L1, n = 7; L2, n = 12; L3, n = 20; L4, n = 16; A, n = 12; *fem-2*: L1, n = 5; L2, n = 16; L3, n = 14; L4, n = 10; A, n = 4; *fem-3*: L1, n = 6; L2, n = 15; L3, n = 10; L4, n = 6; A, n = 10; *tra-1*: L1, n = 7; L2, n = 11; L3, n = 15; L4, n = 16; A, n = 19; *tra-1;fem-1*: L1, n = 1; L2, n = 2; L3, n = 6; L4, n = 17; A, n = 28; *fog-2*: L1, n = 2; L2, n = 14; L3, n = 17; L4, n = 18; A, n = 20; *fog-3*: L1, n = 2; L2, n = 17; L3, n = 36; L4, n = 25; A, n = 15; *fem-3(gf)*: L1, n = 4; L2, n = 17; L3, n = 28; L4, n = 16; A, n = 30; *fbf-1 puf-8*: L1, n = 4; L2, n = 46; L3, n = 34; L4, n = 27; A, n = 18; *gld-1(Mog)*: L1, n = 7; L2, n = 10; L3, n = 26; L4, n = 10; A, n = 6.

Therefore, FOG-1 is not sufficient for sperm specification, but instead marks cells in wild-type animals that are destined for the sperm fate, and takes part in the process of sperm specification.

A

Genotype	# sp	range	n
wild-type	140	115-163	11
gld-1(0)/Bal	122	86-150	12
+/ Bal	163	137-199	10
fbf-1	293	185-385	10

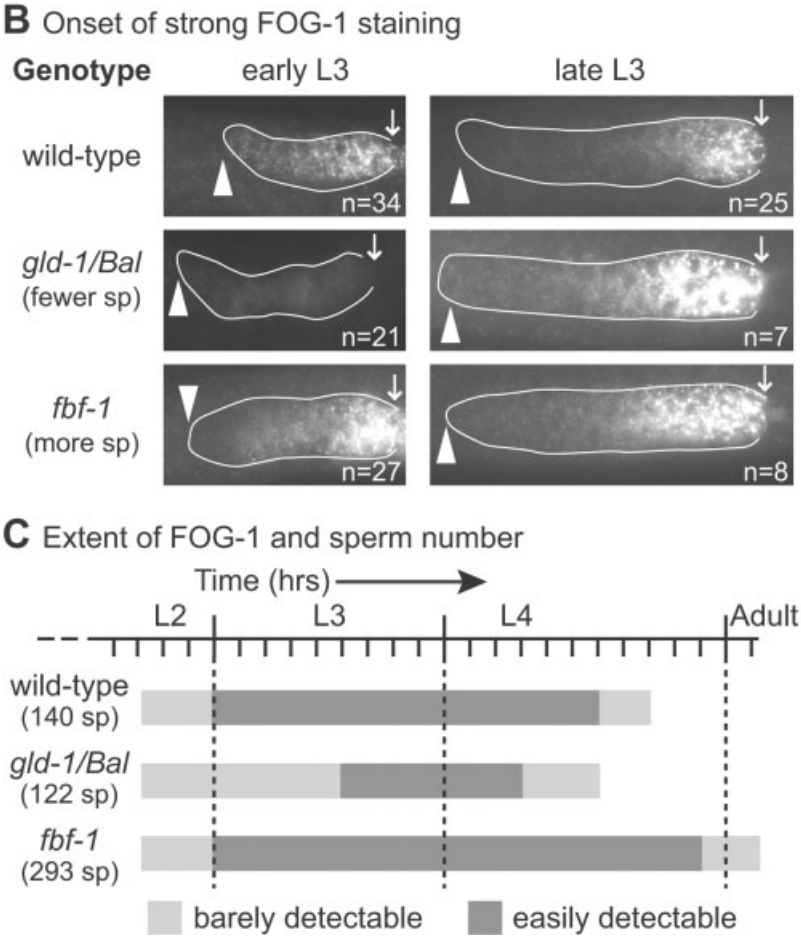

Fig. 4. FOG-1 in mutants with altered sperm number. **A:** Sperm number in wild-type and mutant hermaphrodites. Genotypes were *gld-1(q485)/hT2[qIs48]* balancer, *+/hT2[qIs48]* balancer, and *fbf-1(ok91)* homozygotes; n, number gonadal arms scored. Our sperm counts confirm previous reports: *gld-1/Bal* (Francis et al., 1995), *fbf-1* (Crittenden et al., 2002). **B:** Onset of FOG-1 in wild-type and mutants with altered sperm number. Conventions as in Figure 2A,B. A summary of all germ lines scored is presented in C; the number of germ lines (n) scored for each strain and each stage (time, hours after L3/L4 molt) are as follows: wild-type: 3 hr, n = 17; 6 hr, n = 14; 8 hr, n = 11; 10 hr, n = 25; *gld-1/balancer*: 3 hr, n = 20; 6 hr, n = 17; 8 hr, n = 13; 10 hr, n = 14; *fbf-1*: 3 hr, n = 30; 6 hr, n = 13; 8 hr, n = 18; 10 hr, n = 20; 12 hr, n = 14. **C:** Summary of FOG-1, SP56, and sperm number. Light gray, low FOG-1; dark gray, medium or high FOG-1.

