OXFORD GENETICS

GENETICS, 2025, 229(4), iyae207
https://doi.org/10.1093/genetics/iyae207
Advance Access Publication Date: 12 December 2024
Investigation

Robust sex determination in the *Caenorhabditis nigoni* germ line

Jonathan P. Harbin,[1] Yongquan Shen,[1] Shin-Yi Lin,[2] Kevin Kemper,[1] Eric S. Haag,[3] Erich M. Schwarz ,[4] Ronald E. Ellis[1,2,*]

[1]Department of Molecular Biology, Rowan-Virtua School of Translational Biomedical Engineering and Sciences, Stratford, NJ 08084, USA
[2]Department of Molecular Biology, Rowan-Virtua School of Osteopathic Medicine, Stratford, NJ 08084, USA
[3]Department of Biology, University of Maryland, College Park, MD 20742, USA
[4]Department of Molecular Biology and Genetics, Cornell University, Ithaca, NY 14853, USA

*Corresponding author: Department of Molecular Biology, Rowan-Virtua School of Translational Biomedical Engineering and Sciences, Rowan-Virtua School of Osteopathic Medicine Cell and Molecular Biology, Rowan University SOM Stratford, B303 Science Center, 2 Medical Center Drive, Stratford, NJ 08084, USA. Email: ellisre@rowan.edu

Sexual characteristics and reproductive systems are dynamic traits in many taxa, but the developmental modifications that allow change and innovation are largely unknown. A leading model for this process is the evolution of self-fertile hermaphrodites from male/female ancestors. However, these studies require direct analysis of sex determination in male/female species, as well as in the hermaphroditic species that are related to them. In *Caenorhabditis* nematodes, this has only become possible recently, with the discovery of new species. Here, we use gene editing to characterize major sex determination genes in *Caenorhabditis nigoni*, a sister to the widely studied hermaphroditic species *Caenorhabditis briggsae*. These 2 species are close enough to mate and form partially fertile hybrids. First, we find that *tra-1* functions as the master regulator of sex in *C. nigoni*, in both the soma and the germ line. Surprisingly, these mutants make only sperm, in contrast to *tra-1* mutants in related hermaphroditic species. Moreover, the *XX* mutants display a unique defect in somatic gonad development that is not seen elsewhere in the genus. Second, the *fem-3* gene acts upstream of *tra-1* in *C. nigoni*, and the mutants are females, unlike in the sister species *C. briggsae*, where they develop as hermaphrodites. This result points to a divergence in the role of *fem-3* in the germ line of these species. Third, *tra-2* encodes a transmembrane receptor that acts upstream of *fem-3* in *C. nigoni*. Outside of the germ line, *tra-2* mutations in all species cause a similar pattern of partial masculinization. However, heterozygosity for *tra-2* does not alter germ cell fates in *C. nigoni*, as it can in sensitized backgrounds of 2 hermaphroditic species of *Caenorhabditis*. Finally, the epistatic relationships point to a simple, linear germline pathway in which *tra-2* regulates *fem-3* which regulates *tra-1*, unlike the more complex relationships seen in hermaphrodite germ cell development. Taking these results together, the regulation of sex determination is more robust and streamlined in the male/female species *C. nigoni* than in related species that make self-fertile hermaphrodites, a conclusion supported by studies of interspecies hybrids using sex determination mutations. Thus, we infer that the origin of self-fertility not only required mutations that activated the spermatogenesis program in *XX* germ lines, but prior to these there must have been mutations that decanalized the sex determination process, allowing for subsequent changes to germ cell fates.

Keywords: sex determination; evolution; *Caenorhabditis*; nematode

Introduction

Evolution of mating systems

Reproduction plays a central role in evolution (Schwander *et al.* 2014). Hence, changes in mating systems not only involve numerous sexual traits, but also shape the future course of evolution. For example, many plant (Pannell 2015) and animal species (Weeks 2012) have undergone transitions from obligate female/male systems to ones involving self-fertile hermaphrodites and males. Such changes in mating systems influence many other traits, such as sex ratios (Van Goor *et al.* 2021), outcrossing (Cutter *et al.* 2019), dispersal (Trochet *et al.* 2016), sperm storage (Orr and Brennan 2015), and genome size (Fierst *et al.* 2015; Yin *et al.* 2018).

The importance of these evolutionary transitions has led to a focus on the genetic mechanisms that underlie them in certain groups, including plants of the Brassicaceae (Vekemans *et al.* 2014) and *Caenorhabditis* nematodes (Ellis and Lin 2014; Ellis 2017). In *Caenorhabditis*, 3 species independently evolved self-fertility (Fig. 1a), providing rich opportunities for study and comparison (Kiontke *et al.* 2011).

Self-fertile hermaphrodites

Caenorhabditis hermaphrodites are essentially XX females that have been altered to produce, store, and use sperm (Fig. 1b, Ellis and Lin 2014; Ellis 2017). Since they lack other male somatic traits, they can only self-fertilize or mate with males. This genetic system is particularly easy to work with in the laboratory (Brenner 1974), leading to the wide adoption of *Caenorhabditis elegans* as a model for studying developmental biology and neurobiology.

Received on 19 September 2024; accepted on 08 November 2024

Although there are numerous differences between XX females and XX hermaphrodites in this genus, such as genome size and attraction to mates, the initial transformation to self-fertility might have involved as few as 2 genes, one controlling the sex determination of germ cells and the other controlling sperm activation (Baldi *et al.* 2009). The ability of hermaphrodites to activate their own sperm, allowing self-fertilization, was shown to require the co-option of one of the redundant activation signals used by males (Wei *et al.* 2014b). The other change appears more complex, involving alterations to the regulatory pathway that controls sex determination in the germ line (Ellis 2022).

Nematode sex determination

In *Caenorhabditis* nematodes, sex is determined by a signal transduction pathway that responds to the ratio of *X* chromosomes to autosomes (Meyer 2022). In *C. elegans*, this pathway acts through HER-1, a secreted protein that promotes male fates in both the germ line and soma (Hodgkin 1980; Hunter and Wood 1992; Perry *et al.* 1993), with the ultimate target being the Gli transcription factor TRA-1 (Zarkower and Hodgkin 1992). In *XO* animals, HER-1 binds to and inactivates the TRA-2 receptor, preventing its cleavage by TRA-3 (reviewed by Zarkower 2006). This allows a complex of 3 FEM proteins to work with a cullin to ubiquitinate TRA-1, targeting it for destruction (Starostina *et al.* 2007). As a result, TRA-1 repressor is not made, and male genes can be expressed, leading to male development. By contrast, in XX animals TRA-1 is not degraded, but is instead cleaved (Schvarzstein and Spence 2006) to form a repressor of male genes throughout the body (Berkseth *et al.* 2013) and germ line (Chen and Ellis 2000).

Modifications to genetic pathways in the hermaphrodite germ line

Regulation in the germ line is more complex, probably because XX animals need to produce both sperm and oocytes in a female body (Fig. 1c and d, reviewed by Ellis 2022). Although the core pathway is the same as in the soma, several additional genes are involved, including novel ones like *fog-2* in *C. elegans* (Schedl and Kimble 1988; Clifford *et al.* 2000; Nayak *et al.* 2005) and *she-1* in *Caenorhabditis briggsae* (Guo *et al.* 2009), which were each formed by gene duplication and divergence.

