Genomic Analyses of Sperm Fate Regulator Targets Reveal a Common Set of Oogenic mRNAs in *Caenorhabditis elegans*

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ABSTRACT Germ cell specification as sperm or oocyte is an ancient cell fate decision, but its molecular regulation is poorly understood. In *Caenorhabditis elegans*, the FOG-1 and FOG-3 proteins behave genetically as terminal regulators of sperm fate specification. Both are homologous to well-established RNA regulators, suggesting that FOG-1 and FOG-3 specify the sperm fate post-transcriptionally. We predicted that FOG-1 and FOG-3, as terminal regulators of the sperm fate, might regulate a battery of gamete-specific differentiation genes. Here we test that prediction by exploring on a genomic scale the messenger RNAs (mRNAs) associated with FOG-1 and FOG-3. Immunoprecipitation of the proteins and their associated mRNAs from spermatogenic germlines identifies 81 FOG-1 and 722 FOG-3 putative targets. Importantly, almost all FOG-1 targets are also FOG-3 targets, and these common targets are strongly biased for oogenic mRNAs. The discovery of common target mRNAs suggested that FOG-1 and FOG-3 work together. Consistent with that idea, we find that FOG-1 and FOG-3 proteins co-immunoprecipitate from both intact nematodes and mammalian tissue culture cells and that they colocalize in germ cells. Taking our results together, we propose a model in which FOG-1 and FOG-3 work in a complex to repress oogenic transcripts and thereby promote the sperm fate.

KEYWORDS sperm/oocyte decision; FOG-1; FOG-3; CPEB; Tob

SPECIFICATION of a germ cell as sperm or oocyte lies at the heart of reproduction in virtually all animals. Yet this ancient cell fate decision remains poorly understood. Classically, the sperm/oocyte decision has been viewed as a consequence of somatic sex determination in the early embryo with sexually differentiated somatic tissues signaling to germ cells to become spermatogenic or oogenic (*e.g.*, McLaren 2003). The somatic signaling pathways critical for germline sex determination have been established in worms (*e.g.*, Perry *et al.* 1993; Zarkower 2006; Ellis and Schedl 2007), flies (*e.g.*, Wawersik *et al.* 2005), and mice (*e.g.*, Bowles *et al.* 2006; Koubova *et al.* 2006). Moreover, germ cell autonomous regulators of sex determination are also known in worms (Ellis and Schedl 2007), flies (*e.g.*, Oliver *et al.* 1993; Hashiyama *et al.* 2011), and mice (Tsuda *et al.* 2003; Suzuki and Saga 2008; Saba *et al.* 2014). Among the germ cell intrinsic regulators in these diverse organisms, the FOG-1 and FOG-3 proteins in *Caenorhabditis elegans* stand out as the best candidates for terminal regulators of the sperm fate.

The *fog-1* and *fog-3* genes are essential for sperm fate specification: germ cells that normally make sperm are sexually transformed in *fog-1* and *fog-3* mutants to produce oocytes (Barton and Kimble 1990; Ellis and Kimble 1995) (Figure 1A). Moreover, the *fog-1* and *fog-3* genes behave genetically as terminal regulators of the germline sex determination pathway (Figure 1A). An elaborate regulatory network acts upstream of *fog-1* and *fog-3* with two regulators functioning immediately upstream: TRA-1, the *C. elegans* GLI transcription factor (Zarkower and Hodgkin 1992), and FBF, a *C. elegans* PUF RNA-binding protein (Zhang *et al.* 1997);

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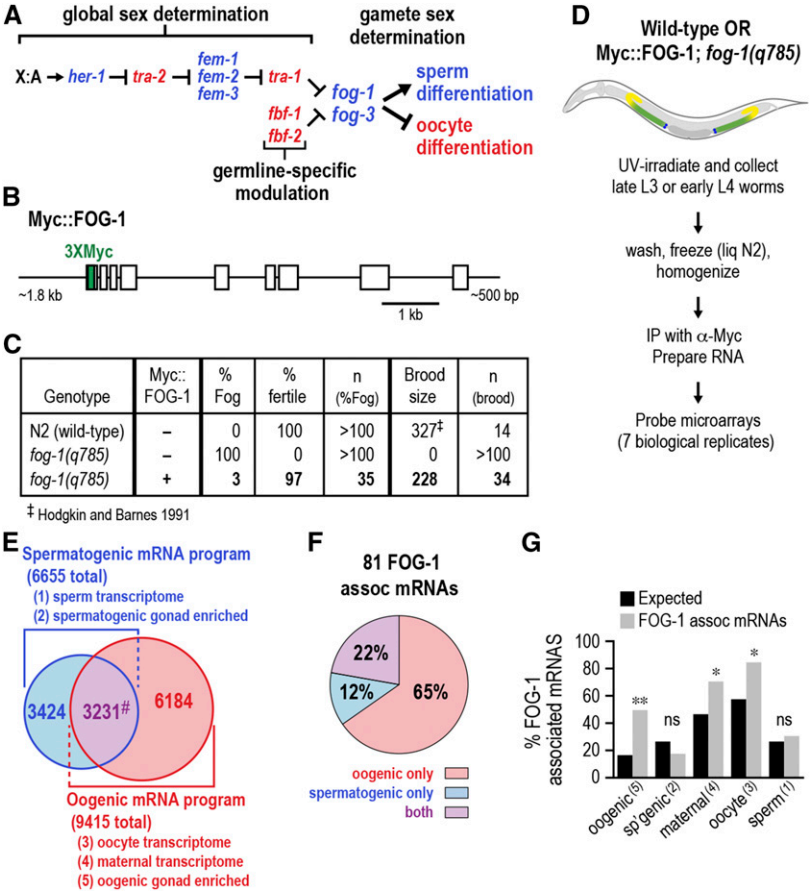


Figure 1 Identification of FOG-1-associated mRNAs. (A) Genetic pathway for regulation of the *C. elegans* sperm/oocyte fate decision. Largely omitted is the elaborate network upstream of *fog-1* and *fog-3* that achieves transient production of sperm in hermaphrodites (see Zarkower 2006 and Ellis and Schedl 2007 for details). (B) Myc::FOG-1 schematic. The Myc::FOG-1 transgene contains the entire *fog-1* locus including all exons and introns, ~1.8 kb upstream of the start codon, ~500 bp downstream of the stop codon, and three copies of the Myc epitope tag at the N terminus; it was introduced into the *C. elegans* genome by *Mos1*-mediated single-copy insertion. (C) Myc::FOG-1 rescues *fog-1(q785)* null mutant hermaphrodites to fertility and supports production of a brood of self-progeny that is ~2/3 of the normal size. (D) RIP-ChIP experimental flow. See text and *Materials and Methods* for details. (E–G) FOG-1-associated mRNAs are enriched for genes in the oogenic mRNA program. (E) Defining the spermatogenic and oogenic mRNA programs. The spermatogenic mRNA program is based on those mRNAs detected by RNA Seq and (1) found in the sperm transcriptome (Ma *et al.* 2014) and (2) enriched in the spermatogenic gonad (Ortiz *et al.* 2014). The oogenic mRNA program is based on those mRNAs (3) found in the oocyte transcriptome by RNA-Seq (Stoeckius *et al.* 2014), (4) found in the maternal transcriptome by microarray (Baugh *et al.* 2003), and (5) enriched in the oogenic gonad by RNA Seq (Ortiz *et al.* 2014). The mRNAs designated as present in both spermatogenic and oogenic mRNA programs (#) also includes mRNAs found in both spermatogenic and oogenic gonads, but not enriched in either one relative to the other (Ortiz *et al.* 2014) and also not found in the sperm, oocyte, or maternal transcriptomes (Baugh *et al.* 2003; Ma *et al.* 2014; Stoeckius *et al.* 2014). By this accounting, 6184 mRNAs are in the oogenic program only; 3424 are in the spermatogenic program only; and 3231 are in both spermatogenic and oogenic programs. (F) Pie chart of FOG-1-associated mRNAs by categories defined in E. Eighty-seven percent of the FOG-1-associated mRNAs belong to the oogenic mRNA program, including 65% that are oogenic only and 22% that are both oogenic and spermatogenic. (G) FOG-1-associated mRNAs are significantly enriched for oogenic but not for spermatogenic transcripts. mRNAs in published data sets (see 1–5 in E) were compared to the FOG-1-associated mRNAs (gray) and to the gonadal transcriptome and to the combined transcriptome of dissected spermatogenic and oogenic XX gonads (expected, black) (Ortiz *et al.* 2014). (*$P < 1E-5$; **$P < 1E-10$; ns, not significant).

both TRA-1 and FBF repress *fog-1* and *fog-3* in hermaphrodites to promote oogenesis (Chen and Ellis 2000; Thompson *et al.* 2005; Berkseth *et al.* 2013; Snow *et al.* 2013) (Figure 1A). Genetic manipulations of TRA-1 and FBF transform germline sexual fate, but their effects rely on the *fog-1* and *fog-3* genes. By contrast, no sperm/oocyte fate regulators have been found downstream of *fog-1* and *fog-3* despite intensive studies over many years. Therefore, *fog-1* and *fog-3* appear to be the terminal regulators of germline sexual fate.

The FOG-1 and FOG-3 proteins both belong to conserved families of post-transcriptional regulators. FOG-1 belongs to the family of CPEB RNA-binding proteins (Luitjens *et al.* 2000; Jin *et al.* 2001a); vertebrate CPEB proteins can function as either translational activators or repressors (Hake and Richter 1994; Stebbins-Boaz *et al.* 1999). FOG-3 belongs to the Tob/BTG family (Chen *et al.* 2000); Tob/BTG proteins are adaptors that recruit deadenylase machinery to mRNAs and repress them (Ikematsu *et al.* 1999; Mauxion *et al.* 2009; Winkler 2010). Intriguingly, CPEB and Tob proteins can interact and function with each other in both vertebrates and *Drosophila* (Hosoda *et al.* 2011; Ogami *et al.* 2014; White-Grindley *et al.* 2014). CPEBs regulate early vertebrate development, the cell cycle, and memory, among other biological roles (Fernandez-Miranda and Mendez 2012; Ivshina *et al.* 2014); Tob/BTG proteins have antiproliferative activity when overexpressed in mammalian tissue culture cells (Jia and Meng 2007; Winkler 2010) and have been implicated as tumor suppressors (Yoshida *et al.* 2003; Yoneda *et al.* 2008). Therefore, Tob/BTG proteins share with CPEB a role in cell cycle control.

