6-1-2024

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Associate editor: Greg Gibson

Abstract

Although evolution is driven by changes in how regulatory pathways control development, we know little about the molecular details underlying these transitions. The TRA-2 domain that mediates contact with TRA-1 is conserved in *Caenorhabditis*. By comparing the interaction of these proteins in two species, we identified a striking change in how sexual development is controlled. Identical mutations in this domain promote oogenesis in *Caenorhabditis elegans* but promote spermatogenesis in *Caenorhabditis briggsae*. Furthermore, the effects of these mutations involve the male-promoting gene *fem-3* in *C. elegans* but are independent of *fem-3* in *C. briggsae*. Finally, reciprocal mutations in these genes show that *C. briggsae* TRA-2 binds TRA-1 to prevent expression of spermatogenesis regulators. By contrast, in *C. elegans* TRA-1 sequesters TRA-2 in the germ line, allowing FEM-3 to initiate spermatogenesis. Thus, we propose that the flow of information within the sex determination pathway has switched directions during evolution. This result has important implications for how evolutionary change can occur.

Key words: **evolution of gene regulation, sex determination, nematodes.**

Introduction

Nematode sex Determination

Because most animal species have two sexes, each individual needs to determine which sex to become, and regulate development to select the appropriate cell fates. For example, the nematode *Caenorhabditis elegans* uses a complex pathway that responds to the ratio of *X* chromosomes to autosomes, so that *XX* animals develop as hermaphrodites and *XO* animals as males (reviewed by Zarkower 2006). This pathway controls the activity of the Gli transcription factor TRA-1, which in turn regulates the expression of hundreds of target genes throughout the body (Berkseth et al. 2013).

How does this pathway work? In the embryo, the *X*/*A* ratio acts through *xol-1* and three *sdc* genes to control the expression of *her-1*, which produces a male sex hormone. In *XO* animals, HER-1 binds the TRA-2 receptor to prevent it from promoting female fates (Fig. 1a). By contrast, in *XX* animals the absence of HER-1 allows the TRA-3 protease to cleave TRA-2 (Barnes and Hodgkin 1996; Sokol and Kuwabara 2000), releasing an intercellular fragment that promotes female development. This TRA-2_{ic} fragment binds FEM-3, preventing a FEM-1/FEM-2/FEM-3 complex from ubiquitinating TRA-1 (Mehra et al. 1999; Starostina et al. 2007). Finally, TRA-1 is cleaved to produce a transcription factor (Zarkower and Hodgkin 1992; Schvarzstein and Spence 2006) that represses male genes (Berkseth et al. 2013), including *fog-1* and *fog-3*, which direct spermatogenesis (Ellis and Kimble 1995; Chen and Ellis 2000; Jin et al. 2001).

Evolution of Self-Fertile Hermaphrodites

This regulatory process has been altered during recent evolution, during the production of new mating systems. Although most *Caenorhabditis* species are male/female, the phylogeny shows that *C. elegans*, *Caenorhabditis briggsae*, and *Caenorhabditis tropicalis* independently evolved *XX* animals that can reproduce as self-fertile hermaphrodites (Fig. 1b, Kiontke et al. 2011). Since a key feature of these hermaphrodites is the ability to make sperm as well as oocytes, each of these three species has altered the germ line to allow a brief period of *XX* spermatogenesis (Ellis 2022, 2016). So far, we only know about these changes in *C. elegans* and *C. briggsae*.

One of the key modifications was the recruitment of novel genes to modify the sex determination pathway in the *XX* germ line. For example, *C. elegans* uses *fog-2* to regulate the activity of *tra-2* in germ cells (Schedl and Kimble 1988). The *fog-2* gene encodes an F-box protein that binds GLD-1, which regulates the expression of *tra-2* mRNA (Clifford et al. 2000). However, because *fog-2* arose through a series of gene duplications and is unique to *C. elegans*, it is not part of this pathway in other species (Nayak et al. 2005). Instead, the *she-1* gene is needed for hermaphrodite spermatogenesis in *C. briggsae* (Guo et al. 2009). It also

Received: March 08, 2024. **Revised:** May 08, 2024. **Accepted:** May 10, 2024

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Discoveries

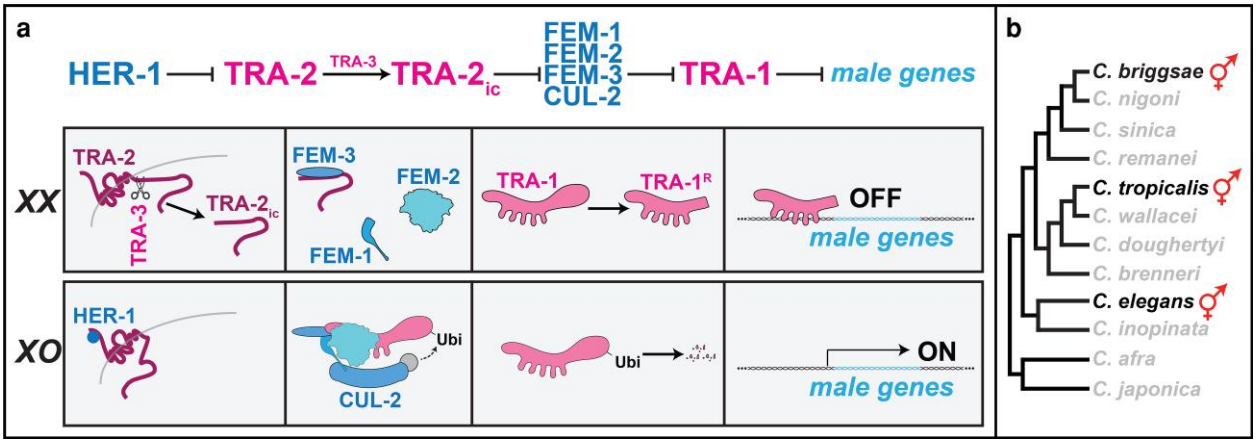

Fig. 1. The core sex determination pathway in *Caenorhabditis* nematodes. a) The top line summarizes the core regulatory interactions that control sexual fates. In *XX* animals, the TRA-2 receptor is free to be cleaved by TRA-3, releasing an intracellular fragment that binds FEM-3, preventing the FEM complex from working. This allows the TRA-1 transcription factor to be cleaved, forming a repressor of male genes. In *XO* animals, the male sex hormone HER-1 binds to and inactivates TRA-2. This leaves the FEM complex free to assemble with CUL-2 and ubiquitinate TRA-1, leading to its degradation. Hence, male genes are expressed. b) The phylogeny shows that ancestral *Caenorhabditis* species were male/female, and three species independently evolved *XX* animals that reproduce as self-fertile hermaphrodites.

arose through a series of gene duplications and acts through TRA-2, but *she-1* is unique to *C. briggsae*.

Other changes to the pathway involve the core genes themselves. For example, *fem-1*, *fem-2*, and *fem-3* encode members of an E3 ubiquitin ligase complex that regulates TRA-1 stability (Starostina et al. 2007). In *C. elegans*, these genes are also required downstream of *tra-1* to initiate spermatogenesis (Hodgkin 1986; Schedl et al. 1989). By contrast, *fem-2* and *fem-3* are not needed for spermatogenesis in *C. briggsae* (Hill et al. 2006).

Finally, some mutations that affect *tra-2* or *fem-3* regulation in *C. elegans* have major effects on hermaphrodite development. Gain-of-function mutations in *fem-3* result in animals that make only sperm (Barton et al. 1987), whereas gain-of-function mutations in *tra-2* cause them to produce only oocytes (Doniach 1986; Schedl and Kimble 1988). These mutations all affect the 3′-untranslated regions of their messages, so they only alter expression, not protein structure (Ahringer and Kimble 1991; Okkema and Kimble 1991; Kuwabara et al. 1992). Analyses of these mutations suggest that one way to achieve hermaphrodite spermatogenesis is by modulating the competition between TRA-2 and FEM-3 to control germ cell fates.

