Recent phylogenetic studies have suggested the surprising possibility that the hermaphroditism of even the closely related *C. elegans* and *C. briggsae* may be convergent (Cho et al., 2004; Kiontke et al., 2004). Since spermatogenesis is the only anatomical difference between females and hermaphrodites, germline sex determination genes must have been the key targets of selection for self-fertility. As *C. elegans* hermaphrodite development is well understood, this offers an excellent opportunity to study the developmental genetics of parallel evolution. In this paper, we present what is, to our knowledge, the first genetic analysis of *C. briggsae* sex determination.

In *C. elegans*, FEM-1, FEM-2, and FEM-3 form part of the core signal transduction pathway that mediates sex determination (Figure 1A) and act in the cytoplasm between the transmembrane receptor TRA-2 and the Gli-related transcription factor TRA-1 to promote male fate in XO animals (Doniach and Hodgkin, 1984; Hodgkin, 1986; Kimble et al., 1984). That all three *fem* genes are also required for hermaphrodite spermatogenesis suggests that they may have been instrumental for the evolution of self-fertility in the *C. elegans* lineage. FEM-1 is composed of ankyrin repeats (Spence et al., 1990). FEM-2 is a P22C phosphatase (Chin-Sang and Spence, 1996; Pilgrim et al., 1995), and FEM-3 is a nematode-specific protein that forms a specific complex with FEM-2 (Ahringer et al., 1992; Chin-Sang and Spence, 1996). Nearly 20 years of research has shown that posttranscriptional controls regulate germline sexual fates in *C. elegans* hermaphrodites (Puoti et al., 1997). The translational repression of *tra-2* by *fog-2* and *gld-1* is required to initiate *fem*-dependent hermaphrodite spermatogenesis (Clifford et al., 2000; Doniach, 1986; Goodwin et al., 1993; Schedl and Kimble, 1988), and similar repression of *fem*-3 by *fbf-1/2* is required for its cessation (Ahringer and Kimble, 1991; Barton et al., 1987; Zhang et al., 1997).

Nearly all *C. elegans* sex determination genes have orthologs in *C. briggsae* (Haag, 2005; Nayak et al., 2005), and *Cb-tra-2* is translationally repressed in a manner consistent with it also being key for the initiation of hermaphrodite spermatogenesis (Jan et al., 1997). However, *fog-2* is specific to *C. elegans*, and RNAI knockdown of *Cb-gld-1* gives an unexpected Mog (masculinization of germline) phenotype (Clifford et al., 2000; Nayak et al., 2005). It is thus unclear at which point *C. briggsae* hermaphrodites regulate the core pathway to initiate XX spermatogenesis. In addition, *Cb-fem-2(RNAi)* (Stothard et al., 2002) and *Cb-fem-3(RNAi)* (Haag et al., 2002) have no effect on hermaphrodites, but they partially feminize XO animals. In the case of *Cb-fem-2*, the XO germline is feminized, but the male soma was left intact (Stothard et al., 2002). For *Cb-fem-3*, the soma is feminized, but the germline remained exclusively male (Haag et al., 2002). These results are intriguing (Stothard and Pilgrim, 2003), but the poor penetrance of RNAI in *C. briggsae* (Haag et al., 2002) limited their interpretation. We sought to rigorously test if spermatogenesis in *C. briggsae* hermaphrodites depends
upon fem activity, and to clarify its roles in males, through analysis of strong loss-of-function mutations.

Results and Discussion

Isolation of Cb-fem-2 and Cb-fem-3 Deletions

We screened for deletion mutations in C. briggsae fem genes with the same method (Figure 1C) used by the C. elegans Knockout Consortium (Edgley et al., 2002). The first screen produced the allele Cb-fem-2(nm27), a 1.6 kbp deletion that removes the entire phosphatase domain as well as part of the 3' UTR. As phosphatase activity is necessary for the sex determination function of C. elegans FEM-2 (Chin-Sang and Spence, 1996), nm27 is likely a null mutation. We used both the polymerase chain reaction (PCR) with primers inside and outside of the deleted region (Figure 1D) and Southern hybridization (A. Doty and E.S.H., unpublished data) to verify that a strain homozygous for Cb-fem-2(nm27) was generated. In addition, no Cb-fem-2 mRNA expression is detected in mutant animals by high-sensitivity in situ hybridization (A. Doty and E.S.H., unpublished data) to verify that a strain homozygous for Cb-fem-2(nm27) was generated. In AF16 (wild-type) animals, the WT-only products are produced, and the flanking primers amplify the full-length product. In all Cb-fem-2(nm27) or Cb-fem-3(nm63) homozygotes, no WT-only product is produced, and flanking primers only generate the deletion-specific products.

