GLD-3, a Bicaudal-C Homolog that Inhibits FBF to Control Germline Sex Determination in C. elegans

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Summary

The FBF RNA binding proteins control multiple aspects of C. elegans germline development, including sex determination. FBF promotes the oocyte fate at the expense of spermatogenesis by binding a regulatory element in the fem-3 3' UTR and repressing this sex-determining gene. Here we report the discovery of GLD-3, a Bicaudal-C homolog and cytoplasmic protein that physically interacts with FBF. Using RNAi and a gld-3 deletion mutant, we show that GLD-3 promotes the sperm fate, a sex determination effect opposite to that of FBF. By epistasis analysis, GLD-3 acts upstream of FBF, and, in a yeast three-hybrid assay, GLD-3 interferes specifically with FBF binding to the fem-3 3' UTR. We propose that GLD-3 binds FBF and thereby inhibits its repression of target mRNAs.

Introduction

Mature mRNAs are extensively regulated during animal development. One type of control involves modification of the basal protein synthetic machinery; for example, phosphorylation of translation initiation factors can regulate both cell growth and specific mRNAs (Dever, 2002). A second mode of control relies on RNA binding proteins that bind specific mRNAs, often through elements in their 3' UTRs, and regulate their translation, stability, or localization (Wickens et al., 2000). This second mechanism is particularly conspicuous in the germline and early embryo. Lesions in the RNA binding protein or its RNA binding site can cause severe developmental defects, including sterility, morphological abnormalities, and lethality. In this paper, we begin to address how specific RNA binding proteins are controlled to regulate mRNAs at the correct time and place of development.

In C. elegans, mRNA regulation plays a major role in the decision between spermatogenesis and oogenesis (Puoti et al., 1997). The specification of a germ cell as sperm or oocyte relies on essentially the same sex determination pathway that governs male or female development in somatic tissues (Meyer, 1997). In the germline, however, that somatic pathway is modified by germline-specific regulators that control mRNA activities (Puoti et al., 1997). Normally, XX animals are hermaphrodites, making sperm first and then oocytes, and XO males make sperm continuously. In both hermaphrodites and males, the fog-1 gene is required for specification of the sperm fate (Barton and Kimble, 1990); intriguingly, fog-1 encodes an RNA binding protein in the CPEB family (Jin et al., 2001; Luitjens et al., 2000). Furthermore, in hermaphrodites, the transient generation of sperm is regulated by a cascade of 3' UTR controls. Thus, the female-promoting tra-2 mRNA is repressed via a 3' UTR element to initiate hermaphrodite spermatogenesis (Goodwin et al., 1993), and the male-promoting fem-3 mRNA is repressed via a distinct 3' UTR element to achieve the hermaphrodite switch from spermatogenesis to oogenesis (Ahringer and Kimble, 1991).

Regulation of the hermaphrodite sperm/oocyte switch has served as a paradigm for analyzing the function of the broadly conserved PUF (Pumilio and FBF) family of RNA binding proteins (Wickens et al., 2002). FBF (fem-3 binding factor) binds the PME regulatory element in the fem-3 3' UTR, the fem-3 male-promoting activity is repressed, and the germline switches from spermatogenesis to oogenesis (Zhang et al., 1997). Other PUF proteins are also 3' UTR regulators. Thus, Drosophila Pumilio binds the NRE regulatory elements in the hunchback (hb) 3' UTR and inhibits hb mRNA translation (Murata and Wharton, 1995; Wharton et al., 1998; Zamore et al., 1997). In S. cerevisiae, PUF-5 binds a 3' UTR regulatory element to control mating type (Tadauchi et al., 2001), a role analogous to that of FBF in C. elegans germline sex determination. In vertebrates, PUF functions remain unknown, but PUF proteins are present in the germline (Nakahata et al., 2001; F. Moore and R. Reijo-Pera, personal communication). Therefore, PUF proteins are broadly conserved and appear to be critical components of an ancient mechanism for mRNA regulation.

FBF is a collective term for the products of two nearly identical genes, fbf-1 and fbf-2, which appear to be redundant (Zhang et al., 1997). Removal of FBF activity abolishes the hermaphrodite sperm/oocyte switch: sperm are made continuously and no oocytes are formed (Zhang et al., 1997). Therefore, FBF normally promotes oogenesis. In addition to its role in germline sex determination, FBF is also required for the process of spermatogenesis (Luitjens et al., 2000) and for maintenance of germline stem cells (Crittenden et al., 2002). Thus, FBF is critical for multiple aspects of germline development.

In this paper, we report the identification and characterization of GLD-3. The GLD-3 acronym (germline development defective) is used here because GLD-3 defects are similar, albeit not identical, to those of two previously described genes, gld-1 and gld-2 (Francis et al., 1995; Kadyk and Kimble, 1998; Wang et al., 2002). The GLD-3 protein was identified in a two-hybrid screen for FBF interactors; it is a KH protein, and belongs to the Bicaudal-C family of RNA binding proteins. We show that GLD-3 is a predominantly cytoplasmic protein and is required for multiple aspects of germline development, including sex determination, and for embryogenesis. Most critical for this work, GLD-3 promotes the sperm fate at the expense of oogenesis, a sex determination function opposite to that of FBF. Genetically, fbf is epistatic to gld-3, suggesting that GLD-3 is a negative
Figure 1. The gld-3 Gene and Its Products

(A) Genetic map position. gld-3 was defined by transcript T07F8.3, which is predicted to map genetically to position +0.28 on chromosome II. Neither of two deficiencies in the region, mnDf30 (Labouesse et al., 1994) and mnDf108 (Zalevsky et al., 1999), removes the locus.

