Phosphorylation state of a Tob/BTG protein, FOG-3, regulates initiation and maintenance of the *Caenorhabditis elegans* sperm fate program

Myon-Hee Lee[a,b,1], Kyung Won Kim[c,1], Clinton T. Morgan[c], Dyan E. Morgan[d], and Judith Kimble[a,c,d,2]

[a]The Howard Hughes Medical Institute, [c]Department of Biochemistry, and [d]Program in Cellular and Molecular Biology, University of Wisconsin, Madison, WI 53706; and [b]Division of Hematology/Oncology, Department of Internal Medicine, Brody School of Medicine at East Carolina University, Greenville, NC 27834

FOG-3, the single *Caenorhabditis elegans* Tob/BTG protein, directs germ cells to adopt the sperm fate at the expense of oogenesis. Importantly, FOG-3 activity must be maintained for the continued production of sperm that is typical of the male sex. Vertebrate Tob proteins have antiproliferative activity and ERK phosphorylation of Tob proteins has been proposed to abrogate "antiproliferative" activity. Here we investigate FOG-3 phosphorylation and its effect on sperm fate specification. We found both phosphorylated and unphosphorylated forms of FOG-3 in nematodes. We then interrogated the role of FOG-3 phosphorylation in sperm fate specification. Specifically, we assayed FOG-3 transgenes for rescue of a *fog-3* null mutant. Wild-type FOG-3 rescued both initiation and maintenance of sperm fate specification. A FOG-3 mutant with its four consensus ERK phosphorylation sites substituted to alanines, called FOG-3(4A), rescued partially: sperm were made transiently but not continuously in both sexes. A different FOG-3 mutant with its sites substituted to glutamates, called FOG-3(4E), had no rescuing activity on its own, but together with FOG-3(4A) rescue was complete. Thus, when FOG-3(4A) and FOG-3(4E) were both introduced into the same animals, sperm fate specification was not only initiated but also maintained, resulting in continuous spermatogenesis in males. Our findings suggest that unphosphorylated FOG-3 initiates the sperm fate program and that phosphorylated FOG-3 maintains that program for continued sperm production typical of males. We discuss implications of our results for Tob/BTG proteins in vertebrates.

sex determination | sperm/oocyte decision | germline

Specification of a germ cell as either sperm or oocyte lies at the heart of reproduction and fertility. In many animals, the germline tissue produces a steady stream of gametes, with germ cells constantly maturing from germline stem cell (GSC) through entry into the meiotic cell cycle into overt differentiation as sperm or oocyte. A prevailing idea has been that germline sex determination relies on commitment to a male or female fate during early development. However, sexual identity can be plastic, even in later development (1). Of particular relevance to this work, the commitment of a germ cell to a sperm or oocyte fate must be continually reinforced in *Caenorhabditis elegans* adults (2).

The *C. elegans* sperm/oocyte fate decision relies on a well-established molecular pathway (Fig. 1*A*) (2, 3). Normally, this small free-living nematode exists as either an XO male or an XX self-fertilizing hermaphrodite: XO males begin to produce sperm during late larval development and continue making sperm as adults (Fig. 1*B*), whereas XX hermaphrodites make sperm transiently during late larval development and then make oocytes in adults (Fig. 1*C*). The initial sex-determining signal, which is the ratio of X chromosomes to autosomes, regulates a "global" sex determination pathway to drive female or male development in all tissues. In the germline, the TRA-1 transcription factor represses expression of the germline–specific *fog-3* (*feminization of germline*) gene to promote oocyte fate specification (4). TRA-1 likely also represses *fog-1* expression (5). Genetically, FOG-1 and

FOG-3 proteins function at the end of the pathway to specify the sperm fate. In *fog-1* and *fog-3* null mutants, germ cells that normally differentiate as sperm are sexually transformed into oocytes (6, 7). For example, *fog-3* XO null mutants have a male soma but produce only oocytes, but *fog-3* XX null mutants have a hermaphrodite soma, even though they also produce only oocytes. Importantly, FOG-3 is not only required for initiating spermatogenesis during larval development, but FOG-3 must remain active in adult males for continued sperm production: adult wild-type males subjected to RNA-mediated interference (RNAi) against *fog-3* cease spermatogenesis and are sexually transformed to produce oocytes (8).

FOG-3 belongs to the Tob/BTG protein family of "antiproliferation" proteins (8). All Tob/BTG proteins possess an N-terminal Tob/BTG domain required for inhibition of cell proliferation (9). The molecular function of Tob/BTG proteins has been implicated in both transcriptional and translational regulation (10, 11). ERK, a member of the MAPK family, phosphorylates human Tob and this phosphorylation has been proposed to interfere with antiproliferative activity (12, 13).

The *C. elegans* ERK homolog, MPK-1, regulates the sperm/oocyte decision in addition to many other developmental roles, including progression through pachytene, oocyte meiotic maturation/ovulation, and somatic development (14–17). Its role in the sperm/oocyte decision is most relevant to this work. A partial loss-of-function *mpk-1* mutant feminizes a small percentage (6%) of XX hermaphrodites so that they make only oocytes and no sperm; a strong loss-of-function *mpk-1* mutant induces female markers in immature germ cells that normally are destined for spermatogenesis (15). Moreover, inhibition of Ras-ERK signaling, using either a chemical inhibitor of MEK or RNAi against *mpk-1*, can reprogram germlines to make oocytes instead of sperm (17). Therefore, Ras-ERK signaling promotes sperm fate specification. The MPK-1/ERK substrates critical for the sperm fate are not yet known. Two proposed substrates are TOE-5 and ZIM-2, but their effects on germline sex determination are poorly characterized (16).