The *tra-2(mx)* Alleles

A separate group of *tra-2* mutations shows more complex effects in *C. elegans*. These *tra-2(mx)* alleles are all missense mutations that alter a conserved region near the C-terminus of the protein (Lum et al. 2000; Wang and Kimble 2001). The *mx* alleles prevent TRA-2 from directly binding the TRA-1 transcription factor (Lum et al. 2000; Wang and Kimble 2001), and they cause most hermaphrodites to produce only oocytes, transforming them into true females (Doniach 1986). Besides this strong effect on germ cells, the *mx* mutations have only weak effects on the soma, which sometimes result in the partial retraction of the hermaphrodite tail, or in the deaths of the HSN neurons. Thus, they strongly promote oogenesis in the germ line, but weakly promote male fates in the soma. Hence, the *tra-2(mx)* alleles seem likely to reveal key aspects of hermaphrodite gene regulation. But how they work and why they have such different effects in the soma and germ line have remained mysteries. We used comparative evolution to answer these questions.

Results

Caenorhabditis briggsae tra-2(mx) Alleles Increase Hermaphrodite Spermatogenesis

Analyzing the sex determination pathway in *C. elegans* germ cells is tricky, because the FEM complex has multiple roles in the germ line, and is absolutely required for spermatogenesis (Hodgkin 1986). By contrast, neither the *fem-2* nor the *fem-3* gene is needed for spermatogenesis in *C. briggsae* (Hill et al. 2006). Since Wang and Kimble (2001) showed that interaction between TRA-2 and TRA-1 is conserved in *C. briggsae*, the fact that the *fem* genes are not needed to produce sperm makes *C. briggsae* an ideal system for studying *tra-2(mx)* mutations.

We began by preparing an alignment of five *Caenorhabditis* TRA-2 proteins, which revealed extensive conservation near the C-terminus, in the *mx* region (Fig. 2a). This part of the protein is released from the membrane following cleavage by the calpain protease TRA-3 (Barnes and Hodgkin 1996) and interacts with TRA-1 in both *C. elegans* (Lum et al. 2000) and *C. briggsae* (Wang and Kimble 2001). There are five missense mutations that cause the special *tra-2(mx)* phenotype in *C. elegans* (Kuwabara et al. 1998). Four of the affected residues are identical in all five species, while the fifth is partially conserved. Thus, we suspected that the role of the

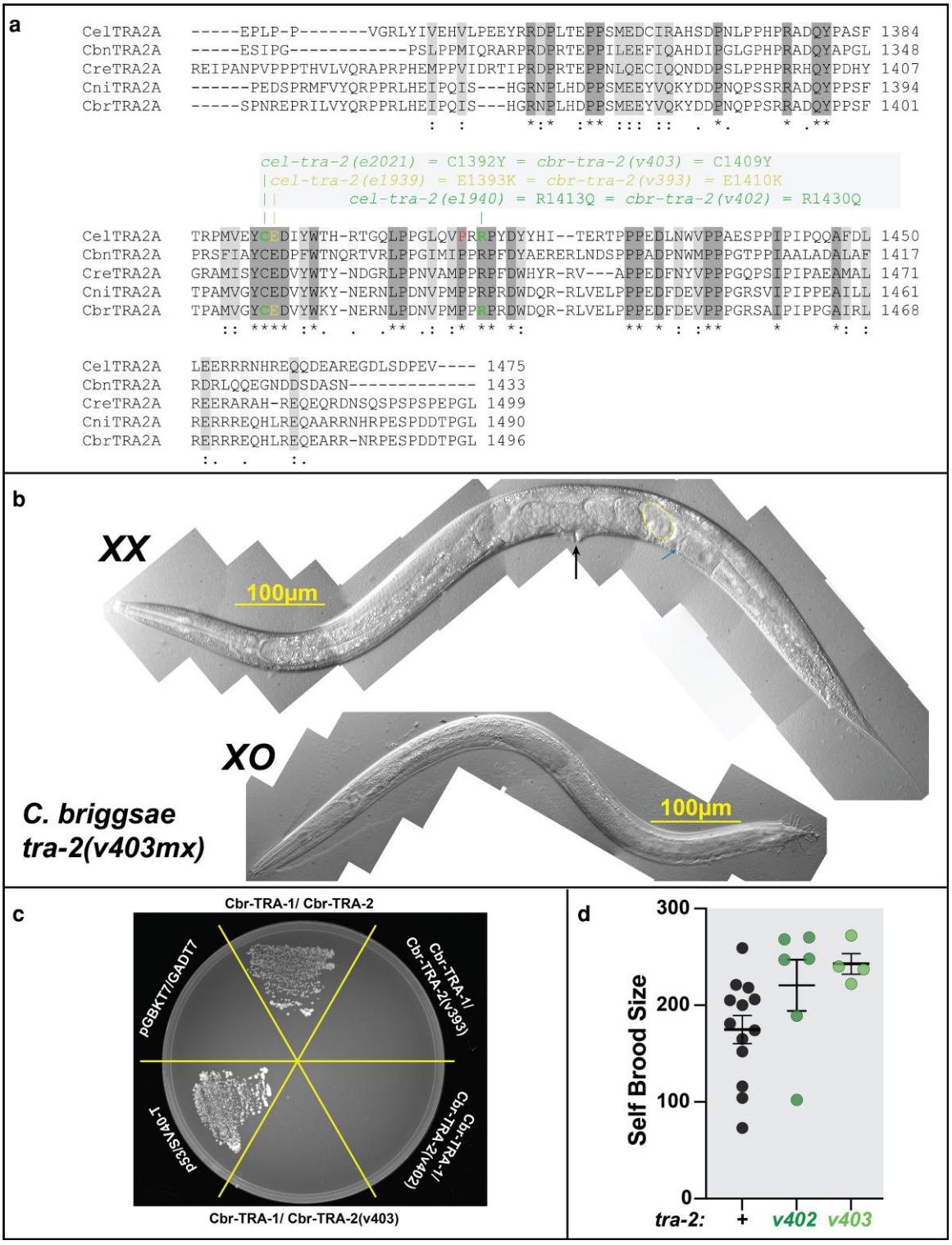

Fig. 2. *Caenorhabditis briggsae tra-2(mx)* alleles do not feminize the germ line. a) Alignment of the C-termini of five *Caenorhabditis* TRA-2 proteins, prepared using MUSCLE. Identical residues are shaded dark gray, and conservative substitutions are shaded light gray. All *C. elegans tra-2(mx)* alleles affect one of five conserved sites in this region. We made identical *C. briggsae* mutations for three of the *C. elegans* alleles (marked in green shades). b) Differential interference contrast photomicrographs of a *Cbr-tra-2(v403)* hermaphrodite and male. The black arrow indicates the vulva, and one of the self-embryos in the uterus is outlined in yellow. The small blue arrow marks two self-sperm. c) Yeast two-hybrid results, with bait constructs listed before prey constructs. d) Self-brood size of hermaphrodites, with error bars indicating the mean and 95% confidence limits.

TRA-2(mx) domain in sex determination might also be conserved.

To learn the effect of *mx* mutations in *C. briggsae*, we made mutations identical to three of the *C. elegans tra-2(mx)* alleles, as shown in Fig. 2a. One of these three alleles, *Cbr-tra-2(v393)*, showed more extensive masculinization than its *C. elegans* ortholog, *tra-2(e1939)* (supplementary fig. S1, Supplementary Material online). The *v393* tail is blunt with several male characteristics, the gonad usually has a single arm ending in the cloaca, and the germ cells all form sperm. These traits suggest that this substitution causes more extensive harm to general TRA-2 function in *C. briggsae* than it does in *C. elegans*.

However, the other two alleles, *cbr-tra-2(v402)* and *cbr-tra-2(v403)*, have only minor effects on the soma, since *XX* animals usually form typical hermaphrodite bodies and *XO* animals are normal males (Fig. 2b). In this respect, these mutants resemble their *C. elegans* orthologs.

We used the yeast two-hybrid assay to confirm that these *C. briggsae* mutations prevent TRA-2 from binding TRA-1 (Fig. 2c). Our assay can detect the normal interaction of TRA-2 with TRA-1 in this species, and control experiments using *C. elegans* proteins confirmed that we can also detect the disruption of binding by the *mx* alleles in that species (data not shown). Thus, the role of the MX domain in TRA-2/TRA-1 interactions is conserved, and this function generally plays little role in somatic sex determination.