(B) Two major gld-3 transcripts, gld-3L and gld-3S. Boxes, exons; connecting lines, introns. Both mRNAs are SL1 trans-spliced to their start codons. An alternate splice site produces gld-3S. gld-3L ends in a 654 nt long 3' UTR, and gld-3S in a 191 nt long 3' UTR. Exons encoding GLD-3 protein motifs are color coded as in Figure 1D. Extent of the 876 bp gld-3(q730) deletion is indicated by a gap.

(C) Northern blot of mRNAs prepared from mixed stage wild-type hermaphrodites (mixed) or adults homozygous for glp-1(q224) raised at restrictive temperature, which have no germline (no GL). A cDNA probe covering exons 1–14 detects gld-3L and gld-3S as well as one major band (asterisks below GLD-3S) corresponding to a double alternatively spliced RNA that removes exons 7–13 and 16-UTR from gld-3L; other alternatively spliced RNAs are represented as minor bands (asterisks). Actin mRNA was used as a loading control; left, molecular weight markers (kb). Both gld-3 transcripts are enriched in germline-containing worms. See Supplemental Data for a description of transcripts marked by asterisks.

(D) Motif organization of Bic-C proteins, drawn to scale. Blue box with white asterisk, KH-like RNA binding domain predicted by PFAM.
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GLD-3 is a regulator of FBF. We provide evidence that GLD-3 interferes with FBF binding to the fem-3 PME regulatory element, and suggest that this change in the FBF/PME interaction is critical for FBF control by GLD-3.

Results

gld-3 Is a Bicaudal-C Homolog
To identify proteins that interact with FBF, we performed a yeast two-hybrid screen using FBF-1 as bait, analyzing 20,000,000 transformants (Kraemer et al., 1999). Among the 19 positives were two partial cDNAs corresponding to the predicted open reading frame T07F8.3; we have dubbed this gene gld-3 (Figure 1A). The gld-3 gene generates several mRNAs (Figures 1B and 1C), which are all trans-spliced to SL1. Of greatest importance here are one long mRNA, gld-3L, and one short mRNA, gld-3S; these share their first 13 exons (Figure 1B). On Northern blots, gld-3L and gld-3S were observed in poly(A)⁺ RNAs prepared from mixed stage animals (Figure 1C, lane 2) and in staged poly(A)⁺ RNAs throughout development (data not shown); however, they are not detected in glp-1 mutant adults, which have essentially no germline (Figure 1C, lane 1). Therefore, gld-3L and gld-3S are likely to be expressed in the germline, an idea that is supported by analysis of GLD-3 proteins (see below). Other gld-3-specific bands detected on Northern blots include a variety of alternatively spliced mRNAs that have been confirmed using RT-PCR (Figure 1C, asterisks; see Supplemental Data at http://www.developmentalcell.com/cgi/content/full/3/5/697/DC1). Analysis of these other gld-3 mRNAs is beyond the scope of this paper.

Immunanalyses (see below) show that the gld-3 locus encodes two major proteins, GLD-3L and GLD-3S, made from gld-3L and gld-3S mRNAs, respectively. Sequence analyses of GLD-3L and GLD-3S reveal a common region with five consecutive KH-related motifs and a serine-rich domain (Figure 1D). C-terminally, GLD-3S ends with 29 unique amino acids, while GLD-3L extends another 395 amino acids. Database searches with the GLD-3 amino acid sequences revealed weak similarity to Bicaudal-C (Bic-C) proteins (Figures 1E and 1F), and no similarity to other proteins. The standard Bic-C architecture includes five KH-related motifs followed by a serine-rich region and a SAM (sterile α motif) domain (Mahone et al., 1995; Wessely and De Robertis, 2000; Wessely et al., 2001). GLD-3 has no SAM domain, but does possess the other diagnostic motifs of the Bicaudal-C family (Figure 1D).

To determine whether the C. elegans genome encodes a more typical Bic-C protein, we searched Wormbase (http://www.wormbase.org) and found three predicted transcripts, M7.3, M7.4, and M7.6, which together might generate a classical Bic-C homolog. By RT-PCR, we showed that M7.3, M7.4, and M7.6 belong to the same transcript; we call this gene bcc-1. Comparison of the KH and SAM motif sequences revealed conservation of key amino acid residues (Figures 1F and 1G). To assess the relationships among GLD-3 and other Bic-C proteins, we examined the five KH-related motifs by exhaustive parsimony analysis (Figure 1E; see Experimental Procedures). Our results, presented as an unrooted distance tree (Figure 1E), show that both C. elegans Bic-C homologs are highly diverged from other Bic-C proteins. We conclude that the C. elegans genome possesses two Bic-C homologs: the more classical bcc-1 and the more divergent gld-3. We focus here on the gld-3 locus.

GLD-3 Is Cytoplasmic and Colocalizes with P Granules in the Early Embryo

To examine GLD-3 protein, we generated two polyclonal antibodies. One, called anti-GLD-3, was raised to the adult GLD-3 amino acid sequence in the KH-like RNA binding region has diverged from other Bic-C family members almost as much as GLD-3. Analysis of the GLD-3 subcellular distribution was investigated by immunocytochemistry. Similar results were obtained using either anti-GLD-3 (Figures 2B–2M) or anti-GLD-3L (data not shown). In most experiments, antibodies to the P granule protein PGL-1 (Kawasaki et al., 1998) were used to highlight germ cells and P granules. The data
Figure 2. GLD-3 Protein during C. elegans Development

(A) Two GLD-3 proteins. A Western blot of proteins prepared from adult animals that were wild-type (wt) hermaphrodites (lane 1), wild-type males (lane 2), glp-1 mutant hermaphrodites raised at restrictive temperature which lack a germline (no GL; lane 3), gld-3(q730) homozygous hermaphrodites (lane 4), and gld-3(q730); smg-1 homozygous hermaphrodites (lane 5), was probed with either of two GLD-3 antibodies: anti-GLD-3 (top blot), which detects both GLD-3L and GLD-3S, or anti-GLD-3L (middle blot). Actin was used as a loading control (lower blot).