Figure 1. Overview of fem Function, PP2C Evolution, and Isolation of Cb-fem-2 and Cb-fem-3 Deletions

(A) The core sex determination pathway of C. elegans, which acts in both soma and germline.

(B) Phylogenetic analysis of C. elegans and C. briggsae PP2C proteins. Aligned phosphatase domains from all predicted family members of each species were analyzed by the neighbor-joining method. Numbers indicate bootstrap support >50%. Parsimony analyses recovered the same orthologous pairs, but with different internal nodes.

(C) Fully nested flanking primers were used to detect gene-specific deletions in pools of genomic DNA from mutagenized nematodes. Primer pairs ("WT only") annealing within the deleted regions were subsequently designed to positively identify the wild-type chromosome.

(D) Deletions were verified by single-worm PCR with flanking and WT-only primers. Each lane represents one of two reactions performed on random single worms of the indicated genotype, with replicates included to demonstrate homozygosity and assay reproducibility. In AF16 (wild-type) animals, the WT-only products are produced, and the flanking primers amplify the full-length product. In all Cb-fem-2(nm27) or Cb-fem-3(nm63) homozygotes, no WT-only product is produced, and flanking primers only generate the deletion-specific products.

function (Ahringer et al., 1992), as well as several short stretches of conservation between species (Haag et al., 2002). Homozygous Cb-fem-3(nm63) XX mutants are also self-fertile hermaphrodites (Figure 2F). It is formally possible that the nm63 mutation is not a null, as it maintains the correct reading frame of the remaining codons if splicing were unaffected. However, we show that nm63 has a strong loss-of-function phenotype in XO animals, indicating that Cb-fem-3, like Cb-fem-2, is not required for hermaphrodite spermatogenesis. The conservation of the FEM-2/FEM-3 interaction in C. briggsae (Stothard and Pilgrim, 2006) left the possibility that only one of them is necessary for hermaphrodite development. However, XX Cb-fem-2(nm27); Cb-fem-3(nm63) worms are also self-fertile (Figure 2H and Table S1; see the Supplemental Data available with this article online).

XO Cb-fem-2 and Cb-fem-3 Mutants Are Hermaphrodites

The 11-member PP2C family has been stable since the divergence of C. elegans and C. briggsae (Figure 1B). An earlier study of a subset of the family (Stothard et al., 2002) indicated that fem-2 evolves faster than other family members. The current, complete analysis generally supports this, but it reveals that a second gene (CE07231/CBP03684) is comparably rapid. The large PP2C family suggested that the lack of Cb-fem-2(nm27) XX feminization could be because Cb-FEM-2 is redundant with another phosphatase, and not
essential for any aspect of sex determination. Similarly, the in-frame deletion of Cb-fem-3(nm63) left open the possibility that an internally truncated Cb-FEM-3 protein with sufficient activity is produced. To address these concerns, we examined the XO phenotype of Cb-fem-2(nm27) by using three genetic assays. We then employed the two most definitive of these assays to similarly characterize Cb-fem-3(nm63). First, various crosses were scored for sex ratio changes indicative of somatic feminization (Table S1). Those that cannot produce Cb-fem-2(nm27) homozygotes had the 35%–40% male frequency characteristic of wild-type crosses (the ratio is not 1:1 due to rare selfing and errant males). However, crosses of Cb-fem-2(nm27) hermaphrodites

Figure 2. Phenotypes of Cb-fem-2 and Cb-fem-3 Mutants

(A) Wild-type XX adult hermaphrodites have two oocyte-producing gonad arms (the anterior visible here); two spermathecae with sperm flank the embryo-containing uterus and medial vulva. The gut is seen in the posterior, under which lies the posterior gonad arm. The tail is tapered. The scale bar = 100 μm.

(B) XO wild-type males make sperm and have a modified tail used in copulation.

(C) Cb-fem-2(nm27) XX animals are self-fertile hermaphrodites. The inset shows the uterus and posterior spermatheca of a virgin adult, with sperm (see also [I]–[L]) and two embryos.

