An RNA-Binding Multimer Specifies Nematode Sperm Fate

Graphical Abstract

Highlights
- FOG-3 crystal structure reveals sites of missense mutations
- FOG-3 assembles into dimers that can multimerize
- FOG-3 binds directly to mRNAs in the oogenic program
- FOG-3 recruited to a reporter mRNA represses its expression

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In Brief
The mechanism of the sperm or oocyte fate decision has been elusive. Aoki et al. report that nematode FOG-3, a Tob/BTG protein driving sperm fate, has evolved from monomeric to multimeric form with acquisition of a divergent Tob/BTG mechanism for mRNA repression.

Data and Software Availability
5TD6
GSE76521
An RNA-Binding Multimer Specifies Nematode Sperm Fate

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SUMMARY

FOG-3 is a master regulator of sperm fate in Caenorhabditis elegans and homologous to Tob/BTG proteins, which in mammals are monomeric adaptors that recruit enzymes to RNA binding proteins. Here, we determine the FOG-3 crystal structure and in vitro demonstrate that FOG-3 forms dimers that can multimerize. The FOG-3 multimeric structure has a basic surface potential, suggestive of binding nucleic acid. Consistent with that prediction, FOG-3 binds directly to nearly 1,000 RNAs in nematode spermatogenic germ cells. Most binding is to the 3' UTR, and most targets (94%) are oogenic mRNAs, even though assayed in spermatogenic cells. When tethered to a reporter mRNA, FOG-3 represses its expression. Together these findings elucidate the molecular mechanism of sperm fate specification and reveal the evolution of a protein from monomeric to multimeric form with acquisition of a distinct mode of mRNA repression.

INTRODUCTION

Gene discovery often paves the way to a molecular understanding of mysterious biological phenomena. But how do we learn molecular functions of newly identified genes? A common method takes advantage of amino acid comparisons to find homologs or at least protein domains that can provide clues. The major challenge is to test the predictions of those clues and tease out the function biochemically. Our focus is molecular functions of newly identified genes? A common method takes advantage of amino acid comparisons to find homologs or at least protein domains that can provide clues.

FOG-3 Is a Divergent Member of the Tob/BTG Family

Like canonical Tob/BTG family members, the FOG-3 primary sequence possesses a predicted N-terminal Tob/BTG domain and a disordered C-terminal region (Figures 1B and S1) (Chen et al., 2000). Comparison of FOG-3 amino acid sequences from several Caenorhabditis species revealed a further nematode-specific conservation that extends ~20 amino acids past the predicted Tob/BTG fold. We used recombinant C. elegans FOG-3 to identify a single domain (amino acids 1–137) that spans the canonical Tob/BTG fold plus this nematode-specific extension (see Supplemental Experimental Procedures and Figures S2A and S2B).

We pursued the FOG-3 crystal structure to gain insight into this putative Tob/BTG protein. FOG-3 crystals were obtained...
FOG-3 is a divergent Tob/BTG protein

(A) FOG-3 is a terminal regulator of the sperm fate, and hence essential for sperm fate specification in both hermaphrodite larvae and males.

(B) Linear diagram of FOG-3 protein. Predicted Tob/BTG fold is N-terminal (gray). C terminus is abbreviated (dashed lines). Vertical lines mark missense mutation sites (also see Figure S1): green, conserved in all orthologs; cyan, conserved in nematode orthologs; magenta, missense mutation generated in this study. Horizontal bars below show extents of subunits in crystal dimer, termed FOG-3A (light blue) and FOG-3B (dark blue).

(C) Asymmetric unit of the FOG-3 crystal structure (PDB: 5TD6). FOG-3A (chain A, light blue) and FOG-3B (chain B, dark blue). Arrows highlight the nematode-specific linker-helix extension.

(D) FOG-3 and human Tob/BTG structures. RMSD of human Tob1 (orange, PDB: 2Z15) compared with FOG-3A and FOG-3B was 1.062 Å and 1.086 Å, respectively. Arrows highlight the linker-helix extension.

(E) Location of FOG-3 missense mutants in the crystal structure. Color scheme matches that in (B).

Details of the FOG-3 crystal structure supported the idea that the two FOG-3 subunits in the asymmetric unit (ASU) represent a bona fide dimer. The buried surface area between the two FOG-3 subunits was large (1,034.2 Å²), favorable for assembly (∆G = −7.2 kcal/mol), and the interface intricate (Figures S2D and S2E). The linker-helix extension of FOG-3B folded around FOG-3A (Figure 1C), making several hydrogen bonds (Figures S2D and S2E). The Tob/BTG folds of FOG-3A and FOG-3B also contacted each other at their N-terminal helices, with hydrogen bonds and arginine planar stacking between conserved residues (Figures S2G and S2H). To ask whether this potential FOG-3 dimer may have biological significance, we analyzed the sites of the missense mutations (Chen et al., 2000). All mutations abolish sperm fate specification and hence render the protein non-functional (Ellis and Kimble, 1995). The FOG-3 structure included all eight missense sites (Figures 1B, 1E, S1, and S2F). Three mutations (P21L, R56Q, P94S) alter residues conserved across Tob/BTG folds, and five others change residues conserved only in FOG-3 and nematode paralogs (Figure S1). The three Tob/BTG fold mutations include two prolines located between helices and an arginine making a hydrogen bond characteristic of Tob/BTG folds (Figures S2G and S2H) (Horiiuchi et al., 2009; Yang et al., 2008). Because of their locations, contacts, and conservation, we speculate that these residues facilitate protein folding. Five other mutations change residues conserved only in nematodes. These include three (E7K, R14K, G33K) in the Tob/BTG fold (Figures S2G and S2H) and two (P125L, A132T) in the nematode-specific linker-helix extension (Figures S2G and S2J). At the potential dimer interface of the Tob/BTG folds, two missense residues (E7K, R14K) map to the interface itself (Figures S2G and S2H) and the third (G33K) maps to a central helix, where a bulky lysine residue could disrupt dimerization via steric hindrance (Figures S2G and S2J). The two mutations outside the Tob fold (P125L, A132T) map to the internal face of the link-helix extension in FOG-3B (Figures S2G and S2J). Thus, the FOG-3 crystal structure and sites of fog-3 mutations provide evidence that FOG-3 has a Tob/BTG fold but is divergent with potential to dimerize.

FOG-3 dimerization and higher-order assembly

The mutations in the linker-helix suggest that this extension is crucial for FOG-3 function. The asymmetrical interaction between the linker-helix of one subunit and the Tob/BTG fold of the other seemed an unusual strategy for dimerization (Figure 1C). We wondered whether the linker-helix might also mediate an interaction between dimers. To explore this idea, we extended the crystal symmetry to visualize FOG-3 dimer-dimer interactions in the structure and found the linker-helix extension tucked neatly into a cleft of the adjacent FOG-3 dimer (Figure 2A). Each dimer was rotated 180° relative to its neighbor in a continuous pattern to form a polymeric-like assembly within the crystal (Figure 2B). Moreover, features of the structure suggested that the dimer-dimer interface may be authentic: its surface area is 1,112.5 Å² (Figure S3A), a value similar to that between subunits in the dimer, and the predicted interface included 10 hydrogen bonds and four salt bridges (Figures S3A–S3C), with many residues conserved among FOG-3 orthologs (Figure S1).
Figure 2. FOG-3 Forms Higher-Order, Multi-dimer Assemblies

(A) Crystal packing of the FOG-3 dimer. FOG-3A and FOG-3B are represented in light and dark blue, respectively.

(B) Model for multimerization of FOG-3 dimers, as observed in the crystal. Image was generated by extending the crystal symmetry. Subunits are colored as in (A).

(C) Negative-stain electron microscopy of recombinant FOG-3 1–137. Note the presence of long rods (arrow). Scale bar, 50 nm.