(B–M) Immunocytochemistry to determine location of GLD-3 protein. Samples shown were stained with anti-GLD-3 antibody. Similar results were obtained with anti-GLD-3L antibodies (data not shown). Epi-fluorescent images (B–H, L, and M); confocal images (I–K).

(B and C) Wild-type L1. Gonadal primordium with the two germline precursors, Z2 and Z3, stained with anti-PGL-1 (B) and anti-GLD-3 (C). For both antibodies, antigen is cytoplasmic; the nucleus is seen as a dark area in the middle of each cell.

(D–G) Extruded germlines of wild-type adult hermaphrodite (D and E) and male (F and G). DAPI (D and F); anti-GLD-3 (E and G). In both sexes, GLD-3 is predominantly cytoplasmic and present throughout the germline, except in secondary spermatocytes and mature sperm. Oocytes stain intensely with anti-GLD-3 (extent of oocytes marked by solid line; most proximal oocyte nucleus marked by small arrowhead). Primary spermatocytes also stain intensely (long arrowheads marked with 1°) and mature sperm (extent marked by dotted line) have no detectable GLD-3. The level of GLD-3 is elevated, but not intense, in the distal germline. GLD-3 is more prominent in male pachytene than hermaphrodite pachytene germ cells, making it easier to visualize GLD-3 in granules surrounding the nuclear membrane (G). Insets in (D) and (E), 3° magnification showing GLD-3 in oocytes and primary spermatocytes. Insets in (F) and (G), 3° magnification showing early meiotic region from germlines of different males.

(F) DAPI-stained nuclei.

(G) Double stained with anti-PGL-1 (green) and anti-GLD-3 (red) antibodies. GLD-3 and PGL-1 overlap in most granules (yellow, arrow), but not all (red, closed arrowhead).

(H–K) Wild-type 12-cell embryo.

(L and M) Twelve-cell gld-3(q730) embryo from gld-3(q730) homozygous mother.
support three main conclusions. First, GLD-3 protein is predominantly cytoplasmic. We found GLD-3 in germ-line cytoplasm throughout development in both sexes (Figures 2B, 2C, 2E, and 2G, and data not shown) and in the cytoplasm of early embryos (Figures 2I–2K). Second, GLD-3 distribution is controlled spatially and temporally. In adult hermaphrodites, GLD-3 was detectable in the transition zone (TZ), where germ cells enter meiotic prophase; it was fainter in the early mitotic region and in pachytene germ cells (Figures 2D and 2E); however, as germ cells entered diakinesis, GLD-3 became abundant (Figures 2D and 2E). During spermatogenesis, GLD-3 was present in primary spermatocytes, but not in secondary spermatocytes or mature sperm (Figures 2D and 2E). A similar pattern was observed in males (Figures 2F and 2G, and data not shown). Third, GLD-3 colocalizes with P granules. P granules mark the germline throughout development, and contain proteins related to mRNA control (Seydoux and Strome, 1999). GLD-3 was found in the cytoplasm of early embryos, and colocalized with P granules from the 1-cell stage through the 64-cell stage; beyond the 100-cell stage of embryogenesis, GLD-3 was no longer detected (Figures 2H–2K, and data not shown). GLD-3 also appeared in particles that were near but not coincident with P granules (Figures 2I–2K, arrowhead). In the germline, GLD-3 was found both diffusely as well as in a more granular form (Figures 2E and 2G). The granular GLD-3 was found primarily at the cytoplasmic side of the nuclear boundary and often colocalized with PGL-1 (Figure 2G, including inset). Mutant embryos derived from gld-3(q730) homozygous mothers could contain apparently normal P granules that appeared to segregate normally to a single blastomere (Figure 2L); however, these gld-3 mutant embryos had no detectable GLD-3 (Figure 2M), consistent with the idea that this mutant removes most gld-3 activity.

**gld-3 Is Required Maternally for Germline Survival and Embryogenesis**

To investigate GLD-3 function, we used RNA-mediated interference (RNAi) and isolated a gld-3 deletion mutant (q730; Figure 1B). Together, these two methods demonstrate that GLD-3 is required for multiple aspects of germline development and for embryogenesis. Injection of gld-3 dsRNA into wild-type hermaphrodite germlines is predicted to reduce both maternal and zygotic gld-3 mRNA in the progeny, which are therefore referred to as gld-3(RNAi) progeny. The two major gld-3(RNAi) defects were lethality and sterility. Lethal defects were variable, ranging from arrest at many stages of embryogenesis to arrest soon after hatching. Defects associated with sterility, on the other hand, were more uniform. The wild-type germline consists of about 1,000 “cells” organized in an elongated gonadal arm (Figures 3A and 3G). By contrast, most gld-3(RNAi) animals had no apparent germline (Figures 3B, 3E, and 3F). An examination of gld-3(RNAi) larvae showed early germline defects: germ-line precursor cells, Z2 and Z3, were smaller than normal in young L1s, divided more slowly than normal, and their descendants remained small and indistinct compared to their wild-type counterparts. A small cluster of faint germ cells was sometimes detected in L4 animals, but was absent in adults (data not shown). The absence of germline cells was confirmed using PGL-1 and NOS-3 antibodies (data not shown). We conclude that the gld-3(RNAi) germline dies in late larvae. To find whether this germline death was dependent on the canonical pathway for programmed cell death (Hengartner, 1997), we injected gld-3 dsRNA into a ced-3 mutant (Ellis and Horvitz, 1986). The ced-3; gld-3(RNAi) animals exhibited the typical gld-3 germline survival defect (data not shown). We term this ced-3-independent germline death the Gls phenotype (germline survival defective). Some non-Gls gld-3(RNAi) hermaphrodites had stacked oocytes in a normal-sized germline (Figures 3C and 3E), a morphology typical of spermatogenesis defects. Finally, some gld-3(RNAi) animals had somatic defects, including a protruding vulva, multiple vulvae, or a reduction in animal size.