Surprisingly, none of the *C. briggsae tra-2(mx)* mutants showed the characteristic Fog phenotype of their *C. elegans* counterparts. Although *cbr-tra-2(v393)* has the opposite phenotype—the animals make only sperm—it also causes *XX* animals to develop intersexual gonads (supplementary fig. S1, Supplementary Material online), which might influence germ cell fates. These somatic effects could be the result of broader problems in TRA-2_{ic} folding, which might also impinge on its ability to bind FEM-3.

Thus, we focused our analyses on the other two *cbr-tra-2(mx)* alleles. To learn whether they reduced sperm number without eliminating it altogether, we measured the self-broods of these animals (Fig. 2d). If these mutants made fewer sperm than normal, they should make fewer self-progeny. Instead, we observed an increase in brood size for both alleles. This result suggested that *v402* and *v403* cause *extra* spermatogenesis in *C. briggsae*, a phenotype opposite that caused by the *C. elegans mx* alleles.

Caenorhabditis briggsae tra-2(mx) Alleles Restore Spermatogenesis to *she-1* Mutants

In *C. elegans*, the *fog-2* gene is required for hermaphrodite spermatogenesis, since *fog-2 XX* mutants develop as females rather than as hermaphrodites, whereas the *XO* mutants are normal males (Schedl and Kimble 1988). By contrast, mutations in the 3′-UTR of *fem-3* increase hermaphrodite spermatogenesis (Barton et al. 1987; Rosenquist and Kimble 1988) and suppress *fog-2* (Schedl and Kimble 1988). This suppression of *fog-2* has been a key diagnostic tool for studying the control of spermatogenesis in *C. elegans* hermaphrodites.

Although *C. briggsae* has no homolog of *fog-2* (Nayak et al. 2005), mutations in *she-1* have a similar effect, transforming *XX* animals into females (Guo et al. 2009). Thus, we tested the ability of *Cbr-tra-2(v402mx)* and *Cbr-tra-2(v403mx)* to suppress *she-1* (Fig. 3a). Both alleles completely restored normal hermaphrodite development to *she-1 XX* animals. These results confirm the idea that *Cbr-tra-2(mx)* mutations promote spermatogenesis in *XX* hermaphrodites. In addition, *tra-2* null mutations are dominant suppressors of *fog-2* in *C. elegans* (Schedl and Kimble 1988) and of *she-1* in *C. briggsae* (Guo et al. 2009), demonstrating the conserved importance of *tra-2* dosage in the *XX* germ line. Although the *C. briggsae tra-2(mx)* alleles are not null, we tested them for dominant suppression of *she-1* in the germ line (Fig. 3b). Our data revealed that they suppress *she-1* almost as well as a null allele does. Furthermore, we included the *Cbr-tra-2(v393mx)* allele in these experiments, since it has no effect on somatic development in heterozygotes, and found that it behaves like the other *mx* alleles do.

In both *C. elegans* and *C. briggsae*, spermatogenesis requires *fog-3* (Ellis and Kimble 1995; Chen et al. 2001), which is a major target for regulation by the transcription factor TRA-1 (Chen and Ellis 2000). We used reverse transcription PCR (RT-PCR) to measure *fog-3* expression in *XX* L4 larvae, during the normal period of spermatogenesis, and found that it was significantly higher in the *cbr-tra-2(mx)* mutants than in the wild type (Fig. 3c, supplementary fig. S2, Supplementary Material online). Thus, these alleles appear to affect spermatogenesis by increasing expression of the key regulator *fog-3*.

Caenorhabditis elegans mutations in *tra-2* and its target *fem-3* have opposite effects on the germ line, which implies that they compete with each other to regulate spermatogenesis (Barton et al. 1987). Hence, the predominant model in the field is that TRA-2 controls sex determination by inactivating FEM-3, which would otherwise direct male development. Although *C. briggsae* FEM-3 is not *required* for spermatogenesis, it remained possible that *C. briggsae tra-2(mx)* mutations promote spermatogenesis by *increasing* the activity of FEM-3 in germ cells. Thus, we tested their ability to suppress *she-1* in a *fem-3* null mutant background (Fig. 3d). As shown previously, a *she-1* mutation feminizes the germ line of *Cbr-fem-3* mutants (Guo et al. 2009). Surprisingly, *Cbr-tra-2(mx)* alleles suppress this feminization. Thus, *C. briggsae tra-2(mx)* alleles do not act through FEM-3 to promote spermatogenesis. Instead, they appear to alter the direct regulation of TRA-1 by TRA-2.

We also measured the effects of *tra-2* mutations on *fog-3* transcript levels in a *fem-3* null mutant background (Fig. 3f, supplementary fig. S3, Supplementary Material online). Even so, the *mx* mutations of *tra-2* caused an increase in *fog-3* expression. Taking these results together, we infer that SHE-1 regulates TRA-2 activity in *C. briggsae* germ cells and that TRA-2 physically interacts with TRA-1 to regulate