Perhaps because the regulatory pathway has been adapted for self-fertility, mutations in some of the core sex determination genes produce complex or intersexual phenotypes.

tra-2

Two aspects of *tra-2* mutants are particularly striking. First, the decision of germ cells to form sperm or oocytes in *C. elegans* and *C. briggsae* responds very strongly to small changes in *tra-2* activity. For example, gain-of-function mutations in the *C. elegans tra-2* 3′-UTR cause all XX germ cells to develop as oocytes (Doniach 1986; Goodwin *et al.* 1993). Furthermore, mutations that prevent TRA-2 from binding TRA-1 cause XX animals to make only oocytes in *C. elegans* (Doniach 1986; Lum *et al.* 2000; Wang and Kimble 2001) but extra sperm in *C. briggsae* (Shen *et al.* 2024a). Moreover, mutations in *fog-2* that create a female/male population of *C. elegans* are often suppressed by haploinsufficiency for *tra-2*, and even by weak, cryptic *tra-2* mutations (Schedl and Kimble 1988; Hu *et al.* 2019). Similarly, *she-1* mutations that create a female/male population of *C. briggsae* are strongly suppressed by haploinsufficiency for *tra-2* (Guo *et al.* 2009).

Second, the somatic effects of *tra-2* are complex. Most of the soma and all of the germ cells undergo male development in *C. elegans* and *C. briggsae tra-2* null mutants (Hodgkin and Brenner 1977; Kelleher *et al.* 2008). However, in both species these XX mutants make defective male tails and are not capable of mating with hermaphrodites.

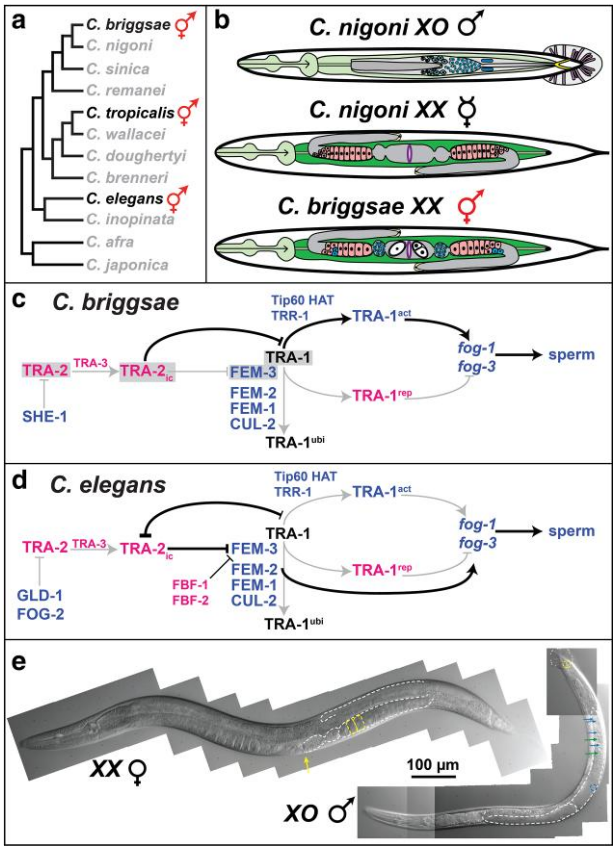

Fig. 1. Evolution of mating systems in *Caenorhabditis*. a) Phylogeny of the *elegans* group of *Caenorhabditis*, based on work by Kiontke *et al.* (2011). Gray font indicates male/female species. b) Schematic diagrams of the sexes discussed in this paper. Males make sperm that remain inactive until mating (small blue cells). Virgin females only make oocytes, which they retain until mating (large pink cells). Hermaphrodites resemble females but produce sperm in larval development, which they activate and store for use in fertilizing oocytes they make as adults (embryos in the uterus are white). c) Outline of gene regulation in the *C. briggsae* germ line. CAPITALS indicate protein names and *lowercase italics* gene names. Items in blue generally promote spermatogenesis and those in red oogenesis. Black lines denote interactions that favor L4 spermatogenesis, and gray lines denote ones that favor oogenesis. Arrows indicate positive regulation and "—|" negative regulation. For details, see text. d) Outline of gene regulation in *C. elegans*. e) DIC photomicrographs of a *C. nigoni* JU1422 female (left) and male (right). Visible portions of the posterior arm of the gonad are outlined with dotted white lines in the female and of the whole gonad in the male. Two oocytes are outlined in yellow, and one primary spermatocyte in blue. A yellow arrow marks the vulva, green arrows mark residual bodies, and blue arrows mark sperm. White dots indicate the 14 out of 18 rays visible in this plane of focus, and a yellow oval marks the spicules.

fem-3

In *C. elegans*, *fem-3* acts upstream of *tra-1* in somatic development but is also required downstream of *tra-1* to control germ cell fates (Hodgkin 1986; Chen and Ellis 2000). By contrast, *C. briggsae fem-3* is not required for spermatogenesis, and both XX and XO *fem-3* mutants are hermaphrodites (Hill *et al.* 2006). However, loss of *fem-3* function is nonetheless partially epistatic to *tra-2* mutations in the germ line (Hill *et al.* 2006) and increases the likelihood that *tra-1* mutants make oocytes (Hill and Haag 2009).

tra-1

In both *C. elegans* and *C. briggsae*, *tra-1* mutations often cause animals to produce sperm and then switch to oogenesis (Hodgkin 1987; Schedl *et al.* 1989; Kelleher *et al.* 2008). This is true for both XX animals (which normally make sperm and oocytes) and XO males (which normally make only sperm). Thus, *tra-1* appears necessary to sustain spermatogenesis, even though its main role is to prevent male gene expression.

These traits might reflect an ancient propensity for the pathway to switch from one sexual state to the other, which could have been a precondition for the evolution of self-fertility in this genus. On the other hand, the ancestral pathway might have been robust, and these unusual phenotypes might have been caused by the modifications that were needed to produce self-fertility.

C. nigoni is the ideal species for studying male/female nematodes

Addressing these questions about how sex determination worked in the male/female ancestor of *Caenorhabditis* requires studying the pathway in a male/female species from the genus. The ideal place to begin is with *C. nigoni*, an XX female/XO male species (Kiontke *et al.* 2011). These animals are so closely related to *C. briggsae* that they can mate with them and produce fertile hybrids (Woodruff *et al.* 2010). Thus, studying *C. nigoni* should provide a window into what the sex determination pathway might have looked like before *C. briggsae* began the path to self-fertility. Finally, the XX hybrids develop as females and so provide a method for testing theories about the control of sex determination in each parent species.

Materials and methods

Strains

We used *C. nigoni* inbred strains JU1422 (Félix *et al.* 2014) and CP168 (below), as well as: *LGII: tra-2(v498)*, *unc-104(v494)*, *dpy-10(v541)*; *LGIII: tra-1(v481)*, *tra-1(v538)*, *dpy-18(v484)*; *LGIV: fem-3(v496)*, *fem-3(v542)*, *fem-3(v395gf)*, *unc-129(v495)*, *unc-33(v543)*. All mutations were first described here except for *dpy-18(v484)* (Harbin and Ellis 2023).

For *C. briggsae*, we used the wild-type strain AF16 (Fodor *et al.* 1983) and the mutations *tra-1(v181) III* (Shen *et al.* 2024b), *tra-2(v440) IV* (Shen *et al.* 2024), *tra-2(v417ts) IV* (this paper), *fem-3(nm63) IV* (Hill *et al.* 2006), and *unc-7(v271) X* (Wei *et al.* 2014a).

Genetics

Unless stated otherwise, worms were raised at 25°C.

Microscopy

Differential interference contrast (DIC) microscopy was performed by standard methods (Wood 1988). Images were captured with a Zeiss Axiocam digital camera and AxioVision 4.8 software.

Single-worm PCR and RT-PCR analyses

For genotyping, individual worms were picked into 2.5 µl worm lysis buffer (Fay and Bender 2008), frozen at –80°C, and incubated at 65°C for 1 h to release the DNA. When multiple reactions had to be run from the same template, additional lysis buffer was used to harvest the animal, and the sample split into fractions after incubation. Finally, PCR reactions were run in standard conditions using hot-start Taq polymerase (New England Biolabs). Individual worms were analyzed by RT-PCR as described by Ly *et al.* (2015). Primers are listed in Table 1.