Here we investigate the sperm fate regulator, FOG-3, as a possible candidate for the MPK-1/ERK substrate essential for sperm fate specification. This hypothesis was based on the findings that MPK-1 promotes the sperm fate in both sexes (15) and that the FOG-3 amino acid sequence harbors a predicted ERK-docking site and consensus phosphoacceptor sites (Fig. 1*D*), like its human homolog Tob, which is an ERK substrate (12, 13). We find that
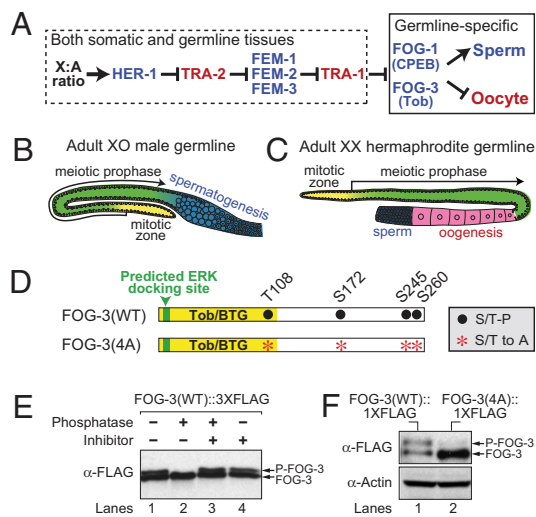
DEVELOPMENTAL BIOLOGY

Fig. 1. FOG-3 is a phosphoprotein in vivo. (*A*) Genetic regulation of the *C. elegans* sperm/oocyte decision (simplified pathway). Boxed with a dashed black line are regulators of sex determination in all tissues; boxed with a solid black line are germline-specific regulators of sex determination, FOG-1 and FOG-3, which are terminal regulators of the sperm fate in both hermaphrodites and males. Blue proteins promote the sperm fate and red proteins promote the oocyte fate. (*B* and *C*) Schematics of adult germlines. Yellow, mitotically dividing germ cells, including germline stem cells; green, germ cells progressing through meiotic prophase I. (*B*) Adult male germlines make sperm (blue) continuously. (*C*) Adult hermaphrodite germlines contain a pool of mature sperm (blue), which were made during larval development, as well as developing oocytes (pale red). (*D*) Schematics of WT and alanine-substituted (4A) FOG-3 proteins. Yellow, Tob/BTG domain; green, predicted ERK docking site; black dots, consensus ERK phosphorylation sites with specific amino acid designated above; red asterisks, alanine substitutions at phosphorylation sites. (*E*) Phosphatase assay. FOG-3(WT)::3XFLAG was immunoprecipitated with anti-FLAG antibody from an extract prepared from *fog-3; him-5; qEx[fog-3(WT)::3xflag]* animals (mixture of XX and XO). The immunoprecipitated FOG-3(WT)::3XFLAG was treated with λ-phosphatase and phosphatase inhibitor, as noted, and analyzed by SDS/PAGE followed by immunoblotting. In α-FLAG panel, arrows mark phosphorylated (P-FOG-3) and unphosphorylated (FOG-3) forms of FOG-3. (*F*) FOG-3 phosphorylation state in vivo assayed by Western blotting. Worm extracts were prepared from *fog-3; him-5; qIs[fog-3(WT)::1xflag]* (lane 1) or *fog-3; him-5; qEx[fog-3(4A)::1xflag]* (lane 2) animals. In lane 2, phosphorylated FOG-3 is significantly reduced and the remaining FOG-3 migrates as if unphosphorylated.

FOG-3 is a phosphoprotein in nematodes, and that the distributions of FOG-3 and active MPK-1 overlap in male germlines. However, our results with *fog-3* transgenes demonstrate that unphosphorylated FOG-3 remains able to initiate sperm fate specification. Therefore, FOG-3 is not likely the primary MPK-1 substrate critical for sperm fate specification. Instead, FOG-3 phosphorylation appears to be required to maintain the continued sperm fate specification typical of adult males. The identification of a role for FOG-3 phosphorylation in maintenance of a cell fate program has important implications for its vertebrate homologs.

Results

FOG-3 Is a Phosphoprotein. We first asked if FOG-3 exists as a phosphoprotein in vivo. Because no FOG-3–specific antibody is available, we generated epitope-tagged *fog-3* transgenes to assess FOG-3 phosphorylation. One transgene, called *fog-3(WT)::3xflag*, harbors the *fog-3* genomic region (1,334 bp of 5′ flanking region, all exons and introns, and 1,387 bp of 3′ flanking region) plus three FLAG tags engineered in frame at the end of the *fog-3* ORF; a second transgene, called *fog-3(WT)::1xflag*, harbors the same DNA but carries only one FLAG tag. The resultant FOG-3 proteins are wild-type, except for the presence of one or three

C-terminal FLAG tags. Both transgenes rescue *fog-3* null mutants to self-fertility (see below) and therefore both make functional FOG-3 protein. The specific transgene used for the various experiments in this article reflects when the transgene was generated; the 3XFLAG version gave a better yield after immunoprecipitation.