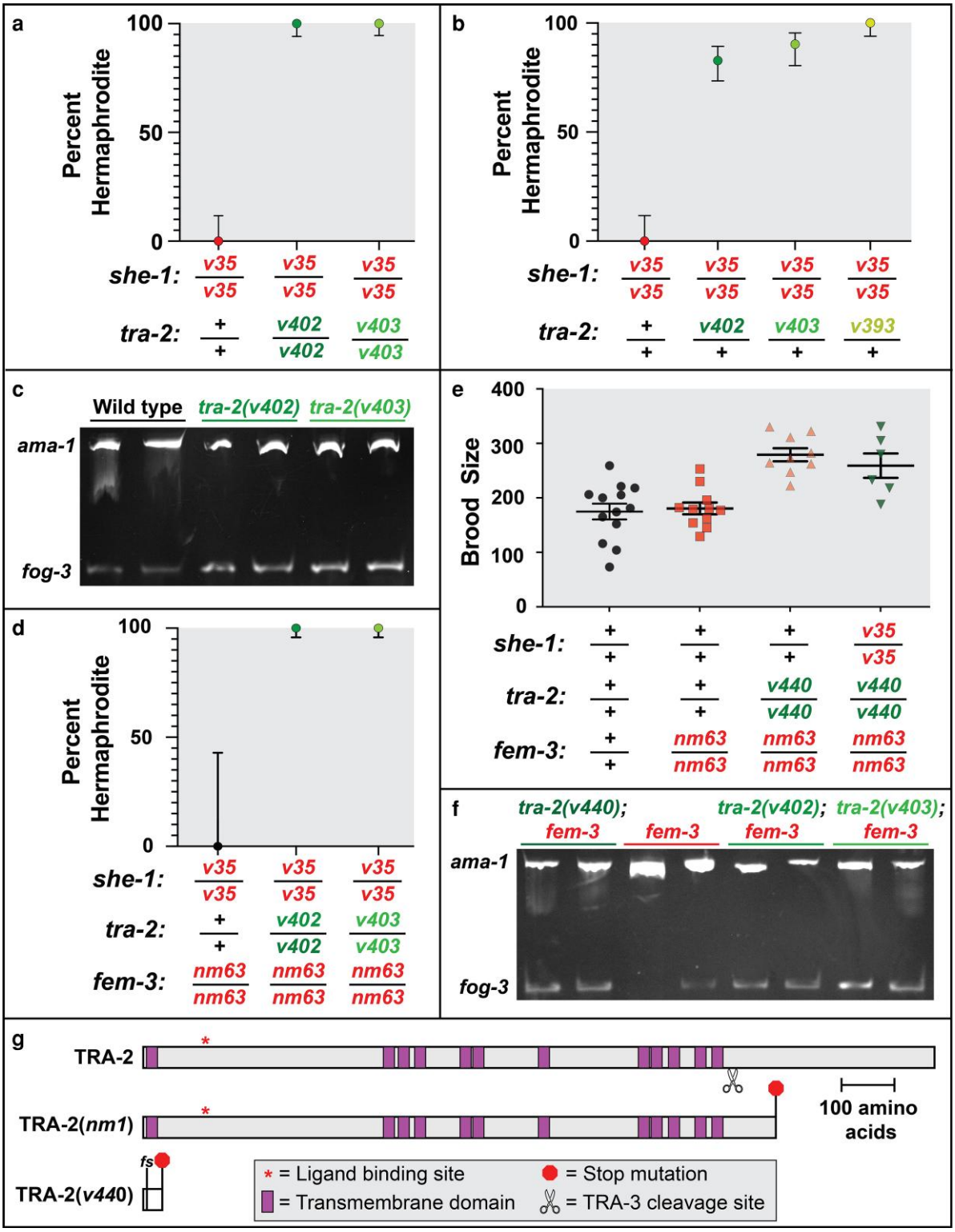

Fig. 3. *Caenorhabditis briggsae tra-2(mx)* mutations suppress *she-1* independent of *fem-3* function. a, b, d) Graphs showing the percent of *XX* animals of each genotype that develop as self-fertile hermaphrodites, rather than as females. The error bars show 95% confidence intervals. a) $N = 29, 62, 67$, respectively. b) $N = 29, 87, 62, 60$. d) $N = 7, 87, 88$. c, f) Semi-quantitative RT-PCR results showing *fog-3* expression (lower band) and control *ama-1* expression (upper band). These two products were produced in separate PCRs (using equivalent amounts of the cDNA template) and run on a single gel. Each lane used cDNA template produced from a mixture of 5 mid-L4 *XX* larvae. e) Self-brood size of hermaphrodites, with error bars indicating the mean and 95% confidence limits. g) Diagram showing the locations of the reference mutation *Cbr-tra-2(nm1)* and the null allele *Cbr-tra-2(v440)*. The *v440* allele truncates the protein prior to the crucial HER-1-binding site and removes the entire intracellular domain, so it represents a molecular null.

fog-3 expression and spermatogenesis, bypassing the need for FEM-3.

These studies suggested that null alleles of *C. briggsae tra-2* should also promote spermatogenesis by preventing an interaction with TRA-1. To test this possibility (Fig. 3, e and f), we began by using gene editing to produce an early frameshift in *C. briggsae tra-2* (Fig. 3g), since other *Cbr-tra-2* alleles are located near the C-terminus and might have residual function. The *Cbr-tra-2(v440)* allele transforms all *XX* animals into pseudo-males, just like *Cbr-tra-2(mn1)* (Kelleher et al. 2008). These *XX* mutants have defective male tails, but normal male gonads and only produce sperm. As expected, this somatic effect is suppressed by a *fem-3* null mutation, which restores normal hermaphrodite development. Thus, *tra-2(v440)* defines the null phenotype.

When we measured brood sizes to assay the number of hermaphrodite sperm that were produced (Singson 2001), we found that the wild type and *fem-3* null mutants made the same number of sperm, but that *tra-2(v440); fem-3* double mutants made extra sperm, confirming that TRA-2 regulates germ cell sexual development even in the complete absence of FEM-3 (Fig. 3e). This increased brood size is also observed in *tra-2(v440); she-1 fem-3* triple mutants (Fig. 3e). As these results predict, *tra-2(v440)* also causes an increase in the expression of *fog-3* (Fig. 3f). We conclude that *C. briggsae* TRA-2 regulates germ cell fates independent of FEM-3. However, SHE-1 cannot regulate germ cell fates without TRA-2 being present and functional.

Blocking the Ability of *C. briggsae* TRA-1 to Bind TRA-2 Also Promotes Spermatogenesis

The TRA-1 transcription factor has a large conserved domain C-terminal to the five zinc fingers (de Bono and Hodgkin 1996), which is known to bind TRA-2 in both *C. elegans* (Lum et al. 2000) and *C. briggsae* (Wang and Kimble 2001). Since we did not know which residues were critical to its function, we made sequential frameshift mutations in *C. briggsae tra-1*, which alter the region of 24 amino acids that lies between them (Fig. 4, a and b). These *Cbr-tra-1(v197 v383)* mutants develop as *XX* hermaphrodites and *XO* males, which indicates that TRA-1's normal function of repressing male development must be unaffected. However, a yeast two-hybrid assay shows that this double mutation does prevent Cbr-TRA-1 from binding TRA-2 (Fig. 4c).

Much like the *Cbr-tra-2(mx)* alleles, this *tra-1* mutation increases hermaphrodite brood size (Fig. 4d). To confirm its effect on hermaphrodite germ cell fates, we directly counted sperm and saw a significant increase in number (Fig. 4e), which is even apparent simply by looking at the gonad (Fig. 4f). As a control, we studied *tra-1(v197)/+* animals, which should make half the normal amount of TRA-1, and they had the wild-type amount of sperm.

As with *tra-2*, we also built double and triple mutants using *she-1*, to confirm that this *tra-1* allele promotes spermatogenesis. As expected, the *tra-1(v197 v383)* mutation suppresses *she-1* (Fig. 4g), and this effect is independent of *fem-3*. However, this suppression is mostly recessive, whereas that by *Cbr-tra-2(mx)* alleles is dominant.

When we build *Cbr-tra-2(mx); tra-1(v197 v383)* animals, some produced many extra sperm, and others had completely masculinized germ lines (supplementary fig. S4, Supplementary Material online). Thus, disrupting the ability of *C. briggsae* TRA-2 to bind TRA-1 causes excess spermatogenesis in hermaphrodites. Since double mutants show a more severe effect, we infer that none of the *tra-2* or *tra-1* single mutants completely disrupts binding on its own.

The *C. elegans tra-2(mx)* Alleles Prevent Sequestration of TRA-2 by TRA-1

These results imply a simple model for how TRA-2 works in *C. briggsae* hermaphrodites. First, TRA-2ic binds FEM-3 in the soma; this interaction protects TRA-1, leaving it free to turn off male genes. Second, TRA-2ic directly binds TRA-1 in germ cells, to help turn off genes that promote spermatogenesis. Although null alleles of *tra-2* block both functions, the *mx* alleles only block its germline activity.

By contrast, the role of TRA-2 and its *mx* mutations in *C. elegans* has always been confusing. Although *C. elegans tra-2(mx)* alleles disrupt the interaction between TRA-2 and the master transcription factor TRA-1 (Lum et al. 2000; Wang and Kimble 2001), they result in *XX* animals that make only oocytes (Doniach 1986). By contrast, *tra-2 (null)* alleles, which also prevent interaction with TRA-1, cause *XX* animals to make only sperm (Hodgkin and Brenner 1977). This comparison implies that something complex is going on in this part of the *C. elegans* sex determination pathway.

This complexity has been hard to address with classical epistasis, since many of the genes that control sex determination act at multiple points in the pathway (Ellis 2022). To address this problem, we carried out experiments looking at the effects of heterozygosity for mutations in the sex determination pathway on *tra-2(mx)* phenotypes. Wang and Kimble (2001) used this approach to study how decreasing TRA-1 dosage affected the developing germ cells of *tra-2(mx)* heterozygotes. Their studies suggest that TRA-1 behaves like a repressor of TRA-2(mx) activity, since lower levels of TRA-1 lead to an increase in the fraction of *XX* females.

We began by studying *tra-2* gene dosage, to learn how *mx* alleles altered TRA-2 activity itself. These experiments were inspired by the small number of *tra-2(mx)/tra-2(null)* animals observed by Doniach (1986). By using PCR or a marker mutation to identify these heterozygotes, we were able to amass large datasets for three *tra-2(mx)* alleles (Fig. 5a). For all three alleles, a *tra-2(mx)* allele in *trans* to a wild-type allele is significantly *more* likely to be female than one in *trans* to a null mutation. Thus, *C. elegans tra-2(mx)* alleles increase the *normal* germline function of TRA-2.

