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A sensitized genetic background reveals evolution near the terminus of the *Caenorhabditis* germline sex determination pathway

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Abstract

Caenorhabditis elegans and *C. briggsae* are both self-fertile hermaphroditic nematodes that evolved independently from male/female ancestors. In *C. elegans*, FEM-1, FEM-2, and FEM-3 specify male fates by promoting proteolysis of the male-repressing transcription factor, TRA-1. Phenotypes of *tra-1* and *fem* mutants are consistent with this simple linear model in the soma, but not in the germ line. While both XX and XO *tra-1(lf)* mutants have functional male somas, they produce both sperm and oocytes. Further, all three *tra-1; fem* double mutants retain the expected male soma, but make only oocytes (the germline *fem* phenotype). Thus, a poorly characterized *tra-1* activity is important for sustained male spermatogenesis, and the *fem* genes affect germline sexual fate independently of their role in regulating TRA-1. *C. briggsae tra-1* mutants are phenotypically identical to their *C. elegans* counterparts, while the *fem* mutants differ in the germline: XX and XO *C. elegans fem* mutants are true females, but in *C. briggsae* they are self-fertile hermaphrodites. To further explore how *C. briggsae* hermaphrodites regulate germline sex, we analyzed *Cb-tra-1/Cb-fem* interactions. *Cb-tra-1* is fully epistatic to *Cb-fem-2* in the germ line, unlike the orthologous *C. elegans* combination. In contrast, *Cb-fem-3* shifts the *Cb-tra-1(lf)* germline phenotype to that of a nearly normal hermaphrodite in the context of a male somatic gonad. This suggests that *Cb-fem-3* is epistatic to *Cb-tra-1(lf)* (as in *C. elegans*), and that the normal control of *C. briggsae* XX spermatogenesis targets *Cb-tra-1*-independent factors downstream of *Cb-fem-3*. The effect of *Cb-fem-3(lf)* on *Cb-tra-1(lf)* is not mediated by change in the expression of *Cb-fog-3*, a likely direct germline target of *Cb-tra-1*. As *Cb-fem-2* and *Cb-fem-3* have identical single mutant phenotypes, *Cb-tra-1* provides a sensitized background that reveals differences in how they promote male germline development. These results represent another way in which *C. briggsae* germline sex determination is incongruent with that of the outwardly similar *C. elegans*.

Introduction

The nematode genus *Caenorhabditis* is emerging as a powerful system for exploring the intersection of development, genetics, and evolution (Haag and Pilgrim 2005; Kammenga et al. 2008). The most striking variable among *Caenorhabditis* is reproductive mode. While *C. elegans* and *C. briggsae* produce males and hermaphrodites, the remaining described species produce males and true females. Hermaphrodites are anatomically female, but produce a limited amount of amoeboid sperm (200–300) during the final larval stage (in *C. elegans*) or in early adulthood (*C. briggsae*) before switching to oocyte production for their remaining life span. Phylogenetic studies suggest hermaphroditism has evolved from male/female

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species numerous times in rhabditid nematodes (Cho et al. 2004; Kiontke et al. 2004; Kiontke and Sudhaus 2006), and recent developmental genetic comparisons have further supported the convergence scenario for *C. elegans* and *C. briggsae* (Hill et al. 2006; Nayak et al. 2005).

The primary determinant of sex in *C. elegans* is the X:A ratio (Madl and Herman 1979; Nigon 1951). A high ratio (XX) represses *xol-1* transcription, resulting in hermaphrodite development. A low ratio (XO) allows high *xol-1* expression, resulting in male development (Rhind et al. 1995). *xol-1* activity controls sexual fate via a negative regulatory pathway (Figure 1, top panel), ultimately resulting in the activation (in hermaphrodites) or repression (in males) of the terminal global transcriptional regulator, TRA-1. At the center of this pathway is a signal transduction cascade consisting of HER-1 (a secreted signaling protein; Perry et al. 1993), TRA-2 (a transmembrane receptor for HER-1; Hamaoka et al. 2004; Kuwabara and Kimble 1995), and TRA-3 (a protease required for TRA-2 function; Barnes and Hodgkin 1996; Sokol and Kuwabara 2000), as well as the three cytoplasmic FEM proteins (Zarkower 2006). The three *tra* genes promote female fate; *her-1* and the *fem* genes promote male fate. The above pathway is used for both somatic and germline sex determination. However, in hermaphrodites additional genes are required for the modulation of activity of core pathway genes to produce both sperm and oocytes (Figure 1, middle panel). In *C. elegans*, *tra-2* and *fem-3* have been most strongly implicated as the targets of this modulation (Kimble and Crittenden 2007). Consistent with this, mutations in any of the three *fem* genes converts XX hermaphrodites into true females (Hodgkin 1986).