To assay its phosphorylation state, we used anti-FLAG antibodies to immunoprecipitate FOG-3(WT)::3XFLAG protein from a *fog-3* rescued transgenic strain that produces both XX hermaphrodites and XO males. Western blots with the anti-FLAG antibodies revealed two distinct FOG-3(WT)::3XFLAG bands (Fig. 1*E*, lane 1). Upon in vitro treatment of the extract with phosphatase, the upper band collapsed to the lower band (Fig. 1*E*, lane 2). Moreover, a phosphatase inhibitor prevented that collapse (Fig. 1*E*, lanes 3 and 4). We conclude that FOG-3(WT)::3XFLAG exists in both phosphorylated and unphosphorylated forms in vivo.

We next asked if the four predicted ERK phosphoacceptor sites in FOG-3 were required for FOG-3 phosphorylation in vivo. To this end, we mutated all four to alanines (T108A, S172A, S245A, and S260A) and generated a strain harboring the *fog-3(4A)::1xflag* transgene (Fig. 1*D*). We then compared the phosphorylation state of FOG-3 protein in *fog-3(WT)::1xflag* and *fog-3(4A)::1xflag* transgenic animals by Western blot analysis. The phosphorylated upper band was dramatically reduced in FOG-3(4A) transgenic animals, compared with FOG-3(WT) (Fig. 1*F*). Therefore, FOG-3 phosphorylation appears to occur at one or more of the four serine and threonine residues that are predicted to be ERK phosphorylation sites.

FOG-3 Is Enriched in Spermatogenic Germlines. We next examined FOG-3(WT)::1XFLAG to ensure its germline expression. FOG-3 (WT)::1XFLAG was visualized with anti-FLAG antibodies, henceforth referred to as FOG-3 staining. In XX hermaphrodites early in the fourth larval stage (L4), when their sperm are being specified, FOG-3 was abundant in the pachytene region (Fig. 2*A*), but in late L4s when their germlines are switching to oogenesis, FOG-3 was no longer detectable (Fig. 2*B*). Therefore, FOG-3 is expressed transiently in XX hermaphrodites.

We next examined XX masculinized germlines. XX *fem-3(gf)* mutants make sperm continuously as adults and fail to switch into oogenesis (18). FOG-3 was also expressed in early *fem-3(gf)* L4s but remained abundant in adults (Fig. 2*C*). In these XX masculinized adult germlines, FOG-3 was expressed from the proximal mitotic zone through the distal pachytene region (Fig. 2*C*).

In XO males, the pattern of FOG-3 was similar to that seen in XX masculinized germlines; FOG-3 was detectable weakly in the proximal mitotic zone and transition zone (leptotene and zygo-
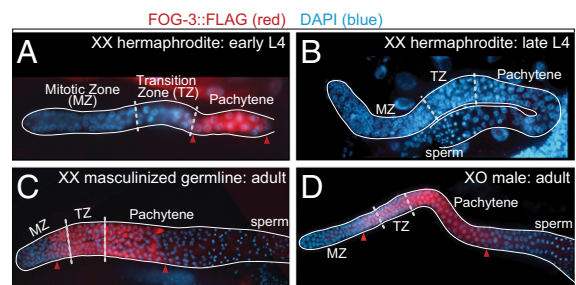
Fig. 2. FOG-3 is enriched in spermatogenic germlines. (*A–D*) Germlines dissected from animals harboring a *fog-3(WT)::1xflag* transgene and stained for FOG-3::1XFLAG (red) and DNA (blue). Dashed lines are mitotic zone/transition zone boundary or transition zone/pachytene boundary; red arrowheads mark the extent of elevated FOG-3::FLAG. (*A*) *fog-3; qIs[fog-3(WT)::1xflag]* XX hermaphrodite early L4. (*B*) *fog-3; qIs[fog-3(WT)::1xflag]* XX hermaphrodite late L4. (*C*) *fog-3; fem-3(gf); qIs[fog-3(WT)::1xflag]* XX hermaphrodite adult with a masculinized germline. (*D*) *fog-3; qIs[fog-3(WT)::1xflag]* XO male adult.

tene), and became abundant more proximally where germ cells progress into pachytene (Fig. 2D). This distribution of FOG-3 in male germlines overlaps that of active MPK-1, which extends from the middle of the transition zone into the distal pachytene region (15). In addition, FOG-3 is predominantly cytoplasmic in both XX and XO animals. We conclude that FOG-3 is enriched in spermatogenic germlines, which is consistent with the role of FOG-3 in sperm fate specification.