Functional Relationship of FOG-1 and FOG-3

The *fog-1* and *fog-3* genes are both germline-specific regulators that act genetically at the end of the germline sex determination pathway (Barton and Kimble, 1990; Ellis and Kimble, 1995). Here, we report that *fog-3* is not required for FOG-1 protein expression. Thus, the timing, localization, and levels of FOG-1 are essentially the same in wild-type and *fog-3* null mutant larvae. Previous work demonstrated that *fog-1* does not control steady-state levels of *fog-3* mRNA (Chen and Ellis, 2000), but analyses of FOG-3 protein are not yet available. An intriguing idea is that FOG-1 protein may control *fog-3* mRNA translation. FOG-1 is a CPEB homolog (Luitjens et al., 2000; Jin et al., 2001b). In *Xenopus*, CPEB binds to CPE regulatory elements in the 3′-untranslated region (UTR) of target mRNAs and can either activate or repress their translation (Richter, 2000). In *C. elegans*, FOG-1 binds the *fog-1* 3′-UTR, which contains CPE-like sequences, and may, therefore, autoregulate its own expression (Jin et al., 2001a). Because multiple putative FOG-1–binding sites exist in the *fog-3* 3′-UTR (J. Kimble, unpublished observations), an attractive possibility is that FOG-1 controls both *fog-1* and *fog-3* mRNAs. Alternatively, FOG-1 and FOG-3 may promote the sperm fate together or in parallel pathways.

Global Sex-Determining Regulators Control FOG-1 Expression

In somatic tissues (e.g., hypodermis), the FEM proteins down-regulate TRA-1 activity, and TRA-1 acts at the end of the global sex determination pathway to repress tissue-specific sexual differentiation genes (Conradt and Horvitz, 1999; Yi and Zarkower, 1999; Yi et al., 2000). In the germ line, *tra-1* controls the sperm/oocyte decision, at least in part by regulating *fog-3* expression (Chen and Ellis, 2000). The presence of four consensus TRA-1–binding sites in the *fog-1* 5′-flanking region indicates that TRA-1 may also repress *fog-1* transcription (Jin et al., 2001a). Our work is consistent with that view. FOG-1 protein is present in *tra-1* mutants, but absent in *fem-1* mutants. Furthermore, FOG-1 is present in *tra-1;fem-1* double mutants, suggesting that TRA-1 repression of *fog-1* transcription is responsible for the absence of FOG-1 in *fem-1* mutants.

FOG-1 and Control of Sperm Number

Many developing systems establish cell clusters of a defined size that are destined for differentiation within a larger group of proliferating cells. Classic examples include somite generation as vertebrate embryos grow posteriorly (Pourquié, 2003), segment allocation as short germ-band insect embryos grow posteriorly (reviewed in

Davis and Patel, 2002), specification of limb elements as the vertebrate limb bud grows distally (Mariani and Martin, 2003), and establishment of root tissues as the root apical meristem elongates (Birnbaum and Benfey, 2004; Ueda et al., 2005). In each of these cases, undifferentiated cells reside at the growing end, while fields of a given size are established proximal to the proliferative zone.

The control of sperm number in the *C. elegans* hermaphrodite germ line provides an unusually well-defined example of this phenomenon of size control. Indeed, hermaphrodite sperm number is under strong selection and must be carefully controlled: mutants making an altered number of sperm (either fewer or more) cannot compete with those making the normal number (Hodgkin and Barnes, 1991). Previous studies have identified several regulators that control the number of prespermatogenic cells (Francis et al., 1995; Crittenden et al., 2002; Eckmann et al., 2002; Lamont et al., 2004). Importantly, all of these regulators converge on control of FOG-1/CPEB, which functions near the end of the network to regulate both proliferation and sperm specification (Barton and Kimble, 1990; Thompson et al., 2005). Therefore, the control of FOG-1 is predicted to have a direct effect on coupling growth and differentiation.