To distinguish between maternal and zygotic effects, we injected gld-3 dsRNA into rde-1 mutants, which are RNAi defective (Tabara et al., 1999). Self-progeny of genotype rde-1; gld-3(RNAi) were viable and fertile, indicating that gld-3(RNAi) relied on rde-1 activity (Figures 3E and 3F). When the injected rde-1 mutant hermaphrodites were crossed with wild-type males, the rde-1/+; gld-3(RNAi) crossprogeny possessed wild-type rde-1, which should render zygotic transcripts sensitive to RNAi, but leave maternal mRNAs intact. These rde-1/+; gld-3(RNAi) progeny were viable and generated a normal-sized germline. The simplest interpretation is that both lethality and Gls sterility, the two main defects typical of gld-3(RNAi) progeny, result from a loss of maternal gld-3 function.

**gld-3 Promotes Spermatogenesis**

To analyze zygotic gld-3 functions, we isolated a chromosomal deletion mutant, gld-3(q730), by sib selection using a PCR-based assay (see Supplemental Procedures). This 876 nt deletion removes nearly three exons from gld-3L and shifts its reading frame; the deletion also removes the cleavage and polyadenylation signal from gld-3S (Figure 1B). All major gld-3 mRNAs were vastly reduced in gld-3(q730) adults as assayed by RT-PCR (data not shown). Similarly, GLD-3 proteins were not detectable on Western blots (Figure 2A) or by immunostaining (Figure 2M). Therefore, gld-3(q730) is likely to be a strong loss-of-function or null mutant.

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(H) DAPI.
(I and L) Anti-PGL-1.
(J and M) Anti-GLD-3.
(K) Merged image of (I) and (J) showing colocalization of GLD-3 with PGL-1 (yellow, arrows). In some granules, GLD-3 does not colocalize with PGL-1 (red, arrowhead). In gld-3(q730) mutant embryos that cleave, P granules are observed (L), but GLD-3 is absent (M).
We first scored *gld-3* mutant hermaphrodites for germline defects. Specifically, we examined young adults, at a time when a wild-type hermaphrodite would have switched from spermatogenesis to oogenesis. In *gld-3* mutant hermaphrodites, most spermatogenic cells were blocked as primary spermatocytes, as judged by...
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size (Figures 3D and 4A), DNA morphology (Figure 4B), and expression of the sperm-specific marker SP56 (Ward et al., 1986; Figure 4C). Arrest at the primary spermatocyte stage was not absolute, however; some secondary spermatocytes and mature sperm were seen in most animals. A similar block at the primary spermatocyte stage was also observed among gld-3(RNAi) hermaphrodites (data not shown). We conclude that GLD-3 promotes maturation of primary spermatocytes to mature sperm.

While scoring gld-3 hermaphrodite germlines, we noted that the number of spermatocytes appeared to be lower than normal. To quantify this effect, we compared the number of spermatogenic cells set aside in wild-type and gld-3 mutants (Figure 4D). In this analysis, we counted primary and secondary spermatocytes as well as mature sperm (defined by cell size, DNA morphology, and SP56 staining) in hermaphrodites with at least one but at most a few mature oocytes; we then calculated the number of primary spermatocytes represented by these various stages (Figure 4D). Whereas wild-type hermaphrodites made an average of 36 primary spermatocytes per gonadal arm (n = 10), gld-3 mutants made an average of only 14 per arm (n = 36). Indeed, some gld-3 gonadal arms made no spermatocytes, but instead made only oocytes. No abnormal cell death was observed in these gld-3 mutant germlines. We interpret the decrease in the number of spermatocytes as an aberrant sperm/oocyte switch that occurred before the usual complement of spermatocytes had been set aside. An alternate interpretation might have been that the decrease in sperm number reflected a change in overall germ cell number or a delay in the onset of spermatogenesis. We have not observed any gross change in germ cell number in gld-3 mutants, but spermatogenesis is in fact delayed by several hours. Nonetheless, in wild-type animals, allocation of normal sperm number is independent of similar delays (Kimble and White, 1981).

Therefore, we conclude that GLD-3 is required during hermaphrodite development to promote the sperm fate and that this function is critical for determining the normal number of hermaphrodite sperm.

We next examined the germlines of gld-3 homozygous males. All adult males produced some spermatogenic cells, most of which progressed from primary spermatocytes to mature sperm, although these sperm were less well defined morphologically than normal and therefore are likely to be aberrant (n = 141). In addition to making some sperm, most adult males produced enlarged granular cells that appeared oocyte-like (Figures 3F, 3H, and 5A). This effect was also observed among rde-1/+; gld-3(RNAi) males (Figure 3F). Within the oocyte-like cells, chromosomal bivalents typical of oocytes were observed (Figure 3J). Similar bivalents are not seen during spermatogenesis (Figure 3I). We further assayed the oocyte-like cells using gamete-specific antibodies, and found that they stained with the oocyte-specific α-RME-2 antibody (Grant and Hirsh, 1999), but did not stain with the sperm-specific SP56 antibody (Figure 5D). Therefore, the germline is feminized in male gld-3 mutants. We conclude that GLD-3 is required during male development for the continued production of sperm and for inhibition of oogenesis.