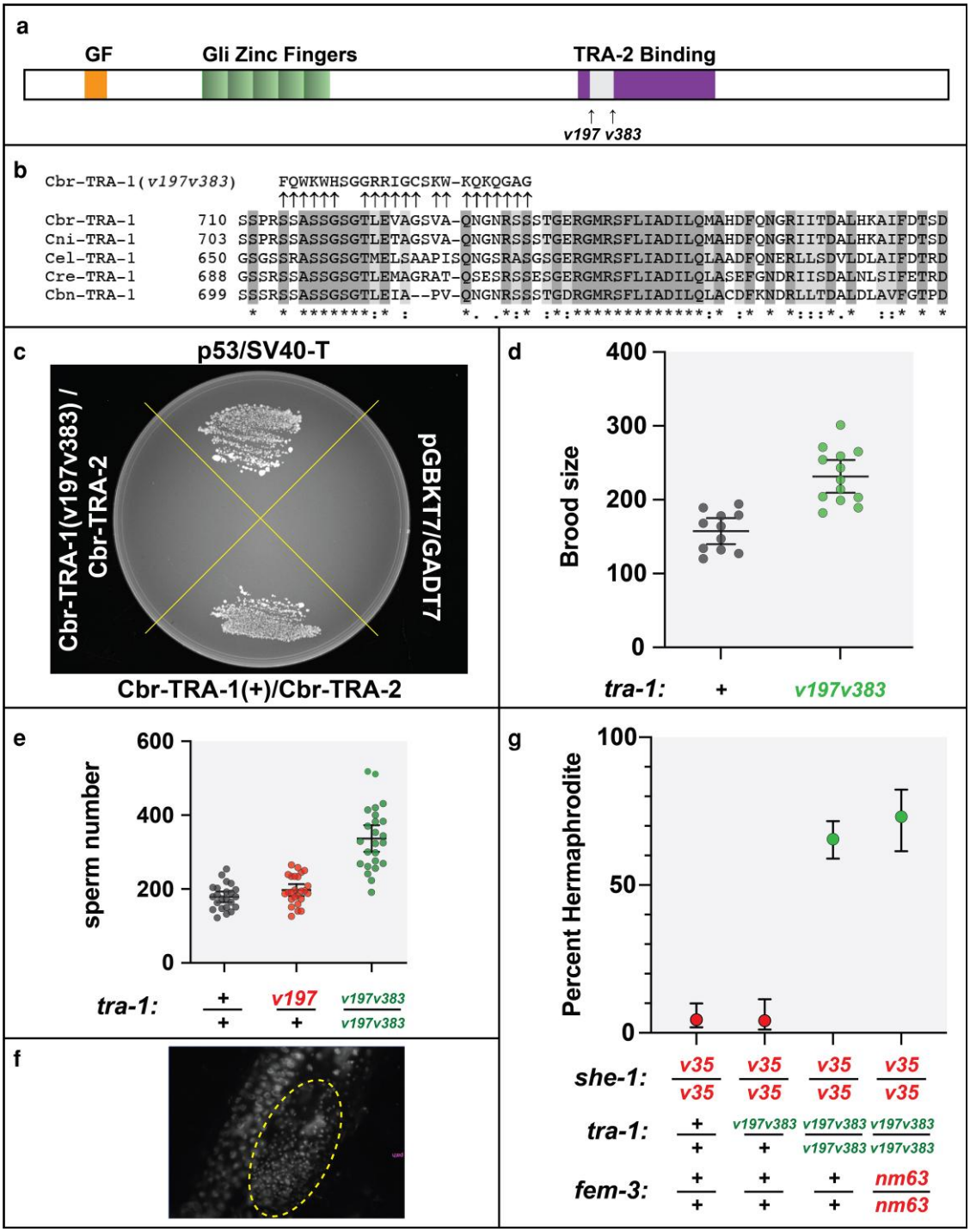

Fig. 4. A *C. briggsae tra-1* mutation that blocks TRA-2-binding favors spermatogenesis. a) Diagram of the structure of *C. briggsae* TRA-1, indicating the location of the double frameshift mutant *v197 v383*. Orange marks the conserved gain-of-function domain, green the five conserved zinc fingers, and purple the conserved TRA-2-binding domain. b) Alignment of five *Caenorhabditis* TRA-1 proteins, showing part of the TRA-2-binding domain. It was prepared using MUSCLE. Identical residues are shaded dark gray, and residues with conservative substitutions are shaded light gray. c) Yeast two-hybrid results, with bait constructs listed before prey constructs. d) The number of self-progeny for animals of the indicated genotypes. The thick line indicates the mean, and the error bars represent 95% confidence intervals. e) The number of sperm in individuals of the indicated genotypes, observed following DAPI staining. f) Extensive sperm in one ovotestis of a *tra-1(v197 v383) XX* animal. g) Graph showing the percent of *XX* animals of each genotype that develop as self-fertile hermaphrodites, rather than as females. The error bars show 95% confidence intervals. $N = 113$, 73, 212, and 67, respectively.

In addition, we studied interactions between *tra-2(mx)* alleles and null alleles of *fem-1*, *fem-2*, or *fem-3*, which encode components of the FEM complex. For these experiments, we used nonsense alleles (Fig. 5b) that are recessive and act zygotically. (By contrast, *fem-1* deletions have a maternal effect due to the absence of germline