(D) Cb-fem-2(nm27) XO animals are also self-fertile hermaphrodites with complete somatic feminization, including the tail (inset). Most are similar to wild-type hermaphrodites and have a completely female soma and two self-fertile gonad arms that produce viable embryos.

(E) Abnormal XO hermaphrodites were seen at low frequency. This individual (shown slightly magnified relative to [A]–[D]) has two spermathecae on the posterior gonad arm (asterisks) and none in the anterior. Sperm can be seen in a broad area of the anterior arm.

(F) Cb-fem-3(nm63) XX mutants are also self-fertile hermaphrodites; an inset at higher magnification reveals the presence of oocytes, sperm, and an embryo of one gonad arm.

(G) Cb-fem-3(nm63) XO mutants are transformed into hermaphrodites with completely feminized soma (the inset shows the tail at higher magnification).

(H) XX Cb-fem-3(nm27); Cb-fem-3(nm63) double mutants (slightly magnified relative to [F] and [G]) are also self-fertile.

(I–L) fem-2(nm27) XX hermaphrodites produce normal sperm. (I) Proximal gonad of a virgin animal; the compact nuclei of sperm (J) are visualized with DAPI staining, and the sperm protein SPE-56 (K) is detected with monoclonal antibody staining. They colocalize (L, merged image) in the spermatheca (indicated by arrowheads). Anterior is to the left, and ventral is down in all panels. Labels: e, embryo; g, gut; o, oocytes; s, sperm; v, vulva.
with Cb-fem-2(nm27)/+ males produced roughly half as many male progeny, as expected if sexual transformation had occurred.

A second test for XO transformation is to suppress male production in a high incidence of males (Him) strain. We serendipitously discovered that unmated hermaphrodites heterozygous for the X chromosome-integrated green fluorescent protein (GFP) reporter transgene syIs802 have small broods that produce 32.5% XO males, presumably due to transgene destabilization of meiotic pairing. In contrast, Cb-fem-2(nm27); syIs802/+ hermaphrodites produce no males (Table S1). Similar Him suppression was seen in the progeny of Cb-fem-3(nm63); syIs802/+ animals. These results also suggest that male development cannot occur in Cb-fem-2 or Cb-fem-3 mutants, but feminized XO animals could not be positively identified.

The most definitive test for putative XO feminization employs genetic markers for outcrossing and karyotype. We scored the progeny of crosses between Cb-dpy(nm4) II; Cb-fem-2(nm27) III hermaphrodites and Cb-fem-2(nm27)/+ III; syIs802[myo-2::GFP] X males. Half of the non-Dpy, non-GFP offspring (XO cross progeny) are Cb-fem-2(nm27) homozygotes that also lack maternal Cb-fem-2 function and are thus potentially feminized, and half are Cb-fem-2(nm27)/+ and are expected to be male. We observed that 59% of non-Dpy, non-GFP progeny (n = 90) were somatically feminized (Figure 2D), while the remainder were normal males. Interestingly, these XO Cb-fem-2(nm27) mutants are self-fertile hermaphrodites (Her), and not females as in C. elegans. They produce viable embryos, but they have small broods and minor defects in female somatic gonad development (e.g., Figure 2E). Cb-fem-2/+ XO animals show late-onset germline feminization similar to that seen with Cb-fem-2(RNAi) (Stothard et al., 2002) (data not shown). As detailed in the Supplemental Data, Cb-fem-2(nm27) exhibits neither the temperature sensitivity nor maternal rescue of Cb-fem-2 mutants (Hodgkin, 1986).

The above-described experiments indicate that Cb-fem-2 is essential for male somatic sex determination, and that it is therefore not generally redundant with other PP2c phosphatases. An identical strategy allowed for the isolation of Cb-fem-3(nm63) XO homozygotes: 59% of XO cross progeny (n = 88) were also self-fertile hermaphrodites (Figure 2G) with small broods and somatic gonad defects similar to those in the Cb-fem-2(nm27)
XO mutants. In *C. elegans*, XO fem mutants are self-sterile because the fem genes act downstream of the tra-2-mediated initiation of spermatogenesis (Goodwin and Ellis, 2002). In contrast, *C. briggsae* XO fem-2 and fem-3 animals execute the sperm-then-oocytes pattern consistent with their transformed somas. This suggests that Cb-fem-2 and Cb-fem-3 act to repress the switch to oogenesis in males, even though they are not required for sperm production in hermaphrodites.