The *C. elegans* *fog-1* and *fog-3* genes also have effects, albeit minor, on the germ cell cycle (Thompson *et al.* 2005; Snow *et al.* 2013) in addition to their essential roles in sperm fate specification. Their proliferation functions depend on genetic context and gene dosage, and their effects can differ. For example, wild-type FOG-1 promotes proliferation in an *fbf-1 fbf-2* mutant background (Thompson *et al.* 2005), whereas wild-type FOG-3 inhibits proliferation in the same background (Snow *et al.* 2013). Nonetheless, both genes affect proliferation, suggesting a physiological link with vertebrate homologs.

Although *fog-1* and *fog-3* satisfy genetic criteria for terminal regulators of the sperm fate, their molecular function as

post-transcriptional regulators does not conform to the widespread notion that terminal cell fate regulators are transcription factors. Examples abound, but the first two discovered in eukaryotes serve as paradigms: the **a** and **α** transcription factors are terminal regulators of *Saccharomyces cerevisiae* mating type (*e.g.*, Johnson and Herskowitz 1985), and the MyoD transcription factor serves that role for vertebrate muscle fate specification (Weintraub *et al.* 1991). In these and other cases, the transcription factor controls a battery of cell type-specific differentiation genes to promote one cell fate at the expense of another.

We predicted that the FOG-1 and FOG-3 proteins, as terminal regulators of the sperm fate, might regulate a battery of gamete-specific differentiation genes. Here we test this prediction by exploring on a genomic scale the mRNAs associated with FOG-1 and FOG-3. Immunoprecipitation of the two proteins and their associated mRNAs identifies 81 FOG-1 and 722 FOG-3 putative targets in spermatogenic germlines. Importantly, almost all FOG-1 targets are also FOG-3 targets, and they are strongly biased for oocyte-specific mRNAs. Given their common targets, we hypothesized that FOG-1 and FOG-3 work together biochemically. Consistent with that idea, FOG-1 and FOG-3 proteins co-immunoprecipitate from both nematodes and mammalian tissue culture, and they colocalize in germ cells. Their genetic role in specifying sperm fate, their genomic bias for oogenic mRNAs, their physical interaction in nematodes plus knowledge from other organisms that their homologs can repress mRNAs, taken together, support the model that FOG-1 and FOG-3 work in a complex to repress the oogenic program.

Materials and Methods

Nematode culture

Strains were maintained at 25°. Wild type was the N2 Bristol strain.

Nematode strains and mutations used in this study

Mutations used were *fog-1(q785)* (Morgan *et al.* 2010) and *fog-3(q520)* (Ellis and Kimble 1995). Strains used were the following: N2—wild type; JK3743—*fog-1(q785) I/hT2[qIs48](I;III)*; JK4266—*fog-3(q520) I/hT2[qIs48](I;III)*; JK4982—*fog-1(q785) fog-3(q520) I/hT2[qIs48](I;III)*; EG4322—*ttTi5605 II*; *unc-119(ed3) III*; EG6699—*ttTi5605 II*; *unc-119(ed3) III*; *oxEx1578*; EG6703—*unc-119(ed3) III*; *cxTi10816 IV*; *oxEx1582*; JK5187—*fog-1(q785) I*; *qSi140[3xMyc::fog-1*; *unc-119(+)] IV* [Myc::FOG-1]; JK4871—*fog-3(q520) I*; *qSi41[fog-3::3xFLAG]*; *unc-119(+) II* [FOG-3::FLAG]; JK5200—*fog-1(q785) fog-3(q520) I*; *qSi41[fog-3::3xFLAG*; *unc-119(+)] II*; *qSi140[3xMyc::fog-1*; *unc-119(+)] IV* [Myc::FOG-1 and FOG-3::FLAG]; JK5028—*qSi77[P_{mex-5}::eGFP::3xFLAG::tbb-1 3′utr::gpd-2 SL2 splice site::mCherry::3xMyc::pgl-1 RGG repeat::tbb-1 3′utr and intergenic region*; *unc-119(+)] II*; *unc-119(ed3) III* [eGFP::FLAG and mCherry::Myc]; DG3913—*lin-41(tn1541[GFP::tev::s::lin-41]) I* (Spike *et al.* 2014); RT408—*unc-119(ed3) III*; *pwIs116[rme-2::GFP + unc-119(+)]* (Kang *et al.* 2011); and TX189—*unc-119(ed3) III*; *teIs1[oma-1::GFP*; *unc-119(+)] IV* (Lin 2003).

Generation of strains carrying epitope-tagged FOG-1 and FOG-3 transgenes

To make an epitope-tagged FOG-1 transgene, we first created pJK1776, which harbors the complete *fog-1* genomic sequence (~1.8 kb upstream of the start codon, all exons and introns, and ~500 bp downstream from the stop codon) plus an insert of 3xMyc at the 5′ end of the coding sequence cloned into pCFJ356. To make an epitope-tagged FOG-3 transgene, we first created pJK1660, which contains the complete *fog-3* genomic sequence (~1.3 kb upstream of the start codon, all exons and introns, and ~1.3 kb downstream of the stop codon) plus an insert of 3xFLAG at the 3′ end of the coding sequence, cloned into pCFJ151. Using the *Mos1*-mediated single-copy insertion (MosSCI) method (Frøkjær-Jensen *et al.* 2012), we used pJK1776 to generate *qSi140* in the *cxTi10816* site on *LGIV* of strain EG6703 and pJK1660 to generate *qSi41* in the *ttTi5605* site on *LGII* of strain EG6699. Transgenes were then introduced into appropriate null mutants to generate strains JK5187, JK4871, and JK5200 with the presence of each mutant verified by PCR for deletion mutants, PCR followed by restriction digest, or phenotype as needed.

Generation of strain carrying epitope-tagged GFP and mCherry transgenes

To create *qSi77*, the pJK1728 plasmid (*mex-5* promoter::GFP with introns::3xFLAG::*tbb-1* 3′ UTR::*gpd-2* SL2 splice site::mCherry with introns::3xMyc::*pgl-1* RGG repeat::*tbb-1* 3′ UTR and intergenic region) was injected into EG6699 along with somatic RFP markers and the germline transposase following a MosSCI direct insertion protocol (Frøkjær-Jensen *et al.* 2012). *unc-119*-rescued transgenic animals lacking somatic RFP reporter expression were recovered as described. Insertion of the transgene into the *ttTi5605* site on *LGII* was validated by PCR, and strains were outcrossed three times with N2.

Immunocytochemistry

Synchronized early L4 animals (1–3 hr past the L4 molt) were cut just behind the pharynx in PBST (PBS plus 0.1% Tween-20) with 0.25 mM Levamisole. Dissected animals were fixed in 3% (wt/vol) paraformaldehyde and 0.1 M K_2HPO_4 for 5 min and permeabilized in 100% methanol at −20° for 5 min. Samples were washed three times in PBST and blocked in PBST plus 0.5% BSA for 30 min at room temperature. Samples were incubated in primary antibodies at 4° overnight in PBST plus 0.5% BSA at the following dilutions: mouse anti-FLAG, 1:1000 (Sigma), and rat anti-Myc, 1:300 (Bio-Rad). Alexa Fluor 488- and 647-conjugated secondary antibodies (Molecular Probes) were used at a 1:1000 dilution in PBST plus 0.5% BSA and DAPI (0.5 ng/μl) for 1 hr at room temperature.

Choice of late L3/early L4 XX larvae for RNA coimmunoprecipitation

Because obtaining the necessary quantity of purely isolated males was impractical, we chose late L3/early L4 XX spermatogenic animals for RNA coimmunoprecipitations (RIPs). This choice was based on a number of factors. First, FOG-1 and FOG-3 are the key terminal regulators of sperm fate both in L3/L4 XX hermaphrodites and in XO males (Barton and Kimble 1990; Ellis and Kimble 1995). Second, animals at the stage and temperature (25°) used for RIP have just begun spermatogenesis and do not show morphological or molecular signs of oogenesis (Supporting Information, File S1 Figure S1). JK5187 and JK4871, the transgenic animals used in this study, were examined by differential interference microscopy (DIC) at 3-hr intervals starting from L3/L4 lethargus. At L3/L4 lethargus, not even primary spermatocytes were seen. At 3 hr past L3/L4 lethargus (early L4 and the oldest animals in samples used for RIP), the most proximal germ cells had just begun overt spermatogenesis with few primary spermatocytes, no secondary spermatocytes, and no mature sperm (Figure S1). At later time points, we found primary and a few secondary spermatocytes at 6 hr (mid L4); primary and secondary spermatocytes plus mature sperm at 9 hr (late L4); and final stages of spermatogenesis plus oocytes at 12 hr (early adult) (Figure S1). We also examined strains carrying transgenes for three oogenic markers (LIN-41::GFP, RME-2::GFP, or OMA-1::GFP) at the same intervals and the same temperature (25°); these were assayed by both DIC and GFP expression. By DIC, all three oogenic marker strains followed the same temporal progression of spermatogenesis and then oogenesis seen with JK5187 and JK4871; moreover, their earliest GFP expression was in late L4s and expression became strong only in early adults, the first time that oocytes could be recognized morphologically (Figure S1). We conclude that late L3/early L4 XX animals used for RIPs were spermatogenic and had not begun oogenesis.