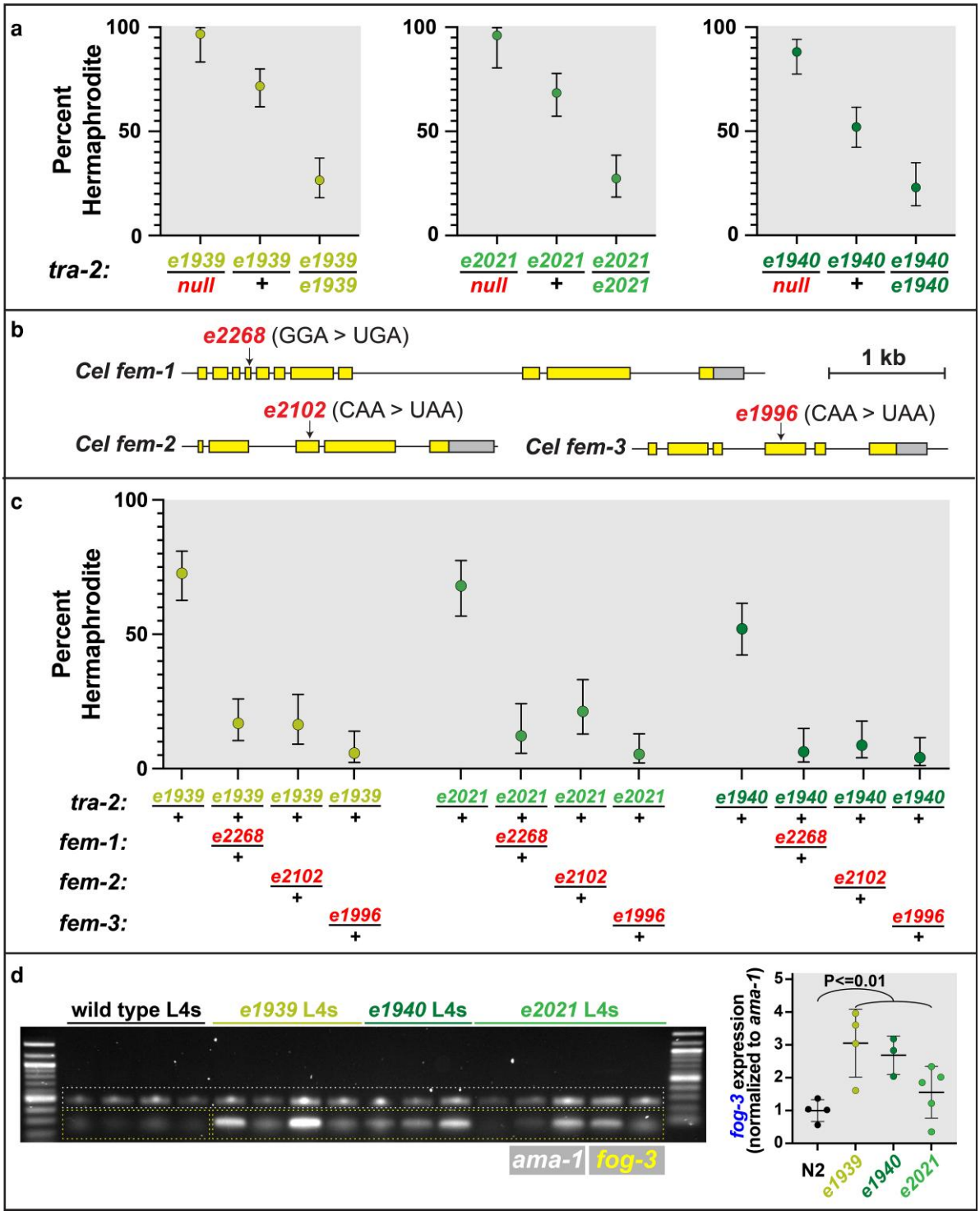

Fig. 5. *Caenorhabditis elegans tra-2(mx)* alleles increase TRA-2's ability to target the FEM complex. a, c) Animals of the indicated genotypes were generated from crosses using *tra-2(mx)* fathers as described in the Materials and Methods and raised at 20 °C. On its own, each *fem* mutation is recessive. The error bars show 95% confidence intervals. a) $N = 30$, 92, 79, 25, 76, 73, 59, 100, and 61, respectively. c) $N = 88$, 89, 61, 69, 75, 49, 61, 75, 100, 64, 69, and 72, respectively. b) Location of the nonsense alleles we used in *C. elegans fem* genes. d) Semi-quantitative RT-PCR results showing *fog-3* expression (lower band) and control *ama-1* expression (upper band). These two products were produced in separate PCRs (using equivalent amounts of the cDNA template) and run on a single gel. Each lane used cDNA template produced from single mid-L4 *XX* larvae. The quantitation on the right was done using Carestream MI software. The *fog-3* values are normalized to the average wild-type values for *ama-1*. (All calculations were done in arbitrary units.) Each genotype shows bars for the mean and standard deviation. The *P*-value was computed for a comparison of wild type with combined mutant data using the Mann–Whitney *U* test.

licensing [Johnson and Spence 2011].) We observed strong genetic interactions between *tra-2(mx)* mutations and lowered expression of each *fem* gene (Fig. 5c). Moreover, these effects were particularly strong for *fem-3*, the direct target of TRA-2. We conclude that *tra-2(mx)* alleles increase inhibition of the FEM complex and that this effect is enhanced by reducing *fem* gene dosage, which is likely to result in fewer FEM complexes to compete with TRA-2.

These results suggest that in *C. elegans* TRA-1 negatively regulates TRA-2, resulting in higher activity of the FEM complex. Since the FEM complex not only targets TRA-1, but also acts downstream of it to promote spermatogenesis (reviewed by Ellis 2022), when *tra-2(mx)* mutations decrease FEM activity, they should favor oogenesis. Putting all these results together, we suggest that TRA-1 plays an important role in sequestering TRA-2 in the *C. elegans* germline, to facilitate hermaphrodite spermatogenesis.

Since this model depends on the downstream activity of the FEM complex, it does not require *fog-3* transcript levels to be low to cause oogenesis in the *tra-2(mx)* mutants. We wondered whether the absence of an interaction with TRA-1 caused an increase in these transcripts, as is observed in *C. briggsae*. RT-PCR analyses show that this is in fact the case (Fig. 5d). Hence, TRA-2 binds TRA-1 in both species to lower the expression of the sperm regulator *fog-3*. However, in *C. elegans* the most important aspect of this interaction is that TRA-1 is sequestering TRA-2 in germ cells, allowing the FEM proteins to direct hermaphrodite spermatogenesis.

Discussion

Evolutionary biologists generally assume that if the genes in a regulatory pathway are conserved, their relationships within that pathway will have been conserved as well. For example, the identification of *ras* genes in *C. elegans* and *Drosophila* led to the hope that they could be used to elucidate the *ras* signal transduction pathway for other animals (Han 1992), a hope that was soon realized (Treisman 1996).

These types of studies often focus on long time scales and distantly related species, but a detailed picture of how evolutionary change occurs requires a narrower focus. Sex determination is a particularly useful trait, because it changes more rapidly than many other traits. In nematodes, these changes not only involve secondary sexual characteristics, but often reproductive traits and even entire mating systems (reviewed by Ellis and Lin 2014; Ellis 2016). To date, evolutionary studies using nematodes have focused on *C. elegans* and *C. briggsae*, because of the ease of using isogenic strains and hermaphrodite genetics to dissect the regulation of sexual traits.

In both species, *tra-2* (Hodgkin and Brenner 1977; Kuwabara et al. 1992; Kuwabara 1996; Kelleher et al. 2008), *fem-3* (Hodgkin 1986; Rosenquist and Kimble 1988; Haag et al. 2002; Hill and Haag 2009), and *tra-1* (Hodgkin and Brenner 1977; Zarkower and Hodgkin 1992; de Bono and Hodgkin 1996; Kelleher et al. 2008) are sex determination genes with conserved structures. Moreover, their somatic functions are strongly conserved. However, *fem-3* is absolutely required for spermatogenesis in *C. elegans*, but not in *C. briggsae* (Hill et al. 2006).

Not only is each gene conserved, the binding interactions between TRA-2 and FEM-3 (Mehra et al. 1999; Haag et al. 2002) and between TRA-2 and TRA-1 (Lum et al. 2000; Wang and Kimble 2001) are conserved as well. But although the TRA-2/TRA-1 interaction involves conserved residues near the C-terminus of TRA-2 (Fig. 2a), we show that the function of this interaction is exactly opposite in the two species. Identical *tra-2(mx)* mutations cause hermaphrodites to make oocytes instead of sperm in *C. elegans*, but sperm instead of oocytes in *C. briggsae*. How has this remarkable difference come to be?

The regulation in *C. briggsae* appears straightforward (Fig. 6). In this species, TRA-2 binds FEM-3 to promote female development throughout the soma, by preventing FEM-3 and its partners from causing TRA-1 degradation. Our data imply that TRA-2 also binds TRA-1 to promote oogenesis in the germ line, since the *Cbr-tra-2(mx)* mutants make extra sperm, and do so even in *fem-3(null)* mutants. Thus, *C. briggsae* TRA-2 promotes female development in both soma and germ line.

We previously suggested that TRA-1, like other Gli transcription factors, makes an activator as well as a cleaved repressor (Guo et al. 2013). Although TRA-1 repressor seems to turn off male genes throughout the body and germ line (Conradt and Horvitz 1999; Chen and Ellis 2000; Yi et al. 2000; Berkseth et al. 2013), our data suggested that TRA-1 activator specifically promotes spermatogenesis. Thus, one simple model is that TRA-2 binds TRA-1 activator in *C. briggsae* to stop it from inducing spermatogenesis. However, other models, such as TRA-2 facilitating the repression of *fog-3* by TRA-1, also remain possible.

The situation in *C. elegans* is more complex, which is why these special *tra-2* alleles were named *mixomorphic*—the mutations cause weak masculinization in the soma, but strongly promote oogenesis in the germ line. Hence, they have opposite effects on sexual development in different tissues. Based on several experiments, we propose that *C. elegans* TRA-1 binds to and sequesters TRA-2 in the germ line (Fig. 5, a and c). (i) Lowering the amount of TRA-1 increases the ability of *tra-2(mx)* alleles to promote oogenesis (Wang and Kimble 2001). This result suggests that TRA-1 could be a negative regulator of TRA-2 in germ cells. (ii) Increasing wild-type TRA-2 function in *tra-2(mx)* heterozygotes increases the likelihood that *XX* animals only make oocytes, which implies that the *mx* alleles augment the normal ability of TRA-2 to promote this fate. (iii) Decreasing the activity of the FEM complex increases the ability of *tra-2(mx)* alleles to promote oogenesis, which suggests that *C. elegans* TRA-2 regulates germ cells fates by inhibiting FEM-3 activity. And (iv) the *tra-2(mx)* mutants produce elevated levels of the sperm regulator *fog-3*, even though they make only oocytes. This final result