In addition to examining the germline of gld-3 mutants, we also examined embryos generated by gld-3 homozygous mothers. Some mothers produced a few fertilized embryos in addition to many unfertilized oocytes. The embryos usually arrested early, with little cleavage or nuclear division (Figures 3I–3K). When gld-3 mothers were crossed with wild-type males, the crossprogeny either arrested during embryogenesis or grew up with no germline, the Gls phenotype. These defects mimicked the effect of gld-3 RNAi, and confirmed their maternal nature. Therefore, GLD-3 is required maternally for early embryogenesis.

gld-3 Acts Upstream of fbf to Control Germline Sex Determination

To explore the regulatory relationship between FBF and GLD-3, we generated animals lacking both activities using either RNAi or deletion mutants. We focused on the epistatic relationship between these two activities in germline sex determination for several reasons. First, both FBF and GLD-3 govern germline sex determination, and they have opposite effects. Second, their sex determination defects are discrete transformations of cell fate and are easily scored. Third, effects on sex determination have been well characterized for both genes (this work; Zhang et al., 1997). These criteria are not met for other gld-3 and fbf defects. One caveat to these experiments might have been that fbf mutants affect
**A** *fbf* is epistatic to *gld-3*

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Figure 5. GLD-3 Is a Negative Regulator of FBF

(A) Epistasis analysis of *gld-3* and *fbf*. Top, hermaphrodites; sp, sperm; oo, oocytes. Bottom, males; sp, sperm; oo, oocyte-like cells; n, number of germline arms scored; for *fbf-1 fbf-2* double mutant hermaphrodites, data are from Crittenden et al. (2002). RNAi progeny were scored 12–36 hr postinjection (16/H11034C); triple mutants were scored at 20°C; *gld-3(q730)* homozygotes were derived from *gld-3(q730)/H11001*.

(B and C) Whole animals; arrows, primary spermatocytes; dashed line, border of region with mature sperm.

(B) *fbf-1(ok91) fbf-2(q704) gld-3(q730)* triple mutant hermaphrodite, Nomarski.

(C) *fbf-1(ok91) fbf-2(q704) gld-3(q730)* triple mutant male, DAPI stained.

(D and E) Extruded germlines stained with both an oocyte-specific antibody (α-RME-2) and a sperm-specific antibody (SP56). Arrowhead marks the proximal end of germline tissue.

(D) *gld-3(q730)* male germline. Oocyte-like cells predominate; four sperm were detected using the SP56 antibody, but they are not visible in this image.

(E) *fbf-1(ok91) fbf-2(q704) gld-3(q730)* triple mutant male. Sperm extend throughout the germline; no oocytes are made. The *fbf-1(ok91) fbf-2(q704)* double mutant has the same staining pattern (not shown).

germline proliferation (Crittenden et al., 2002). However, sufficient germ cells are made in both hermaphrodites and males to assess the sperm/oocyte effect.

In both sexes, we find that *fbf* is epistatic to *gld-3* (Figure 5). Thus, whereas *gld-3* mutant hermaphrodites normally make sperm and oocytes (this work) and *fbf*-depleted animals make only sperm (Zhang et al., 1997), the *fbf-1 fbf-2 gld-3* triple mutant hermaphrodites make only sperm (Figure 5A). These *fbf-1 fbf-2 gld-3* hermaphrodite germlines stain positively for the sperm-specific antibody SP56, and fail to stain with the oocyte-specific antibody α-RME-2, confirming the switch in sexual fate (data not shown). In males, *gld-3* mutants often make oocyte-like cells and never make sperm continuously (Figure 5H), but the *fbf-1 fbf-2 gld-3* males make only sperm (Figure 5C). Furthermore, whereas *gld-3* mutant males stain positively with the oocyte-specific antibody (Figure 5D), the *fbf-1 fbf-2 gld-3* males stain positively with the sperm-specific antibody and fail to stain with the oocyte-specific antibody (Figure 5E). We conclude that *fbf* germline masculinization is epistatic to *gld-3* germline feminization.

We also examined the maturation of spermatocytes in the *fbf-1 fbf-2 gld-3* triple mutant hermaphrodites. In *fbf*-deficient animals, sperm appear to mature normally (Zhang et al., 1997), whereas in *gld-3* mutants, hermaphrodite sperm are blocked as primary spermatocytes (Figures 3 and 4). In both *gld-3*; *fbf(RNAi)* and *fbf-1 fbf-2 gld-3* triple mutant hermaphrodites, sperm are no longer arrested, but appear to mature normally (Figure 5B). Therefore, a lack of *fbf* suppresses the spermatocyte block typical of *gld-3* mutant hermaphrodites, suggesting...
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Figure 6. GLD-3 Interacts Specifically with FBF-1

(A) GLD-3L, GLD-3S, FBF-1, FBF-2, PUF-8 (amino acids 143–535), dmPum (amino acids 1091–1426), FOG-1, and Lamin were fused to either the LexA DNA binding domain or the Gal4 transcriptional activation domain (AD) and assayed for interaction using the yeast two-hybrid system. β-galactosidase activity is given in Miller units.

(B) In vitro binding of GLD-3 and FBF. GST/GLD-3L fusion protein and GST alone were immobilized on Sepharose-conjugated glutathione beads and incubated with radiolabeled in vitro-translated FBF-1 or FOG-1. FBF-1 was specifically retained by the GST/GLD-3L fusion protein (left panel). This interaction appears to be RNA independent (right panel). The rabbit reticulocyte lysate was either treated with RNase A or mock incubated without the nuclease before incubation with GST/GLD-3L.