RNA coimmunoprecipitations

XX larval hermaphrodites of genotype JK5187 or JK4871 were grown at 25° and collected 3 hr after the first group of animals in the population had entered L3/L4 lethargus. Developmental stages were assessed for all sample preparations by standard measures (stage of vulval development before, after, or in lethargus) and quantified for the first three replicates (~60% early L4; ~20% L3/L4 lethargus; ~20% late L3). The 3xMyc::FOG-1 RIP samples were prepared as follows. Wild-type and JK5187 XX animals were synchronized by bleaching and plating worms on NGM agar plates as synchronized L1s using standard methods and aged to late L3/early L4 stage at 25°. Collected larvae were washed four times in M9 buffer and then exposed to 400 mJ/cm^2 UV radiation on 10-cm plates, washed into 2-ml Eppendorf tubes, and spun down. Approximately 100 μl of packed animals was resuspended in lysis buffer [50 mM HEPES (pH 7.5), 10 mM KCl, 100 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 10% glycerol, 0.1% Tween20, EDTA-free protease inhibitor cocktail (Roche), Protector RNAse inhibitor cocktail (Roche)]. Worms were frozen immediately in liquid nitrogen and stored at −80°. Frozen samples were lysed and homogenized using a mixer mill (Retsch MM400) and cleared by centrifugation at 13,200 × *g* for 10 min at 4°. For each immunoprecipitation, 4 mg of the extract was incubated overnight at 4° with 20 μl Myc affinity gel (Pierce). The beads were washed six times with wash buffer [50 mM Tris–HCl (pH 7.5), 10 mM KCl, 200 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 10% glycerol, 0.1% Tween-20, 1 mM DTT, EDTA-free protease inhibitor cocktail, Protector RNAse inhibitor cocktail (Roche)]. The coimmunoprecipitate was digested with proteinase K and extracted using Trizol (Invitrogen) and the RNeasy Micro kit (Qiagen). Purified RNA samples were run on a BioAnalyzer 2000 (Agilent) to confirm RNA integrity.

The FOG-3::3xFLAG RIP samples were prepared as described above, except we used wild-type and JK4871 worms, extract was incubated with 20 μl FLAG affinity gel (Sigma), and wash buffer contained 500 mM NaCl.

Microarrays

3xMyc::FOG-1 RIP-ChIP: For each of 14 biological replicates (7 each of wild type and JK5187), 50% of total immunoprecipitation (IP) RNA was subjected to one round of linear amplification and labeling with the MessageAMP Premier Amplification Kit (Ambion). For all biological replicates, equal volumes of amplified labeled cRNA were fragmented and hybridized to *C. elegans* whole-genome GeneChip Arrays (Affymetrix). Arrays were stained using the AFX450 Fluidics Station (Affymetrix) and scanned using the AFX GC3000 G7 scanner (Affymetrix) according to established Affymetrix protocols. The data were extracted at the University of Wisconsin Biotechnology Center Gene Expression Center (GEC) using the AFX Expression Console v 1.2 software.

FOG-3::3xFLAG RIP-ChIP: For each of 14 biological replicates (7 each of wild type and JK4871), RIP–chromatin immunoprecipitation (ChIP) was performed as described above, except that data were extracted at the GEC using AFX Expression Console v 1.1 software. The v 1.1 and v 1.2 Console versions generate comparable data.

Microarray analyses

Unscaled intensities were extracted and exported to Bioconductor software. Array data were background-corrected by Robust Multi-Array Average and normalized by Variance Stabilization, both available in Bioconductor package Affy (affy 1.42.2). To identify transcripts enriched in RIP samples, background-corrected and normalized data were analyzed with the Bioconductor package Siggenes (Siggenes 1.38.0) (Schwender *et al.* 2006) using two-class significance analysis of microarrays (SAM) function. Biomart package was used for conversion of Affy IDs to WormBase IDs.

Immunoprecipitation of FOG-1 and FOG-3 in vivo

JK5028 and JK5200 were grown to early L4 stage prior to 1% formaldehyde cross-linking (Fisher), washing in M9 buffer, and freezing. Worms were defrosted in IP buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA, 5 mM $MgCl_2$, 1% Triton-X, 1 M urea) and lysed by mortar/pestle with complete EDTA-free protease inhibitors (Roche). Lysis was clarified by centrifugation (20 min, 16,100 × *g*, 4°), and the supernatant was treated with 10 μg/ml RNaseA (Sigma). The treated supernatant was incubated with wash FLAG or Myc affinity gel for 4 hr at 4° with rotation. Beads were washed six times prior to elution with acid (100 mM glycine, pH 3.0). Samples were run on a 10% polyacrylamide stacking gel, transferred to PVDF (Millipore), and probed with FLAG (Sigma), Myc (Sigma), and actin (C4, Millipore) primary and goat anti-mouse HRP (Jackson Laboratory) secondary antibodies. Immunoblots were developed with ECL Western blotting substrate (Pierce) and exposed on film (Kodak).

In vitro binding analyses of FOG-1 and FOG-3

FOG-1 and FOG-3 coding sequences were amplified from mixed-stage N2 complementary DNA generated from random hexamers (Ambion) and reverse transcription (Invitrogen). HA-maltose binding protein (MBP) and FLAG tags were added by PCR and cloned into pcDNA™3.1 (Invitrogen) by ligation-independent cloning (Aslanidis and De Jong 1990) to get HA-MBP-FOG-1 and FOG-3-FLAG. Deletions of HA-MBP-FOG-1 were obtained by inverse PCR and blunt-end ligation (New England BioLabs). All clones were Sanger-sequenced to confirm identity. Plasmids were calcium-phosphate-transfected (Green *et al.* 2012) in HEK 293T and collected 24 hr later. Cell pellets were lysed in cell lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton-X, 0.5 M urea), clarified by centrifugation (20 min, 16,100 × *g*, 4°), treated with 10 μg/ml RNaseA, and incubated with FLAG antibody agarose resin for 1 hr. Beads were washed with cell lysis buffer and eluted with 100 mM glycine pH 3.0. Samples were run on a 10% polyacrylamide stacking gel, transferred to PVDF (Millipore), and probed with FLAG (Sigma), HA (Covance), and actin primary and goat anti-mouse HRP secondary antibodies. Immunoblots were developed with ECL Western blotting substrate and exposed on film.

Data availability

Raw reads and messenger RNA (mRNA) expression data are available at the Gene Expression Omnibus under accession no. GSE73070.

Results

Identification of FOG-1-associated mRNAs

To identify FOG-1 target mRNAs on a genome-wide scale, we generated a MosSCI of an epitope-tagged Myc::FOG-1 transgene (Figure 1B). This transgenic Myc::FOG-1 mimicked endogenous FOG-1 in that it rescued *fog-1(q785)* null mutants to fertility (Figure 1C) and was expressed in the same germline pattern seen previously for endogenous FOG-1 (Figure S2) (Thompson *et al.* 2005; Lamont and Kimble 2007). Our experimental design is shown in Figure 1D. Briefly, we cross-linked proteins and RNA by UV-irradiating late L3/early L4 XX hermaphrodites (for details see *Materials and Methods*) that had just begun overt spermatogenesis and had not yet switched into oogenesis (Figure S1). We then immunoprecipitated Myc::FOG-1 protein and its associated RNAs using anti-Myc antibodies and probed microarrays (Figure 1D) (for details see *Materials and Methods*). Parallel experiments were done with a rescued *fog-1(q785)*; Myc::FOG-1 transgenic strain and wild-type animals as a control, with seven biological replicates for each strain. The array data were analyzed using SAM (Tusher *et al.* 2001), which assigns a score for each probe set on the array and estimates false discovery rates (FDR). We then used two criteria (FDR of <0.01 and threefold enrichment over control) to identify 81 putative FOG-1 mRNA targets.

We next asked if FOG-1-associated mRNAs were biased for oogenic or spermatogenic transcripts. To this end, we first defined oogenic and spermatogenic mRNA programs using data from a series of independently obtained transcriptomes (Figure 1E). The oogenic mRNA program was compiled using data from the maternal transcriptome (RIP-ChIP of early embryos before zygotic transcription) (Baugh *et al.* 2003) and oocyte transcriptome (RNA-Seq of isolated oocytes) (Stoeckius *et al.* 2014) as well as mRNAs enriched in oogenic young adult XX gonads (RNA-Seq of dissected oogenic and spermatogenic gonads) (Ortiz *et al.* 2014); conversely, the spermatogenic mRNA program was compiled using data from the sperm transcriptome (RNA-Seq of isolated sperm) (Ma *et al.* 2014) as well as mRNAs enriched in spermatogenic gonads compared to oogenic XX gonads (Ortiz *et al.* 2014). These various data sets revealed 6184 mRNAs found or enriched in oogenic germlines, 3424 mRNAs found or enriched in spermatogenic germlines, and 3231 mRNAs in both. Thus the oogenic program contains a total of 9415 mRNAs (Figure 1E; Table S1), and the spermatogenic program contains a total of 6655 mRNAs (Figure 1E; Table S2).

Comparisons of the 81 FOG-1-associated mRNAs (Table S3) to the oogenic and spermatogenic mRNA programs (Figure 1E; Table S1 and Table S2) revealed that despite being immunoprecipitated from spermatogenic germlines, the vast majority (87%) belonged to the oogenic mRNA program, and the majority (65%) were mRNAs detected exclusively or enriched in the oogenic program (Figure 1F; Table S3). Only 12% were mRNAs detected exclusively or enriched in the spermatogenic program (Figure 1F). Thus the FOG-1-associated mRNAs were significantly enriched for oogenic program transcripts and not for spermatogenic program transcripts (hypergeometric distribution, FDR <1E-5; Figure 1G).