TRA-1 is a Gli/Ci family zinc-finger transcription factor (Zarkower and Hodgkin 1992). The *tra-1* gene encodes two transcripts, one producing the protein TRA-1A (1109 amino acids) and another the smaller TRA-1B (287 amino acids). TRA-1A contains five C2H2 zinc fingers and binds DNA, while TRA-1B has only the first two zinc fingers, does not bind DNA, and has no known function (Zarkower and Hodgkin 1992; Zarkower and Hodgkin 1993). Most TRA-1A is proteolytically processed in hermaphrodites to a truncated form known as TRA-1¹⁰⁰, which accumulates to high levels and is presumably responsible for *tra-1*'s male-repressing activity (Schwarzstein and Spence 2006). Starostina et al. (2007) have shown that TRA-1¹⁰⁰ levels are directly regulated by the FEM proteins through their participation in a CUL-2-based ubiquitin ligase complex. FEM-1 (an ankyrin-repeat protein; Spence et al. 1990) is the substrate-recognition subunit, and FEM-2 (a protein phosphatase; Chin-Sang and Spence 1996; Pilgrim et al. 1995) and FEM-3 (a novel, rapidly evolving protein; Haag et al. 2002; Rosenquist and Kimble 1988) act as cofactors. The activity of the FEM complex is inhibited by direct interaction between FEM-3 and cytoplasmic C-terminus of TRA-2 (Mehra et al. 1999), and this inhibition is in turn thought to be abrogated in males by binding of HER-1 to TRA-2 (Hamaoka et al. 2004; Hunter and Wood 1992).

TRA-1 is known to negatively regulate four male-specific genes, *egl-1*, *mab-3*, *dmd-3*, and *fog-3* (Chen and Ellis 2000; Conradt and Horvitz 1999; Mason et al. 2008; Yi et al. 2000), suggesting repression of male fates is crucial to the control of sex determination. Consistent with this, strong loss-of-function alleles of *tra-1* convert XX hermaphrodites into well formed males that are capable of mating and siring progeny (Hodgkin 1987; Hodgkin and Brenner 1977). However, both XX and XO *tra-1* males cannot consistently sustain spermatogenesis, and often make oocytes of variable quality in a male somatic gonad (Hodgkin 1987; Schedl et al. 1989). This suggests that a poorly characterized activity of *tra-1* is necessary for the promotion of male fates in the germ line. In addition, *fem*; *tra-1* double mutants display a male soma (like *tra-1* alone), but a completely feminized germline (the phenotype of the *fem* genes; Hodgkin 1986). This implies that a *tra-1*-independent role exists for the *fem* genes in the control of germline sex (Ellis and Schedl 2007).

C. briggsae tra-1 mutants have both the complete XX somatic masculinization and incomplete XO germline feminization of their *C. elegans* counterparts (Kelleher et al. 2008), indicating that the potentially complex regulation of *tra-1* is conserved. In contrast, XX *C. briggsae fem* mutants are normal hermaphrodites, and XO *Cb-fem-2* and *Cb-fem-3* mutants are completely sex-reversed to hermaphrodites, rather than to females as in *C. elegans* (Hill et al. 2006). The modulations in germline sex determination used by *C. briggsae* hermaphrodites are thus distinct from those of *C. elegans*, and are likely to act downstream of the *Cb-fem* genes (Figure 1, bottom panel). One goal in this study was to assess whether this different regulation of hermaphrodite spermatogenesis seen in *C. briggsae* would be reflected in the genetic interaction between *Cb-tra-1* and *Cb-fems*. We also sought further evidence for the existence of a *tra-1*-independent role of the *fem* genes that may be a general feature in *Caenorhabditis*.