In this work, we report that changes in FOG-1 expression correlate with changes in sperm number. Mutants that reduce sperm number also reduce FOG-1, both in length of time expressed and overall abundance, while mutants that increase sperm number correspondingly increase FOG-1 levels. By contrast, the larval expression of regulators acting upstream of FOG-1 (i.e., FBF-2, GLD-1) remains unchanged in these same mutants—both in pattern and abundance. We propose that the regulation of *fog-1* expression plays a key role in determining cell number. Other factors of course also impinge on sperm number (e.g., regulators of FOG-1 or FOG-3 activity). Indeed, FOG-3 protein awaits characterization, but given *fog-3* mRNA analyses (Chen and Ellis, 2000), the prediction is that FOG-3 expression may similarly be regulated to determine sperm cell number.

EXPERIMENTAL PROCEDURES

Nematode Strains

All strains were derived from Bristol strain N2 and grown by standard procedures at 20°C unless specified (Brenner, 1974). Mutations and balancers used in this work include the following: *LGI*: *gld-1(q93, q485)* (Francis et al., 1995), *fog-3(q520)* (Ellis and Kimble, 1995; Chen and Ellis, 2000), *hT2[qIs48]* balancer; *LGII*: *fbf-1(ok91)* (Crittenden et al., 2002), *puf-8(q725)* (Bachorik and Kimble, 2005), *mnIn1[mIs14 dpy-10(e128)]* balancer; *LGIII*: *fem-2(b245)* (Kimble et al., 1984), *tra-1(e1099, e1834)* (Hodgkin, 1993), *eDp6* balancer; *LGIV*: *fem-1(hc17)* (Nelson et al., 1978), *fem-3(e1996 och, q96 gf)* (Hodgkin, 1986; Barton et al., 1987; Ahringer and Kimble, 1991; Ahringer et al., 1992), *nT1[qIs51]* balancer; *LGV*: *fog-2(q71)* (Schedl and Kimble, 1988). *fem-3(q96 gf)* strains were maintained at 15°C; their phenotype was scored after a shift to 25°C for 12 hr. *tra-1; fem-1(ts)* strains were maintained at 15°C; their germ lines were scored in progeny of adult hermaphrodites shifted to 25°C.

FOG-1 Staining

To assess the onset of FOG-1 staining during early larval development, whole larvae were costained with a polyclonal antibody specific for FOG-1 (Thompson et al., 2005), the DNA dye 4′,6-diamidine-2-phenylidole-dihydrochloride (DAPI), and MH27, a monoclonal antibody that highlights the hypodermis, vulva precursor cells (VPCs), and their descendants (Francis and Waterston, 1991; Hoier et al., 2000; Köppen et al., 2001). In these experiments, we scored germ lines using a Zeiss Axio Imager.D1 epifluorescent microscope and compared wild-type and mutant images both by eye and using Openlabs software to examine both images at equivalent settings. FOG-1 staining was scored as "none," "barely detectable," or "easily detectable," where "none" refers to the absence of detectable FOG-1; "barely detectable" refers to the presence of FOG-1 at a level just above background, which is associated with mitotically dividing germ cells (Thompson et al., 2005); and "easily detectable" refers to the presence of FOG-1 at a level well above background, which is associated with sperm specification (Thompson et al., 2005). We also note that "easily detectable" staining includes what we subjectively code as moderate to intense staining. L1, L2, and L3 larvae were staged roughly by size and more precisely by staining with anti-MH27 (1:50) antibodies. In the first half of L1, P cells reside laterally, but in the last half of L1, they have moved ventrally. In the first half of L2, VPCs are oval and separated from each other, whereas in late L2, they have flattened and touch each other. In L3, VPCs are undivided for the first 2 hr, VPC daughters are present in the next 3-hr interval, and VPC granddaughters mark the final 3- to 4-hr period (Sulston and Horvitz, 1977). For progression through L4, we first picked larvae at L3 lethargus, an ~1-hr period of quiescence just before the molt into L4, incubated them for defined intervals (0 hr, 3 hr, 6 hr, 8 hr, 10 hr, 12 hr, 14 hr) and then scored FOG-1 in extruded germ lines.

Immunocytochemistry and Sperm Counts

Germ lines of mixed larval stages (L1–L4) were prepared using standard whole-mount techniques (Finney and Ruvkun, 1990; Miller and Shakes, 1995). L4 and adult gonads were extruded from the body and prepared as described (Crittenden and Kimble, 2006). Germ lines were stained using rat anti-FBF-1 (1:5; Zhang et al., 1997), rat anti-FOG-1 (1:5; Thompson et al., 2005), mouse anti-MH27 (Francis and Waterston, 1991), rabbit anti-RME-2 (1:500; Grant and Hirsh, 1999), rabbit anti–PGL-1 antibodies (Kawasaki et al., 1998), and mouse anti-SP56 (1:100; Ward et al., 1986), and DNA was visualized with DAPI (4′,6-diamidino-2-phenylindole). Sperm were counted in DAPI-stained young adults that contained oocytes, but no embryos (Kadyk and Kimble, 1998).

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