Production of CP168

The *C. nigoni* wild isolate EG5268 was originally isolated by Joel Ehrenkranz, from human-disturbed soil in 2008 in Shinkolobwe, Katanga Province, Democratic Republic of the Congo, and was obtained from the *Caenorhabditis* Genetics Center at the University of Minnesota (https://cgc.umn.edu/strain/EG5268). It was inbred as follows: 3 individual female adults that had already mated (as judged by the presence of a copulatory plug and embryos) were used to establish 3 isofemale lines on 2.2% agar NGM plates. The

Table 1. Primers used in these experiments.

Use	Primer name	Sequence
Genotyping *Cbr-tra-2(v440)*	JH9-Cbr-Tra2-E-F	AAAGAAGCCAGAAGCAGACC
	JH10-Cbr-Tra2-E-R	GTGTTCCTTCAAGAAATGGAGC
Genotyping *Cni-tra-2*	JH65-Cni-tra-2-F	TGGAGCAATGAAGTTGGCAT
	JH66-Cni-tra-2-R	AATAGAGAAGCCAGAAGCAGACC
Genotyping *Cni-dpy-10*	Cni-dpy-10-F	AACTATTCGCGTCAGATGACGTA
	Cni-dpy-10-R	TTGAGTGGACAGGTCTGATTTGG
Genotyping *Cni-unc-104*	JH71-Cni-unc-104-F	AAAACCCAGGGATGGACTCG
	JH72-Cni-unc-104-R	ACGTATTCGGAACCTTGACCA
Genotyping *Cni-tra-1(v481)*	JH26-Cni-tra1-F	CTATCTGGCACCACCACAT
	JH27-Cni-tra1-R	GCATCACACAGAGGGAGTAT
Genotyping *Cni-tra-1(v538)*	JH116-Cni-tra-1.1 F	GCCTTGTTCCCAATAGCTTG
	JH117-Cni-tra-1.1 R	TTGATGGCTCAGCTGTTGAA
Genotyping *Cni-dpy-18*	JH34-Cni-dpy18-F	TAGCACTTCTCGTGCTGGC
	JH35-Cni-dpy18-R	GTTGCTGCATATCCGCAATCG
Genotyping *Cni-fem-3*	JH75-Cni-fem-3-F	TACCGGATGATGTGGAACCC
	JH76-Cni-fem-3-R	TCCAGGCCATTTATTCCCCC
Genotyping *Cni-unc-129*	JH78-Cni-unc-129-F	GACGACTCCCAATCGTCTCG
	JH79-Cni-unc-129-R	ACTCACTTGGATCCGAACTCT
Genotyping *Cni-unc-33*	JH128-Cni-unc-33 F	GATGCGTCTGGAAAACTTCT
	JH129-Cni-unc-33 R	GCATCCAGTTGAAATATCGTC
Identifying JU1422 vs CP168 X chromosomes	JH112-Cni-SNPX1 F	AAGATCAACTTCCTGCAAGC
	JH113-Cni-SNPX1 R	TCAGAGCTCATACATTCCCA
Identifying Cni vs Cbr DNA	JH114-Cni-SNPX2 F	TGATTTCGTGTGGTGCTCTA
	JH115-Cni-SNPX2 R	ACGGGTTTTTCCAAATCACA
Cni-her-1 RT-PCR	JH88-Her-1.2 F	CGTTACGAATGTTGTATGGATATGAT
	JH89-Her-1.2 R	AATTGCATGCAACAGGAACT

number of sires of was unknown. After 2 to 3 generations of growth, a single mated female was again chosen to found the next plate. This process was continued for 10 rounds of single-female bottlenecks. All 3 lines persisted, and 1 was chosen to be CP168.

Yeast two-hybrid analysis

Clones were constructed and tested as described by Shen *et al.* (2024a) using pGBKT7 as a bait vector and pGADT7 as a prey vector.

Genome analysis

High-molecular-weight genomic DNA was extracted from a mixed-stage culture of CP168 using a conventional proteinase K/phenol–chloroform procedure, with modifications to prevent shearing. These were described in detail by Yin *et al.* (2018). CP168 genomic DNA was sequenced in paired-end 2 × 150-nt Illumina reads by Novogene USA; separate runs were pooled into a single set of read pairs. Pooled reads were quality-filtered and trimmed with fastp 0.23.1 (Chen 2023) using the arguments "*–length_required 75 –max_len1 150 –detect_adapter_for_pe –dedup –merge –correction*." This yielded a filtered set of paired-end, merged, and unpaired reads with a genome coverage of 26.3×. The fastp-filtered and merged reads were assembled with SPAdes 3.15.3 (Prjibelski *et al.* 2020) using the arguments "*–isolate –pe1-1 [pair-end 1 read file] –pe1-2 [pair-end read 2 file] –pe1-m [merged read file] –pe1-s [unpaired read file]*." Reference-based chromosomal scaffolding of the SPAdes contigs was done with RagTag 2.1.0 (Alonge *et al.* 2022) using the arguments "*scaffold –r*" and using the *C. nigoni* JU1422 assembly (Yin *et al.* 2018) as a reference genome. Protein-coding gene annotations were lifted over from the JU1422 genome assembly to the final CP168 genome assembly with LiftOff 1.6.2 (Shumate and Salzberg 2021) using the arguments "*-copies -polish –cds*." Protein and DNA sequences were extracted from the lifted-over gene annotations and the final CP168 genome assembly with gffread 0.12.7 (Pertea and Pertea 2020) using the arguments "*-W -C -x [CDS DNA FASTA file] -y [translated protein FASTA file] -o/dev/null*."

Results

Isolation of *C. nigoni* sex determination mutants

To learn how sex determination is regulated in male/female species of nematodes, we identified the *C. nigoni* orthologs of 3 critical sex determination genes known from the male/hermaphrodite species *C. elegans* and *C. briggsae* (Supplementary Figs. 1–3, Ellis 2022) and used gene editing to make null alleles in each (Harbin and Ellis 2023). Because we suspected these mutations could not be maintained as homozygous strains, we also made mutations in nearby marker genes that could be used as genetic balancers (Wei *et al.* 2014a).

C. nigoni tra-1 promotes female development in both the soma and germ line

TRA-1 is a Gli transcription factor with 5 conserved zinc fingers, and directly controls the expression of hundreds of genes responsible for male development in *C. elegans* (Fig. 2a, Berkseth *et al.* 2013). To produce *C. nigoni tra-1* null mutations, we made frameshift alleles just before or after the zinc fingers. Similar mutations behave as null alleles in *C. elegans* and *C. briggsae* (Hodgkin and Brenner 1977; Hodgkin 1987; Zarkower and Hodgkin 1992; Kelleher *et al.* 2008).

We began by studying the *C. nigoni* strain JU1422, which had been produced from the wild isolate JU1325 by 25 generations of inbreeding (Félix *et al.* 2014). After injecting gravid females with CRISPR/Cas9/sgRNA solutions (Harbin and Ellis 2023), we identified a *tra-1* mutation among the progeny by PCR analysis. Because this mutation could not reproduce as a homozygous strain, we used *dpy-18* (Harbin and Ellis 2023) to balance it (Fig. 2b). This allele, *cni-tra-1(v481)*, causes a frame shift and truncation shortly after the zinc fingers (Supplementary Fig. 1).

To analyze homozygous *tra-1* mutants, we studied the progeny produced by our balanced strain and then determined which were *tra-1* XX or XO animals. First, we used PCR to determine genotype and then single-animal RT-PCR analysis to determine karyotype. Individual XX animals had low *her-1* transcript levels, and XO animals had high levels (Figs. 2c and d, Trent *et al.* 1991; Ly *et al.* 2015). The *tra-1* XX animals developed male bodies with fully developed male tails that included 18 rays and 2 spicules (Fig. 2c). However, their gonads failed to develop (*N* = 28/28). By contrast, the homozygous *tra-1* XO animals developed as normal males with mature gonads and made sperm throughout their lives (*N* = 17/17 5-to-7-day-old adults, 25°C). These results suggest that *C. nigoni* TRA-1 is not required to sustain spermatogenesis, unlike TRA-1 in *C. elegans* (Hodgkin 1987; Schedl *et al.* 1989) or *C. briggsae* (Kelleher *et al.* 2008).