(D) Coomassie-stained gel of modified FOG-3 recombinant protein incubated in the presence of long rods (arrow). Scale bar, 50 nm.

(E) Mutation of a key residue, E126K, transforms the germline from hermaphrodites (Figures S3L–S3N) for similar results in hermaphrodites. The E126K mutation may be unstable. We turned next to negative-stain electron microscopy (Figures S3J and S3K). We reasoned that changing E126 to a positively charged amino acid (E126K) should disrupt the dimer-dimer interface specifically. Indeed, an E126K lysine variant still formed dimers, but not higher-order, multimeric species (Figures 2D and S3I). To ask whether dimer-dimer contact was critical for sperm fate specification in vivo, we introduced E126K in the endogenous fog-3 gene (see Supplemental Experimental Procedures). Animals homozygous for either of two independently generated E126K alleles failed to make sperm and instead had a fully penetrant Fog phenotype in both males (Figures 2E–2G) and hermaphrodites (Figures S3L–S3N). The E126K mutation may affect FOG-3 in ways other than multimerization, but the simplest explanation is that FOG-3 functions in vivo as a multimer of dimers to promote sperm fate.

FOG-3 binds RNA directly to 3′ UTRs of Oogenic-Associated Transcripts

The electrostatic surface potential of the FOG-3 3′ multimer is highly basic (Figures S4A and S4B) and prompted us to ask whether FOG-3 binds directly to RNA. In nematode spermatogenic germ cells, FOG-3 immunoprecipitated radiolabeled RNA after UV crosslinking (Figures S4C–S4F), a treatment creating covalent bonds between protein and RNA (Huppertz et al., 2014). Using in vivo crosslinking and immunoprecipitation (iCLIP) (Huppertz et al., 2014), we identified RNAs crosslinked to FOG-3 (Figures S4G–S4I; Tables S2 and S3) and sites of FOG-3 binding within those RNAs. After normalization to a negative control (see Supplemental Experimental Procedures), FOG-3 enriched for 955 mRNA targets and 38 non-coding RNAs (Figure 3A). Remarkably, ~94% of FOG-3-bound mRNAs belonged to the oogenesis program (Figures S3B and S4J), despite immunoprecipitation from sperm-fated germ cells. The FOG-3 targets identified by iCLIP overlapped with those identified by microarray (Figures S4J and S4K; p < 1 × 10^{-208}), but iCLIP significantly increased both number of targets as well as their enrichment for oogenic RNAs (Figures S4J and S4K). Because iCLIP is more stringent than microarray methods (Wang et al., 2009), we suggest that iCLIP improved the signal-to-noise ratio of RNAs immunoprecipitating with FOG-3. We conclude that FOG-3 binds directly to RNAs that belong largely to the oogenesis program.

The vast majority of FOG-3 binding sites mapped to 3′ UTRs (Figures 3C and S4L–S4N), implying that FOG-3 binding is largely restricted. A multimer is expected to leave an extensive footprint. Consistent with this idea, 624 of 955 protein-coding genes (65.3%) had two or more sequence peaks in their 3′ UTRs (Figures 3D, 3E, and S4O). This pattern is reminiscent of multi-site RNA binding proteins, like HuR (Lebedeva et al., 2011). Gaps between peaks might signify authentic binding absences from breaks in FOG-3 multimerization or binding by
independent dimers. These gaps may also be sites of preferred enzymatic digestion during iCLIP (see Supplemental Experimental Procedures). Regardless, we conclude that FOG-3 binds the 3′ UTRs of its target mRNAs and that most of its targets belong to the oogenic program, despite being immunoprecipitated from spermatogenic cells.

Tethered FOG-3 Represses Expression of a Reporter mRNA

Genetically, FOG-3 is a master regulator of sperm fate (Ellis and Kimble, 1995) and FOG-3 binds many oogenic mRNAs directly in spermatogenic germ cells (this work). The simplest model is that FOG-3 specifies the sperm fate by repression of oogenic mRNAs. One cannot remove FOG-3 to test this idea because germ cells make oocytes rather than sperm without FOG-3. As an alternative approach, we turned to a protein-mRNA tethering reporter assay used in other systems (Coller and Wickens, 2002) and C. elegans (Wedeles et al., 2013), which takes advantage of λN22 peptide binding to boxB RNA hairpins (Baron-Benhamou et al., 2004) (Figure 4A). Endogenous FOG-3 was engineered to include a C-terminal 3xFLAG epitope tag, with or without λN22 (see Supplemental Experimental Procedures). The reporter mRNA expressed GFP-tagged histone and carried three boxB hairpins in its 3′ UTR (Figure 4A). Both engineered FOG-3 proteins promoted the sperm fate and were expressed as expected (Figures 4B and 4F). Thus, our modifications did not affect FOG-3 function or expression.

We compared fluorescence in germ cells expressing engineered FOG-3 and the reporter. GFP was easily detected throughout the male germline when FOG-3::3xFLAG was not fused to λN22 (Figures 4B–4E). However, when FOG-3::3xFLAG was fused to λN22, GFP fluorescence decreased in the region of FOG-3 expression (Figures 4F–4I). Therefore, FOG-3 represses GFP expression when tethered to a reporter mRNA.

DISCUSSION

Our findings support a model for the molecular mechanism of sperm fate regulation (Figure 4J). We propose that the functional form of FOG-3 is multimeric, that FOG-3 multimers bind directly to the 3′ UTRs of target mRNAs, and that FOG-3 is a broad-spectrum repressor of the oogenesis program. Because FOG-3 and its orthologs specify sperm fate in both C. elegans sexes and related dioecious species (Chen et al., 2001), this mechanism is likely fundamental to nematode sperm fate specification. Protein expression appears sufficient for such function; FOG-3 is readily observed by immunoblot and its staining is granular (Noble et al., 2016), implying that FOG-3 may be further concentrated within the cytoplasm for multimerization. Our results challenge the idea that FOG-3 regulates RNAs via the mechanism elucidated for mammalian Tob/BTG proteins, which function as monomeric adapters. Although FOG-3 might also function as a monomeric adaptor, such a canonical Tob/BTG mechanism is unlikely for its role in sperm fate. Terminal regulators of mammalian germ cell fate are not yet known, so we cannot exclude the possibility that the biological role of Tob/BTG proteins may have been conserved in germ cells.
The restricted footprint of FOG-3 to 3′ UTRs raises questions about the regulation of FOG-3 binding. FOG-3 may itself provide RNA binding specificity, although no sequence element could be detected beneath its iCLIP peaks. More likely, another sequence-specific RNA-binding protein seeds FOG-3 assembly (Figure 4J). FOG-1/CPEB is a strong candidate for such a seed protein, because it also drives sperm fate specification (Barton and Kimble, 1990), binds to FOG-3, and associates with oogenic mRNAs (Noble et al., 2016). The mechanism of repression remains a challenge for the future. The FOG-3 multimer might recruit effector complexes to modify the mRNA, similar to mammalian Tob/BTG. If true, details are likely different because residues corresponding to the deadenylase binding interface of human Tob (Horiuchi et al., 2009) are not conserved in FOG-3 and no missense mutations mapped to that potential interface. Other possibilities include competition with an activator or moving mRNAs to sites of repression within the cell.