Cb-tra-1(nm2) was the allele employed to generate *Cb-tra-1; Cb-fem* double mutants for this analysis. This is a strong loss-of-function allele caused by a nonsense mutation at codon 512. This eliminates approximately 50% of the full-length TRA-1A protein, but retains the DNA-binding zinc finger domain. The mutants exhibit robust mating behavior, and young XX *nm2* males can sire cross-progeny, although at much lower levels than wild-type males (Kelleher et al. 2008). The comparable *C. elegans* TRA-1 mutation, *e1781*, has a nonsense mutation in the same region (Zarkower and Hodgkin 1992) and also has a fully transformed soma with an intersexual germ line (Hodgkin 1987). However, a true null allele of *Ce-tra-1*, *e1099*, has additional defects in somatic gonad development (Hodgkin 1987; Mathies et al. 2004), suggesting that *tra-1(e1781)* and *Cb-tra-1(nm2)* may both retain residual function in gonad development. We also present some further analyses of germline phenotypes of the *Cb-tra-1(nm30)* allele, a mutation in the 5'splice site of Intron 2 characterized by an incomplete male tail and well formed oocytes within a male somatic gonad (Kelleher et al. 2008).

An obvious potential complication to analysis of *Cb-tra-1; Cb-fem* double mutants (and in contrast to *C. elegans*) is the lack of distinct phenotypes between the single mutants. The single *Cb-fem* mutants have a normal hermaphroditic germline, producing both sperm and oocytes in a double-armed gonad (Hill et al. 2006). *Cb-tra-1 (nm2)* single mutants have an intersexual germline that also produces both sperm and oocytes, albeit in the context of a male somatic gonad (Kelleher et al. 2008). It was therefore crucial to define germ line phenotypes quantitatively. Because the three *fem* mutants from a given species produce identical germline phenotypes, we predicted that any effects *Cb-fem-2* and *Cb-fem-3* had on the *Cb-tra-1* phenotype would be the same. Surprisingly, they are not.

Methods

Mutant strains

Mutant alleles used in this study include *C. elegans tra-1(e1099) III* and *tra-1(e1781) III*, and *C. briggsae Cb-tra-1(nm2) III*, *Cb-tra-1(nm30) III*, *Cb-let(nm28) III*, *Cb-fem-2(nm27) III*, and *Cb-fem-3(nm63) IV*. All genetic experiments were conducted at 20°C using standard *C. elegans* media (Wood 1988), with the exception of increasing the agar for NGM plates to 2.2% to discourage burrowing.

To produce *Cb-fem; Cb-tra-1 (nm2)* double mutants, the first step was to cross *Cb-tra-1(nm2)/Cb-let(nm28)* hermaphrodites with AF16 (wild-type) hermaphrodites. Males from this cross, half of which carry the *nm2* allele, were mated to homozygous *Cb-fem-2* or *Cb-fem-3* mothers. Hermaphrodite progeny were singled as virgins, allowed to lay most of their progeny, and then genotyped to confirm *Cb-fem* heterozygosity as described in Hill et al. (2006). Mothers that were also *Cb-tra-1(nm2)/+* produced approximately one-quarter

Tra pseudomale progeny and were retained. Hermaphrodite offspring from plates founded by double heterozygous mothers were again singled, allowed to lay progeny, and then genotyped by PCR to find animals that were both homozygous for the *Cb-fem* deletion allele and segregating Tra animals *Cb-tra-1(nm2)/+*. For *Cb-fem-2* this required a recombination event, but as it is roughly 30 cM from *Cb-tra-1* this was not an impediment. The resulting strains, *Cb-fem-2(nm27)*; *Cb-tra-1(nm2)/+* and *Cb-fem-3(nm63)*; *Cb-tra-1(nm2)/+*, were maintained during the experiments by occasional sib selection.

Phenotypic scoring

For differential interference contrast (DIC) microscopy, worms were immobilized with 50mM sodium azide in M9 salts (Wood 1988) and mounted on 2% agar pads. For Hoechst 33258 staining, worms were rinsed three times with M9 salts with centrifugation for three minutes at 3400 rpm between each rinse. 400 μ l of -20°C methanol was added to the worms, which were then incubated at -20°C for a minimum of 10 min. After 3 more rinses in M9, worms were incubated for 45 min. at room temperature in the dark with 200 μ l of 7.5 μM Hoechst 33258 in M9. Following 3 further rinses in M9 salts, 30 μ l of worm suspension was mixed with 10 μ l of vectashield (Vector Laboratories) and mounted onto 3-4 agar pads (2%) for fluorescence microscopy. All images were captured with a Zeiss Axiocam digital camera and Open Lab software (Improvision). Phenotypic categories were based on those used for *C. elegans tra-1* by Schedl et al (1989).