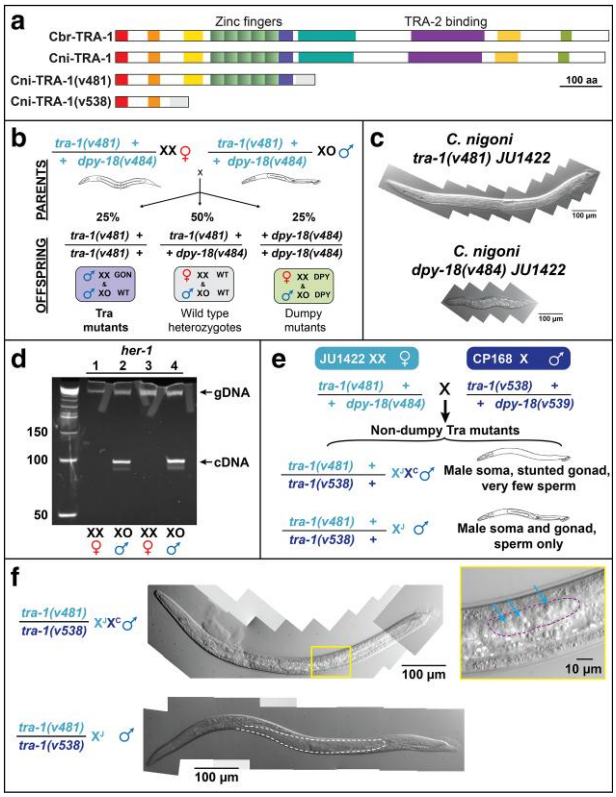

Fig. 2. *C. nigoni tra-1* mutations transform XX animals into males with underdeveloped gonads. a) Comparison of *C. briggsae* and *C. nigoni* TRA-1 proteins, showing regions of high homology as colored domains. The *C. nigoni* TRA-1 mutant proteins are indicated below, with gray highlighting sequences after the frameshift but before the stop codon. b) Mating strategy to generate homozygous mutants from a balanced strain. Light blue indicates JU1422 sequences. c) DIC photomicrographs of: (top) a *tra-1(v481)* XX animal displaying typical male soma with tiny gonad and (bottom) *dpy-18(v484)* homozygote. Anterior is left and ventral up. d) Single-worm RT-PCR analysis to identify karyotype by the level of *her-1* expression. e) Interstrain cross to produce *tra-1* null mutants in a uniform JU1422/CP168 background. Dark blue marks CP168 sequences. f) Phenotypes of *tra-1* mutants in interstrain hybrids. XX animals are males with small gonads (top); the few germ cells develop as sperm (blue arrows, inset). XO animals are normal males (bottom). Anterior is left and ventral up.

The gonad problems in these *tra-1 XX* mutants were unexpected. However, male/female species of *Caenorhabditis* have a high degree of standing genetic variation (e.g. Dey *et al.* 2013), and the inbreeding of large haplotypes required to make a mutation homozygous in a new strain can produce artificial phenotypes unrelated to the focal mutation itself (Yin *et al.* 2018). We thus wondered if this phenotype had been influenced by the genetic background of the inbred JU1422 strain. To address this concern, we made another inbred *C. nigoni* strain with a different genetic origin, CP168, for comparative analysis. We then assembled and annotated a draft genome of CP168 to facilitate the design of guide RNAs and primers.

Finally, we made a new *tra-1* null allele in this strain, *Cni-tra-1(v538)*, which causes a frame shift and a truncation before the zinc fingers (Supplementary Fig. 1). Once again, we saw that *tra-1 XX* mutants had male bodies ($N = 32/32$), but that their gonads failed to develop normally. However, these *XX tra-1* gonads appeared slightly larger than in the JU1422 genetic background (Supplementary Fig. 4). The *tra-1 XO* animals were normal males and made only sperm ($N = 14/14$, 6-day-old adults, 25°C).

To confirm our identification of the *tra-1* null phenotype, we studied JU1422/CP168 interstrain hybrids that were heterozygous for these 2 null alleles (Fig. 2e). This approach provides a uniform genetic background without the potential complication of homozygous recessive mutations from inbreeding. Furthermore, it allowed us to determine karyotypes with a simple PCR test (*e.g.* Fig. 3c). In these hybrids, the *tra-1 XX* animals still developed male bodies with defective gonads ($N = 8/8$). However, these gonads were slightly larger than in either JU1422 or CP168 and occasionally produced sperm (Fig. 2f). These rare sperm in the mutants show that *tra-1* normally promotes oogenesis as well as female somatic fates in *C. nigoni XX* animals.

By contrast, the hybrid *XO* animals were normal males that made only sperm (5/5, 7-day-old adults, 25°C), as we had observed for *tra-1(v481)* and *tra-1(v538)* homozygous *XO* mutants. Thus, *tra-1* is not needed in *C. nigoni XO* animals to sustain spermatogenesis. This result suggests that the role of TRA-1 in determining germ cell fates differs between the female/male species *C. nigoni* and the 2 hermaphrodite/male species studied previously.

C. nigoni fem-3 promotes male development in both soma and germ line

TRA-1's stability is controlled by a ubiquitin ligase complex that includes 3 FEM proteins and CUL-2 (Starostina *et al.* 2007). We selected FEM-3 for detailed analysis because it is essential for the complex to function and does not have pleiotropic activities like FEM-2. To work with *C. nigoni fem-3(null)* mutants, we used *unc-129* as a balancer in the JU1422 background, and *unc-33* in the CP168 background. The *unc-129* mutants were extremely sick when homozygous, producing mainly dead larvae. The *unc-33* homozygotes were healthier and often survived to adulthood, but the females did not mate efficiently.

Both the JU1422 *fem-3(v496)* mutation and the CP168 *fem-3(v542)* mutation are early frameshifts that eliminate most of the protein (Fig. 3a; Supplementary Fig. 2). Furthermore, both mutations completely transformed *XO* individuals into females ($N = 19/19$), but did not affect *XX* animals ($N = 31/31$) (Supplementary Fig. 5). We confirmed these phenotypes by studying interstrain hybrids (Fig. 3b) and identified the genotypes and karyotypes of individual animals using PCR (Fig. 3c).

The *fem-3* mutants not only developed female bodies, but even *fem-3 XO* animals produced oocytes throughout their lives