FOG-3(4A) Can Support Sperm Fate Specification Transiently. We next investigated the role of FOG-3 phosphorylation in sperm fate specification with a series of transgenes encoding either wild-type FOG-3 or mutants substituted at its predicted phosphorylation sites. Each transgene was assayed for its ability to drive the sperm fate in both XX and XO fog-3 null mutants, which otherwise made only oocytes (Fig. 3A, line 1) (7). The fog-3(WT)::1xflag transgene, which makes wild-type FLAG-tagged FOG-3 protein, rescued both XX and XO fog-3 mutants. The rescued XX hermaphrodites

made sperm in larvae, switched to oogenesis, and became self-fertile adults; the rescued XO males made sperm continuously and were cross-fertile [Fig. 3 A (line 2) and B]. Therefore, the sperm-promoting activity of the FLAG-tagged FOG-3 mimics that of endogenous FOG-3 in both sexes.

We then tested fog-3 mutant transgenes for fog-3 rescue. Each transgene harbored one or more alanine substitutions of the four predicted phosphorylation sites (T108A, S172A, S245A, and S260A). The fog-3(4A)::1xflag transgene generates a mutant protein with alanine substitutions at all four phosphorylation sites and is largely unphosphorylated (Fig. 1F); the analogous alanine-substituted form of human Tob appears to mimic the unphosphorylated state (12, 13). When assayed by immunocytochemistry, all of the alanine-substituted FOG-3 proteins were predominantly cytoplasmic and expressed in a pattern within the germline tissue that was virtually identical to FOG-3(WT) (Fig. 2A and Fig. S1); however, they did not have equivalent rescuing activity (Fig. 3A, lines 3–8).
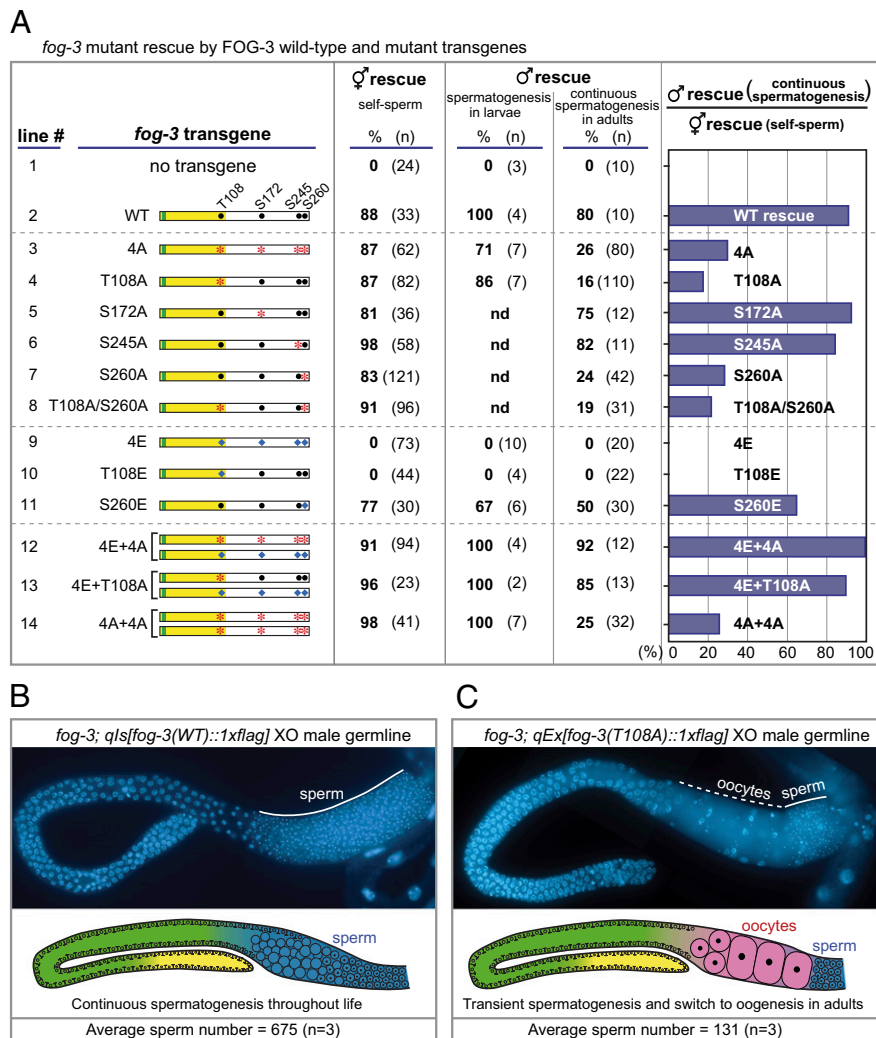
Fig. 3. A fog-3 mutant rescue by FOG-3 wild-type and FOG-3 mutant transgenes. (A) Rescuing activity was scored in fog-3 null mutants harboring the transgene shown on left. Transgene schematics use the same conventions as detailed in Fig. 1D. Rescuing activity was scored in XX hermaphrodite adults (Left), XO male L4 larvae (Center), and XO male adults (Right). Hermaphrodite rescue indicates the percentage of self-fertile fog-3 XX animals, which made their own sperm (Left); larval male rescue indicates the percentage of fog-3 XO L4 larvae with any sperm (Center); adult male rescue indicates the percentage of fog-3 XO adults with continuous spermatogenesis (Right). Bar graphs on right depict the ratio of XO male rescue (continuous spermatogenesis in adults) to XX hermaphrodite rescue. See Table S1 for the rescue of each transgenic line. nd, not determined. (B and C) fog-3 mutant rescue by (B) fog-3(WT)::1xflag or (C) fog-3(T108A)::1xflag transgene. DAPI-stained adult male germlines (Top); schematics of the germlines (Middle); the average number of sperm produced at 1-d-old in the adult male germline (Bottom).