Fundamental aspects of nematode FOG-3 function diverge from mammalian Tob/BTG. Although mRNA repression is shared, FOG-3 functions as a multimer and binds RNA directly, whereas mammalian Tob/BTG proteins function as monomeric adaptors that link sequence-specific RNA binding proteins to enzymes (Hosoda et al., 2011; Ogami et al., 2014; Yu et al., 2016). Consistent with this divergence, FOG-3 possesses a nematode-specific linker-helix extension that mediates dimer-dimer interactions, and those dimer-dimer interactions are integral to sperm fate specification. This strategy of FOG-3 assembly is reminiscent of certain viral RNA binding proteins that package RNA viral genomes using a core domain plus a C-terminal linker-helix or linker-sheet extension to drive multimerization (Harrison, 2017). In an analogous but distinct strategy, yeast RNA-binding protein Rim4 assembles into amyloid-like fibers to repress translation of mRNAs required for gametogenesis (Berchowitz et al., 2015). Collaborative assembly with mRNA is common among viral assembly proteins and other nucleic acid binding polymers (Ghosal and Löwe, 2015). FOG-3 may similarly require an mRNA scaffold for higher-order assembly, given that FOG-3 dimers could not multimerize into higher-order assemblies at low protein concentration.

Our work on FOG-3 highlights the concept that evolution can usurp a well-conserved domain to form multimers with only modest changes to its primary sequence and structural fold. Multimerization appears to be a unique feature of FOG-3-related Tob/BTG proteins. The critical residues at the dimer interface are conserved in nematodes, but not in mammalian Tob/BTG proteins, and mammalian homologs show no evidence of multimerization (Horiuchi et al., 2009; Yang et al., 2008). Because only a few Tob/BTG proteins have been characterized biochemically, FOG-3 might yet have a vertebrate counterpart. However, we favor instead the idea that an existing protein fold was adapted during evolution to transform a protein that acts as a monomer into a multimeric repressor with RNA binding properties. The transformation from monomer to multimer required evolution of inter-subunit interacting elements. Stated differently, evolution can shape a well-conserved primary sequence to drive an intermolecular interaction.

Figure 4. FOG-3 Represses mRNA Reporter Expression When Tethered in Nematodes

(A) Summary of the protein-mRNA tethering assay. With N22, FOG-3 can bind and regulate the protein expression of a GFP::histone reporter transcript containing 3xboxB hairpins in its 3′ UTR.

(B–I) Maximum intensity projections from confocal images of representative male adult germlines expressing both modified FOG-3 and the GFP reporter. FOG-3::3xFLAG (B–E) or FOG-3::N22:3xFLAG (F–I) with the GFP::histone reporter were imaged by fluorescent confocal microscopy for (B and F) FLAG, (C and G) GFP, (D and H) FLAG and GFP overlay, and (E and I) DNA (DAPI). DNA images reduced in size 2.5-fold. Dashed white line outlines male germlines. Scale bar, 20 μm.

(J) Model of the FOG-3 molecular mechanism. Multimers of FOG-3 dimers bind 3′ UTRs and promote the sperm fate by repressing mRNAs in the oogenic program. FOG-3 may find its targets by interacting with a distinct sequence-specific seed protein, one candidate being FOG-1 (see Discussion).
surfaces to form dimers and evolution of inter-dimer interacting surfaces to permit multimerization. In the case of FOG-3, the inter-dimer interface was created by adding a linker-helix extension that fits into the cleft of its neighbor, adding a binding surface to one side of the dimer. This extension provides directionality for multimer assembly. Therefore, FOG-3 provides an elegant example of protein evolution, in which a broadly conserved protein domain is redeployed to acquire a distinct mode of mRNA repression.

EXPERIMENTAL PROCEDURES

Crystalization and Structure Determination

Crystalization conditions were screened with sitting drop trays set up using the Mosquito (TTP Labtech, Cambridge, MA, USA). We obtained crystals using recombinant FOG-3 (1–137 H48N C117A) with an intact histidine tag and incubating our trays at 4 °C. Coordinates, reflection data, and further experimental details are available at RCSB (PDB: 5TD6) and in the Supplemental Experimental Procedures.

Negative-Stain Electron Microscopy

EM was performed with recombinant FOG-3 (1–137 H48N C117A) as previously described (Bozzola and Russell, 1999). See Supplemental Experimental Procedures.

Molecular Genetics

Worm Maintenance

C. elegans were maintained as described previously (Brenner, 1974). Strains used were JA1515: weSi2 II; unc-119 III; JK2739: hT2[pIs48](I;III)/lin-6(e1466) dpy-5(e61); JK4871: fog-3(q847) I; q541[fog-3::3xFLAG] II; JK5437: fog-3(q847) I/hT2[q548](III); JK5439: fog-3(q849) I/hT2[q548](III); JK5921: qSi375 [mex-5 promoter:EGFP::linker::his-58::3xboxB::tbb-2 3'(UTR)] II; JK5942: fog-3(q873)I; qSi375 II; and N2 Bristol. Strains are available at the Caenorhabditis Genetics Center (https://cbs.umn.edu/cgc/home) or upon request. See Supplemental Experimental Procedures for details on generation of the fog-3 alleles and in vivo fluorescent reporter.

Fertility and Fog Phenotype

Heterozygous and homozygous fog-3(q847), or fog-3(q849), were singed onto plates as L4 larvae. After 3 and 4 days, worms were scored for the presence of L1 larvae and Fog phenotype.

Imaging

Live worms were imaged by differential interference contrast (DIC) microscopy with 0.1 mM levamisole (Sigma, St. Louis, MO, USA). For fluorescent imaging, germines were extruded, fixed, permeabilized with Triton-X (0.5%), and stained as previously described (Crittenden et al., 2017). See Supplemental Experimental Procedures for further details.

iCLIP

In vivo crosslinking and immunoprecipitation (iCLIP) was carried out essentially as described (Huppertz et al., 2014), with modifications to worm growth, crosslinking, lysis, RNase digestion, and data analysis described in the Supplemental Experimental Procedures. All reads within a gene had their position randomized to empirically determine a cluster p value. A Benjamin-Hochberg (BH) correction was applied (1% false discovery rate [FDR]). Only overlapping clusters called independently as significant in at least two replicates were retained. Fisher’s exact test was used to compare our results with previous FOG-3 RIP results (Noble et al., 2016).

DATA AND SOFTWARE AVAILABILITY

The accession number for the structure reported in this paper is PDB: 5TD6. The accession number for the raw sequence files of all replicates reported in this paper is GEO: GSE76521 (https://www.ncbi.nlm.nih.gov/geo/).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and three tables and can be found with this article online at https://doi.org/10.1016/j.cellrep.2018.05.095.

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AUTHOR CONTRIBUTIONS

S.T.A. designed and performed experiments, analyzed the data, and wrote the paper; D.F.P. and A.P. designed and performed experiments and analyzed the data; C.A.B. acquired and analyzed data; M.W. analyzed the data and drafted the manuscript; and J.K. analyzed the data and wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Supplemental Information

An RNA-Binding Multimer

Specifies Nematode Sperm Fate

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Supplemental Information

Supplemental Experimental Procedures

Biochemistry and crystallography

Protein expression and purification
Full length FOG-3 1-263 was amplified from *C. elegans* N2 cDNA with primers that included a six-histidine tag, stop codon and 12 nucleotides suitable for annealing with ligation independent cloning (LIC) (Aslanidis and de Jong, 1990). Mixed stage N2 cDNA was generated by reverse transcription (SuperScript® II Reverse Transcriptase, Thermo Fisher; Waltham, MA) with oligo-dT (Invitrogen; Carlsbad, CA). The FOG-3 PCR product was cloned into a pET21a (EMD Millipore; Billerica, MA) bacterial expression plasmid by LIC. From this plasmid, histidine-tagged FOG-3 1-238 was amplified by PCR and inserted into pET21a using LIC. For FOG-3 1-137, an *E. coli* codon-optimized sequence for histidine-tagged FOG-3 1-137 was ordered as a gBlock (Integrated DNA Technologies (IDT); Coralville, IA) and cloned into pET21a by LIC. All further changes to the pET21a histidine-tagged FOG-3 1-137 were by Gibson assembly cloning (Gibson, 2009). Residues 1-137 gave good yields, but solubility and stability remained issues at high concentration. We improved solubility by retaining the histidine tag and mutating non-conserved residues to amino acids in related FOG-3 orthologs (H47N and C117A) (Figure S1). For chemical crosslinking, we modified this optimized, histidine-tagged FOG-3 1-137 expression plasmid to include mutations (R22K, L64K, I112K, R82K) and extended the C-terminus three amino acids (138-140) to permit histidine tag cleavage (not used). This lysine substitution FOG-3 plasmid was later modified to include mutations R14K or E126K. Final plasmids used for protein expression listed (below).