(C) Identification of the region within GLD-3L that binds FBF-1. GLD-3L truncations were fused to the LexA DNA binding domain and tested for their interaction with FBF-1 (amino acids 121–614) fused to the Gal4 transcriptional activation domain (AD). Filter assayed yeast are shown and rated according to their blue color, indicative of β-galactosidase expression levels (+ and − represent strong and weak binding, respectively). GLD-3 motifs follow the codes in Figure 1. A novel 90 amino acid region in the C-terminal region of GLD-3L binds FBF-1.

(D) Identification of the region within FBF-1 that binds to GLD-3L. FBF-1 truncations were fused to the Gal4 activation domain and tested for their interaction with full-length GLD-3L fused to the LexA DNA binding domain. Open boxes, PUF repeats; black and gray ovals, flanking Csp domains. All FBF-1 variants were verified for expression on Western blots.

that a major role of GLD-3 in the maturation of hermaphrodite sperm involves negative regulation of FBF.

GLD-3 and FBF Proteins Interact Specifically

GLD-3 was first identified in a yeast two-hybrid screen as a protein that interacts with FBF-1. To examine the specificity of this interaction, we tested both GLD-3L and GLD-3S for interactions with a variety of PUF proteins (Figure 6A). We found that GLD-3L, but not GLD-3S, was able to bind both FBF-1 and FBF-2. Indeed, the interaction was seen when GLD-3L was fused to the LexA DNA binding domain and FBF-1 to the Gal4 activation domain (AD), or vice versa. Binding of GLD-3L is likely to be similar to FBF-1 and FBF-2: FBF-1 was expressed at about 10-fold higher levels than FBF-2 in two-hybrid tests (Figure 6A and data not shown), perhaps accounting in part for the lower lacZ activity with FBF-2 versus FBF-1. Importantly, GLD-3L did not bind either of two other PUF proteins, C. elegans PUF-8 or Drosophila Pumilio, or to another C. elegans RNA binding protein, FOG-1. Therefore, the GLD-3L does not bind PUF proteins nonspecifically, but instead binds to FBF specifically.

We next examined the binding of GLD-3L to FBF-1 in vitro. To this end, we first generated a fusion protein with glutathione S-transferase (GST) fused to GLD-3L. Purified GST/GLD-3L was incubated either with in vitro-translated radiolabeled FBF-1 or another RNA binding protein, FOG-1, and retained proteins were examined by gel electrophoresis (Figure 6B, left panel). We found that GLD-3L retained FBF, but did not retain FOG-1. Therefore, GLD-3L appears to bind FBF specifically in vitro. To test the possibility that the presence of RNA molecules might mediate the observed binding between GLD-3L and FBF-1, we treated the lysate containing 35S-labeled FBF-1 with RNase A before incubation with GST/
GLD-3L. Although virtually all RNA has been removed, the physical interaction between GLD-3 and FBF-1 was still observed (Figure 6B, right panel). We conclude that GLD-3 binds specifically to FBF in an RNA-independent fashion.

Finally, we investigated the regions within GLD-3 and FBF-1 critical for their interaction. First, various GLD-3 fragments were tested with FBF-1, defining a domain of 90 amino acids unique to GLD-3L that retained strong FBF-1 binding (Figure 6C). This short FBF binding region bears a novel amino acid sequence that is not found elsewhere in the C. elegans genome. Second, various FBF-1 fragments were tested with GLD-3L, defining Puf repeats 3–8 plus the C-terminal Csp domain as essential for the interaction (Figure 6D).

GLD-3 Antagonizes FBF Binding to the fem-3 3’UTR Regulatory Element

How might GLD-3 repress FBF activity? One possibility is that GLD-3 might interfere with FBF binding to the PME regulatory element in the fem-3 3’ UTR. To test this idea, we employed a yeast three-hybrid assay (Sen-Gupta et al., 1996). Briefly, this assay uses three different hybrid molecules to drive transcription of a reporter gene: one hybrid protein carries both a DNA binding domain (e.g., LexA) and an RNA binding domain (e.g., MS2); a second hybrid protein fuses a second RNA binding protein (e.g., FBF-1) to the Gal4 activation domain; and finally, a hybrid RNA (e.g., MS2/PME) bridges the two hybrid RNA binding proteins. We first assayed the binding of FBF-1, Drosophila Pumilio, and GLD-3L to either the PME regulatory element of fem-3 or the NRE of hb mRNA. As shown previously (Sonoda and Wharton, 1999; Zhang et al., 1997), FBF-1 binds the PME, but not the NRE (Figure 7A, lanes 1 and 2), and Pumilio binds the NRE, but not the PME (Figure 7A, lanes 3 and 4). Importantly, GLD-3L bound neither the PME nor the NRE (Figure 7A, lanes 5 and 6). Therefore, GLD-3L on its own does not bind specifically to the PME regulatory element of the fem-3 3’ UTR.

We next used a modified three-hybrid assay to learn whether GLD-3L could interfere with the FBF-1/PME comparisons. Numbered lanes and bars in this panel correspond to numbered lanes in expression controls provided in (D). Gal4 AD fusion proteins: FBF-1 is fragment 12 from Figure 6D; dmPUM is a fragment of Drosophila Pumilio similar to FBF-1.2. Test proteins: PK is pyruvate kinase, and FO-1 is a CPEB-1-related RNA binding protein. Hybrid RNAs: N, NRE; P, PME; lane 1, FBF-1 does not bind the NRE; lane 2, FBF-1 binds the PME; lane 3, GLD-3L reduces FBF-1 RNA binding to the PME by about 2-fold; lanes 4 and 5, neither FO-1 nor pyruvate kinase (PK) affects the FBF-1/PME interaction; lane 6, dmPUM binds the NRE; lane 7, GLD-3L does not affect dmPUM/NRE binding.