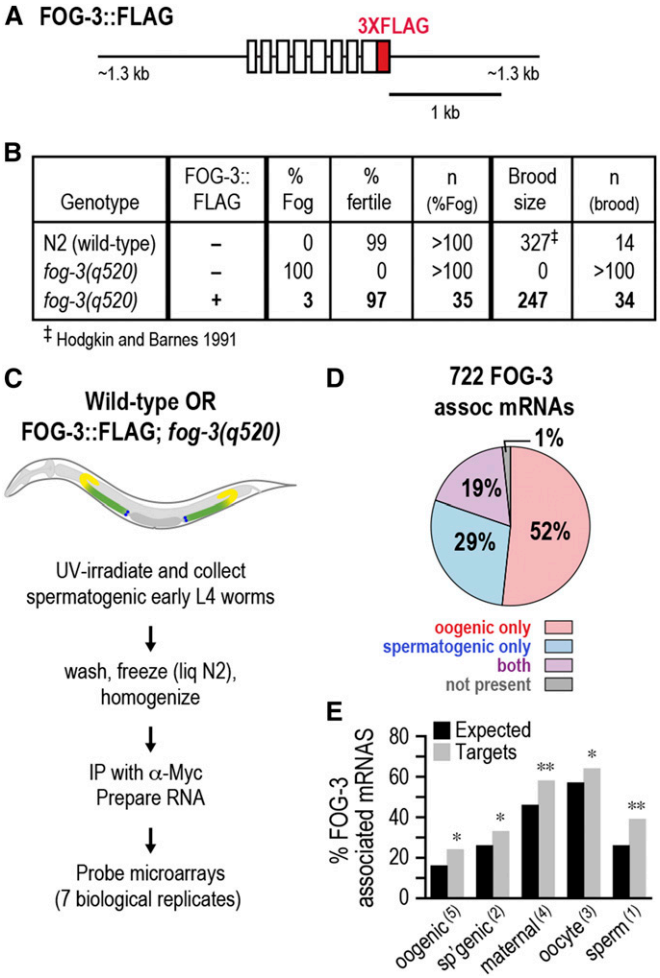
We analyzed the 81 FOG-1-associated mRNAs for enrichment of motifs and functional classes and also examined them for genes of interest. FOG-1 has a CPEB-like RNA-binding domain (Luitjens *et al.* 2000; Jin *et al.* 2001a; Merkel

et al. 2013; Figure 5A), although its RNA-binding element is not yet known. To query for enrichment of 3′ UTR motifs among its putative targets, we used the algorithms MEME (Bailey and Elkan 1994) and DREME (Bailey 2011), but found no enrichment for canonical cytoplasmic polyadenylation elements or other U-rich elements. To query for functional classes, we used DAVID (Huang *et al.* 2009a,b), which revealed enrichment for genes functioning in the cell cycle, embryogenesis, reproduction, and oogenesis. The FOG-1-associated mRNAs included the *fog-1* mRNA itself, which was previously implicated as a likely FOG-1 target in a proposed autoregulatory loop (Jin *et al.* 2001b). Among the cell cycle regulators were two cyclin B genes (*cyb-2.1* and *cyb-2.2*), which are conserved metazoan CPEB targets (de Moor and Richter 1999). Notably absent were other regulators of germline sexual fate and mitosis/meiosis decisions (*e.g.*, *tra-1*, *gld-1*, *gld-2*, *gld-3*, *fog-3*, *fbf-1*, *fbf-2*, *nos-3*) (Ellis and Schedl 2007; Kimble and Crittenden 2007); also absent was *spe-44*, which is a key regulator of the spermatogenesis differentiation program (Kulkarni *et al.* 2012). We conclude that in XX spermatogenic germlines FOG-1 protein is associated predominantly with oogenic mRNAs.

Identification of FOG-3-associated mRNAs

To identify FOG-3-associated mRNAs on a genome-wide scale, we conducted a second group of RIP-ChIP experiments of similar design. In this case, we generated a single-copy insertion of an epitope-tagged FOG-3::FLAG transgene (Figure 2A), which rescued *fog-3(q520)* null mutants to fertility (Figure 2B) and was expressed in the same pattern seen previously for a different rescuing *fog-3* transgene (Figure S1) (Lee *et al.* 2011). Again, parallel immunoprecipitations were done using a rescued *fog-3(q520)*; FOG-3::FLAG transgenic strain and wild-type animals as a control, with seven biological replicates for each (Figure 2C). Using the same methods and stringent criteria described above, we identified 722 putative FOG-3 mRNA targets.

We again queried the oogenic and spermatogenic mRNA programs (Figure 1E; Table S1; Table S2) to ask if FOG-3-associated mRNAs (Table S4) were biased for either program. Nearly all were expressed in the gonad or gametes (714/722), and the majority belonged to the oogenic program (508/722; 70%): more than half (374/722; 52%) were mRNAs detected exclusively or enriched in the oogenic program (Figure 2D). Yet many also belonged to the spermatogenic mRNA program (340/722; 47%), with a substantial number being mRNAs detected exclusively or enriched in the spermatogenic program (206/722; 29%). Indeed, unlike FOG-1, FOG-3-associated mRNAs were significantly enriched for both oogenic and spermatogenic transcriptomes (hypergeometric distribution, FDR < 1E-5; Figure 2E). Analysis by DAVID showed an enrichment of cell cycle, embryonic development, phosphate metabolism, and oogenesis (listed in order of enrichment). Notably, the FOG-1-associated mRNAs *fog-1*, *cyb-2.1*, and *cyb-2.2* were also associated with FOG-3. In addition, FOG-3-associated mRNAs included several regulators of germline sex determination and the mitosis/meiosis decision (*e.g.*, *gld-1*, *gld-3*, *fog-3*, *nos-1*, *rnp-8*). We conclude that, unlike FOG-1, FOG-3 is associated with both oogenic and spermatogenic mRNAs.



Genotype	FOG-3::FLAG	% Fog	% fertile	n (%Fog)	Brood size	n (brood)
N2 (wild-type)	–	0	99	>100	327‡	14
fog-3(q520)	–	100	0	>100	0	>100
fog-3(q520)	+	**3**	**97**	**35**	**247**	**34**

‡ Hodgkin and Barnes 1991

Figure 2 Identification of FOG-3-associated mRNAs. (A) FOG-3::FLAG schematic. The FOG-3::FLAG transgene contains the entire *fog-3* locus, including all exons and introns, ~1.3 kb upstream of the start codon, ~1.3 kb downstream of the stop codon, and three copies of the FLAG epitope tag at the C terminus; it was introduced into the *C. elegans* genome by *Mos1*-mediated single-copy insertion. (B) FOG-3::FLAG rescues *fog-3(q520)* null mutant hermaphrodites to fertility and supports production of a brood of self-progeny ~2/3 of the normal size. (C) RIP-ChIP experimental flow. See text and *Materials and Methods* for details. (D and E) FOG-3-associated mRNAs are enriched for genes in the oogenic and spermatogenic mRNA programs. (D) Pie chart of FOG-3-associated mRNAs by categories defined in Figure 1E. (E) FOG-3-associated mRNAs are significantly enriched for both oogenic and spermatogenic transcripts. As described in Figure 1G, published mRNA data sets were compared to FOG-3-associated mRNAs (gray) and the gonadal transcriptome (expected, black). (*P < 1E-5; **P < 1E-10).

Common FOG-1- and FOG-3-associated mRNAs

A comparison of FOG-1- and FOG-3-associated mRNAs revealed 76 in common (Figure 3A; Table S5), which is unlikely by chance (hypergeometric distribution, FDR < 1E-80). This nonrandom association did not correspond to

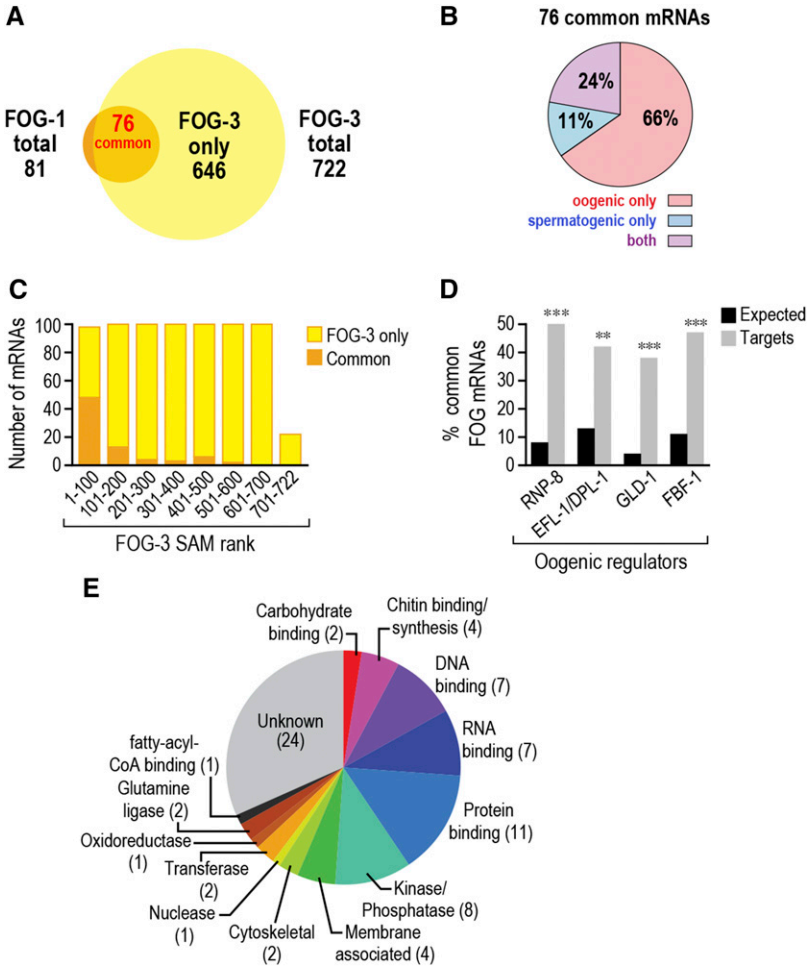


Figure 3 Common FOG-1- and FOG-3-associated mRNAs. (A) Comparison of FOG-1- and FOG-3-associated mRNAs identifies 76 mRNAs in common. (B) Pie chart of common mRNAs by categories defined in Figure 1E. (C) Common mRNAs are biased toward more significant FOG-3 SAM ranks. (D) Common mRNAs are also targets of oogenic regulators. Common FOG mRNAs are enriched for RNP-8 target mRNAs (Kim *et al.* 2010), EFL-1/DPL-1 target genes (Kudron *et al.* 2013), GLD-1 target mRNAs (Jungkamp *et al.* 2011), and FBF-1 target mRNAs. As described in Figure 1G, the gonadal transcriptome was used to define expected values (Ortiz *et al.* 2014). (**$P < 1E-10$; ***$P < 1E-50$). (E) The common mRNAs encode proteins with diverse molecular functions.

abundance in the input samples and therefore likely is of biological significance. The mRNAs in common (common mRNAs) included nearly all (~94%) FOG-1-associated mRNAs and hence had essentially the same transcriptome overlaps and enrichments, with most (~90%) belonging to the oogenic mRNA program (Figure 3B). To learn whether this bias might have resulted from use of XX germlines, we queried the L4/adult XO male transcriptome (Thomas *et al.* 2012) and found 75/76 of the common mRNAs (the single remaining mRNA was not in their XX data set either). To ensure that the FOG-1- and FOG-3-associated mRNAs were not biased toward abundant mRNAs, we analyzed microarray data from whole-worm input samples. We found none of these common mRNAs within the 1000 most abundant mRNAs. The common mRNAs were also strongly biased for those FOG-3-associated mRNAs with a high SAM rank (Figure 3C), suggesting that they are *bona fide* targets of both proteins.