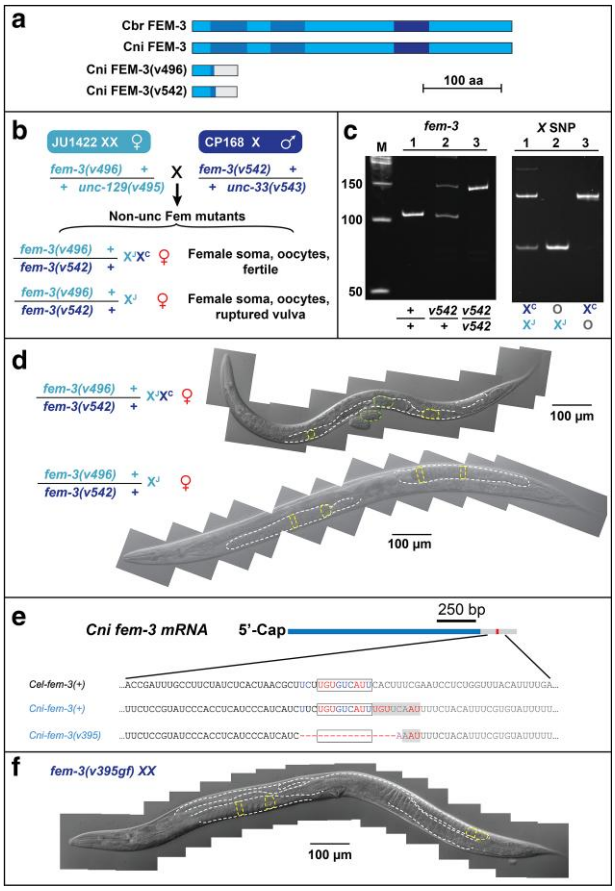

Fig. 3. *C. nigoni* FEM-3 directs male development in both the germ line and soma. a) Diagram comparing wild-type *C. nigoni* and *C. briggsae* FEM-3 proteins. Darker shades of blue indicate higher levels of conservation. The sizes of both *C. nigoni* mutant proteins are indicated below, with gray highlighting sequences after the frameshift but before the stop codon. b) Cross for producing interstrain hybrid mutants shown below. c) PCR tests to confirm *fem-3* genotype (left) and karyotype (right). d) Phenotypes of *fem-3* mutants in interstrain hybrids. *XX* animals are normal females (top). Anterior is left and ventral is down. *XO* animals are females, but do not produce progeny in crosses (bottom). Anterior is left and ventral is facing up. e) Identification of conserved PUF regulatory sites in the 3′-UTR of *C. nigoni fem-3* mRNAs. A gray box highlights the site first defined in *C. elegans* (Ahringer and Kimble 1991; Bernstein *et al.* 2005) and gray shading a duplicate site located adjacent to it in *C. nigoni*. Critical residues are in red. These two sites are destroyed by the *cni-fem-3(v395)* mutation. f) The *fem-3(v395) XX* animals develop as normal females. Anterior is left and ventral down.

($N = 11/11$, 7-day-old adults, 25°C). By contrast, in the sister species *C. briggsae*, *fem-3 XX* and *XO* animals are self-fertile hermaphrodites (Hill *et al.* 2006). Thus, *fem-3* completely controls germ cell fates in *C. nigoni*, whereas it is dispensable for spermatogenesis in *C. briggsae*.

We tested young adult females for their ability to reproduce by mating individual animals with 3 wild-type males from strain *JU1422*. Although the homozygous *XX* animals could mate and produce progeny, the homozygous *XO* animals did not produce offspring in male/female crosses ($N = 29/29$), despite developing as females. This fertility problem resembles that of *C. briggsae fem-3 XO* mutants (Hill *et al.* 2006) and of *C. elegans her-1 XO* mutants (Hodgkin 1980), which both produce tiny self-broods. Thus, we suspect that the lack of progeny from these *C. nigoni fem-3 XO* animals reflects the harmful effects of an *XO* karyotype on the expression of oogenesis genes (Strome *et al.* 2014).

While studying *C. nigoni fem-3*, we identified a conserved site in its 3′-UTR, which is orthologous to the FBF binding site in *C. elegans fem-3* (Ahringer and Kimble 1991; Zhang *et al.* 1997; Bernstein *et al.* 2005). In *C. elegans*, mutations at this site cause *XX* animals to make sperm instead of oocytes (Barton *et al.* 1987; Ahringer and Kimble 1991), so we used gene editing to remove it (Fig. 3e). Despite this change, the *Cni-fem-3(v395)* mutants developed as *XX* females (Fig. 3f) and *XO* males. The females made only oocytes and did not display any signs of incipient spermatogenesis, such as laying unfertilized oocytes ($N = 14/14$). Thus, this regulatory site is conserved in sequence, but appears to have different functions in *C. nigoni* and *C. elegans*.

C. nigoni tra-2 promotes female development in soma and germ line

The activities of both FEM-3 and TRA-1 are regulated by binding a fragment of the TRA-2 receptor, which is related to Patched (Kuwabara *et al.* 1992; Kagawa *et al.* 2011). Thus, the final gene we targeted was *Cni-tra-2*. We made 2 alleles in the JU1422 background, *v498* and *v499* (Fig. 4a; Supplementary Fig. 3). Since both cause an early frameshift and truncation that removes most of the protein, we focused our analyses on *v498*. In the sister species *C. briggsae*, a similar mutation behaves as if it causes a complete loss of function (Shen *et al.* 2024a).

To balance *tra-2(v498)*, we generated *Cni-unc-104(v494)* in the JU1422 background. UNC-104 is a kinesin involved in synaptic vesicle transport within axons (Hall and Hedgecock 1991), and the *cni-unc-104* mutants often coiled or arrested in early larval development. Finally, the 2 lines were crossed to create the balanced *JU1422* strain: *Cni-tra-2(v498) + / + Cni-unc-104(v494)*. We also backcrossed *tra-2(v498)* 10 times into the CP168 genetic background. To balance it, we generated a recessive mutation of *C. nigoni dpy-10* in CP168.

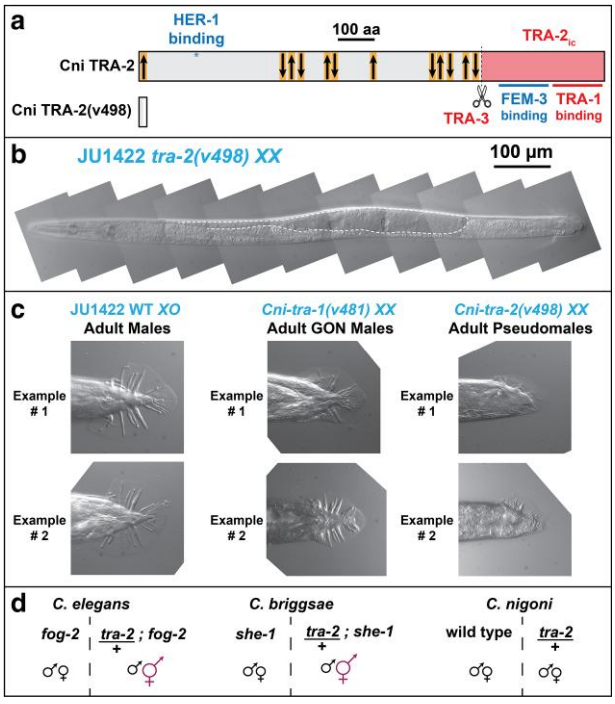

Fig. 4. *C. nigoni* TRA-2 directs female development in both germ line and soma. a) Diagram showing wild-type and mutant *C. nigoni* TRA-2, with white highlighting sequences after the frameshift but before the stop codon. Arrows indicate predicted transmembrane domains, with up indicating those pointing out of the cell. b) DIC photomicrograph of *C. nigoni tra-2(v498) XX* pseudomale. Anterior is left and ventral facing out. The dotted line outlines the visible part of the male gonad. c) DIC photomicrographs of male tails in wild-type *XO* and *tra-1 XX* males and defective tails in *tra-2 XX* mutants, which have abnormal or missing rays. d) Haploinsufficiency for *tra-2* induces spermatogenesis in *XX* females for *C. elegans fog-2* and *C. briggsae she-1* mutants, but not for *C. nigoni* strains.

Finally, we studied *C. nigoni tra-2(v498)* in the *JU1422* background (Fig. 4b), the CP168 background, and hybrids. In all cases, it partially masculinized somatic tissues and completely masculinized the germ cells of *XX* animals ($N = 33/33$, 19/19, and 5/5, respectively). The partial masculinization was restricted to the tail, which had reduced or incomplete male rays, a smaller fan and stubbier tail section than in *XO* males. Indeed, these stunted tails resembled those of *C. elegans* and *C. briggsae tra-2 XX* mutants (Fig. 4c). Because of these defects, *tra-2 XX* animals cannot mate and are referred to as pseudomales (Hodgkin and Brenner 1977). By contrast, the *C. nigoni tra-2 XO* mutants were normal males. Thus, *C. nigoni* TRA-2 promotes female cell fates, but does so imperfectly in the tail. We infer that some aspects of *tra-1* activity in the tail do not require *tra-2*.