Quantitative RT-PCR

Worms of the appropriate genotype and within their first day of adulthood were picked in groups of 5 into 10 μ l drops of nuclease-free water in the caps of 0.6 ml microcentrifuge tubes and then spun into the bottom of the tubes after gently capping them. This was repeated twice for hermaphrodite worms or thrice for males and Tra animals (i.e. 10 or 15 animals per tube). Worms were then mixed with 150 μ l Tri Reagent (Molecular Research Center) and frozen at -80°C for at least overnight. After thawing, the worms were lysed with four cycles of grinding and pelleting with disposable plastic pestles. Total RNA was isolated from lysates according to manufacturer's instructions, with the addition of 2 μ l of Polyacryl carrier (Molecular Research Center). RNA pellets were resuspended in the water and oligo-dT primer components of the Transcriptor cDNA synthesis kit (Roche) and reverse-transcribed according to manufacturer's instructions.

2 μ l of each cDNA preparation was used as template for quantitative PCR using the Light Cycler 480 SYBR Green reagent (Roche) and the Roche Light Cycler 480 machine. A 232 nt *Cb-fog-3* cDNA fragment was amplified with the oligonucleotide primers EH36 (5' GGATGTTGGCTTGAACGTGAAC 3') and EH39 (5' ATACTGATTACACTATCAGCC 3'). EH36 is wholly contained within Exon 7, while EH39 anneals to the Exon 4/5 junction. For the actin internal standard, a 222 nt actin fragment was amplified using the primers EH37 (5' TACCTCATGAAGATCCTCACCG 3') and EH38 (5' CATACCCAAGAAGGATGGCTGG 3'). Because little is known about sex-specific regulation of actin family members in *C. briggsae*, EH37 and EH38 were designed using an alignment of all *C. briggsae* actin gene predictions in WormBase (<http://www.wormbase.org>) so as to anneal to essentially all family members without amplicon length variation (ESH, unpublished data). Threshold cycle number was determined using the second derivative maximum method. Amplification efficiencies for actin and *Cb-fog-3* were determined directly from multiple amplification curves (Ramakers et al. 2003) to be 0.57-0.58. Threshold cycle numbers and efficiencies were then used to produce normalized linear expression values (Livak and Schmittgen 2001).

Results

Oogenesis in *Cb-tra-1(nm2)* mutants: variable onset and endomitosis

To establish the range of phenotypes, we first scored over 200 *Cb-tra-1(nm2)* mutant animals by DIC microscopy. As noted by Kelleher et al. (2008), most XX pseudomales produced only sperm into the first day of adulthood, while older animals switched to an abnormal oogenesis. However, the age at which oogenesis begins varies from as early as the L4 (last larval) stage (Figure 2A) to not at all (not shown). In addition to oocyte production, some mutants exhibited poorly organized germ lines, often consisting of a granular, apparently acellular cytoplasm. This material was found most often between regions of sperm and oocytes (Figure 2B), although in some older adults it extended throughout most of the proximal gonad arm.

Staining of mutants with Hoechst 33258 revealed that both well-defined oocytes and the apparently acellular granular material often harbored swollen, endoduplicated nuclei (Figure 2C, D). This endomitotic oocyte (Emo) phenotype has been shown in *C. elegans* to be due to inappropriate activation of oocyte mitotic cell cycle in the absence of ovulation (Greenstein 2005). A second *Cb-tra-1* allele, *nm30*, is also frequently Emo (Table 1). While oocytes have long been recognized in the gonads of *C. elegans tra-1* mutants (Hodgkin 1987; Schedl et al. 1989), they have not been reported to be Emo. We stained the null allele of *C. elegans tra-1*, *e1099*, as well an *nm2*-like allele, *e1781*, and found that in both cases they can also be Emo (Figure 2E, F; Table 1). *e1781* animals are frequently Emo, but *e1099* mutants only rarely so, presumably because the latter only rarely produces oocytes to begin with (Hodgkin 1987; Schedl et al. 1989).

Double mutant analysis of *Cb-tra-1* and the *Cb-fems*

A major goal of this study was to determine whether loss of *Cb-fem-2* or *Cb-fem-3* could feminize (or otherwise modify) the variably intersexual germline phenotype of *Cb-tra-1*. Due to the similarities between *Cb-tra-1* and *Cb-fem-2/3* mutants, we scored many animals to produce phenotypic distributions that could potentially reveal quantitative shifts. Because of its ability to reveal small numbers of sperm and endomitotic oocytes that are difficult to see by transmitted light microscopy, we used Hoechst 33258 staining for this purpose. That this method is generally accurate is supported by the similarity of our analysis of two *C. elegans tra-1* alleles to that of Schedl et al. (1989; Figure 3).