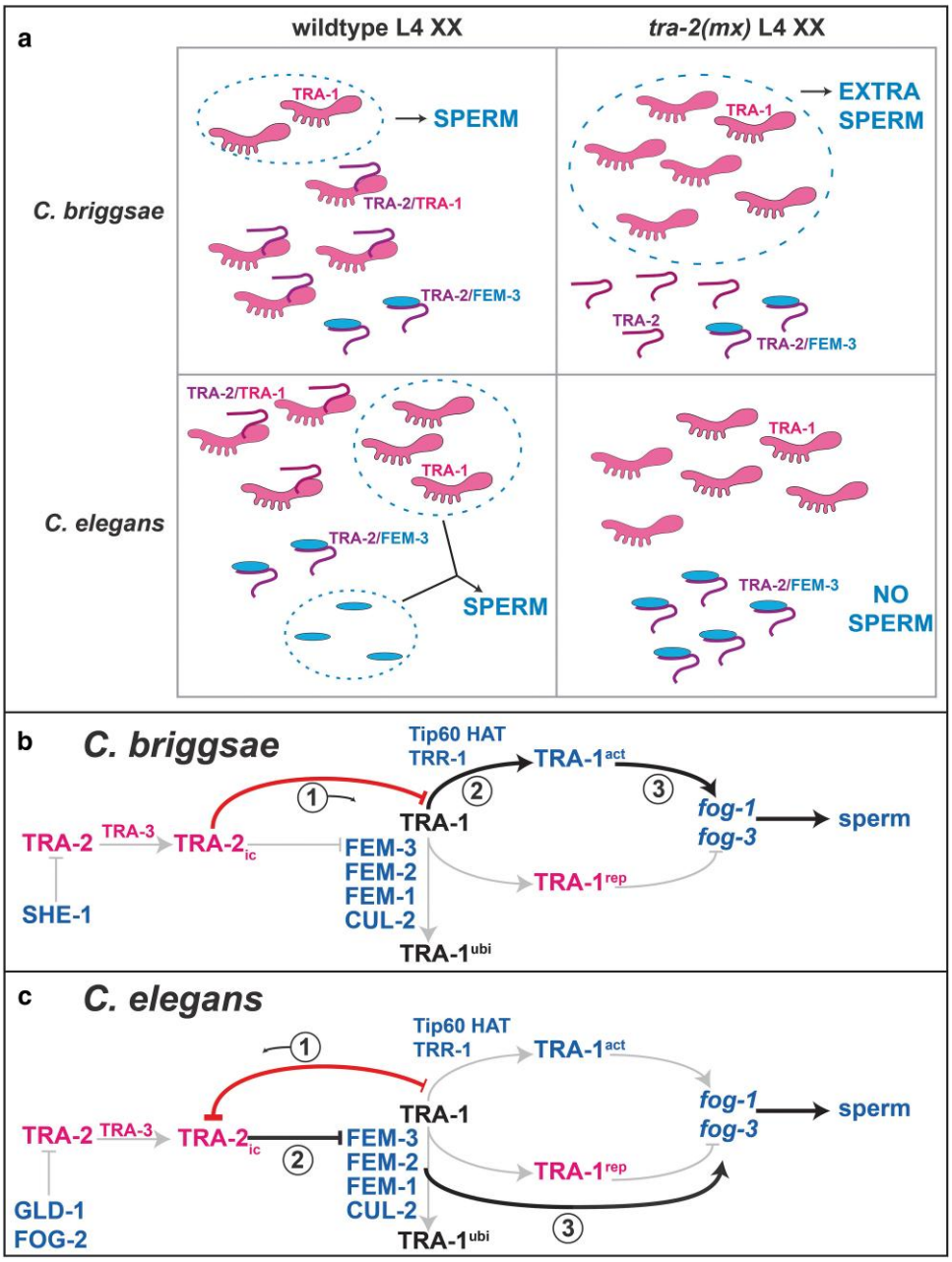

Fig. 6. Recent evolutionary changes have rewired *Caenorhabditis* sex determination. a) Models for how the relative concentrations of TRA-2ic (purple loop), TRA-1 (pink), and FEM-3 (blue) specify germ cell fates. The stippled ovals enclose factors that promote spermatogenesis. In *C. briggsae*, TRA-2_{ic} binds TRA-1 to prevent spermatogenesis. We present one possible mechanism by which it might act, in which full-length TRA-1 *promotes* the expression of sperm genes and TRA-2_{ic} blocks this activation step. (Although TRA-1 repressor also participates, we omit it for clarity.) By contrast, *C. elegans* FEM-3 plays an essential role in spermatogenesis downstream of TRA-1. Thus, if TRA-2_{ic} fails to bind TRA-1, it is free to inactivate FEM-3 and prevent spermatogenesis. b) Model for sex determination in the germ cells of larval *C. briggsae* hermaphrodites. Genes or proteins that promote male fates are in blue, and those that promote female fates in red. Proteins are in capitals and genes in lowercase italics. Arrows denote positive interactions, and "—|" denotes negative ones. Critical relationships in the regulation of spermatogenesis are highlighted in black and the key TRA-2/TRA-1 interaction in red. c) Similar model for *C. elegans*.

suggests that the key factor that determines phenotype is low *fem-3* activity, which is needed downstream of *fog-3* transcription to allow spermatogenesis (Chen and Ellis 2000). Thus, we suggest that in *C. elegans* germ cells, TRA-1 regulates TRA-2 activity by sequestration and that this interaction prevents TRA-2 from binding FEM-3.

This situation would not be stable in *C. elegans*, if the FEM complex regulated germ cell fates only by controlling TRA-1. However, the three *fem* genes also have an essential spermatogenesis function downstream of TRA-1. In *C. elegans*, *tra-1; fem* double mutants make male bodies, the *tra-1* phenotype, but only produce oocytes, the *fem* germ line phenotype (Hodgkin 1986). Furthermore, these double mutants have elevated expression of the critical spermatogenesis gene *fog-3*, a direct TRA-1 target, even though they make oocytes (Chen and Ellis 2000).

Thus, the FEM complex regulates a downstream spermatogenesis factor, in addition to controlling TRA-1. This essential role for the *C. elegans* FEM complex in spermatogenesis means that regulation of TRA-2 levels by TRA-1 could indeed act through the FEM complex to control spermatogenesis.

Some evidence suggests that *C. briggsae fem-3* retains the ability to promote spermatogenesis downstream of *Cbr-tra-1* (Hill and Haag 2009), but it is not essential. Indeed, null mutants of *Cbr-fem-2* or *Cbr-fem-3* make sperm normally (Hill et al. 2006), and *Cbr-fem-3* hermaphrodites produce the correct number of sperm before switching to oogenesis (Fig. 3e).

To date, the only molecular function known for the FEM complex is regulation of TRA-1 stability. Thus, an important goal for future research is identifying the downstream target of these proteins in *C. elegans*, and determining how the FEM complex controls its activity and why this control has been altered during recent evolution.

Our data show that *C. briggsae she-1* controls hermaphrodite spermatogenesis by acting through *tra-2* to control *tra-1*, since this regulation can occur even in *fem-3* null mutants. By contrast, *C. elegans fog-2* primarily acts through *tra-2* to control *fem-3* activity. These differences show that pathways with multiple branches are prone to changes that decrease or even eliminate the function of one of those branches. Despite the important changes we have observed, the sex determination pathway still retains multiple roles for the key genes *tra-2*, *fem-3*, and *tra-1* in both species. However, the directionality and significance of their interactions have changed dramatically.

If TRA-1 indeed regulates the activity of its binding partner TRA-2 by sequestration, this result opens up new possibilities for the control of development by transcription factors. In some situations, they might determine cell fates not only by regulating the expression of target genes, but also by directly altering the levels of other binding factors. This would allow for multiple regulatory branches in a heretofore unanticipated manner.

Taken together, our results and those of previous studies suggest that hermaphrodite development is based on fundamentally different approaches in *C. elegans* and *C. briggsae*.