FOG-3(4A) rescued both XX and XO *fog-3* mutants to make some sperm (Fig. 3*A*, line 3). Therefore, FOG-3(4A) makes a functional protein and the four predicted FOG-3 phosphorylation sites are not required in either sex for sperm specification per se. However, rescue differed in the two sexes. FOG-3(4A) rendered hermaphrodites self-fertile, and did so with the same penetrance as the wild-type *fog-3* transgene (Fig. 3*A*, line 3). In contrast, FOG-3(4A) only allowed XO male larvae to make sperm transiently; most adults switched to oogenesis (Fig. 3*A*, line 3).

Among the proteins harboring individually substituted phosphorylation sites, FOG-3(T108A) and FOG-3(S260A) behaved like FOG-3(4A) in the *fog-3* rescue assay, whereas FOG-3(S172A) and FOG-3(S245A) behaved like FOG-3(WT) [Fig. 3 *A* (lines 4–7) and *C*]. For example, *fog-3* hermaphrodites carrying FOG-3(T108A) produced sperm transiently during larval development as does FOG-3(WT); the average sperm number was similar to that of FOG-3(WT) [FOG-3(WT) = 89 on average (*n* = 12); FOG-3(T108A) = 109 on average (*n* = 5)]. Unlike the full rescue observed with the FOG-3(WT) transgene in males, *fog-3* males carrying FOG-3(T108A) produced sperm only transiently and switched into oogenesis within 24 h of adulthood; the average sperm number was much lower than that of FOG-3(WT) [FOG-3(WT) = 675 on average (*n* = 3); FOG-3(T108A) = 131 on average (*n* = 3)] (Fig. 3 *B* and *C*). Therefore, two FOG-3 residues, T108 and S260, emerge as critical for the continued spermatogenesis typical of males. The FOG-3(T108A/S260A) double-alanine substitution had essentially the same penetrance as either single mutant (Fig. 3*A*, line 8), suggesting that these two sites work together. Therefore, a mutant FOG-3 lacking the T108 and S260 phosphorylation site can drive germ cells into the sperm fate but cannot maintain commitment to that male-specific program in adult males. The simplest explanation is that FOG-3 phosphorylation at T108 and S260 is not required to specify the initial sperm fate, but instead is required to maintain spermatogenesis in males.

FOG-3(4E) Cannot Support Sperm Fate Specification on its Own. We next tested a second set of *fog-3* mutant transgenes for *fog-3* rescue. Each transgene harbored one or more glutamate substitutions of the four predicted phosphorylation sites (T108E, S172E, S245E, and S260E). The *fog-3(4E)::1xflag* transgene generates a mutant protein, FOG-3(4E), with glutamate substitutions at all four phosphorylation sites; the analogous glutamate-substituted form of human Tob appears to mimic the phosphorylated state (12, 13). When assayed by immunocytochemistry, the glutamate-substituted FOG-3 proteins were predominantly cytoplasmic and expressed in a pattern within the germline tissue that was virtually identical to FOG-3(WT) (Fig. 2*A* and Fig. S1). However, FOG-3(4E) failed to drive any germ cells into the sperm fate: both XX and XO *fog-3* mutant germlines expressing FOG-3(4E) made only oocytes and no sperm (Fig. 3*A*, line 9). Therefore, FOG-3(4E) was not capable of rescuing either XX or XO *fog-3* mutants.

For individual phosphorylation sites, we assayed FOG-3(T108E) and FOG-3(S260E) mutant transgenes, because the corresponding alanine substitutions had dramatically reduced continued spermatogenesis in adult males (see above). Like FOG-3(4E), the FOG-3(T108E) individual substitution failed to rescue *fog-3(0)* mutants in either sex; however, FOG-3(S260E) behaved like FOG-3(WT) and rescued *fog-3(0)* in both sexes (Fig. 3*A*, lines 10 and 11). Therefore, FOG-3(T108E), which may mimic a constitutively phosphorylated FOG-3 at T108, fails to drive the sperm fate when assayed by *fog-3* rescue.