Expression plasmids were transformed into Rosetta™2(DE3) cells (EMD Millipore; Billerica, MA) and grown in LB (MP Biomedicals; Santa Ana, CA) for 5 hours at 37°C until A600 = ~0.8. The culture was then induced with 0.1 mM IPTG (MP Biomedicals; Santa Ana, CA) and grown at 16°C for 16-20 hours prior to collection, centrifugation, washing and freezing in liquid nitrogen. Cells were defrosted on ice and reconstituted in lysis buffer (20 mM NaPO4, pH 7.4, 300 mM NaCl, 10 mM imidazole, 5 mM β-mercaptoethanol) with cOmplete protease inhibitors (Roche; Indianapolis, IN). Cells were lysed with a French Press, centrifuged (3,220 g, 20 hours prior to collection, 4°C) and 10,000 x g) to remove unlysed cells and precipitate, and incubated with Nickel-NTA beads (Thermo Fisher; Waltham, MA) for 2 hours at 4°C with rocking. Beads were washed with lysis buffer and eluted with an imidazole step gradient (imidazole at 20, 40, 60, 80, 100, 250 mM) in elution buffer (20 mM NaPO4, pH 7.4, 300 mM NaCl, 5 mM β-mercaptoethanol). Protein used for biochemical experiments was dialyzed in FOG-3 buffer (20 mM HEPES pH 7.0, 50 mM NaCl, 0.5 mM (tris(2-carboxyethyl)phosphine) (TCEP)), while protein for crystallization was dialyzed in crystallization buffer (20 mM HEPES pH 7.0, 100 mM MgSO4, 0.5 mM TCEP). Samples were concentrated with Amicon Ultra-4 3000 MW concentrators (EMD Millipore; Billerica, MA) and run on a Superdex 200 (GE Healthcare; Pittsburgh, PA). Recombinant protein was again concentrated with Amicon Ultra-4 3000 MW concentrators and protein concentration estimated by A280.

Plasmids
pJK2069: pET21a vector backbone, *C. elegans* FOG-3 1-238::six histidines
pJK2070: pET21a vector backbone, *E. coli* codon-optimized *C. elegans* FOG-3 1-137 H47N C117A::six histidines
pJK2071: pET21a vector backbone, *E. coli* codon-optimized *C. elegans* FOG-3 1-140 H47N C117A, lysine substitutions (R22K, I112K, L64K, R82K)::six histidines
pJK2072: pET21a vector backbone, *E. coli* codon-optimized *C. elegans* FOG-3 1-140 R14K H47N C117A, lysine substitutions (R22K, I112K, L64K, R82K)::six histidines
pJK1910: pDD162 vector backbone, CRISPR-Cas9 sgRNA targeting fog-3 (caatcagcccccagtaec)
pJK1925: pDD162 vector backbone, CRISPR-Cas9 sgRNA targeting fog-3 (ggggcggcgaagctgct)

Crystallization, data collection and refinement
Initial crystallization trials proved unfruitful. Thinking that a cofactor was missing, we performed a thermal folding assay (Ericsson et al., 2006) with various additives (see below). Magnesium and sulfate improved protein thermostability (Figure S2C) so we reasoned that they might aid stability during crystallization. Crystallization conditions were screened with sitting drop trays set up using the Mosquito (TTP Labtech; Cambridge, MA). We obtained crystals using recombinant FOG-3 (1-137 H47N C117A) with an intact histidine tag and incubating our
trays at 4°C. After 3 weeks, rhomboid crystals were observed in conditions A (0.1 M sodium citrate pH 5.6, 10% (vol/vol) isopropanol, 10% (wt/vol) PEG 4000) and B (0.1 M magnesium acetate, 0.1 M sodium citrate pH 5.6, 8% (wt/vol) PEG 10000). UV scanning with a UVEX-M (280 nm excitation, 350 nm emission; JANSi; Seattle, WA) identified these to be protein crystals. Both conditions were reproducible. We were able to collect complete datasets from the crystals grown directly from the condition B screening trays. Phasing was accomplished with molecular replacement using Phaser (McCoy et al., 2007) and a human Tob homolog (PDB ID: 2Z15) as a starting model. Model building and refinement were done in Phenix (Adams et al., 2010) and Coot (Emsley and Cowtan, 2004). Water molecules were first modeled by Phenix before being checked manually. Three densities were too big to be water molecules. We could model one of the densities with sulfate. Two densities were observed in the solvent-accessible area adjacent to residues 52-56 in both copies in the ASU. Density is observed at FoFc contour levels past 6 σ. We attempted modeling of acetate (too small) and citrate (too large), both molecules that were present in the crystallization conditions, but the fit was unsatisfactory. Thus, the final uploaded model does not account for these two large densities. Analyses of protein assemblies, dimer interactions and free energy estimations were done in PISA (Krissinel and Henrick, 2007). Structural images were generated in PyMol (The PyMOL Molecular Graphics System, Version 2.0, 1 Schrödinger, LLC).

The FOG-3 missense mutations were modeled in Coot (Emsley and Cowtan, 2004). Their disruption of FOG-3 folding, dimerization or multimerization was inferred based on mutant residue disruption of hydrogen bonds, salt bridges and hydrophobic packing (steric hindrance) in the structure. The lysine substitution sites were chosen based on whether the locations were close enough for intra-dimer (R22K, I112K) and dimer-dimer (L64K, R82K) BS3 crosslinking, sequence variability (a lack of sequence conservation, Figure S1) and their expected tolerance for a lysine mutation. R22 was conserved in FOG-3 paralogs, but lysine was easily modeled. Two mutations changed hydrophobic residues, but lysines at these positions were found in other FOG-3 orthologs and could be modeled.

**FOG-3 protease cleavage**

Comparison of FOG-3 sequences from several *Caenorhabditis* species reveals further nematode-specific conservation that extends ~20 amino acids past the predicted Tob/BTG fold. We used recombinant *C. elegans* FOG-3 protein and proteases to identify a single domain spanning the canonical Tob/BTG fold and a nematode-specific extension. Recombinant FOG-3 1-238 with a C-terminal histidine tag was incubated with trypsin (Sigma-Aldrich; St. Louis, MO) and elastase (Sigma-Aldrich; St. Louis, MO) at room temperature prior to SDS-PAGE. The gel was stained with Coomassie to visualize cleavage products. The proteases generated ~15 kDa protected fragments (Figure S2B). Samples were also cleaved with trypsin or elastase for 45 minutes at room temperature (~20°C) and submitted for in-solution mass spectrometry (University of Wisconsin-Madison Biotechnology Center). The mass spectrometry fragment that most closely matched the SDS-PAGE band mapped to residues 1-135 for trypsin and 1-142 for elastase. Both fragments included the predicted Tob/BTG fold plus the nematode-specific extension (Figure 1B). FOG-3 1-137 exhibited a broad elution peak at higher versus lower concentrations (Figure S2A) that could be attributed to different dimer versus monomer states.