In *C. elegans*, the levels of TRA-2 and FEM-3 in the germ cells are tightly balanced, so that a change in either one can alter the decision to produce sperm or oocytes (Doniach 1986; Barton et al. 1987; Schedl and Kimble 1988). Furthermore, recent studies suggest that TRA-2 protein levels in the germ line are very low, so low as to be almost undetectable (Hu et al. 2019). These observations point to a model in which the germline expression of both *tra-2* and *fem-3* was lowered and balanced during the evolution of self-fertility. This arrangement would make it easier for the same germ line to produce sperm early in life, and later switch to oogenesis.

By contrast, *C. briggsae* hermaphrodites appear to have low or nonexistent expression of *fem-3* in germ cells, and the regulation of spermatogenesis is instead controlled by the modulation of TRA-2 and TRA-1. As a consequence, *fem-3* null mutations have no effect on hermaphrodite reproduction in this species (Hill et al. 2006). Indeed, their effects can only be discerned if TRA-2 is completely absent.

Although we now have a good picture of the differences between *C. elegans* and *C. briggsae*, we need to dissect the process of sex determination in their male/female relatives to be able to figure out the times and directions of the major events that shaped these species. Such studies had previously been impractical, but the advent of rapid gene editing should soon lead to results.

Materials and Methods

Strains

The following strains were used: *C. elegans*: N2, *dpy-10 (e128)* (Brenner 1974) *dpy-10(cn64)* (Levy et al. 1993) *tra-2(e1939mx)*, *tra-2(e1940mx)* and *tra-2(e2021mx) II* (Doniach 1986), *tra-1(e1099) III* (Hodgkin and Brenner 1977), *fem-1(e2268)* (Johnson and Spence 2011), *fem-2(e2102)* (Hodgkin 1986), and *fem-3(e1996)* (Hodgkin 1986). *C. briggsae*: AF16 (Fodor et al. 1983), *tra-2(v440) II*, *tra-2(v393mx) II*, *tra-2(v402mx) II* and *tra-2(v403mx)*, *tra-1(v197v383) III* (this paper), *dpy-18(mf104) III* (Winter et al. 2007), *she-1(v35)* IV (Guo et al. 2009), and *fem-3(nm63) IV* (Hill et al. 2006).

Genetics

Animals were maintained at 20 °C on plates seeded with AMA1004, unless otherwise indicated. Procedures for raising animals and building double mutants were based on work by Brenner (1974). If necessary, genotypes were tested using single-worm PCR. For Fig. 5a, we studied *C. elegans tra-2(mx)* homozygotes from a male/female strain, heterozygotes from a cross of *tra-2(mx)* males by *dpy-18 unc-32* hermaphrodites, and *tra-2(mx)/tra-2(e1095*null) animals from crosses of *tra-2(mx)* males by *tra-2(e1095)/dpy-10* hermaphrodites. All animals were raised at 20 °C. For Fig. 5c, we crossed *tra-2(mx)* males by null mutants in the *fem* genes and observed the heterozygous progeny for enhancement of feminization.

Genome Editing

Mutations were made using gene editing with TALENs (Wood et al. 2011; Wei et al. 2014) or CRISPR (Arribere et al. 2014; Farboud et al. 2019). Specific edits were made using single-strand oligos as repair templates. All solutions were injected into the germline syncytium of young adult hermaphrodites, and F_1 progeny screened for mutations by the PCR on acrylamide minigels, after they had successfully reproduced. Homozygotes were isolated from among their progeny.

Phenotype Assays

To identify hermaphrodites or females, individual L4 larvae were picked onto separate plates and scored as hermaphrodite by the production of eggs, or as female by the absence of eggs and the presence of stacked oocytes in the gonad. If necessary, genotypes were determined by single-worm PCR analysis. To assess dominant suppression of *she-1* female phenotypes, *she-1(v35)* males were crossed with the appropriate double mutant *XX* animals, after which the F_1 were raised and scored at 25 °C. If necessary, genotypes were determined by single-worm PCR analysis after their phenotypes had been determined.

Brood Counts

Individual L4 hermaphrodites were plated at 20 °C and passed to new plates every 8 to 14 h. After transfer, eggs and larvae were counted. Animals that did not survive through the period of sperm exhaustion were not included in the assays.

Sperm Counts

Young adults were stained with DAPI, their germ lines were photographed at four separate focal planes using fluorescent microscopy, and then, sperm were counted.

Quantitative RT-PCR

For *C. briggsae*, groups of five animals of the indicated ages were picked into 2 µl of sterile water in the lid of a microcentrifuge tube and then spun into a mixture of 50 µl of RNAzol (MRC Inc.), 16 µl of water, and 3 µl of precipitation carrier (MRC Inc.). The worms were lysed by freezing at −70 °C, thawing at 65 °C, and 30″ sonication on power 9 with a Misonix cup-horn sonicator. Afterward, we followed the MRC protocol for RNA preparation. The final pellet was resuspended in 20 µl of water, and 10 µl was used for reverse transcription, yielding 20 µl of cDNA. From this total, 1.5 µl of template was used for individual PCRs. The number of cycles was limited so that one-third and one-ninth dilutions of template were clearly distinguishable.

For *C. elegans*, we pushed the technology to study samples from individual worms. The only change we made was to add a proteinase K lysis step (Ly et al. 2015) before proceeding with the RNAzol procedure, as described above. For single animals, we resuspended the RNA in 10 µl and used all of it for reverse transcription.

Yeast Two-Hybrid Assays

The C-terminus of cDNA encoding TRA-1 was cloned into the bait vector pGBKT7 (Promega), to be fused with the GAL4 DNA-binding domain. The C-termini of cDNAs encoding different alleles of TRA-2 were cloned into the prey vector pGADT7 (Promega), to be fused with the GAL4 DNA activation domain. Each insertion was confirmed by DNA sequencing. Yeast transformations were performed using standard protocols (Lundblad 2001) with bait constructs into strain AH109 and prey into strain Y187. Afterward, the transformed AH109 and Y187 strains were crossed to generate progeny that contained both bait and prey vectors, using SD-Trp/-Leu dropout selection. The pGBKT7-p53/pGADT7-SV40-T–antigen pair was used as a positive control and empty bait/prey vector as a negative control. To test TRA-1/TRA-2 interactions, the established strains containing bait and prey were streaked on SD-Trp/-Leu dropout plates and incubated at 30 °C for 2 d, until the clones had grown to 1 to 2 mm^2. Next, the appropriate clones were picked into H_2O and 5 µl of 10^5/µl cells were streaked on SD-Trp/-Leu/-His/-Ade dropout plates and incubated at 30 °C for 3 to 5 d. The end-point was when colonies had grown up for the positive but not negative controls.

For Cbr-TRA-1, we used the C-terminus (residues 661-1146 of TRA-1A), and for Cel-TRA-1 we used the corresponding region (residues 624-1110). For Cbr-TRA-2, we used residues 1102-1496 of TRA-2A, and for Cel-TRA-2, we used residues 1089-1475.

Table of Primers

Cbr-fog-3	**cbr-fog-3RT-F2**	**TTCCACTCGCGTTGGAGAAG**
...	**cbr-fog-3RT-R2**	**CGGATGTTGGCTTGAACGTG**
Cbr-ama-1	**RE1041**	**CGACAACCCACTCTCCATAA**
...	**RE1042**	**GCCAATCGATGAAGATGTCAC**
Cel-fog-3	**RE1033**	**TTTGGCGCTGAACTTGGAAA**
...	**RE1034**	**CATCGCAGTTCACATCTCCA**
Cel-ama-1	**RE1049**	**CCGACTCTCCACAAAATGTCA**
...	**RE1050**	**GGACGGCGCAGAGAGTATC**

Supplementary Material

Supplementary material is available at *Molecular Biology and Evolution* online.

Acknowledgments

The authors thank D. Pilgrim and J. Hodgkin for strains. Additional strains were provided by the CGC, which was funded by the NIH Office of Research Infrastructure Programs (P40 OD010440). The authors also thank NIH Grants R01GM118836 and R01GM121688 for funding and the American Cancer Society for a Postdoctoral Award to support Shin-Yi Lin (126627-PF-15-228-01-DDC).

Data Availability

The data underlying this article are available in the article and in its online supplementary material. Strains are available upon request.

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