Together, FOG-3(4A) and FOG-3(4E) Provide Full Rescue. The inability of FOG-3(4E) or FOG-3(T108E) mutant proteins to drive any sperm fate specification was puzzling. We considered three explanations: (*i*) these proteins might be nonfunctional; (*ii*) they might actively interfere with sperm fate specification; or (*iii*) they

may be unable to initiate the sperm fate. To distinguish among these possibilities, we introduced both FOG-3(4A) and FOG-3(4E) transgenes into the same animal. The result supports the third explanation. A *fog-3* mutant germline expressing both FOG-3(4A) plus FOG-3(4E) was fully rescued: both XX and XO germlines initiated spermatogenesis and XO germlines continued spermatogenesis in adults (Fig. 3*A*, line 12). Full rescue was also obtained with the individually substituted FOG-3(T108A) together with FOG-3(4E) (Fig. 3*A*, line 13). To control for a potential effect of extra FOG-3 dosage, we introduced two FOG-3 (4A) mutants with different tags; however, a germline expressing both FOG-3(4A)::FLAG and FOG-3(4A)::HA was not capable of continued spermatogenesis (Fig. 3*A*, line 14). We conclude that the glutamate-substituted FOG-3 mutant is a functional protein and that it does not interfere with sperm fate specification, at least when expressed with FOG-3(4A) or FOG-3(T108A). Although FOG-3(4E) cannot on its own instigate the sperm fate (Fig. 3*A*, line 9), it is required for the continued production of sperm in adult males. Furthermore, given that FOG-3(T108E) failed to drive spermatogenesis in either sex (Fig. 3*A*, line 10), we suggest that FOG-3 must remain "unphosphorylated" at T108 to initiate the sperm fate.

Discussion

FOG-3 Phosphorylation and Sperm Fate Specification. FOG-3 is a key regulator of sperm fate specification (7) and a member of the Tob/BTG family of "antiproliferation" proteins (8). Here we provide evidence that the state of FOG-3 phosphorylation has a dual role in germline sex determination and present a model for this dual role (Fig. 4). FOG-3(4A) mutant protein lacks its four phosphorylation sites but still triggers germ cells to adopt the sperm fate; however, that mutant cannot maintain commitment of subsequent germ cells to adopt the sperm fate. By contrast, FOG-3(4E), in which the same sites are substituted to glutamate and therefore are likely to mimic "constitutive" phosphorylation, cannot trigger initial commitment to the sperm fate but can maintain that commitment when expressed together with FOG-3(4A). Indeed, two individual residues, T108 and S260, were critical for promoting the sperm fate. The simplest explanation is that the state of FOG-3 phosphorylation has a dual role: phosphorylation must be prevented at T108 to initiate the spermatogenesis program during larval development, but phosphorylation must occur at T108/S260 to maintain that program in adult males (Fig. 4).

How does the state of FOG-3 phosphorylation affect its activity? We do not know, but propose that phosphorylated FOG-3
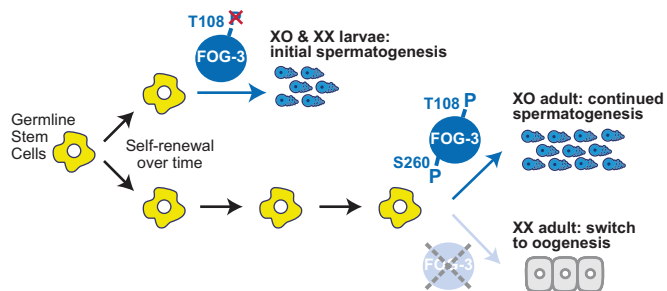
Fig. 4. Model for control of the sperm fate specification. GSCs continuously produce progeny that differentiate as sperm or oocytes. Upper branch: The first set of GSC progeny adopts a sperm fate in both sexes. We propose that FOG-3 must be unphosphorylated at T108 to trigger this initial commitment. Lower branch: In males, GSC progeny continuously adopt the sperm fate in adults, whereas in hermaphrodites, germline sex switches to make oocytes in adults. We propose that FOG-3 must be phosphorylated at T108 and S260 in adult males to continue sperm fate specification and prevent the switch to oogenesis. FOG-3 could either be turned off as shown here (X), or left in its unphosphorylated state in adult hermaphrodites.

could stabilize sperm fate regulators or inhibit oogenesis regulators. T108 is located within the Tob/BTG domain, which spans the N-terminal 116 amino acids of FOG-3 protein (8). Given that T108 must remain "unphosphorylated" for initiation and be "phosphorylated" for maintenance of continued sperm fate specification, we suggest that the state of T108 phosphorylation provides the FOG-3 Tob/BTG domain with distinct activities. One speculative but plausible idea is that the phosphorylation state directs the formation of discrete complexes. Another possibility is that phosphorylation state directs distinct subcellular locales, although both FOG-3(4A) and FOG-3(4E) appear predominantly cytoplasmic. Regardless, the two forms appear to have functions specialized for initiation (unphosphorylated form at T108) or maintenance (phosphorylated form at T108/S260) of the sperm fate program.

What Are the Regulators of FOG-3 Phosphorylation? This work began with the idea that FOG-3 protein might be the primary substrate of MPK-1 to control sperm fate specification. That hypothesis was based on the requirement for MPK-1 in sperm fate specification (15) and the presence of a predicted ERK docking site and four consensus phosphoacceptor sites in the FOG-3 protein (12). However, our results did not support the idea. Instead, unphosphorylated FOG-3 drives sperm fate specification, at least transiently, and phosphorylated FOG-3 maintains commitment to the sperm fate. Because the distributions of active MPK-1 and FOG-3 overlap in male germlines (present study and ref. 15), FOG-3 may indeed be an MPK-1 substrate but that possibility remains untested. The specific kinases and phosphatases required to regulate the FOG-3 phosphorylation state remain a challenge for the future.