**Protein folding assay**

The protein folding assay followed published protocols (Ericsson et al., 2006). Briefly, recombinant FOG-3 1-137 with histidine tag was incubated with 90x concentrated SYPRO orange (5000x stock, Invitrogen; Carlsbad, CA) in FOG-3 buffer. 18 µl of the protein-dye mix was mixed with 2 µl Additive Screen (Hampton Research; Aliso Viejo, CA) and heated in a 7500 Real-Time PCR System thermocycler (Applied Biosystems; Foster City, CA) at 0.1°C/s from 20°C to 70°C while monitoring A405. SYPRO orange dye bound to unfolded protein. Thus, FOG-3 unfolded at a certain temperature, allowing dye binding and increasing A405 absorbance. The additive was judged as enhancing thermostability based upon the shift in the melting curve to the right, or requiring higher temperatures for signal. This assay was performed twice with similar results.

**Negative-stain electron microscopy**

Samples were negative stained with Nano-W (Nanoprobes; Yaphank, NY) using the two-step method. A 2 µl droplet of samples was placed on a Pioloform (T. Pella) coated 300 mesh Cu Thin-Bar grid (EMS; Hatfield, PA), coating side down. The excess was wicked with filter paper and allowed to barely dry. A 2 µl droplet of Nano-W was applied, wicked again with clean new filter paper, and allowed to dry. The sample was viewed on a Philips CM120 transmission electron microscope at 80 kV and documented with a SIS (Olympus / Soft Imaging Systems; Münster, Germany) MegaView III digital camera.

**Protein crosslinking**
Bis[sulfosuccinimidyl] suberate (BS3) (Thermo Scientific; Waltham, MA) was diluted in crosslinking buffer (20 mM HEPES pH 7.0, 150 mM NaCl) and added to recombinant protein for a final concentration of 0.5 mg/ml protein and 0.5, 0.25, 0.125, 0.0625, 0.03125 mM BS3 crosslinker. Buffer alone was added as a negative control. After 30 minutes at room temperature (~20°C), the reaction was quenched with 1M Tris pH 6.8 (50 mM final concentration). Samples were analyzed by SDS-PAGE and coomassie staining. Experiments were performed twice in these conditions with similar results.

**C. elegans E126K alleles**
CRISPR-Cas9 gene editing of the endogenous fog-3 gene was achieved using a dpy-10 roller co-injection strategy (Arribere et al., 2014). Briefly, an sgRNA construct containing the U6 promoter and sgRNA scaffold from pDD162 (Dickinson et al., 2013) along with the targeting sequences caatcagtccccgagtacg (pJK1910) and ggttctgaccacgtactcg (pJK1925) was cloned into the Xmal site of pUC19 using one step isothermal DNA assembly. The repair template was a ssDNA oligo (Integrated DNA Technologies (IDT); Coralville, IA) that inserted an E126K mutation and removed an Aval restriction site. See Table (below) for CRISPR-Cas9 target sequences and repair oligos used. Injections were performed in young N2 hermaphrodite *C. elegans*, using either 1) fog-3 sgRNA plasmids, dpy-10 sgRNA plasmid, fog-3 E126K repair template, and Cas9 plasmid as described (Arribere et al., 2014), or 2) fog-3 crRNA, dpy-10 crRNA, fog-3 E126K repair template and recombinant Cas9 protein as described (Paix et al., 2015) (see Table below for reagent sequences), and F1 rollers were screened for the desired mutation by PCR and Aval digest. Alleles were recovered from separate injected animals and therefore represent independent editing events. We verified the fog-3 mutations by Sanger sequencing. Homozygous mutants had a Fog phenotype and thus could only produce oocytes. These worms were outcrossed twice with N2 before crossing with JK2739 containing balancer hT2[qIs48](I;III).

**CRISPR-Cas9 guide RNAs and repair oligos**

| Name                  | Type                   | Strain targeted | Sequence                                      |
|-----------------------|------------------------|-----------------|-----------------------------------------------|
| CRISPR-Cas9 guide RNAs: |                        |                 |                                               |
| fog-3 5 crRNA         | CRISPR-Cas9 RNA        | N2              | target sequence: taatactgggaatattaaaaa        |
| fog-3 crRNA 8         | CRISPR-Cas9 RNA        | N2              | target sequence: cgtttgcacagctctgg            |
| his-58 tbb-2 crRNA 1  | CRISPR-Cas9 RNA        | JA1515          | target sequence: aggatccttgcatTTACTTGGC      |

single stranded DNA repair oligos:

| Name                  | Type                   | Strain targeted | Sequence                                      |
|-----------------------|------------------------|-----------------|-----------------------------------------------|
| fog-3 E126K repair    | ssDNA repair oligo     | N2              | ataaatatattaatttttaaatgtttccagctcaaatcagtcaccACgttCgtCgagtGAGGA |
| fog-3 FLAG3x repair   | ssDNA repair oligo     | N2              | tcaacgacaccaaatagatattttctccctcttgAGGAGCAAG |
| fog-3 lambda repair   | ssDNA repair oligo     | N2              | CAA GGC GTG CAC CAA GTA TAC TAC TAC TAG CAA GTA ATG CAT AAG TCC AAG TAC TAC TAC TAC TAC TAC |
| his-58 tbb-2 boxB repair | ssDNA repair oligo | JA1515          | CAA GGC GTG CAC CAA GTA TAC TAC TAC TAG CAA GTA ATG CAT AAG TCC AAG TAC TAC TAC TAC TAC TAC |

Reporter design and application
The goal was to develop a reporter that could take advantage of the lambda/boxb mRNA tethering system (Baron-Benhamou et al., 2004) and a germline fluorescent expression reporter. However, the published boxb-containing reporters and our own MosSCI-generated reporters could not be detected, most likely due to weak signal in the
worms yielded ~12 mg/mL of total protein, and we used 10 mg total protein per biological replicate. Double RNase determined with the Direct Detect spectrometer (EMD Millipore; Billerica, MA). Our pellets containing 250,000 was cleared by centrifugation for 15 minutes at 16,100 × 4 minutes. Worm lysis was confirmed by observing a small aliquot of final lysate on a dissection scope. The lysate immersion in liquid nitrogen for 1 minute, then returning setting of 30 Hz, with four 400 MM mill mixer (Ve
1 minute and washed 3 times with ice cold lysis buffer. Lysis buffer was added to the pellet along with a 5 mm glycerol, 0.05% (vol/vol) Tween 20) and frozen with liquid nitrogen. Pellets were stored at −80°C for 1 hour. After thawing, the pellets were washed with 50 mM Tris pH 7.5, 150 mM NaCl, 10% (wt/vol) glycerol, 0.05% (vol/vol) Tween 20 and frozen with liquid nitrogen. Pellets were stored at −80°C until use.

Imaging

For fluorescent imaging, germlines were extruded, fixed and permeabilized with Triton-X (0.5%) as previously described (Crittenden et al., 2017). Germlines were incubated with primary antibodies to FLAG (M2® (mouse), Sigma; St. Louis, MO) and GFP (Rabbit anti-GFP, Invitrogen; Carlsbad, CA) overnight, stained with fluorescein-labeled secondary antibodies (Alexa 555 Donkey anti-Mouse, Alexa 488 Goat anti-Rabbit; Invitrogen; Carlsbad, CA) and DAPI (Invitrogen; Carlsbad, CA), washed, and mounted in Vectashield (Vector Laboratories; Burlingame, CA). Germlines were imaged by confocal microscopy on a Leica SP8 scanning laser confocal microscope, taking 1 μm slices in sequence. Maximum intensity stack projections were generated with ImageJ (Schindelin et al., 2015) and brightness adjusted with Photoshop (Adobe; San Jose, CA). All images were equally treated in ImageJ and Photoshop, with the exception of the reported DAPI images. Imaging experiments were repeated at least twice with similar results.

iCLIP

In vivo crosslinking and immunoprecipitation (iCLIP) was carried out essentially as described (Huppertz et al., 2014), with modifications to worm growth, crosslinking, lysis, RNase digestion, and data analysis as described.