We compared the common mRNAs to transcripts regulated by other regulators of oogenesis or spermatogenesis. The translational regulators FBF-1, GLD-1, and RNP-8 promote the oocyte fate (Zhang *et al.* 1997; Kim *et al.* 2009); GLD-1 and RNP-8 also promote the process of oogenesis (Francis *et al.* 1995; Kim *et al.* 2010), as does the transcriptional regulator DPL-1/EFL-1 (Chi and Reinke 2006). As might be expected given their bias for oogenic mRNAs, the common FOG-1- and FOG-3-associated mRNAs are highly enriched for targets of RNP-8 (Kim *et al.* 2010), EFL-1/DPL-1 (Kudron *et al.* 2013), GLD-1 (Jungkamp *et al.* 2011), and FBF-1 (Kershner and Kimble 2010) (Figure 3D).

The common FOG-1- and FOG-3-associated mRNAs encode proteins with diverse molecular functions plus a substantial proportion [~30% (24/76)] with no known molecular function (Figure 3E). Two of the largest groups with a known molecular function are nucleic acid-binding proteins (18%). The DNA-binding proteins included two paralogs of CENP-A, which is a histone H3 variant critical for kinetochore assembly (HCP-3 and CPAR-1); three proteins involved in DNA repair (DVC-1, W02F12.4, CKU-80); a Rad-21-like cohesion protein (SCC-1); and a component of the origin recognition complex (MCM-5). The RNA-binding proteins included FOG-1 itself, two quaking paralogs (K07H8.9, Y69A2AR.32), and four PUF paralogs (PUF-6, -7, -10, and -11). One possibility might have been that FOG-1 and FOG-3 promote the sperm fate by regulating transcription factors that are the actual terminal regulators of the sperm fate. Genetics might not have found such transcription factors due to either redundancy or pleiotropy. Yet transcription factors were notably absent from the list of

common FOG-1- and FOG-3-associated mRNAs. Therefore, FOG-1 and FOG-3 remain our best candidates for terminal regulators of the sperm fate regulatory pathway.

FOG-1 and FOG-3 proteins interact in nematodes

The strong overlap of FOG-1- and FOG-3-associated mRNAs suggested that these two regulators might work together in nematode germ cells. To test this idea, we generated a strain carrying both Myc::FOG-1 and FOG-3::FLAG transgenes in a *fog-1(q785) fog-3(q520)* double mutant. This strain ensured analysis of functional proteins, because the two transgenes rescued the double mutant to fertility (Figure 4A). We first assayed the proteins cytologically. Both were expressed in early meiotic prophase germ cells as expected (Figure S2). More importantly, Myc::FOG-1 colocalized with FOG-3::FLAG in puncta at the nuclear periphery (Figure 4B), consistent with formation of a protein complex *in vivo*. We then assayed them on Western blots after RNase treatment and IP. Myc::FOG-1 coimmunoprecipitated with FOG-3::FLAG (Figure 4C, left) and vice versa (Figure 4C, right). By contrast, epitope-tagged GFP::FLAG and mCherry::Myc did not co-immunoprecipitate (Figure 4C). These experiments also show that more FOG-3 protein was present than FOG-1 on Western blots (Figure 4C, Input) and that more FOG-3 was immunoprecipitated than FOG-1 (Figure 4C, Elute), which may explain why the FOG-3 pull downs identified more mRNAs than the FOG-1 pull downs. We conclude that nematode FOG-1 and FOG-3 interact in an RNA-independent fashion and likely function within the same protein complex.

FOG-1 and FOG-3 interact in tissue culture cells

To explore the interaction between FOG-1/CPEB and FOG-3/Tob further, we turned to HEK 293T human tissue culture cells. We first asked which FOG-1 domain was responsible for the FOG-3 interaction. FOG-1 harbors an N-terminal region lacking motifs and a C-terminal RNA-binding domain (Figure 5A). We coexpressed FLAG-tagged FOG-3 with variants of MBP-tagged FOG-1 and assayed their interaction by coimmunoprecipitation. The FOG-3::FLAG IP enriched for full-length MBP-FOG-1 (Figure 5B, right, lane 8), but not for MBP alone (Figure 5B, right, lane 9). FOG-3::FLAG IP also enriched for FOG-1 lacking its N-terminal domain, but not for variants lacking any part of the RNA-binding domain (Figure 5B, right, lanes 11-14) even though all were expressed (Figure 5B, left). Therefore, the FOG-1–FOG-3 interaction requires the FOG-1 RNA-binding domain.

The *C. elegans* genome encodes four CPEB paralogs: FOG-1 is essential for sperm fate specification; CPB-1 promotes the process of spermatogenesis; CPB-3 functions in oocyte production; and CPB-2 has no known function but is expressed during spermatogenesis (Barton and Kimble 1990; Luitjens *et al.* 2000; Hasegawa *et al.* 2006). To probe the specificity of FOG-3 interaction with diverse CPEBs, we coexpressed FOG-3::FLAG with the RNA-binding domains of all four *C. elegans* CPEBs. The FOG-3::FLAG IP enriched for FOG-1 and CPB-1 RNA-binding domains, but not for CPB-2 or CPB-3 RNA-binding domains (Figure 5C, right), even though all were expressed (Figure 5C, left). Therefore, the FOG-3–CPEB interaction is specific for FOG-1 and CPB-1 among the four CPEB paralogs.

A

Genotype	Myc::FOG-1	FOG-3::FLAG	% Fog	% fertile	n (%Fog)	Brood size	n (brood)
fog-1(0) fog-3(0)	–	–	100	0	>100	0	>100
fog-1(0) fog-3(0)	+	+	2	98	50	187	50

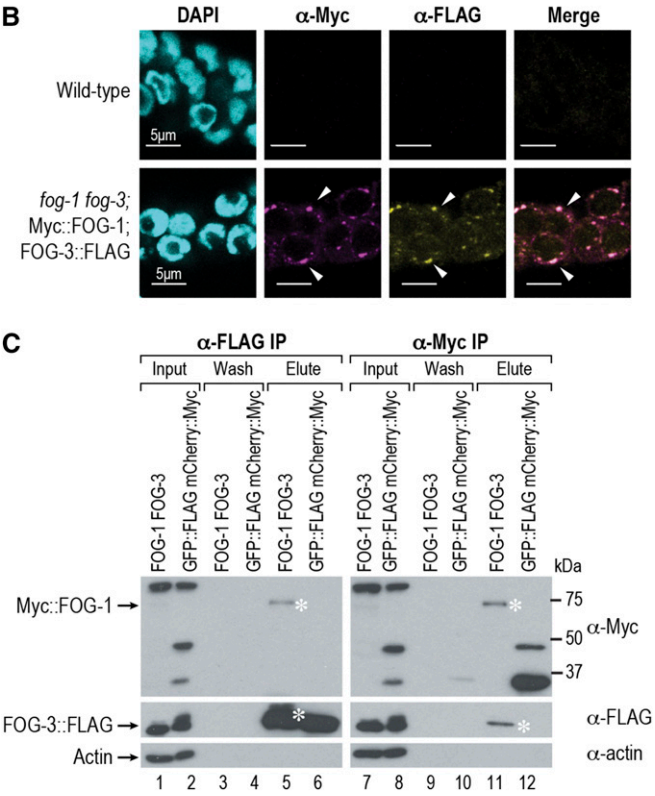


Figure 4 FOG-1 and FOG-3 proteins interact *in vivo*. (A) The Myc::FOG-1 and FOG-3::FLAG single-copy transgenes rescue the *fog-1 fog-3* double-mutant phenotype in hermaphrodites. (B) FOG-1 and FOG-3 costain in the early L4 hermaphrodite germline. The germline was immunostained using anti-Myc antibodies to visualize Myc::FOG-1 (magenta), anti-FLAG antibodies to visualize FOG-3::FLAG (yellow), and DAPI to visualize the DNA (blue). Both FOG-1 and FOG-3 form puncta around the germ cell nucleus. The merged image shows colocalization of the FOG-1 and FOG-3 puncta (white) around the nucleus. (C) Western blots of FOG-1 and FOG-3 reciprocal coimmunoprecipitations from rescued *fog-1 fog-3* mutant hermaphrodites carrying Myc::FOG-1 and FOG-3::FLAG transgenes (JK5200). Control coimmunoprecipitations were done in parallel from hermaphrodites carrying GFP::FLAG and mCherry::Myc transgenes (JK5028). (Left) Anti-FLAG agarose beads used to immunoprecipitate FLAG-tagged protein from lysates of early L4 transgenic animals and blotted to detect both FOG-1 and FOG-3 (asterisks). (Right) Anti-Myc agarose beads used to immunoprecipitate FOG-1 and blotted to detect both FOG-1 and FOG-3 (asterisks).