Although the germ line is completely masculinized in *C. nigoni tra-2 XX* homozygotes, we saw no masculinization in *tra-2* heterozygotes (Fig. 4d). By contrast, when *C. elegans* or *C. briggsae XX* animals are converted into true females by the loss of *fog-2* or *she-1*, respectively, heterozygosity for *tra-2* restores spermatogenesis (Schedl and Kimble 1988; Guo *et al.* 2009). Finally, we tested *C. nigoni tra-2* heterozygotes for signs of partial masculinization of the germ line by seeing if any young adult virgin females laid unfertilized oocytes, which would indicate the presence of sperm proteins even in the absence of functional sperm, but saw no effect ($N = 15/15$).

Epistasis tests show that sex determination is the same in the *C. nigoni* soma and germ line

Our results with *tra-1*, *fem-3*, and *tra-2* suggested that control of sex determination in the germ line is more robust in *C. nigoni* than in either of the hermaphroditic species. To test this idea, we examined double mutants, since some double mutants have differing effects on germ cells and the soma in the 2 hermaphroditic species (Hodgkin 1986; Hill *et al.* 2006).

The fact that *C. nigoni* is a female/male species meant that we could not maintain homozygous double mutant strains. Furthermore, making strains in which 2 sex determination genes were present and both balanced by marker mutations would be difficult. Thus, we set up crosses between animals heterozygous for 2 sex determination genes and then studied their progeny by DIC microscopy (Fig. 5). Afterward, we determined the genotype and karyotype of each animal by PCR. To simplify karyotyping, we used the *JU1422* strain to introduce one gene into the cross and CP168 to introduce the other gene. This procedure only allowed us to distinguish *XO* and *XX* animals among half of the progeny, and the others remained ambiguous.

First, we studied *tra-1; fem-3* double mutants. In *C. elegans*, these animals develop male bodies but produce only oocytes (Hodgkin 1986). In *C. briggsae*, they develop male bodies and usually make both sperm and oocytes (Hill *et al.* 2006). However, in *C. nigoni* they formed male bodies that only made sperm (Fig. 5a and b). Since the *fem-3* mutation did not rescue gonad development in the *XX* double mutants, we saw both *tra-1; fem-3* animals with small gonads and sperm (presumed to be XX) and *tra-1; fem-3* animals with normal male gonads and sperm (some presumed and some proven to be XO, Fig. 5b). Thus, *tra-1* is completely epistatic to *fem-3* in both the soma and germ line of

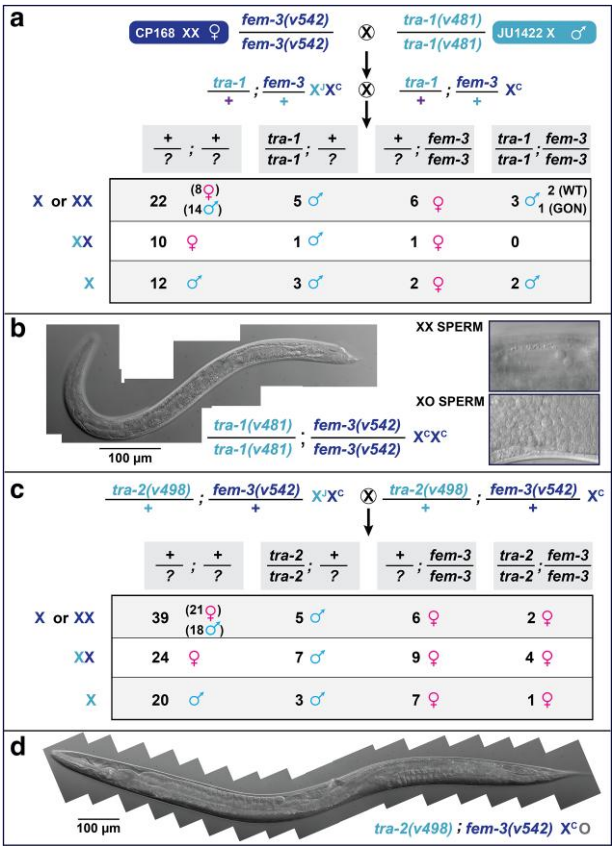

Fig. 5. In *C. nigoni*, sex determination occurs in a linear regulatory pathway. a) Cross showing how *tra-1; fem-3* double mutants were generated, and the sexual phenotypes of all the progeny. b) DIC photomicrograph of a *tra-1; fem-3 XX* double mutant, showing normal male development and spermatogenesis. Anterior is left and ventral up. The karyotype was inferred from the observation that the animal lacks a gonad. Expanded views of spermatogenesis in both *tra-1; fem-3 XX* and *XO* animals (right). c) Cross showing how *tra-2; fem-3* double mutants were generated, and the sexual phenotypes of all the progeny. d) DIC photomicrograph of a *tra-2; fem-3* double mutant, showing normal female development and a gonad populated only with oocytes. Anterior is left and ventral up.

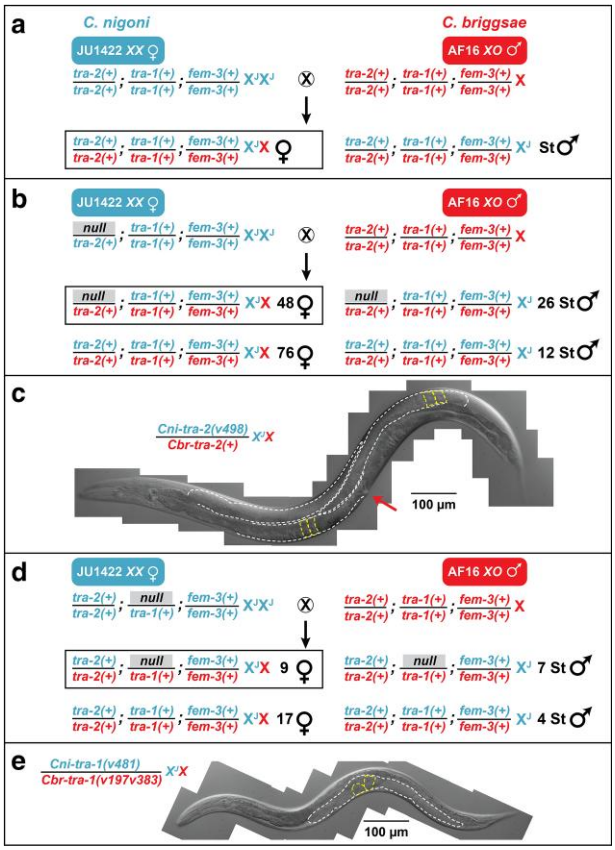

Fig. 6. *C. nigoni/C. briggsae XX* hybrids have robust sex determination in germ cells. a) Summary of crosses that produce intraspecies hybrids (Woodruff *et al.* 2010). Genes derived from *C. nigoni* are in blue and those from *C. briggsae* in red. "St" denotes sterile. b) Diagram of crosses that produce intraspecies hybrids lacking a functional *C. nigoni tra-2* allele. c) DIC photomicrograph of a hybrid *XX* animal lacking *C. nigoni tra-2*. Note that it develops a female body and female gonad (outlined in white) and produces oocytes (four outlined in yellow). The red arrow indicates the vulva. Anterior is left and ventral down. d) Diagram of crosses that produce intraspecies hybrids lacking a functional *C. nigoni tra-1* allele. e) DIC photomicrograph of a hybrid *XX* animal that is *Cni-tra-1(null)/Cbr-tra-1(v197 v383)*. Note that it develops a female body with an abnormally shaped gonad (outlined in white) but does produce 2 oocytes (outlined in yellow). Anterior is left and ventral down.