### Germline Expression of fem Genes Is Conserved

Despite the differences in the germline phenotype of fem-2 and fem-3 mutants between *C. elegans* and *C. briggsae*, we find that adult hermaphrodites of the two species both produce germline fem-2 mRNA at comparable levels, as judged by in situ hybridization (Figure 3). This expression is first detectable as oocytes exit pachytene and begin gametogenesis, and it is strongest in mature diakinesis oocytes. This is consistent with the maternal rescue of sexual transformation and the minor requirement for Ce-fem-2 in late embryogenesis (Piekny et al., 2000). Maternal Cb-fem-2 expression may have a similar embryonic function, but we have not observed embryogenesis defects like those reported for Ce-fem-2 mutants.

### Cb-fem-3 mRNA Is Expressed in the Germ Line in a Graded, Proximal-Biased Manner Similar to that seen in Cb-fem-2 (Figures 3F and 3G), While Cb-fem-2 Staining Is Strong and Consistently Cytoplasmic in Mature Oocytes, Cb-fem-3 Staining Is Often Perinuclear. Cb-fem-3 Staining Is Also Observed in Somatic Tissues, Including the Gut. Germline Expression of Ce-fem-3 Is Largely Similar (A. Puoti, personal communication). Thus, as with fem-2, differences in fem-3 function cannot be explained by changes in germline transcription. However, Northern analysis of *C. elegans* mutants lacking germ-lines (Rosenquist and Kimble, 1988) indicates that somatic expression of Ce-fem-3 is much lower than for Cb-fem-3.

### Isolation of Cb-fem Genes as Cb-tra-2(ts) Suppressors

Have other *C. briggsae* genes taken on a fem-like role of promoting spermatogenesis in the XX germ line? In *C. elegans*, the first fem-3 mutations were isolated as genetic suppressors of tra-3 and tra-2 (Hodgkin, 1986). We undertook similar screens for mutants that suppress the somatic masculinization of two different temperature-sensitive alleles of Cb-tra-2 (C.E.D.C., M. Layton, J.S., E.S.H., and D.P., unpublished data; Figure 4). A total of 75 F2 suppressors were isolated from 760,000 suppressors of Cb-tra-2(ts) Muta-

### Figure 4. Suppressors of Cb-tra-2(ts) Mutations Are Self-Fertile

(A) At 15°C, XX Cb-tra-2(tnm9ts) animals are self-fertile hermaphrodites with a whip-like tail (arrowhead) and numerous embryos (e). (B) At 25°, they are transformed into Tra pseudomales with an imperfect male tail (arrowhead) and abundant sperm (s). (C) All suppressor mutations that restore a female soma to two such Cb-tra-2(ts) alleles are self-fertile. The different recovery rates for suppressors of the ed23ts and nm9ts alleles are due to methodological differences in the two screens (see Experimental Procedures). (D) Comparison of *C. elegans* and *C. briggsae* XX germ line sex determination. Conserved components of the core sex determination pathway, which act in both males and hermaphrodites, are shown in black. In *C. elegans* (top), hermaphrodite spermatogenesis is initiated through the repression of tra-2 by the factors shown in green, and it is terminated by repression of fem-3 by the factors shown in red. A tra-1/fog-3-independent role for the Ce-fem genes and a possible sperm-promoting role for tra-1 have been omitted for simplicity (Chen and Ellis, 2000; Hodgkin, 1987; Schedl et al., 1989). In *C. briggsae* (bottom), unknown factors acting downstream of the Cb-fem genes start and stop sperm production. The masculinizing activity that starts spermatogenesis is suggested here to repress Cb-tra-1 and/or stimulate Cb-fog-1/3, while the sperm-oocyte switch would depend upon either stimulating Cb-tra-1 and/or repressing Cb-fog-1/3. *C. briggsae* orthologs of xol-1 (Lu et al., 2003), tra-3, and the sdc genes (not shown) exist, but they have not been functionally characterized. Evidence for conservation of the remaining genes has recently been reviewed (Haag, 2005).
haploid genomes screened. At least five failed to complement the Cb-fem-2(nm27) mutation, and the remaining alleles fell into at least two more complementation groups (C.E.d.C. and D.P., unpublished data). While the alleles varied in their ability to suppress the somatic and germline masculinization of the Cb-tra-2 alleles, all Cb-tra-2; sup double mutant strains can be maintained as self-fertile XX hermaphrodites at the restrictive temperature. No Cb-tra-2; sup XX females were isolated, although the large number of genomes screened and the isolation of multiple alleles of the suppressors suggest that the screen was close to saturation. Importantly, XX Cb-tra-2; Cb-fem-2 double mutants are self-fertile hermaphrodites (data not shown). This demonstrates that, despite the lack of an XX phenotype on its own, Cb-fem-2 acts downstream of tra-2 in both the soma and germline in C. briggsae, as in C. elegans.