Nematode culture and UV crosslinking for iCLIP

L1 larvae from C. elegans strain JK4871 were obtained by bleaching and synchronizing via standard methods (Stiernagle, 2006). Larvae were plated onto 10 cm OP50 plates (~50,000 per plate) and propagated at 20°C for ~40-46 hours until most worms were at the early L4 stage when FOG-3 expression is greatest. Worms were washed with M9 (42.3 mM NaH2PO4, 22 mM KH2PO4, 85.6 mM NaCl, 1 mM MgSO4), pooled into groups of 250,000 living worms, placed on a 10 cm NGM agarose plate and liquid removed. Animals were irradiated two times sequentially at 254 nm with 0.9999 J/cm² in a XL-1000 UV Crosslinker (Spectrolinker, Thomas Scientific; Swedesboro, NJ). Non-crosslinked samples were incubated at room temperature as a negative control for the radiolabeled gel (Figure S4E). For the iCLIP negative control, we performed the pulldown of crosslinked JK4871 worm lysate with beads alone (no antibody). Worms were rinsed from plates with cold M9, washed once, and transferred to a 2 mL Eppendorf tube. The pellet was washed again in freezing buffer (50 mM Tris pH 7.5, 150 mM NaCl, 10% (wt/vol) glycerol, 0.05% (vol/vol) Tween 20) and frozen with liquid nitrogen. Pellets were stored at -80°C until use.

Lysis and RNA digestion

C. elegans pellets were thawed by adding ice cold lysis buffer (50 mM Tris pH 7.5, 100 mM NaCl, 1% Pierce NP-40, 0.1% SDS, 0.5% sodium deoxycholate, Roche cOmplete EDTA-free Protease Inhibitor Cocktail, Ambion ANTI-RNase) and incubated for 20 minutes at 4°C with rocking. The thawed pellets were centrifuged at 1,000 x g, 4°C for 1 minute and washed 3 times with ice cold lysis buffer. Lysis buffer was added to the pellet along with a 5 mm Retsch stainless steel ball (Verder Scientific; Newtown, PA). Lysis was performed in the cold room using a Retsch 400 MM mill mixer (Verder Scientific; Newtown, PA). Lysis was completed after three 10-minute cycles at a setting of 30 Hz, with four-minute freeze-thaws after the first and second cycles. Freeze-thaws were performed by immersion in liquid nitrogen for 1 minute, then returning to liquid state by immersion in room temperature water for 4 minutes. Worm lysis was confirmed by observing a small aliquot of final lysate on a dissection scope. The lysate was cleared by centrifugation for 15 minutes at 16,100 x g, 4°C. Protein concentration of the cleared lysate was determined with the Direct Detect spectrometer (EMD Millipore; Billerica, MA). Our pellets containing 250,000 worms yielded ~12 mg/mL of total protein, and we used 10 mg total protein per biological replicate. Double RNase
digestion of protein-RNA complexes was performed as previously described (Spitzer et al., 2014). For the first digestion, which occurred immediately after lysis and just prior to immunoprecipitation, guanosine specific RNase T1 (Thermo Fisher; Waltham, MA) was added to the cleared lysate at a final concentration of 1 Unit/µL. The sample was incubated in a Thermomixer for 15 minutes at 22°C, 1,100 rpm and then cooled on ice for 5 minutes.

Protein G Dynabeads (Life Technologies; Waltham, MA) were aliquoted to a fresh RNase-free roundbottom tube (USA Scientific; Ocala, FL). The tube was placed on a Dynal magnet (Invitrogen; Carlsbad, CA), the existing buffer was removed, and M2 FLAG antibody (Sigma-Aldrich; St. Louis, MO) added at 20 µg antibody to 3 mg Protein G Dynabeads in PBS-T (PBS pH 7.2 (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, 0.02% Tween-20). The beads plus antibody solution was incubated at room temperature on a rotator for 45 minutes. The tube was again placed on the magnet, the antibody solution removed, and the cleared lysate added. Immunoprecipitation was carried out overnight at 4°C. As a negative control, we performed the pulldown of crosslinked JK4871 worm lysate with beads alone (no antibody).

Following immunoprecipitation, the beads were washed as described (Huppertz et al., 2014), with minor modifications. We performed washes in the cold room (~4°C) with two wash buffers: a high-salt wash buffer (50 mM Tris-HCl pH 7.5, 1 M NaCl, 1 mM EDTA, 1% Pierce NP-40, 0.1% SDS, 0.5% sodium deoxycholate) and PNK buffer (20 mM Tris-HCl pH 7.5, 10 mM MgCl2, 0.2% Tween 20). The second RNase T1 digestion was then performed on the washed beads at a final concentration of 100 Units/µL in PNK buffer. Samples were incubated in a Thermomixer for 15 minutes at 22°C shaking at 1,100 rpm, cooled on ice for 5 minutes, and then processed through the remaining iCLIP protocol as described (Huppertz et al., 2014). We confirmed that FOG-3:3xFLAG crosslinked to RNA by visualizing 5’ radioactively labeled RNA bound to the FOG-3 protein when antibody was present on the beads (Figure S4E). We confirmed immunoprecipitation of FOG-3 from experimental versus negative control samples by immunoblot with an M2 FLAG antibody (Figure S4F).

Single-end sequencing was performed on an Illumina HiSeq 2000 (University of Wisconsin Biotechnology Center; Madison, WI). The cDNA library of each replicate was prepared with a unique “Rclip” reverse transcription primer (as in Huppertz et al., 2014), which contained a partially randomized sequence (i.e., a “barcode”). The constant portion of the barcode enabled each read to be identified by replicate and allowed for replicate multiplexing. The randomized portion of the barcode allowed for computational filtering of artifacts from individual reads caused by PCR amplification of the cDNAs, such as read duplication. After high-throughput sequencing, the barcode sequence preceded the cDNA sequence and thus could be easily identified and removed prior to read mapping.

**iCLIP sequence analysis**

Reads (Table S2, Tab 1, column B) were aligned to the WS235 genome using STAR (Dobin et al., 2013) and previously described parameters (Kassuhn et al., 2016), except for the parameter --alignEndsType Local (mismatches at the ends of reads are tolerated). Multi-mapping reads were removed, and high-confidence mappings were selected as those with alignment scores of at least 20 (Table S2, Tab 1, column C). PCR duplicates were collapsed to unique reads (Table S2, Tab 1, column E) using the method described in Weyn-Vanhentenryck et al. (2014). Reads were assigned to genes using HTSeq (Anders et al., 2015). CIMS (crosslinking induced mutation sites) and CITS (crosslink induced truncation sites) analyses were performed as described (Weyn-Vanhentenryck et al., 2014), except we did not require CIMS to reproduce between replicates, and are included in Table S3, tab 3. For peak analysis, “clusters” were defined as regions of overlapping reads. Using the reads indicated in Table S2, tab 1, column E, all reads within a gene had their position randomized 1000 times to empirically determine a cluster p value as the odds of having a cluster with the given maximum read depth from randomized read positions. This is similar to the local Poisson method (Zisoulis et al., 2010), as the Poisson approximates of read scrambling. A Benjamini-Hochberg (BH) correction for multiple hypothesis testing was then applied at 1% FDR, resulting in the cluster numbers in Table S2, tab 2, column F. Finally, only overlapping clusters called independently as significant in at least 2 of the 4 replicates were retained as reproducible clusters, resulting in the cluster numbers in Table S2, tab 2, column G. Final clusters for FOG-3 and control samples are given in Table S3. While this is a simple method that does not account for background RNA abundance, it resulted in only 6 clusters for the negative control samples, suggesting it is effective at removing background in our datasets. We define peaks as all maxima at least 5 reads deep and at least 5% of the highest peak in the given gene; we counted neighboring peaks as distinct only if signal dropped to 50% or less of the lower peak maxima. Our definition of peaks differs from our definition of clusters, which are regions of continuous read coverage that pass the 1% FDR threshold. Clusters extend until iCLIP coverage drops to zero, thereby containing any number of distinct signal concentrations, and motivating our separate definitions of peaks and clusters.
To compare our results with previous FOG-3 RIP results (Noble et al., 2016), we calculated overlap with the top 722 FOG-3 targets, and evaluated significance by Fisher’s exact test. To determine whether FOG-3 targets were associated with oogenesis, spermatogenesis, or mitosis, we used the method described previously (Noble et al., 2016), with significance evaluated by Fisher’s exact test. Figures depicting iCLIP results (Figure S4M,N) were generated using Matplotlib (Hunter, 2007) and Python scripts available at https://github.com/dfporter.