By both qualitative and quantitative criteria, *Cb-tra-1(nm2); Cb-fem-2(nm27)* mutants were indistinguishable from *Cb-tra-1(nm2)* alone. They manifested both the temporally variable switch to oogenesis at a similar frequency (Figure 3) and produced endomitotic oocytes (Figure 4A, B). In contrast, *Cb-tra-1(nm2); Cb-fem-3(nm63)* mutants differed from both *Cb-tra-1(nm2)* and *Cb-tra-1(nm2); Cb-fem-2(nm27)* both qualitatively and quantitatively. First, oocytes were never made by L4 *Cb-tra-1(nm2); Cb-fem-3(nm63)* mutants (not shown), yet appeared in over 90% of young adults (Figure 2). In contrast, over half of both *Cb-tra-1(nm2)* and *Cb-tra-1(nm2); Cb-fem-2(nm27)* young adults made only sperm (Figure 3). When oocytes were made in *Cb-tra-1(nm2); Cb-fem-3(nm63)* animals, they were noticeably more robust than in *Cb-tra-1(nm2)* (Figure 4C), and frequently became endomitotic (Figure 4E, F).

In addition to the differences in gamete development described above, 8% of *Cb-tra-1(nm2); Cb-fem-3(nm63)* animals scored with DIC microscopy had unexpected somatic gonad abnormalities, in many cases including the presence of both a robust, testis-like posterior arm and a smaller anterior arm (Figure 4D). These abnormalities were less apparent in Hoechst 33258-stained specimens (Figure 3), but were not seen in *Cb-*

tra-1(nm2) or *Cb-tra-1(nm2)*; *Cb-fem-2(nm27)* animals even when scored by DIC microscopy (data not shown).

Transcript levels of *fog-3* do not correlate with phenotypic shifts

The only known direct target of TRA-1 in the germ line in *C. elegans* is *fog-3* (Chen and Ellis 2000). As *fog-3* is conserved in *C. briggsae* (Chen et al. 2001), we sought to test whether the feminization of the *Cb-tra-1(nm2)* germ line by loss of *Cb-fem-3* activity might be due to reduced *fog-3* expression. As obtaining large numbers of double homozygotes is not feasible, we turned to quantitative PCR of cDNA prepared from small pools of animals. As shown in Figure 5, wild-type males, *Cb-tra-1(nm2)* pseudomales, and *Cb-tra-1(nm2)*; *Cb-fem-2/3* double mutants produce large amounts of *fog-3* transcript, while wild-type hermaphrodites and *Cb-fem-2/3* single mutants produce very little. Expression levels between these two classes are significantly different from each other (e.g. 2-tailed T-test for difference between *Cb-tra-1(nm2)*; *Cb-fem-3(nm63)* and *Cb-fem-3(nm63)*, $p=0.038$), but not within them. We conclude that *Cb-fog-3* transcript levels in double mutants are not distinguishable from each other nor from *Cb-tra-1(nm2)* alone.

Discussion

tra-1(lf) oocytes are often endomitotic

C. elegans hermaphrodites lacking function of factors involved in muscle physiology, cell cycle regulation, and signaling have been reported to be Emo (Aono et al. 2004; Hajnal and Berset 2002; Inoue et al. 2006; Iwasaki et al. 1996; Kostic et al. 2003; Kuwabara et al. 2000; Ono and Ono 2004; Wissmann et al. 1999). Ablations of somatic gonad cells that block ovulation also produce endomitotic oocytes (McCarter et al. 1997). Although *tra-1* oocytes have not previously been described as endomitotic, the data presented here clearly show that *tra-1(lf)* germ cells from two different species frequently are.