(D) Expression and loading controls. Lane numbers as in (C). Protein assays: above, Western blot probed for single HA tag fused to each protein. Note that FO-1 and FBF-1 fusion proteins are almost exactly the same size and appear as one intense band in lane 4. Below, actin provided a loading control. RNA assays: above, Northern blot of total RNA probed for hybrid RNAs. Note that the PME hybrid is slightly larger than the NRE hybrid; doublets are from promiscuous termination by RNA polymerase III. Below, 18S ribosomal RNA provided a loading control.
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Figure 8. GLD-3 Negatively Regulates FBF to Promote Spermatogenesis

(A) Active fem-3 mRNA produces FEM-3 protein, which specifies the sperm fate. The PME is a regulatory element located in the fem-3 3'UTR.
(B) FBF binds the PME, represses fem-3 mRNA activity, and blocks production of FEM-3 protein. As a result, spermatogenesis stops and oogenesis begins.
(C) GLD-3 negatively regulates FBF, which derepresses fem-3 mRNA and promotes spermatogenesis.

interaction. To this end, we introduced into the three-hybrid system a fourth component: a plasmid encoding a test protein (e.g., GLD-3; Figure 7B). We found that FBF-1 bound robustly to the PME as normal in the modified assay (Figure 7C, lane 2), but that binding was attenuated in the presence of GLD-3L (Figure 7C, lane 3). Quantitation showed that introduction of GLD-3L reproducibly reduced β-galactosidase expression by about 2-fold (Figure 7C, bars). Three controls were done to demonstrate that this reduction is specific. First, inhibition required GLD-3L and was not observed when the test protein was an unrelated RNA binding protein (FOG-1; Figure 7C, lane 4) or a different overexpressed protein (PK; Figure 7C, lane 5). Second, GLD-3L did not have a major effect on binding by fly Pumilio to the NRE (Figure 7C, lanes 6 and 7), or binding by yeast Mpt5p/ GLD-3 Antagonizes FBF to Control Germline Sex Determination

GLD-3 Antagonizes FBF to Control Germline Sex Determination

Normally in C. elegans, the hermaphrodite germline makes sperm and then oocytes, whereas males make sperm continuously. In hermaphrodites, the number of sperm made before the transition to oogenesis is roughly the same from individual to individual. The gld-3 gene promotes the sperm fate: in males, gld-3 governs the continued production of sperm, and in hermaphrodites, gld-3 is critical for making the normal number of sperm. We suggest here that gld-3 exerts its control over germline sex determination by modulating FBF activity.

The FBF RNA binding proteins normally promote oogenesis by inhibiting fem-3 mRNA, which encodes a sperm-promoting activity (Zhang et al., 1997). In the absence of FBF, fem-3 mRNA makes FEM-3 protein, which directs the sperm fate (Figure 8A); in the presence of FBF, fem-3 mRNA is repressed and oogenesis ensues (Figure 8B). GLD-3 promotes the sperm fate, an effect opposite to that of FBF. Double mutant experiments show that FBF is epistatic to GLD-3: when FBF is removed, GLD-3 is no longer required for specification of the sperm fate. Therefore, GLD-3 is likely to act upstream of FBF to inhibit or antagonize FBF activity (Figure 8C).

Discussion

This paper identifies the C. elegans GLD-3 protein, characterizes its role in regulating germline development, and explores its functional relationship with two other germine regulators, FBF-1 and FBF-2. GLD-3 belongs to the Bicaudal-C (Bic-C) family of RNA binding proteins. Like Bic-C of Drosophila (Saffman et al., 1998 and references therein), GLD-3 has multiple roles in germline development and is also required for embryogenesis. GLD-3 was initially discovered as a two-hybrid interactor with FBF-1, an RNA binding protein of the PUF family. Like GLD-3L, FBF also controls multiple aspects of germline development, including sex determination (Zhang et al., 1997), spermatogenesis (Luijten et al., 2000), and maintenance of germline stem cells (Critten-den et al., 2002). We have focused on germline sex determination to explore the functional relationship between FBF and GLD-3.
The FBF regulation by GLD-3 plays a key role in controlling germline sexual fates. FBF normally remains active in the mature germline to promote stem cells (Crittenden et al., 2002) and therefore must be inhibited to make sperm. We suggest that GLD-3 is a major inhibitor of FBF, and that GLD-3 normally inhibits FBF to permit spermatogenesis in both sexes. However, other FBF inhibitors are likely to exist, because most hermaphrodites and males make some sperm before switching to oogenesis in the absence of GLD-3.

**FBF and GLD-3 Control Overlapping, But Not Identical, Aspects of Development**

The biological processes regulated by GLD-3 and FBF are overlapping, but not identical. Maternally, GLD-3 is required for both embryogenesis and germline survival; zygotically, GLD-3 is required for germline sex determination (see above) and for the processes of both oogenesis and spermatogenesis. By contrast, FBF has no known role in embryogenesis or oogenesis; instead, FBF controls germline stem cell maintenance (Crittenden et al., 2002) and germline sex determination (see above), and appears to be critical for the process of spermatogenesis (Luijten et al., 2000).

The maternal GLD-3 functions in early embryogenesis, and germline survival cannot be easily explained by a relationship with FBF. FBF has no known effect in early embryogenesis, and FBF protein is not detectable in oocytes or early embryos. Furthermore, although FBF has been implicated in germline survival (Subramaniam and Seydoux, 1999), its germline survival defects were observed in animals depleted for FBF plus four other PUF proteins. One possible explanation for the *gld-3* germline survival defect may be that maternal GLD-3 acts in the P4 blastomere or its descendants for early embryogenesis. Consistent with this idea, GLD-3 colocalizes with P granules, which have been implicated in germline specification (Seydoux and Strome, 1999).