Discussion

FOG-1/CPEB and FOG-3/Tob associate with a battery of oogenic mRNAs

FOG-1/CPEB and FOG-3/Tob behave genetically as terminal regulators of the sperm/oocyte fate decision and are

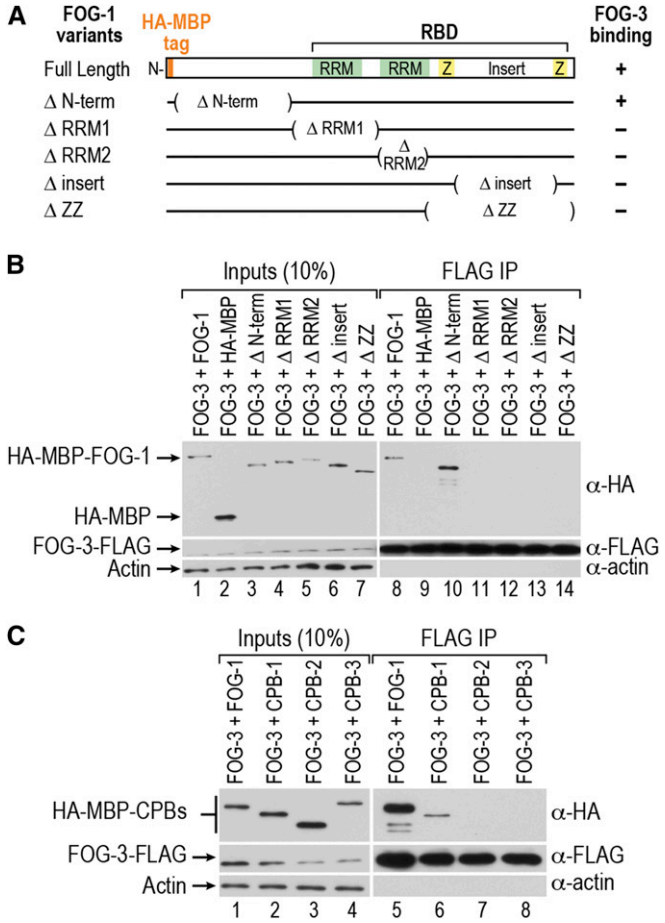


Figure 5 FOG-1 and FOG-3 proteins interact *in vitro*. (A) Domain structure of HA-MBP-FOG-1 and its variants tested for FOG-3 association. RRM, RNA recognition motif; ZZ, cysteine/histidine-rich motif. See Merkel *et al.* (2013) for ZZ motif. (B) FOG-1 associates with FOG-3 via the FOG-1-predicted RNA-binding domain. HEK 293T cells were transfected with FOG-3::FLAG and one of six HA-MBP-FOG-1 variants (see A). Western blots show that all proteins were expressed (left). Full-length FOG-1 associates with FOG-3 as does the FOG-1 variant lacking the N-terminal domain, but variants lacking any part of the predicted RNA-binding region (RBD) fail to associate with FOG-3 (right). Actin served as a loading control. (C) Specific FOG-1–FOG-3 binding. HEK 293T cells were transfected with FOG-3::FLAG and one of four HA-MBP-CPEBs (RBD). Western blots show that all proteins were expressed (left), but that only FOG-1 and CPB-1 associated with FOG-3 (right). Actin served as loading control.

predicted to regulate gene expression post-transcriptionally (see Introduction). To gain insight into how FOG-1 and FOG-3 regulate germ cell fate, we investigated their associated mRNAs on a genomic scale. We found 81 mRNAs associated with FOG-1 and 722 mRNAs associated with FOG-3; these mRNAs are putative targets, and henceforth we refer to them as "targets" for simplicity. As with any genomic study, these targets likely include false positives, but four findings suggest that they may largely represent *bona fide* targets. First, almost all FOG-1 targets (94%) were also FOG-3 targets, which is unlikely by chance (see *Results*) and suggests that FOG-1 and FOG-3 work together to regulate a battery of common targets. Second, the common targets include the *fog-1* mRNA, which was previously proposed as a likely FOG-1 target (Jin *et al.* 2001b). Third, the common targets include mRNAs encoding cyclin B (*cyb-2.1* and *cyb-2.2*), which is a phylogenetically conserved CPEB target (de Moor and Richter 1999; Pique *et al.* 2008; Igea and Méndez 2010).

Finally and most intriguingly, the common targets are heavily biased for mRNAs expressed during oogenesis (~90%), and most (66%) belong uniquely to the oogenic mRNA program (*i.e.*, they are missing from the spermatogenic mRNA program). This striking bias for oogenic mRNAs is of particular note because immunoprecipitations were done from spermatogenic germlines. One caveat might have been our use of XX larval spermatogenic germlines in this study, which was done for technical reasons (see *Materials and Methods*). However, the XX germlines were validated as spermatogenic and not oogenic (Figure S1) and virtually all oogenic-biased common mRNAs were also present in the XO male L4/adult transcriptome (Thomas *et al.* 2012). Given that FOG-1 and FOG-3 function similarly in both sexes (Barton and Kimble 1990; Ellis and Kimble 1995), the most likely scenario is that they associate with oogenic mRNAs in both XX and XO germlines. In addition to the common mRNAs, FOG-3 on its own was associated with many more oogenic transcripts. Indeed, the FOG-3-only associated mRNAs introduce an additional 440 transcripts that belong to the oogenic mRNA program, including 324 belonging uniquely to the oogenic mRNA program. One simple explanation is that the FOG-3-only oogenic mRNAs are in fact FOG-1–FOG-3 common mRNAs that escaped detection with FOG-1. Regardless, we conclude that both FOG-1 and FOG-3 are heavily biased for association with oogenic mRNAs.

We scoured the FOG-1 and FOG-3 putative targets for key regulators of germ cell fates. Regulators of the sperm or oocyte fates were not among the common targets, except for *fog-1* itself. This finding is consistent with genetic epistasis experiments placing the *fog-1* and *fog-3* genes at the end of the sperm/oocyte fate determination pathway (Barton and Kimble 1990; Ellis and Kimble 1995). A second function of FOG-1 and FOG-3 is cell cycle control, although their effects on germ cell proliferation are complex (Thompson *et al.* 2005; Snow *et al.* 2013) (see Introduction). The presence of key cell cycle regulators among their common targets (*e.g.*, cyclin B) and their enrichment for the term "cell cycle" according to DAVID suggest an intimate relationship between sperm fate regulation and the cell cycle. Investigating that relationship is likely complex and remains a challenge for the future. We conclude that FOG-1 and FOG-3 associate with and likely regulate a battery of oocyte-specific differentiation mRNAs as well as cell cycle regulators. This finding is consistent with FOG-1 and FOG-3 being terminal regulators of the sperm fate.

FOG-1/CPEB and FOG-3/Tob proteins work together to control germ cell fates

One molecular model consistent with *fog-1* and *fog-3* genetics—identical null phenotypes and identical genetic positions in

the sex determination pathway—is that the FOG-1 and FOG-3 proteins work together in a complex to specify the sperm fate. Here we provide evidence for that idea. FOG-1 and FOG-3 proteins coimmunoprecipitate with a set of common target mRNAs, and the proteins also coimmunoprecipitate with each other; they colocalize in germ cells; and they coimmunoprecipitate from mammalian cells. An interaction between CPEB and Tob proteins has also been observed in other organisms. For example, human homologs CPEB and Tob interact with each other to regulate cell growth in tissue culture (Hosoda *et al.* 2011; Ogami *et al.* 2014), and *Drosophila* homologs Orb2/CPEB and Erb2/Tob interact to regulate memory formation (White-Grindley *et al.* 2014). Moreover, their interaction relies on the CPEB RNA-binding region of both human and worm counterparts (Hosoda *et al.* 2011; this work). A CPEB–Tob biochemical relationship therefore appears to be broadly conserved.

How does the FOG-1–FOG-3 complex regulate mRNAs? Molecular clues come from studies in other systems. *Xenopus* CPEB binds specifically to its targets and recruits either poly (A) polymerases or the PARN deadenylase complex, which in turn either activate or repress RNAs (Kim and Richter 2006). Human Tob recruits the CCR4-NOT deadenylase and represses mRNAs (Miyasaka *et al.* 2008; Winkler 2010). Perhaps most relevant here, the CPEB–Tob complex represses mRNAs, presumably by recruitment of a deadenylase (Hosoda *et al.* 2011; Ogami *et al.* 2014). By analogy, we suggest that the FOG-1/CPEB RNA-binding protein functions as a specificity factor that guides FOG-3/Tob to common targets and that the FOG-1–FOG-3 complex represses those mRNAs, perhaps by recruitment of a deadenylase (Figure 6, A and B). One test of this model for the *C. elegans* proteins might have been to compare expression of target 3′ UTR reporters in wild-type and mutant germlines; however, FOG-1 and FOG-3 normally function in germlines destined to be spermatogenic, and their removal drives oogenesis. At the current time, no mutant background has been found that permits spermatogenesis in the absence of FOG-1 and FOG-3, a fact that precludes comparison in the same type of cells. Another test of the model would compare expression of reporters harboring 3′ UTRs with wild-type or mutant FOG-1-binding elements; however, that element has not yet been identified. Vertebrate CPEBs bind U-rich 3′ UTR elements, but most *C. elegans* 3′ UTRs are U-rich, and no U-rich motif was enriched in the target 3′ UTRs. Testing this model must therefore await delineation of the FOG-1-binding element. Regardless, our model for FOG-1–FOG-3 control of sperm fate specification is based on several lines of evidence: their genetic role in specifying sperm fate, their heavy bias for association with oogenic mRNAs in a spermatogenic germline, their physical interaction in nematodes, and knowledge from other organisms that their homologs can repress mRNAs. Together, these findings support the notion that FOG-1 and FOG-3 work in a complex to repress the oogenic mRNA program (Figure 6A). This model follows the same molecular logic used by other terminal cell fate regulators, which control batteries of genes encoding products required for cell-specific differentiation (*e.g.*, Johnson and Herskowitz 1985; also see below).