C. nigoni. This relationship differs from that seen in both of the hermaphroditic species.

Next, we studied *tra-2; fem-3* double mutants. Although *fem-3* is completely epistatic to *tra-2* in *C. elegans* (Hodgkin 1986), the *tra-2; fem-3* double mutants are self-fertile hermaphrodites in *C. briggsae* (Hill *et al.* 2006), which is hard to interpret on its own. However, further tests showed that *C. briggsae she-1 fem-3 XX* animals are female, but *tra-2; she-1 fem-3* animals are self-fertile hermaphrodites (Guo *et al.* 2009; Shen *et al.* 2024a), suggesting that *tra-2* acts downstream of *she-1* and *fem-3* in the germ line.

When we studied *C. nigoni*, we saw that *fem-3* was completely epistatic to *tra-2* in both the soma and the germ line (Fig. 5c and d). This result was true for both *XX* and *XO* double mutants. Thus, in *C. nigoni*, it appears that TRA-2 acts through FEM-3 to control germ cell fates. By contrast, in *C. briggsae*, the critical step is the direct interaction of TRA-2 with TRA-1 (Shen *et al.* 2024a). Putting these results together, it appears that sex determination in the *C. nigoni* germ line is not only robust, but that the critical interactions occur in a simple linear pathway.

Heterozygosity for *C. nigoni* mutations does not affect germ cells in interspecies hybrids

A simple explanation for the robust regulation of sexual development we observe in *C. nigoni* would be that TRA-1 or TRA-2 activity is very high in *XX* animals relative to that of FEM-3 and its partners, but very low in *XO* animals. To probe this idea, we studied the effects of heterozygosity for *tra-1* or *tra-2* in interspecies hybrids between *C. nigoni* and *C. briggsae*. Normally, these *XX* animals make only oocytes, just like *C. nigoni XX* animals (Fig. 6a; Woodruff *et al.* 2010). We wondered if high TRA-2 or TRA-1 activity from the *C. nigoni* genes was the reason these *XX* animals only produce oocytes.

First, we crossed *C. briggsae* wild-type *XO* males with *C. nigoni tra-2/+* females, studied their progeny, and used PCR to confirm the genotype and karyotype of each hybrid. Both the *+/+* and the *Cni-tra-2/+ XX* animals were female ($N = 78/78$ and 48/48, respectively, Fig. 6b and c). As expected, those *XO* animals that survived were sterile males. Thus, even hybrids that only express *C. briggsae tra-2* make no sperm and develop as females. This result implies that high *C. nigoni* TRA-2 levels are not the only factor causing oogenesis in the interspecies hybrids.

Second, we crossed *C. briggsae* XO males with *C. nigoni tra-1*/+ females. Here too, we saw that both the +/+ and *Cni-tra-1*/+ XX animals were female ($N = 17/17$ and 9/9, respectively, Fig. 6d). Again, the XO animals that survived were sterile males. Thus, hybrids that express only *C. briggsae tra-1* are also female and do not make sperm. Since *tra-1* acts at the end of the sex determination pathway, this result implies that upstream factors from *C. nigoni* are able to drive oogenesis in the hybrid XX animals, by acting through *C. briggsae tra-1*.

Since these results implied that *C. nigoni* TRA-1 should be able to interact with *C. briggsae* TRA-2, and vice versa, we tested protein interactions for both *C. nigoni* and for cross-species binding using the yeast two-hybrid system. We detected TRA-1/TRA-2 and TRA-2/FEM-3 interactions in *C. nigoni* and in all the possible cross-species arrangements (Supplementary Fig. 6).

Third, we studied hybrids in which the only *tra-1* allele present is one that promotes *extra* spermatogenesis in *C. briggsae* XX animals (Shen *et al.* 2024a). Even among these hybrid progeny, we saw only one animal that might have made sperm. Most had abnormal gonads and made either germline tumors, or oocytes, or both (Supplementary Fig. 7; Fig. 6e).

Finally, we carried out a series of experiments using a temperature-sensitive allele of *C. briggsae tra-2* that we produced by gene editing. *Cbr-tra-2(v417ts)* is a missense mutation that changes A2629 to C, causing the substitution Aspartic Acid 1210 to Alanine. This alters a rare, conserved residue in an otherwise rapidly evolving part of the FEM-3-binding region (Fig. 7a).

C. briggsae tra-2(v417ts) XX animals were normal hermaphrodites at 20°C, but showed strong masculinization at 25°C. Most notably, at the restrictive temperature all animals showed partially masculinized tails ($N = 11/11$). Their gonad development ranged from hermaphroditic ($N = 1/11$) to fully male ($N = 2/11$; Fig. 7b) with the remainder being severely disorganized. As a consequence of the partial transformation of the gonad, we observed a protruding vulva in some animals, which would be induced by a hermaphroditic anchor cell ($N = 4/11$). Finally, all animals made sperm, but only a few also made oocytes ($N = 3/11$).

We observed a similar range of phenotypes in *C. briggsae tra-2(v417ts)/tra-2(v440null)* XX mutants (Fig. 7c). Thus, we propose that *v417* causes a strong but incomplete reduction of TRA-2 function at the restrictive temperature of 25°C.

To study the effects of low TRA-2 activity in intraspecies hybrids, we examined *Cbr-tra-2(v417ts)/Cni-tra-2(v498null)* XX animals at 25°C (Fig. 7d). Surprisingly, all developed as normal females and produced only oocytes ($N = 9/9$). Thus, we infer that when Cbr-TRA-2(v417) works in the context of *C. briggsae* development, it is unable to block FEM activity at 25°C. However, when it works in a hybrid context, the robust regulation from the *C. nigoni* members of the regulatory pathway restores normal sex determination.

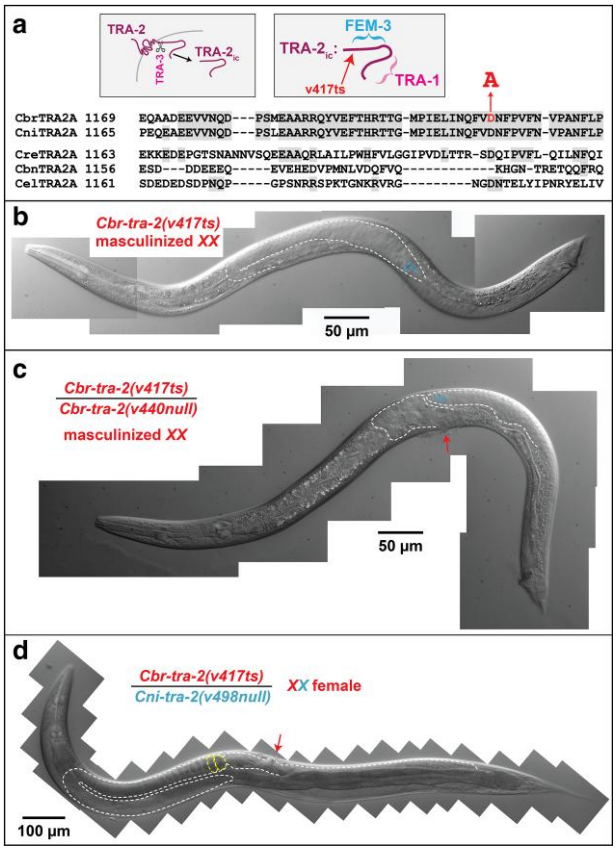

Fig. 7. The temperature-sensitive mutation *tra-2(v417)* is suppressed in hybrids. a) Location of the *Cbr-tra-2(v417)* mutation. Alignment of a portion of the TRA-2 proteins from 5 *Caenorhabditis* species, with residues conserved between *C. nigoni* and any of the other species highlighted in gray. The residue affected by *v417* in *C. briggsae* is in red. The left inset shows the position of TRA-2 in the membrane and the release of its intracellular fragment by cleavage. The right inset shows the location of the residue altered by *v417* on the intracellular fragment, along with the FEM-3 and TRA-1 binding domains. b) DIC photomicrograph of a *Cbr-tra-2(v417ts)* XX animal at 25°C. The visible parts of the male gonad are outlined in white, and 3 sperm are indicated with blue arrows. Anterior is left and ventral down. The frames showing the head and tail were taken in a different plane of focus from the others. c) DIC photomicrograph of a *Cbr-tra-2(v417ts)/Cbr-tra-2(v440)* XX mutant. The abnormal gonad is outlined in white, and 2 sperm are indicated with blue arrows. The red arrow marks the protruding vulva. d) DIC photomicrograph of a *Cbr-tra-2(v417ts)/Cni-tra-2(v498)* XX mutant. Note that it develops a female body and female gonad (outlined in white) and produces oocytes (2 outlined in yellow). The red arrow indicates the vulva. Anterior is left and ventral up.