Although no other PUF protein has been identified to date as a GLD-3 interactor (C.R.E., unpublished data), we cannot exclude the possibility that GLD-3 may antagonize other PUF proteins for these maternal functions. Alternatively, GLD-3 may act independently of PUF proteins in the early embryo. A parallel study has revealed that GLD-3 binds to another germline regulator, GLD-2, which colocalizes with P granules and is required maternally for early embryogenesis (Wang et al., 2002). Therefore, GLD-3 may act with GLD-2 rather than with FBF in the early embryo.

**How Does GLD-3 Inhibit FBF at the Molecular Level?**

The molecular mechanism by which GLD-3 opposes FBF is not understood, but several findings provide clues. First, GLD-3 and FBF are both cytoplasmic proteins that are expressed in the germline (this work; Zhang et al., 1997). Although FBF is expressed in the germline mitotic region (Crittenden et al., 2002) and GLD-3 is expressed in the transition zone and oocytes (Figure 2), their expression overlaps in the proximal mitotic region and transition zone, indicating that GLD-3 may interact directly with FBF in *C. elegans*. The narrow region of overlap suggests that only a minority of FBF and GLD-3 protein molecules interact. Second, GLD-3 binds specifically to FBF. For example, GLD-3 binds both FBF-1 and FBF-2, but not other PUF proteins (Figure 6). Third, GLD-3 appears to interfere with FBF binding to the fem-3 PME 3’UTR regulatory element (Figure 7). Putting these findings together, we suggest that GLD-3 antagonizes FBF by a mechanism relying on direct interaction between the two proteins. The GLD-3/FBF interaction may release target mRNAs from FBF repression, or it may alter FBF binding to RNA or other interactors (e.g., NOS-3; Kraemer et al., 1999). A more radical possibility is that GLD-3 may be recruited to target mRNAs by FBF and then actively promote translation, reversing the effect of FBF. Recent studies have shown that GLD-3 binds and stimulates the activity of another germline regulatory protein called GLD-2, a regulator cytoplasmic poly(A) polymerase (Wang et al., 2002). Therefore, GLD-3 may antagonize FBF by promoting polyadenylation of its target mRNAs. Challenges for the future include determining the mechanism of antagonism and learning the extent to which the regulatory relationship between PUF and Bic-C family members has been conserved.

**Experimental Procedures**

**Strains**

The following mutations were used: LGI: smg-1(r681), LGII: fbf-1(ok91), fbf-2(e704), gld-3(q730); LGIII: unc-32(e109), gld-1(q224); LGIV: ced-3(j717); LGV: rde-1(ne219). Rearrangements: mnhk1[nls14 dpy-10(e128)] (LGI).

**Sequence Analysis of gld-3 and bcc-1**

*gld-3L*, gld-3S, and bcc-1 correspond to the predicted genes T07F8.3a, T07F8.3b, and M7.3, respectively. The intron/exon boundaries and the SLI trans-spliced leader were determined by sequencing cDNAs of *gld-3* (yk35111, yk34563, GLS[puk848]) and various RT-PCR-generated clones for *gld-3* and bcc-1. RT-PCR experiments and phylogenetic analysis are explained in Supplemental Procedures.

**RNA Interference**

For RNAi of *gld-3*, we injected a double-stranded (ds) RNA corresponding to nucleotides (nt) 1–2847 into wild-type adult hermaphrodites and crossed them with *unc-32(e189)* and *smg-1(r681)*, respectively. The intron/exon boundaries and the SLI trans-spliced leader were determined by sequencing cDNAs of *gld-3* (nt 363–1842) and *gld-3* (nt 1–1719) at a final concentration of 0.5 mg/ml each. Detailed information is in Supplemental Procedures.

**Two-Hybrid Screen/Assays and GST-FBF Protein Affinity Chromatography**

The initial yeast two-hybrid screen with FBF-1 has been described previously (Kraemer et al., 1999). Derivatives of *gld-3L* in pBTM116 and FBF-1 in pACTII were made by utilizing convenient restriction sites. Affinity chromatography experiments were performed essentially as described (Kraemer et al., 1999). *“S“-radiolabeled FBF-1 and FOG-1 were prepared by priming the T7 TNT-coupled reticulocyte lysate transcription/translation system (Promega) separately with cDNAs inserted into the pCITE-4 vector system (Novagen).

**Three-Hybrid Assays**

Three-hybrid experiments and assays were performed as described (Bernstein et al., 2002). The RNA sequences for PME in *pilhA/MS2-2* and NRE in *pilhA/MS2-2* and HO 3’UTR/MS2-2 were described previously (Sonoda and Wharton, 1999; Tadauchi et al., 2001; Zhang et al., 1997). These RNAs were cotransformed with either FBF-1 (amino acids 163–814), *dmFUM* (amino acids 1091–1426), or GLD-3L (amino acids 1–949) in pACT/pACTII vectors into YBZ-1.
Hybrid protein and hybrid RNA constructs to test proteins interfering with the observed RNA:protein interactions are explained in Supplemental Procedures. All constructs expressing GLD-3L, element binding protein. Development Biology.

GLD-3 Antibodies

Polyclonal antibodies were made against the N-terminal peptide (amino acids 2–24; anti-GLD-3) and the C-terminal fragment of GLD-3L (amino acids 582–949; anti-GLD-3L) fused to GST. Further information is available in Supplemental Procedures.

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