Our observations are consistent with mutant animals being increasingly likely to be Emo as they age. Endomitotic cells are also typically proximal (“downstream”) to oocytes that are in diakinesis of meiosis I, the stage at which normal oocytes arrest prior to fertilization. It is thus likely that the Tra Emo phenotype results from activation of the cell cycle in essentially mature oocytes in the absence of fertilization and ovulation, just as it does in Emo hermaphrodites. Oocyte activation in *C. elegans* hermaphrodites is normally mediated by the sperm-derived MSP signal (Miller et al. 2001; Miller et al. 2003). As *tra-1(lf)* mutant males almost always produce at least a few sperm, these sperm may signal to oocytes much as they would in a hermaphrodite gonad. Activation is presumably not accompanied by fertilization, however, as embryos with eggshells are never seen. As *tra-1(lf)* sperm are capable of cross-fertilization with hermaphrodites, this may be due to a general inability of fertilization to occur in a male somatic gonad, perhaps more specifically to failure of sperm that are otherwise competent to undergo activation inside the testis. This activation is accomplished in males by passage through the vas deferens during ejaculation, and in *C. elegans* hermaphrodites by contact with the spermatheca (L’Hernault 1997; Ward and Carrel 1979). Neither of these mechanisms would be available to *tra-1(lf)* spermatids with respect to their ability to fertilize adjacent oocytes in the same gonad.

tra-1 background separates *Cb-fem-2* and *Cb-fem-3* phenotypes

In our previous analyses of *Cb-fem-2* and *Cb-fem-3* mutants, we saw no differences in their single mutant germline phenotypes (Hill et al. 2006). Given the common participation of their *C. elegans* orthologs in a complex that acts to regulate TRA-1 proteolysis (Starostina et al. 2007; Tan et al. 2001), this was not surprising. However, all three *C. elegans fem* mutations are epistatic to *tra-1* in the germ line, indicating that, at least in that species, there

must be some additional male-promoting role for the *fem* genes other than their regulation of TRA-1 levels. We therefore sought to determine whether this was a general feature of *Caenorhabditis* sex determination by examining the equivalent mutants in *C. briggsae*. The *Cb-fem-3(nm63)* mutation eliminates the L4 oogenesis seen in *Cb-tra-1(nm2)* mutants, produces near-complete production of sperm and eggs early in adulthood, and qualitatively enhances oocyte development relative to *tra-1(nm2)*. This represents the closest thing to a well-organized hermaphrodite germ line as a Tra male could possibly make. We therefore suggest that *Cb-fem-3* is epistatic to *Cb-tra-1(nm2)* in the germ line, similar to what is observed in *C. elegans* (but with a different phenotype). Whether this is due to direct effects of the loss of *Cb-fem-3* in germ cell nuclei, or indirect effects mediated by the surrounding somatic gonad is unclear. That most *Cb-fem-3*; *Cb-tra-1* animals have normal male gonads suggests *Cb-fem-3* epistasis is a germ cell property, but the low level of two-armed gonads seen is consistent with somatic influences.

Surprisingly, there is no indication that *Cb-fem-2(nm27)* has the same “hermaphroditizing” effect on *Cb-tra-1(nm2)* observed for *Cb-fem-3(nm63)*. By both qualitative and quantitative criteria, there are no differences between the single and double mutants. This represents a departure from the uniform behavior of the *fem* genes in both single and double mutant analyses in *C. elegans*, and from single mutant phenotypes in *C. briggsae*. There are two potential reasons for the difference. First, the *nm2* allele is not likely to represent a complete functional null. It may therefore be that the two *Cb-fem* mutations have different effects on residual TRA-1 product, such that their loss has different consequences. Alternatively, given the essentially perfect somatic masculinization seen in *Cb-tra-1(nm2)*, it is possible that any residual TRA-1 product it produces is insufficient to impact sex determination (though it may effect somatic gonad development; Kelleher et al. 2008; Mathies et al. 2004). In this scenario, the feminization seen when *Cb-tra-1(nm2)* is combined with *Cb-fem-3(nm63)* must result from a *tra-1*-independent role of *Cb-fem-3* in promoting male germline fates. If we interpret the low level of two-armed gonads seen in *Cb-tra-1(nm2)*; *Cb-fem-3(nm63)* as feminization, this effect extends (albeit weakly) to the soma as well.

The quantitative RT-PCR results shown in Figure 4 suggest that the putative *Cb-tra-1*-independent promotion of sperm fate by *Cb-fem-3* does not work through transcriptional regulation of *Cb-fog-3*, the ortholog of the sole known germline target of *C. elegans tra-1*. Chen and Ellis (2000) examined the effects of *tra-1* and *fem* mutations on *fog-3* transcript levels in *C. elegans*. They found that XX *tra-1* mutants have elevated *fog-3* mRNA levels, *fem* mutants have reduced levels, and *tra-1*; *fem* double mutants have levels of *fog-3* mRNA comparable to (or slightly higher than) wild-type hermaphrodites, even though no sperm are made. We observed no downshift of *Cb-fog-3* mRNA levels when *Cb-fem-3* is combined with *Cb-tra-1*, even though strong feminization is observed. It thus appears that in both species cells that are fated to be oocytes can still express *fog-3* if they lack *fem-3*.