In addition to phosphorylated FOG-3, two other sex-determination regulators normally regulate the continued spermatogenesis typical of male germlines. One regulator is TRA-1, the sole *C. elegans* GLI transcription factor (19); *tra-1* null mutants are sexually transformed into males that make sperm transiently and then switch to oogenesis (20, 21). The other relevant regulator is FOG-1, a cytoplasmic polyadenylation element binding (CPEB) RNA-binding protein (5, 22); *fog-1* males make oocytes rather than sperm but *fog-1*/+ males make sperm transiently and then switch to oogenesis (6). A simple but speculative idea is that TRA-1 and FOG-1 promote continued spermatogenesis in males via an effect on FOG-3 phosphorylation. However, they are not likely direct regulators of FOG-3 phosphorylation as neither encodes a kinase.

Implications for Tob Regulation. What implications might our analysis of FOG-3 phosphorylation have for vertebrate Tob/BTG proteins? Vertebrate Tob proteins have antiproliferative activity; it has been proposed that the ERK phosphorylation of Tob abrogates its "antiproliferative" activity (12, 13). Both wild-type Tob and an alanine-substituted unphosphorylatable Tob can restore antiproliferative activity to $tob^{-/-}$ cells, much like both wild-type FOG-3 and alanine-substituted FOG-3 restore sperm-promoting activity to *fog-3* null mutants. Moreover, a glutamate-substituted and likely phosphomimetic Tob lacks antiproliferative activity in $tob^{-/-}$ cells, much as the analogous FOG-3(4E) lacks sperm fate-promoting activity in *fog-3* null mutants. The conclusion from this line of experiments has been that vertebrate Tob phosphorylation inhibits its antiproliferative activity.

Our work introduces the idea that unphosphorylated and phosphorylated forms of FOG-3 have distinct roles in the sperm/oocyte decision. We posit that a similar effect may hold true for Tob. Evidence already demonstrates that unphosphorylated Tob retains antiproliferative activity. By analogy with our FOG-3 findings, we speculate that unphosphorylated Tob might initiate an antiproliferative state and phosphorylated Tob might maintain that state. Such a maintenance function would not be easily detected in tissue culture cells, but instead would require analysis in an actively developing or cancerous tissue. If Tob phosphorylation maintains antiproliferative activity, that state would have important clinical implications in the treatment of cancer.

Tob/BTG proteins affect multiple aspects of vertebrate development (9). Given the key role of FOG-3 in germline sex determination, one might ask if Tob/BTG proteins similarly govern vertebrate spermatogenesis. The simple answer is that this question remains unexplored. However, multiple Tob/BTG family members are expressed in the mammalian testis, suggesting a possible role in that organ (9). Intriguingly, murine BMP8B regulates both initiation and maintenance of sperm differentiation at the expense of apoptosis (23), and Tob represses BMP signaling in osteoblasts (24). An attractive idea, although clearly speculative, is that Tob also influences vertebrate spermatogenesis, perhaps via an effect on BMP signaling.

Materials and Methods

***C. elegans* Strains and Transgenes.** The following mutants and balancers were used: LG I: *fog-3(q520)*, *hT2[qIs48]*; LG IV: *fem-3(q20ts, gf)*; LG V: *him-5(e1490)*.

To construct *fog-3::flag* transgenes, *flag* DNA (GAT TAC AAG GAT GAC GAC GAT AAG) was inserted as either one or three copies in frame, immediately before the *fog-3* stop codon, into the wild-type *fog-3* gene, including 1,334 bp of 5′ flanking sequence, all exons and introns, and 1,387 bp of 3′ flanking sequence [pRE11 (8)]. To generate *fog-3* transgenic animals, *fog-3* plasmid (1 ng/μL, linearized using XhoI restriction enzyme) with either pTG96 [*Psur-5::gfp* (25); 1 ng/μL, linearized using SphI restriction enzyme] or pCFJ104 [*Pmyo-3::mCherry* (26); 1 ng/μL] were microinjected into *fog-3(q520)/hT2[qIs48]* animals with yeast genomic DNA (60 ng/μL, cut with PvuII). Injected animals and all transgenic lines were maintained at 25 °C to minimize cosuppression (27). At least two lines for each transgene were examined for phenotype analyses (Table S1). Plasmids, transgenes, and transgenic lines reported here are listed in Table S2.

Immunoprecipitation and Phosphatase Assay. FOG-3(WT)::3XFLAG was immunoprecipitated using anti-FLAG M2 Affinity Gel (Sigma-Aldrich) from *fog-3; him-5; qEx[fog-3(WT)::3xflag]* adult worm lysate, as previously described (28), with minor modifications. Bound proteins were eluted from the FLAG gel by competition with 3XFLAG peptide (Sigma-Aldrich). Eluted proteins were treated with 2 units/μL λ-protein phosphatase (New England Biolabs) for 30 min at 30 °C, in the absence or presence of Halt Phosphatase Inhibitor Mixture (Thermo Scientific). The reactions were separated on a polyacrylamide gel and then analyzed by immunoblotting using anti-FLAG M2 antibody (1:1,000 dilution; Sigma-Aldrich).