FOG-3 may have a FOG-1-independent function in spermatogenesis

The FOG-1- and FOG-3-associated mRNAs have distinct sexual signatures. The vast majority of FOG-1 targets as well as the common targets of the two FOG regulators belong to the oogenic program (90%), as discussed above, but a substantial percentage of the targets unique to FOG-3 belong to the spermatogenic program and do not overlap with the oogenic program (29%). One simple explanation might be that these FOG-3-associated spermatogenic mRNAs are false positives. Yet our cutoff was stringent, and the spermatogenic targets were not enriched for abundant mRNAs. An alternative explanation is that FOG-3 functions during the process of spermatogenesis in addition to its role in sperm fate specification. Such a *fog-3* role has not been suggested before, but the *fog-3* mutant germline is sexually transformed from spermatogenic to oogenic (Ellis and Kimble 1995), precluding discovery of a role during spermatogenesis.

How might FOG-3 regulate mRNAs during spermatogenesis? One idea is suggested by our result (Figure 5C) that FOG-3 binds a second *C. elegans* CPEB paralog, called CPB-1, which is expressed in spermatogenic cells and required for spermatogenesis (Luitjens *et al.* 2000). CPB-1 may act as a specificity factor that guides FOG-3 to its spermatogenic targets. A simple prediction of this idea, which can be tested once tools are available for CPB-1, is that FOG-3 and CPB-1 share a set of common spermatogenic mRNAs. If, indeed, CPB-1–FOG-3 controls spermatogenic mRNAs, one initially might think that the complex would activate them. Although plausible, a repressive mechanism (Figure 6C) is more in line with the functions of conserved homologs (Hosoda *et al.* 2011; Ogami *et al.* 2014) and also more in line with the likely FOG-1–FOG-3 repression of oogenic mRNAs (see above). A CPB-1–FOG-3 repressive complex might delay translation of spermatogenic mRNAs until the appropriate stage of spermatogenesis or store them for delivery to the embryo as paternal mRNAs. Distinguishing among these possibilities must await identification of FOG-3 partners of spermatogenic mRNAs and, if CPB-1, the analysis of CPB-1–FOG-3 targets and functional assays.

Post-transcriptional control of the sperm/oocyte decision: A conserved theme?

Many common FOG-1–FOG-3 target mRNAs encode oocyte differentiation proteins that function in oocyte maturation and embryonic viability: *egg-4* contributes to the oocyte/embryo transition (Parry *et al.* 2009); *cbd-1*, *cpg-1*, and *chs-1* function in osmotic integrity of the embryo (Zhang *et al.* 2005; Olson *et al.* 2006; Johnston *et al.* 2010); and *rme-2* drives yolk transport (Grant and Hirsh 1999). An attractive model is that the FOG-1–FOG-3 RNA regulatory complex specifies the sperm fate, at least in part, by repression of

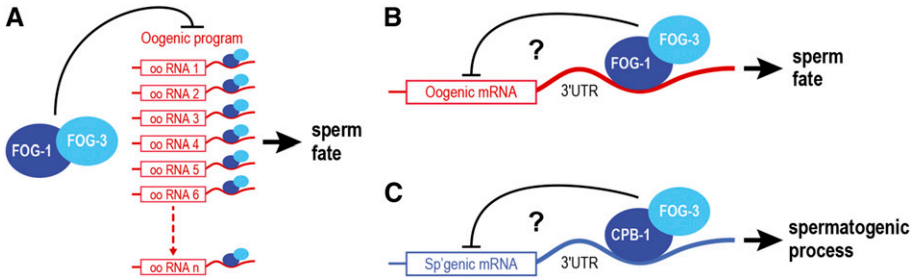


Figure 6 Models for FOG-1 and FOG-3 regulation of target mRNAs and cell fate. See text for explanations. (A) Model for regulation of the sperm fate. We propose that a FOG-1–FOG-3 complex represses a battery of oogenic differentiation mRNAs and that this broad-spectrum repression leads germ cells to adopt the sperm fate. (B) Model for FOG-1–FOG-3 repression of oogenesis mRNAs. We propose that FOG-1 binds in a sequence-specific fashion to regulatory elements in its oogenic mRNA targets and thereby guides FOG-3 to those targets. (C) Model for CPB-1–FOG-3 control of spermatogenic mRNAs. We propose that CPB-1 may function with FOG-3 to regulate spermatogenic mRNAs; alternatively, FOG-3 may function with other partners not yet known. The idea that a CPB-1–FOG-3 complex represses spermatogenic mRNAs is counterintuitive, but we regard it as most likely (see text for rationale) if CPB-1 is indeed the FOG-3 partner at spermatogenic mRNAs.

the oogenic mRNA program (Figure 6A). This model brings together the established biological role of FOG-1 and FOG-3, which is to specify the sperm fate (Barton and Kimble 1990; Ellis and Kimble 1995), with the discovery that FOG-1 and FOG-3 associate primarily with oogenic mRNAs (this work). The alternative idea—that the FOG-1–FOG-3 complex activates oogenic mRNAs rather than represses them—seems unlikely for two reasons. First, FOG-1 and FOG-3 are not required to make functional oocytes (Barton and Kimble 1990; Ellis and Kimble 1995); second, FOG-1 protein disappears from germ cells within 2 hr of being chemically switched from a spermatogenic to oogenic fate and that disappearance occurs many hours before the switched germ cells overtly begin oogenesis (Morgan *et al.* 2013). The biochemical mechanism of this proposed mRNA repression is unknown. Vertebrate CPEB and Tob/BTG recruit the CCR4-Not deadenylase to shorten poly(A) tail length (Ogami *et al.* 2014), but FOG-1 and FOG-3 are divergent homologs (Luitjens *et al.* 2000; Jin *et al.* 2001b) and their biochemical mode of action must await experimentation.

Regulation of mating type in *S. cerevisiae* was the first and arguably remains the best understood example of cell fate regulation (*e.g.*, Johnson and Herskowitz 1985), and the logic used for mating-type control extends to a variety of somatic cell fates (*e.g.*, muscle, neurogenein neuron, pancreatic β-cell). In each case, a key transcription factor controls a battery of cell type-specific differentiation genes to promote one cell fate at the expense of another (Buckingham and Relaix 2007; Mastracci and Sussel 2012; Wilkinson *et al.* 2013; Cano *et al.* 2014; Comai and Tajbakhsh 2014). Our FOG-1–FOG-3 model follows the same regulatory logic, but employs a key RNA regulator rather than a transcription factor (Figure 6A). The finding that terminal regulators of cell fate act at an RNA rather than a DNA level is unusual. The only other metazoan example is control of the mitosis/meiosis decision, which also occurs in germ cells (see Kimble 2011 for review). In this case, the FBF RNA-binding protein represses mRNAs that encode key regulators of meiotic entry as well as mRNAs that encode meiosis differentiation proteins. Mitosis/meiosis regulators, however, could be relegated to cell cycle rather than cell fate regulation. FOG-1–FOG-3, by contrast, definitively expand the molecular repertoire of metazoan cell fate regulators to include RNA regulation.

A major question is whether terminal regulators of the sperm/oocyte fate decision act at a post-transcriptional level in other animal phyla. The *Drosophila* RNA-binding protein Sex lethal (Sxl) initiates and maintains the female germ cell fate (Hashiyama *et al.* 2011; Shapiro-Kulnane *et al.* 2015). Moreover, *Sxl* down-regulates Nanos to enable differentiation of germline stem cells (Chau *et al.* 2012). In mammals, the RNA-binding protein Nanos2 is required for male-specific gene expression in testis germ cells (Suzuki and Saga 2008; Suzuki *et al.* 2014) and drives ectopic male-specific gene expression in ovary germ cells (Suzuki and Saga 2008). Yet the positions of *Drosophila* Sxl and murine Nanos2 in their respective regulatory pathways of germline sex determination remain unknown. Importantly, the *C. elegans* *fog-1* and *fog-3* mRNAs are themselves subject to post-transcriptional regulation, and mutations in their upstream regulators also sexually transform the germline (Zhang *et al.* 1997; Thompson *et al.* 2005; Kershner and Kimble 2010; Snow *et al.* 2013). For example, the *C. elegans* Nanos2 homolog, *nos-3*, promotes the sperm fate, but does so as a partner of RNA-binding protein FBF, which acts upstream of the *fog-1* and *fog-3* mRNAs (Kraemer *et al.* 1999; Thompson *et al.* 2005; Snow *et al.* 2013). Therefore, intriguing parallels exist between worms, flies, and mammals in that RNA regulation influences the sperm/oocyte fate decision. A major challenge is identification of the terminal sperm/oocyte fate regulators in other metazoans. Only then will we know if the *C. elegans* sperm fate regulators are evolutionary deviants or prophets of ancient rites.

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GENETICS

Supporting Information
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Genomic Analyses of Sperm Fate Regulator Targets Reveal a Common Set of Oogenic mRNAs in *Caenorhabditis elegans*

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Timing	Developmental stage	L3/L4	early L4	mid L4	late L4	early A
	Hrs after L3/L4 lethargus	0 hrs	3 hrs	6 hrs	9 hrs	12 hrs
Assay for germ cell differentiation	spermatogenesis DIC	–	+	+	+	+
	oogenesis DIC	–	–	–	–	+
	LIN-41::GFP	–	–	–	+	+
	RME-2::GFP	–	–	–	–	+
	OMA-1::GFP	–	–	–	–	+

Figure S1 Early L4 larvae (3 hours after L3/L4 lethargus) have just begun spermatogenesis and do not start making oocytes for another 9 hours. Results of a 12-hour time course assaying germ cell differentiation. All assays were done at 25° with at least 5 germlines examined for each time point and each strain. DIC assays: transgenic strains central to this work (JK5187 [Myc::FOG-1] and JK4871 [FOG-3::FLAG]) were examined for both spermatogenesis (primary spermatocytes, secondary spermatocytes or mature sperm) and oogenesis (immature early stage oocytes distal to spermatogenic region), which were scored as either present (+) or absent (-). Oocytes were only present at the final stage scored. In addition, we scored three molecular markers specific for oogenesis, including an early oogenesis marker, LIN-41::GFP (DG3913) (Spike *et al*. 2014), a later oogenesis marker, RME-2::GFP (RT408) (Kang *et al*. 2011), and an even later-stage oogenesis marker, OMA-1::GFP (TX189) (Lin 2003). For all strains, DIC revealed that primary spermatocytes were first seen at 3 hours after L3/L4 lethargus (the latest stage used for RIP) and spermatogenesis remained in progress at 9 hours after L3/L4 lethargus.