Conclusions and Prospects
The hermaphrodites of C. elegans and C. briggsae both produce 200–300 sperm around the L4-adult molt and then switch to oogenesis. Our results indicate that this outwardly similar phenotype is produced by intervention at different levels of the core sex determination pathway (Figure 4D). In C. elegans, the control of hermaphrodite spermatogenesis targets tra-2 (”sperm on”) and fem-3 (”sperm off”), and, consequently, the only loss-of-function mutations that convert XO males into hermaphrodites (the Her phenotype) affect her-1, which lies immediately upstream of tra-2. In contrast, we find that XO mutants lacking at least two of the Cb-fem genes are Her, and that no readily mutable C. briggsae fem-like gene (as defined by being downstream of Cb-tra-2) is required for normal XX spermatogenesis. The control of hermaphrodite spermatogenesis therefore operates downstream of the fem genes, implicating Cb-tra-1 and/or its targets. One likely Cb-tra-1 target, Cb-fog-3, is expressed during and is required for both male and hermaphrodite spermatogenesis (Chen et al., 2001). Since both its promoter and coding sequence can support the rescue of Ce-fog-3 germline feminization, this control may target Cb-tra-1.

While it is formally possible that the last common ancestor of C. elegans and C. briggsae was hermaphroditic, our findings are most consistent with convergent evolution of selfing in Caenorhabditis, and they indicate the existence of flexibility in how it is achieved (True and Haag, 2001). Other studies of convergent evolution have often found the striking reuse of key genes (Colosimo et al., 2004; Cresko et al., 2004; Eizirik et al., 2003; Gompel and Carroll, 2003; Protas et al., 2006; Shapiro et al., 2004; Sucena et al., 2003; Yamamoto et al., 2004). However, a few cases of genetically distinct convergence have also been found (e.g., Hoekstra and Nachman, 2003; Wittkopp et al., 2003). Nematode hermaphroditism represents a new example of the latter, but it is distinguished by its basis in sex determination, its germline specificity, and the posttranscriptional regulation that accompanies them.

Why might hermaphroditism evolve via distinct genetic changes? One possibility is that there are many routes to self-fertility; thus, independent use of any particular one is unlikely. Another is that differences in initial conditions greatly influence subsequent events. The closest known relative of C. briggsae is the gonochoristic (male/female) C. remanei (Cho et al., 2004; Kiontke et al., 2004). Germline sex determination in the gonochoristic C. remanei/C. briggsae common ancestor may have already diverged somewhat from that of the C. elegans lineage, such that evolution of hermaphroditism in C. briggsae necessarily required distinct modifications. These scenarios may be distinguished through further forward and reverse genetic characterization of C. briggsae and C. remanei sex determination, a task made much easier by the C. briggsae genomic sequence (Stein and others, 2003).

Experimental Procedures
Nematode Culture, Strains, and Genetics
C. briggsae strains were cultured by using standard C. elegans conditions (Wood, 1988), with the use of 2.2% agar plates to discourage burrowing. All mutants were derived from the wild isolate AF16. The Cb-fem-2 deletion allele nm27 and the Cb-fem-3 deletion allele nm63 were isolated from different libraries of EMS-mutagenized worms (1.1 million haploid genomes each) by using standard C. elegans methods (Edgley et al., 2002) and without the “poison primer” modification (primer sequences are in Supplementary Data). Cb-dpy(nm4), Cb-tra-2(nm9ts), and Cb-tra-2(ed23ts) were isolated in conventional F2 screens for recessive mutants that will be described elsewhere. Evidence that nm9ts and ed23ts are alleles of Cb-tra-2 includes tight linkage to a Cb-tra-2 polymorphism, RNAi phenocopy (Haag et al., 2002; Kuwabara, 1996), missense mutations in the Cb-tra-2 open reading frame, and mutual noncomplementation. syls802, an X-integrated array consisting of a wild-type C. elegansdaf-4 genomic fragment and the myo-2::GFP reporter construct, was kindly provided by T. Inouye and P. Sternberg (CalTech). All mut- tant alleles were outcrossed with wild-type AF16 males (and verified by PCR where necessary) at least four times prior to use in building strains.