Whether the phenotypes of the double mutants analyzed here differ because of differential regulation of residual *Cb-tra-1* function in *nm2* mutants or to a *tra-1*-independent role for *fem-3* alone, another mystery is what molecular mechanism would enable them to have distinct phenotypes. One possibility relates to intrinsic conditional expression of phenotype and maternal effects. In *C. elegans*, null alleles of the three *fem* genes manifest distinct degrees of temperature sensitivity and maternal rescue. Full expression of feminization by *fem-2* mutants requires that the homozygous mutant both lack maternal product and be grown at elevated temperature, while *fem-3* is zygotic and largely temperature-independent (Hodgkin 1986). Differences in their phenotype could, in principle, be due to different levels of maternal rescue or temperature dependence. However, as we analyzed stocks that had been homozygous for both *Cb-fem* mutations for multiple generation prior to analysis, and since earlier work found no evidence of maternal rescue or temperature sensitivity (Hill et

al. 2006), this is not likely. A more likely possibility is that *Cb-fem-3* has a biochemical activity that does not rely upon the presence of the other *Cb-fem* genes, while *Cb-fem-2* does not. This putative activity may nevertheless normally occur in the context of the FEM complex, but it also possible that it participates in one or more as-yet-uncharacterized alternative interactions.

That *Cb-fem-3* would have a particularly important role relative to other *Cb-fem* genes has some precedent in the *C. elegans* literature. For example, overexpression of *fem-3* is sufficient for masculinization of XX animals, while that of *fem-1* and *fem-2* are not (Lee and Portman 2007; Mehra et al. 1999). Also, Schedl et al. (1989) reported that *fem-3(gf)* masculinized the germline of *tra-1(lf)* mutants, which would again indicate that *fem-3* has a *tra-1*-independent effect on sexual fate. An important subject for future research will therefore be the discovery of this mechanism.

Significance for *C. briggsae* hermaphrodite development

Cb-tra-2; *Cb-fem* double mutants are self-fertile hermaphrodites (Hill et al. 2006), and thus, unlike in *C. elegans*, neither *Cb-tra-2* nor *Cb-fem-3* are required for the normal physiological control of hermaphrodite spermatogenesis. This was interpreted by Hill et al. (2006) as evidence that the control of XX spermatogenesis acts further downstream in the sex determination pathway (e.g. *Cb-tra-1*, *Cb-fog-3*, *Cb-fog-1*, and perhaps unknown factors). In this study, simultaneous loss of *Cb-fem-3* and *Cb-tra-1* also produced a temporally regulated bisexual gonad (although in a male soma). The absence of *Cb-fem-3* activity thus appears to be sufficient to trigger the hermaphrodite germline program, even in the absence (or near-absence) of *Cb-tra-1*. We also note that the ability of a well-formed male somatic gonad to house a vigorous hermaphrodite germ line in *C. briggsae* argues against the importance for soma-germ line interactions in determining sexual fate that has been reported in *C. elegans* (McCarter et al. 1997). Overall, the available data suggest that *tra-1*-independent mechanisms of sex determination are important in the germ line of both *C. elegans* and *C. briggsae*, and that in *C. briggsae* (but not *C. elegans*) these mechanisms were the locus of regulatory changes that allowed the evolution of hermaphroditism.

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Figure 1.

Comparison of the somatic and germ line sex determination pathways of *C. elegans* and *C. briggsae*. The top panel shows the so-called core pathway, which controls somatic sexual dimorphism all known *Caenorhabditis* species. The middle panel depicts how the core pathway is modified in the *C. elegans* germ line, with germline-specific controls of the XX hermaphrodite shown in gray. Those targeting *tra-2* are required for XX spermatogenesis, while those targeting *fem-3* are required for the switch to oogenesis. While both *fog-1* and *fog-3* both lie downstream of *tra-1* by genetic criteria, only *fog-3* has been shown to be directly regulated by *tra-1*. The bottom panel shows what is known about *C. briggsae* germ line sex determination. The locus of the regulation of XX spermatogenesis proposed by Hill et al. (2006) is shown to target *tra-1* or points downstream, with the unknown factors promoting spermatogenesis depicted above the core pathway, and those promoting the switch to oogenesis below. The potential existence of a *Cb-tra-1*-independent sperm-promoting function for one or more *Cb-fem* genes (examined in this paper) is indicated with a dashed line.