We could not find enriched sequence motifs at FOG-3 binding sites. Instead, we sought motifs enriched near the binding sites and found enrichment of a CUCAC motif (Figure S4P, p value < 1.8 x 10^{-26}). CUCA is part of the GLD-1/STAR signature motif (Ryder et al., 2004). GLD-1 regulates germline sex determination, but it can promote either the sperm or oocyte fate (Francis et al., 1995).

Raw sequence files of all replicates are available through Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/; GEO accession GSE76521).

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Figure S1. Alignment of FOG-3 ortholog sequences, related to Figure 1. Amino acid sequence alignment of FOG-3 orthologs, including human Tob and BTG proteins. Nematode orthologs include FOG-3 from *C. elegans* (Ce), *C. briggsae* (Cb), *C. brenneri* (Cbr), *C. remanei* (Cr), and *C. japonica* (Cj). Alignment by T-Coffee (Di Tommaso et al., 2011). Conservation noted by identity (*) plus high (:) or moderate (.) similarity. Missense alleles are labeled with their amino acid changes (Chen et al., 2000; this work); allele labels are colored to mark conservation among most metazoan orthologs (green), conservation among most nematode orthologs (blue) and a mutation generated in this study (magenta). Boundary of the canonical Tob/BTG fold is marked with a dashed line; extents of dimer subunits are shown below, including subunit A (light blue) and subunit B (dark blue). Amino acids highlighted indicate dimer subunit-subunit contacts (red), dimer-dimer contacts (orange), and both (blue); these contacts include both hydrogen bonds and salt bridges.
Figure S2

A: Chromatogram showing elution volumes (Vol) for FOG-3 subunits.

B: SDS-PAGE gels showing Trypsin (left) and Elastase (right) digestion of FOG-3 subunits.

C: A405 absorbance over thermal cycle for MgCl₂, NH₄SO₄, and control samples.

D: Diagram of FOG-3 subunits A and B.

E: Table of H-bonds between subunits A and B:

| A  | B   |
|----|-----|
| E7 | N10 |
| N10| E7  |
| S41| R135|
| Y121| Q122|

F: Table of mutation sites, conservation, site in structure, and proposed defect:

| Mutation | Conservation | Site in structure | Proposed defect |
|----------|--------------|-------------------|-----------------|
| P21L     | All Tob/BTG folds | Tob/BTG fold | Tob/BTG Folding |
| R56Q     | All Tob/BTG folds | Tob/BTG fold | Tob/BTG Folding |
| P94S     | All Tob/BTG folds | Tob/BTG fold | Tob/BTG Folding |
| E7K      | FOG-3 Tob/BTG fold | Dimer interface | Dimerization |
| G33K     | FOG-3 Tob/BTG fold | Dimer interface | Dimerization |
| R14K     | FOG-3 Tob/BTG fold | Dimer interface | Dimerization |
| P125L    | FOG-3 extension | Linker-helix | Dimer/polymer |
| A132T    | FOG-3 extension | Linker-helix | Dimer/polymer |
| E126K    | FOG-3 extension | Linker-helix | Polymerization |

G: Diagram showing 90° rotation of FOG-3 subunits.

H: Enlarged view of hydrogen bonds H7-E7 and N10-R14.

I: Enlarged view of P21L and R56Q mutations.

J: Enlarged view of A132T and P125L mutations.
Figure S2. FOG-3 biochemical characterization, dimer subunit protein contacts and missense mutants, related to Figure 1. (A) Size exclusion chromatography elution profile of recombinant FOG-3 protein. Red, amino acids 1-238 with its histidine tag at 10 mg/ml; blue, amino acids 1-137 with histidine tag at 10 mg/ml; green, amino acids 1-137 with histidine tag at 0.2 mg/ml. Position of max peaks labeled. A280 milliabsorbance units, mAU. (B) Protease mapping the FOG-3 Tob/BTG-containing domain. FOG-3 1-238 was incubated with either trypsin or elastase, and samples were collected over time. Incubation with either protease produced a cleavage product of ~15 kDa. Protein incubated without protease labeled as “0.” (C) Thermal-folding assay reveals domain stabilization with magnesium and sulfate. See Supplemental Experimental Procedures and main text for further details. (D) Contact surface between FOG-3 dimer subunits. Residues making hydrogen bonds (pink) and other contacting residues (yellow) based on distance. (E) Table of subunit-subunit hydrogen bonds. (F) Summary of FOG-3 missense mutations. (G) FOG-3 dimer with boxed regions enlarged in H-J. (H-J) Residues from FOG-3A are colored light blue and those from FOG-3B are dark blue. (H) Sites of fog-3 missense mutants at the Tob/BTG domain dimer interface. (I) Sites of fog-3 missense mutants conserved across FOG-3 orthologs and human Tob proteins. (J) Sites of fog-3 missense mutants in linker-helix extension, including one generated in this study (magenta).
**Figure S3**

**A**

Dimer-dimer H-bonds

| Dimer 1 | Dimer 2 |
|---------|---------|
| A:F11   | B:R130  |
| A:E103  | A:S123  |
| A:N104  | A:D118  |
| A:N104  | A:Y121  |
| A:N104  | A:Q122  |
| A:G105  | A:Q122  |
| A:G105  | B:Q122  |
| A:E107  | B:Q122  |
| A:E107  | A:V124  |
| A:T108  | B:V124  |

**B**

Dimer-dimer salt bridges

| Dimer 1 | Dimer 2 |
|---------|---------|
| A:R14   | B:E126  |
| B:R14   | B:E126  |
| B:H19   | A:E31   |
| B:R82   | A:E72   |

**C**

**D**

Intra-dimer crosslink

**E**

Inter-dimer crosslink

**F**

**G**

**H**

WT* R14K E126K

**I**

Xlink: WT* R14K E126K

**J**

**K**

**L**

| Genotype      | % fertile | % Fog | n  |
|---------------|-----------|-------|----|
| fog-3(q847)/nT1 | 100       | 0     | 105|
| fog-3(q847)/(q847) | 0         | 100   | 106|
| fog-3(q849)/nT1 | 100       | 0     | 107|
| fog-3(q849)/(q849) | 0         | 100   | 110|