Cb-dpy(nm4); II; Cb-fem-2(nm27)+ III hermaphrodites were the progeny of a cross between Cb-dpy(nm4); Cb-fem-2(nm27) (strain CP48) and AF16 males. CP48 was produced as follows: AF16 males crossed with Cb-fem-2(nm27) III hermaphrodites produce Cb-fem-2(nm27)+ III males, which were then mated with dpy(nm4) II hermaphrodites. Many Dpy F2 were singed and PCR genotyped for Cb-fem-2 after they had laid most of their progeny. Cb-fem-2(nm27)+ III; syls802[myo-2::GFP] X males were the GFP+ male progeny of a cross between AF16 males and Cb-fem-2(nm27) III; syls802[myo-2::GFP] X hermaphrodites. These hermaphrodites were, in turn, produced from mating syls802[myo-2::GFP] X males (produced spontaneously by syls802[myo-2::GFP] X+ hermaphrodites) with Cb-fem-2(nm27) III hermaphrodites and PCR genotyping GFP+ F2 for Cb-fem-2. Similar methods were used to build the analogous Cb-fem-3(nm63) strains.

F2 suppressors of Cb-tra-2(nm9ts) and Cb-tra-2(ed23ts) were identified by plating the F1 progeny of EMS-mutagenized L4 hermaphrodite larvae at 15º (400 per 6 cm plate for ed23ts, 150 per 20 cm plate for nm9ts). F2 embryos were shifted to 25º and scored for rare mutants with normal female somas. Although the suppressors analyzed thus far are recessive, the collection may include dominant Cb-tra-1 alleles, similar to those found in C. elegans tra-3(sup) screens (Hodgkin, 1986). The ed23ts screen probably undercounted suppressors for two reasons: at the higher density, the youngest and sickly worms produce few progeny, and only a single F2 suppressor was retained per plate in both screens to ensure independence of mutations. A total of 74 of 75 mutants were verified as second-site mutations via outcrossing with AF16 and recovery of the Tra phenotype in the F2.

Single-Worm PCR
Single-worm PCR was carried out as described (Haag et al., 2002). For the assay of a single worm with two primer sets, PCR was first performed with the outer flanking primers, after which this outer reaction was diluted 1:250 for use in inner reactions with either the inner flanking or WT-only primers.
Phylogenetic Analysis

All C. elegans PP2C phosphatases, as predicted by Pfam profile searches, were obtained from Wormbase (www.wormbase.org). After splice variants were removed, the set was used to perform BLAST searches on the predicted C. briggsae proteome (CB25 release), and all hits with E values below 0.01 were scrutinized for PP2C domains. This quickly established the largest possible set of C. briggsae family members. PP2C domains were then excised and aligned with Clustal W as implemented in Vector NTI (Invitrogen), and the alignment was validated by recovery of the conserved sequence blocks described by Pilgrim et al. (2006). Neighbor-joining and maximum parsimony analyses were performed with PAUP 4b10 (Swofford, 2002).

In Situ Hybridization

Single-stranded digoxigenin-labeled DNA probes were produced as described (Patel et al., 1992) from a 1.1 kb Cb-fem-2 cDNA clone covering the phosphatase domain, a complete 2.0 kb Cb-fem-2 cDNA plasmid, pDP1DH20 (D. Hansen and D.P., unpublished data), and the complete Cb-fem-3 coding sequence cDNA in pJK769. Dissection of gonads, fixation, hybridization, and detection were generally performed as previously described (Jones et al., 1996), although the concentration of proteinase K was routinely increased to 100 μg/ml, used for the experiments in Figures 3C and 3D, completely extruded gonads were fragmented. Staining of nuclei was with 0.5 μg/ml 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI).

Supplemental Data

Supplemental Data including Table S1 and primer sequences are available at http://www.developmentalcell.com/cgi/content/full/10/4/531/DC1/.

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