Figure 2.

C. briggsae and *C. elegans tra-1(lf)* mutants produce endomitotic oocytes. A. *Cb-tra-1(nm2)* XX Tra males produce oocytes as early at the L4 larval stage. Inset shows the tail of the same animal, which is beginning tip retraction. g, gut; o, oocytes; sc, spermatocytes; sv, seminal vesicle; vd, vas deferens. B. Adult XX *Cb-tra-1(nm2)* animals often have a mixture of sperm (sp), oocytes, and acellular material (am). C, D. Matched pair of DIC and Hoechst 33258 stained *Cb-tra-1(nm2)* animals (D) shows that the acellular material in *Cb-tra-1(nm2)* gonads corresponds to endomitotic oocytes (eo) that have lost their plasma membrane integrity. In the upper animal, immature spermatocytes (sc) are seen in the Emo gonad, while the lower animal is producing only sperm. E, F. Matched pair of an XX *C. elegans tra-1(e1781)* Emo mutant. In all panels, anterior is to the right.

Figure 3.

Summary of somatic gonad and germline phenotypes. All animals were scored by Hoechst 33258 staining in the first day of adulthood. “Spermatogenesis then oogenesis” includes both Emo and non-Emo animals. “Abnormal germline” includes a change in the location and number of sperm, spermatocytes, oocytes and germline stem cells. “Abnormal gonad” includes animals with abnormally shaped gonad arms and double-armed gonads. The phenotypic distributions of *Cb-tra-1(nm2)* and *Cb-tra-1(nm2); Cb-fem-2(nm27)* are not significantly different (chi-square, omitting abnormal gonad category, $p=0.667$, $df=2$), while both of these are significantly different from *Cb-tra-1(nm2); Cb-fem-3(nm63)* (*Cb-tra-1(nm2)* vs. *Cb-tra-1(nm2); Cb-fem-3(nm63)*: $p<.001$, $df=2$. *Cb-tra-1(nm2); Cb-fem-2(nm27)* vs. *Cb-tra-1(nm2); Cb-fem-3(nm63)*: $p<.001$, $df=2$). The number of animals scored is indicated in the upper right corner of each panel.

Figure 4.

Cb-tra-1; Cb-fem double mutant phenotypes. A, B are a matched pair of a Hoechst 33258-stained *Cb-tra-1(nm2); Cb-fem-2(nm27)* Tra male under DIC (A) or epifluorescence (B). Both sperm (sp) and endomitotic oocytes (eo) can be observed. Asterisk indicates the male tail. C. DIC microscopy of a live adult *Cb-tra-1(nm2); Cb-fem-3(nm63)* mutant, showing well defined mature oocytes (mo), endomitotic oocytes proximal to them (eo), and mature sperm (sp) in the seminal vesicle. D. Two adult *Cb-tra-1(nm2); Cb-fem-3(nm63)* mutants under DIC. The more reflexed animal (above and to the right) has both a normal testis whose distal tip is indicated (dt), but also a smaller anterior gonad (ag) arm with oocytes in it. The less reflexed animal (below and to the left) has only a testis, although it is also making oocytes. E, F. Matched pair of Hoechst 33258-stained *Cb-tra-1(nm2); Cb-fem-3(nm63)* double mutant, showing the testis with its distal tip indicated in E and a large number of endomitotic oocytes.

Figure 5. Quantitative RT-PCR analysis of *Cb-fog-3* transcript levels in various mutants. *Cb-fog-3* expression was normalized to a multi-gene actin amplicon amplified from the same cDNA prep and run in the same microtiter plate. Error bars represent standard errors of the mean for 6 biological replicates. See text for more information on statistical tests.

Table 1

Frequency of endomitotic oocyte (Emo) phenotype in various *C. briggsae* and *C. elegans tra-1* XX pseudomales.

genotype	percent Emo	total scored
<i>Cb-tra-1(nm2)</i>	25.6	348
<i>Cb-tra-1(nm30)</i>	7.7	194
<i>Cb-tra-1(nm2); Cb-fem-2(nm27)</i>	24.1	228
<i>Cb-tra-1(nm2); Cb-fem-3(nm63)</i>	81.1	185
<i>Ce-tra-1(e1099)</i>	2.5	161
<i>Ce-tra-1(e1781)</i>	58.1	155