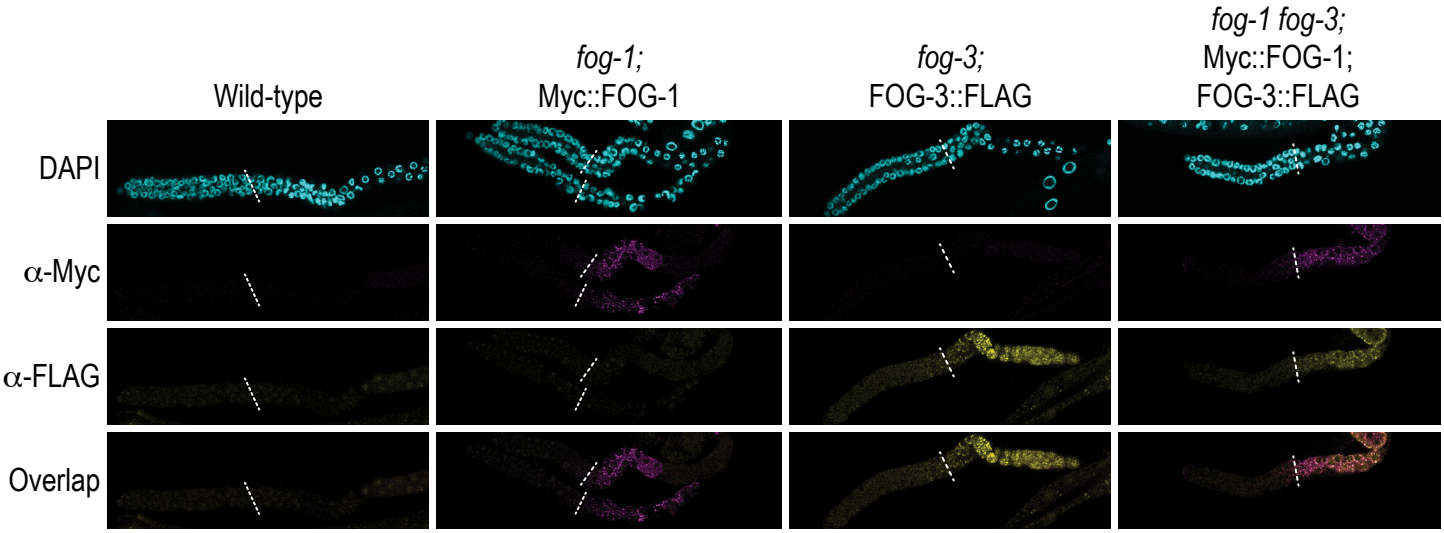

Figure S2 Expression of Myc::FOG-1 and FOG-3::FLAG in early L4 spermatogenic hermaphrodite germlines. Stained gonads were dissected from animals of the following genotypes: (1) N2 (wild type), (2) *fog-1;* Myc::FOG-1, (3) *fog-3;* FOG-3::FLAG, and (4) *fog-1 fog-3;* FOG-3::FLAG; Myc::FOG-1. Staining was done with anti-Myc antibodies (magenta), anti-FLAG antibodies (yellow), and DAPI (blue). Dashed white lines mark the boundary between the mitotic and transition zones, where germ cells enter meiotic prophase and differentiate as sperm or oocyte (Francis *et al*. 1995a). Both Myc::FOG-1 and FOG-3::FLAG are expressed in the transition zone and also more proximally in their respective single transgenic animals and in the double transgenic. Both Myc::FOG-1 and FOG-3::FLAG are expressed in a pattern similar to that seen in previous publications (Lamont and Kimble 2007; Lee *et al*. 2011).

Table S1 Transcripts in oogenic mRNA program (Figure 1E). Curated list of mRNAs found in the maternal transcriptome (Baugh *et al.* 2003), found in the oocyte transcriptome (Stoeckius *et al.* 2014) and enriched in the oogenic gonad (Ortiz *et al.* 2014). (.xlsx, 389KB)

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Table S2 Transcripts in spermatogenic mRNA program (Figure 1E). List of mRNAs in program described in Figure 1E. Curated set of mRNAs found in the sperm transcriptome (Ma *et al.* 2014) and enriched in the spermatogenic gonad (Ortiz et al. 2014). (.xlsx, 251 KB)

Available for download as a .xlsx file at
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Table S3 FOG-1–associated mRNAs identified by RIP-ChIP. Table of 81 FOG-1–associated mRNAs annotated with the overlaps with the following datasets: Sp-gonad (enriched in the spermatogenic gonad); Oo-gonad (enriched in the oogenic gonad); GN-gonad (gender neutral in the gonad); Maternal (maternal transcriptome); Oocyte (oocyte transcriptome); Sperm (sperm transcriptome); RNP-8 (RNP-8–associated mRNAs); EFL/DPL (EFL-1/DPL-1 target genes); FBF-1 (FBF-1–associated mRNAs); GLD-1 (GLD-1–associated mRNAs); Common (common FOG-1– and FOG-3–associated mRNAs); spermatogenic mRNA program; oogenic mRNA program. (.xlsx, 18 KB)

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Table S4 FOG-3–associated mRNAs identified by RIP-ChIP. Table of 722 FOG-3–associated mRNAs annotated with the overlaps with the following datasets: Sp-gonad (enriched in the spermatogenic gonad); Oo-gonad (enriched in the oogenic gonad); GN-gonad (gender neutral in the gonad); Maternal (maternal transcriptome); Oocyte (oocyte transcriptome); Sperm (sperm transcriptome); RNP-8 (RNP-8–associated mRNAs); EFL/DPL (EFL-1/DPL-1 target genes); FBF-1 (FBF-1–associated mRNAs); GLD-1 (GLD-1–associated mRNAs); Common (common FOG-1– and FOG-3–associated mRNAs); spermatogenic mRNA program; oogenic mRNA program. (.xlsx, 96 KB)

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www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.182592/-/DC1/TableS4.xlsx

Table S5 Common FOG-1– and FOG-3–associated mRNAs identified by RIP-ChIP. Table of 76 common mRNAs annotated for overlaps with the following datasets: Sp-gonad (enriched in the spermatogenic gonad); Oo-gonad (enriched in the oogenic gonad); GN-gonad (gender neutral in the gonad); Maternal (maternal transcriptome); Oocyte (oocyte transcriptome); Sperm (sperm transcriptome); RNP-8 (RNP-8–associated mRNAs); EFL/DPL (EFL-1/DPL-1 target genes); FBF-1 (FBF-1–associated mRNAs); GLD-1 (GLD-1–associated mRNAs); spermatogenic program; oogenic program. (.xlsx, 15 KB)

Available for download as a .xlsx file at
www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.182592/-/DC1/TableS5.xlsx

Table S1. Transcripts in oogenic mRNA program (Figure 1E). Curated list of mRNAs found in the maternal transcriptome (Baugh *et al.* 2003), found in the oocyte transcriptome (Stoeckius *et al.* 2014) and enriched in the oogenic gonad (Ortiz *et al.* 2014).

Table S2. Transcripts in spermatogenic mRNA program (Figure 1E). List of mRNAs in program described in Figure 1E. Curated set of mRNAs found in the sperm transcriptome (Ma *et al.* 2014) and enriched in the spermatogenic gonad (Ortiz *et al.* 2014).

Table S3. FOG-1–associated mRNAs identified by RIP-ChIP. Table of 81 FOG-1–associated mRNAs annotated with the overlaps with the following datasets: Sp-gonad (enriched in the spermatogenic gonad); Oo-gonad (enriched in the oogenic gonad); GN-gonad (gender neutral in the gonad); Maternal (maternal transcriptome); Oocyte (oocyte transcriptome); Sperm (sperm transcriptome); RNP-8 (RNP-8–associated mRNAs); EFL/DPL (EFL-1/DPL-1 target genes); FBF-1 (FBF-1–associated mRNAs); GLD-1 (GLD-1–associated mRNAs); Common (common FOG-1– and FOG-3–associated mRNAs); spermatogenic mRNA program; oogenic mRNA program.

Table S4. FOG-3–associated mRNAs identified by RIP-ChIP. Table of 722 FOG-3–associated mRNAs annotated with the overlaps with the following datasets: Sp-gonad (enriched in the spermatogenic gonad); Oo-gonad (enriched in the oogenic gonad); GN-gonad (gender neutral in the gonad); Maternal (maternal transcriptome); Oocyte (oocyte transcriptome); Sperm (sperm transcriptome); RNP-8 (RNP-8–associated mRNAs); EFL/DPL (EFL-1/DPL-1 target genes); FBF-1 (FBF-1–associated mRNAs); GLD-1

(GLD-1–associated mRNAs); Common (common FOG-1– and FOG-3–associated mRNAs); spermatogenic mRNA program; oogenic mRNA program.

Table S5. Common FOG-1– and FOG-3–associated mRNAs identified by RIP-ChIP. Table of 76 common mRNAs annotated for overlaps with the following datasets: Sp-gonad (enriched in the spermatogenic gonad); Oo-gonad (enriched in the oogenic gonad); GN-gonad (gender neutral in the gonad); Maternal (maternal transcriptome); Oocyte (oocyte transcriptome); Sperm (sperm transcriptome); RNP-8 (RNP-8–associated mRNAs); EFL/DPL (EFL-1/DPL-1 target genes); FBF-1 (FBF-1–associated mRNAs); GLD-1 (GLD-1–associated mRNAs); spermatogenic program; oogenic program.

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