Immunohistochemistry. For FOG-3::1XFLAG, dissected gonads were fixed in 3% paraformaldehyde/6 mM K_2HPO_4 (pH 7.2)/75% methanol for 10 min at −20 °C. After blocking for 1 h with 0.5% BSA in 1XPBS containing 0.1% Tween 20, fixed gonads were incubated 1 h at room temperature with a mouse anti-FLAG M2 antibody (1:200; Sigma-Aldrich) followed by 1 h at room temperature with secondary antibody. DAPI staining followed standard methods.

1. DeFalco T, Capel B (2009) Gonad morphogenesis in vertebrates: Divergent means to a convergent end. *Annu Rev Cell Dev Biol* 25:457–482.
2. Kimble J, Crittenden SL (2007) Controls of germline stem cells, entry into meiosis, and the sperm/oocyte decision in *Caenorhabditis elegans*. *Annu Rev Cell Dev Biol* 23: 405–433.
3. Ellis R, Schedl T (2007) Sex determination in the germ line. *WormBook,* ed The *C. elegans* Research Community. Available at: www.wormbook.org, 10.1895/wormbook.1.82.2.
4. Chen P-J, Ellis RE (2000) TRA-1A regulates transcription of *fog-3*, which controls germ cell fate in *C. elegans*. *Development* 127:3119–3129.
5. Jin S-W, Kimble J, Ellis RE (2001) Regulation of cell fate in *Caenorhabditis elegans* by a novel cytoplasmic polyadenylation element binding protein. *Dev Biol* 229: 537–553.
6. Barton MK, Kimble J (1990) *fog-1*, a regulatory gene required for specification of spermatogenesis in the germ line of *Caenorhabditis elegans*. *Genetics* 125:29–39.

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

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

9. Jia S, Meng A (2007) Tob genes in development and homeostasis. *Dev Dyn* 236:913–921.

10. Mauxion F, Chen CY, Séraphin B, Shyu AB (2009) BTG/TOB factors impact deadenylases. *Trends Biochem Sci* 34:640–647.

11. Winkler GS (2010) The mammalian anti-proliferative BTG/Tob protein family. *J Cell Physiol* 222:66–72.

12. Maekawa M, Nishida E, Tanoue T (2002) Identification of the anti-proliferative protein Tob as a MAPK substrate. *J Biol Chem* 277:37783–37787.

13. Suzuki T, et al. (2002) Phosphorylation of three regulatory serines of Tob by Erk1 and Erk2 is required for Ras-mediated cell proliferation and transformation. *Genes Dev* 16:1356–1370.

14. Sundaram MV (2006) RTK/Ras/MAPK signaling. *WormBook,* ed The *C. elegans* Research Community. Available at: http://www.wormbook.org, 10.1895/wormbook.1.80.1.

15. Lee M-H, et al. (2007) Multiple functions and dynamic activation of MPK-1 extracellular signal-regulated kinase signaling in *Caenorhabditis elegans* germline development. *Genetics* 177:2039–2062.

16. Arur S, et al. (2009) Multiple ERK substrates execute single biological processes in *Caenorhabditis elegans* germ-line development. *Proc Natl Acad Sci USA* 106:4776–4781.

17. Morgan CT, Lee MH, Kimble J (2010) Chemical reprogramming of *Caenorhabditis elegans* germ cell fate. *Nat Chem Biol* 6:102–104.

18. Barton MK, Schedl TB, Kimble J (1987) Gain-of-function mutations of *fem-3*, a sex-determination gene in *Caenorhabditis elegans*. *Genetics* 115:107–119.

19. Zarkower D, Hodgkin J (1992) Molecular analysis of the *C. elegans* sex-determining gene *tra-1*: A gene encoding two zinc finger proteins. *Cell* 70:237–249.

20. Schedl T, Graham PL, Barton MK, Kimble J (1989) Analysis of the role of *tra-1* in germline sex determination in the nematode *Caenorhabditis elegans*. *Genetics* 123:755–769.

21. Hodgkin J (1987) A genetic analysis of the sex-determining gene, *tra-1*, in the nematode *Caenorhabditis elegans*. *Genes Dev* 1:731–745.

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

23. Zhao GQ, Deng K, Labosky PA, Liaw L, Hogan BL (1996) The gene encoding bone morphogenetic protein 8B is required for the initiation and maintenance of spermatogenesis in the mouse. *Genes Dev* 10:1657–1669.

24. Yoshida Y, et al. (2000) Negative regulation of BMP/Smad signaling by Tob in osteoblasts. *Cell* 103:1085–1097.

25. Yochem J, Gu T, Han M (1998) A new marker for mosaic analysis in *Caenorhabditis elegans* indicates a fusion between hyp6 and hyp7, two major components of the hypodermis. *Genetics* 149:1323–1334.

26. Frøkjaer-Jensen C, et al. (2008) Single-copy insertion of transgenes in *Caenorhabditis elegans*. *Nat Genet* 40:1375–1383.

27. Dernburg AF, Zalevsky J, Colaiácovo MP, Villeneuve AM (2000) Transgene-mediated cosuppression in the *C. elegans* germ line. *Genes Dev* 14:1578–1583.

28. Kim KW, et al. (2009) Antagonism between GLD-2 binding partners controls gamete sex. *Dev Cell* 16:723–733.