**M**

Embryos oocytes

**N**

Stacked, unfertilized oocytes
Figure S3. Dimer-dimer subunit protein contacts and supplemental biochemical analyses, related to Figure 2. (A) Interacting residues between FOG-3 dimers. Salt bridges (magenta), hydrogen bonds (pink), and contacting residues (yellow). (B) Table of predicted dimer-dimer hydrogen bonds. (C) Table of predicted dimer-dimer salt bridges. (D-F) Lysine substitution of recombinant FOG-3 generates residues permitting intra- and inter-dimer crosslinks. Location of lysine substitutions in the context of two FOG-3 dimers (D), which are enlarged in E (red box) and F (orange box). (E) Lysine substitutions R22K and I112K facilitate an intra-dimer crosslink. (F) Lysine substitutions L64K and R82K facilitate an inter-dimer crosslink. (G) Coomassie-stained gel of purified FOG-3 recombinant proteins. Left, "wild type" (WT*) FOG-3 (1-140, H47N C117A) with lysine substitutions; middle, missense mutant R14K predicted to abrogate dimer formation; right, missense mutant E126K predicted to abrogate polymer formation. All proteins (10 µg each) ran at ~15 kDa (large arrow). In addition, a minor ~25 kDa contaminant was observed (small arrow). (H) Size exclusion chromatography elution profile of WT*, R14K, and E126K recombinant FOG-3 (0.2 mg/ml). A280 milli-absorbance units, mAU. (I) Different exposure of Figure 2D. Coomassie-stained gel of modified FOG-3 recombinant protein incubated with increasing amounts of BS3 crosslinker. "-" represents no BS3 included. (J) Crystal packing of the FOG-3 dimer. FOG-3A and FOG-3B in the asymmetric unit are represented in light and dark blue, respectively. Each dimer buries the helix extension in FOG-3B into the adjacent dimer. The helix extension (red box) is enlarged in K. (K) Packing of the helix extension of one FOG-3 subunit into the adjacent dimer. Amino acids in subunits colored as in J, except for red residues from helix extension of the adjacent dimer. (L) Mutation of a key residue in the helix extension sexually transforms the germline (Fog phenotype). Two identical but independently-generated CRISPR-Cas9 alleles (q847 and q849) mutated glutamate 126 to a lysine (E126K). Alleles were placed over a GFP-expressing balancer (nT1). Heterozygous (green) and homozygous (non-green) L4 worms were singled and analyzed 3 and 4 days later for fertility and the Fog phenotype. (M-N) Representative DIC images of hermaphrodite adults, heterozygous (M) or homozygous (N) for the E126K mutation. The embryos in heterozygotes demonstrate fertility, while oocytes stacking in homozygotes demonstrate sterility due to lack of sperm and hence lack of embryo production.
UV-irradiate and collect spermatogenic early L4 worms
wash, freeze (liq N2), homogenize
IP +/- αFLAG Ab
iCLIP (4 biological replicates)

FOG-3::FLAG; fog-3(q520)

FOG-3 sample 1
8441710 (84006/32%)
6609 (2095)

FOG-3 sample 2
2950477 (48568/36%)
3851 (1175)

FOG-3 sample 3
2491607 (33010/43%)
2363 (671)

FOG-3 sample 4
2038150 (35809/40%)
2688 (760)

control sample 1
3257478 (24134/67%)
575 (89)

control sample 2
761755 (6444/77%)
596 (4)

control sample 3
463501 (6357/84%)
279 (5)

control sample 4
463501 (2840/85%)
273 (2)

Total reads (unique mapped/% rRNA)
Clusters (1% FDR)

3'UTR 5'UTR CDS

FOG-3 footprint (nt)

Number of genes

RIP-chip

FOG-3 iCLIP replicate
Figure S4. FOG-3 binds RNA in vivo, related to Figure 3 and Experimental Procedures. (A) Model of FOG-3 multimer composed of three dimers; subunits colored as in Figure 1. (B) Electrostatic surface potential of multimer modeled in A. Blue, basic; red, acidic. (C) Diagram of rescuing, epitope-tagged FOG-3::3xFLAG transgene, adapted from (Noble et al., 2016). (D) Outline of FOG-3 iCLIP protocol. See Supplemental Experimental Procedures for details. (E–F) FOG-3 crosslinks with RNA in vivo. Living worms expressing FOG-3::3xFLAG were UV-crosslinked (+) or mock treated (-), and then immunoprecipitated with αFLAG antibody. Bound sample 5’ radiolabeled and run on SDS-PAGE. (E) Overnight exposure of radiolabeled samples run on SDS-PAGE. (F) Immunoblot of FOG-3::3xFLAG immunoprecipitation samples, visualized with αFLAG antibody. FOG-3 and heavy and light antibody chains labeled. (G) Gel analysis of samples used for iCLIP. Each sample was immunoprecipitated with (+) or without (-) αFLAG antibody, radiolabeled and run on the SDS-PAGE gel. The region above the expected size for FOG-3::3xFLAG (red bracket) was used for iCLIP processing and sequencing. Samples include four biological replicates and their paired controls. (H) FOG-3 iCLIP reads and cluster (regions of overlapping reads) statistics. Unique mapped reads (middle column) were determined by mapping with STAR, filtering out multimapping reads and low confidence alignments, and collapsing duplicate reads (see Supplemental Experimental Procedures). The fraction of unique mapped reads that mapped to tRNA is given as a percentage. The number of significant clusters at FDR 1% (right column) is highly dependent on FOG-3 purification. See Supplemental Experimental Procedures for further details. (I) Targets (black) identified for separate FOG-3 replicates. (J) Comparison of FOG-3 targets identified using FOG-3 iCLIP versus FOG-3 RIP-chip (microarray). Targets belonging to the oogenic program include mRNAs found only in oogenic germlines as well as mRNAs found in both oogenic and spermatogenic germlines, as described (Noble et al., 2016). (K) Venn diagram of mRNA target overlap between FOG-3 iCLIP (this study) and FOG-3 RIP-chip (FOG-3 IP with microarray analysis of associated RNAs) (Noble et al., 2016). (L) Percentages of FOG-3 iCLIP reads in mRNA regions. (M–N) FOG-3 binding sites are represented on a heat map, from no signal (white) to strong signal (red). Only genes with annotated 3’UTRs of at least 50 nt were included. (M) FOG-3 binding sites are at the 3’ end of mRNAs, which are arranged by predicted nucleotide (nt) length from 5’ to 3’. Dashed line marks the 5’ end. Note prevalence of FOG-3 binding sites at the 3’ ends of transcripts. (N) Binding sites occur throughout 3’UTRs, which are arranged by predicted 3’UTR nucleotide (nt) length from stop codon (dashed line) to 3’ end. (O) FOG-3 iCLIP footprint on target transcripts. Coverage includes transcript regions above two reads deep. (P) Motif analysis of iCLIP clusters. Analysis and image generated by MEME (Bailey et al., 2015), except T was replaced by U.
Table S1. Data collection and refinement statistics, related to Figure 1.

|                              | \textit{C. elegans} FOG-3 |
|------------------------------|---------------------------|
| PDB ID                       | 5TD6                      |
| Wavelength (Å…)              | 0.9537                    |
| Resolution range (Å…)        | 29.22 - 2.034 (2.106 - 2.034) |
| Space group                  | P 3 1 2 1                 |
| Unit cell                    | 64.992 64.992 133.57 90 90 120 |
| Total reflections            | 147208 (14689)            |
| Unique reflections           | 21617 (2091)              |
| Multiplicity                 | 6.8 (7.0)                 |
| Completeness (%)             | 99.79 (98.18)             |
| Mean I/sigma(I)              | 24.12 (2.56)              |
| Wilson B-factor              | 39.54                     |
| R-merge                      | 0.0498 (0.7383)           |
| R-meas                       | 0.05399                   |
| CC1/2                        | 1 (0.789)                 |
| CC*                          | 1 (0.939)                 |
| Reflections used for R-free | 1993                      |
| R-work                       | 0.1769 (0.2255)           |
| R-free                       | 0.2285 (0.2886)           |
| Number of non-hydrogen atoms| 2210                      |
| macromolecules               | 2067                      |
| ligands                      | 5                         |
| water                        | 138                       |
|                             |       |
|-----------------------------|-------|
| Protein residues            | 260   |
| RMS(bonds)                  | 0.009 |
| RMS(angles)                 | 1.10  |
| Ramachandran favored (%)    | 98    |
| Ramachandran allowed (%)    | 2     |
| Ramachandran outliers (%)   | 0     |
| Clashscore                  | 3.89  |
| Average B-factor            | 46.60 |
| macromolecules              | 46.70 |
| ligands                     | 32.50 |
| solvent                     | 45.50 |

Statistics for the highest-resolution shell are shown in parentheses.