Discussion

First sex determination mutants described in a male/female species of nematode

Because of the power and simplicity of hermaphroditic genetics, enormous progress has been made using nematodes to model animal development and behavior (see WormBook.org). However, these studies cannot address critical aspects of female development and behavior, or male/female interactions. They also focus on species that are highly inbred (Dolgin *et al.* 2007; Gimond *et al.* 2013), which could have produced unusual genetic traits or regulatory systems.

Addressing this problem requires studying female/male species. Previously, such studies depended on imprecise techniques like RNA interference (Haag and Kimble 2000; Chen *et al.* 2001). However, the ability to make mutations and genetic balancers using gene editing has made more precise genetic studies possible (e.g. Yin *et al.* 2018). Here, we significantly extend these analyses by isolating and studying single and double mutants for 3 critical sex determination genes, none of which could be maintained as a homozygous line. We anticipate a flowering of similar work on female/male species.

C. nigoni has a robust sex determination pathway for germ cells

Both *C. elegans* and *C. briggsae* produce XX animals with male and female germ cells, making them self-fertile. Moreover, mutations

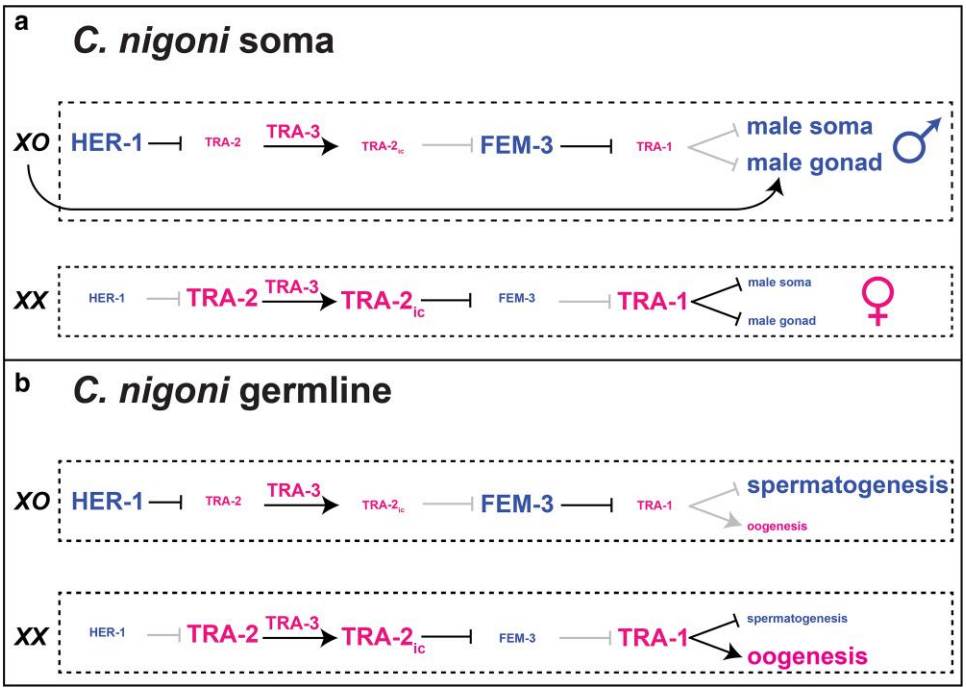

Fig. 8. Sex determination in *C. nigoni*. a) Outline of gene regulation in the *C. nigoni* soma. CAPITALS indicate protein names. Items in blue generally promote male fates and those in red female fates. Larger fonts and black lines denote strong activity, whereas smaller fonts and gray line denote weak activity. Arrows indicate positive regulation and "—|" negative regulation. Because *C. nigoni tra-1(lf)* XX males have incomplete gonadogenesis, there must be a separate regulatory interaction that promotes male gonad development, in addition to that of TRA-1. b) Outline of gene regulation in the *C. nigoni* germ line.

in the genes that control this sexual trait reveal considerable flexibility in the decision to produce sperm or oocytes. For example, *tra-1* null mutations cause both spermatogenesis and oogenesis. Furthermore, *tra-2* mutations reveal extreme sensitivity to gene dosage. And finally, the *fem-3* mutations reveal an essential role in spermatogenesis for *C. elegans*, but almost no role in *C. briggsae*.

By contrast, our *C. nigoni* mutations have clear, simple germline phenotypes. The *tra-2* null mutants make only sperm. The *fem-3* null mutants and *tra-2; fem-3* double mutants make only oocytes. Finally, the *tra-1* null mutants and *tra-1; fem-3* double mutants make only sperm. Moreover, a *fem-3* regulatory mutation that causes *C. elegans* to produce sperm has no effect on *C. nigoni*. These results suggest that the binary nature of sex determination in the *C. nigoni* germ line is inherently robust (Fig. 8). As a further test for robustness of sex determination in *C. nigoni* germ cells, we studied hybrids with the sister species *C. briggsae*. Even those XX hybrids with only *C. briggsae tra-1* activity, or with only diminished *C. briggsae tra-2* activity, developed as females. Thus, the *C. nigoni* sex determination pathway still causes all XX germ cells to become oocytes in these hybrids, even when key *C. nigoni* genes are not present.

Robustness is a critical trait for many biological systems (reviewed by Kitano 2004; Félix and Barkoulas 2015), including the robust suppression of variable phenotypes (Hong *et al.* 2016; Katsanos *et al.* 2017). Thus, regulatory processes are often structured so that small changes in gene activity do not result in large changes in phenotype, or in extremely variable phenotypes. As a result, development and homeostasis are buffered from minor genetic variation or environmental effects. However, such robustness might also be an impediment to the origin of new traits during evolution. We have been studying the frequent, independent origin of self-fertile hermaphrodites in nematodes as a model for this process.

One major question for the origin of self-fertility has been which preexisting traits made this transition possible and capable of independent repetition. One such trait has been known for decades—the use of an XX:XO system to determine sex. Since the XX females have all the genetic information needed for male development in their genomes, regulatory changes that activate male programs in the opposite sex are possible. By contrast, in XX:XY systems, the XX females typically lack many Y genes needed for reproduction (e.g. Hennig *et al.* 1989; Chandley 1998), so only a complex series of genetic changes could (in theory) lead to sperm production. Here, we tested the possibility that flexibility in the sex determination pathway also provided a precondition for the origin of self-fertility. For example, if low *tra-1* activity always caused animals to make both sperm and oocytes, even in female/male species, then small regulatory changes that affected *tra-1* function might have precipitated the origin of hermaphrodites.

Our observations do not support this model. Although changes in the sex determination pathway are clearly a major part of the evolution of self-fertility, the natural robustness of the pathway in *C. nigoni* implies that these changes must fall into 2 categories. First, some changes impaired the robust sexual canalization of germ cells, making it easier for individual animals to switch to producing both sperm and oocytes. Second, other changes directly altered germ cells fates in XX animals, so that they make sperm as well as oocytes. Each of these classes might have involved multiple mutations and a long process of refinement.

This result helps clarify the function of genes like *fog-2* in *C. elegans* and *she-1* in *C briggsae*. Both genes are unique and were produced by recent gene duplications, and both are required for XX animals to develop as hermaphrodites rather than females (Schedl and Kimble 1988; Clifford *et al.* 2000; Nayak *et al.* 2005; Guo *et al.* 2009). As a result, *fog-2* and *she-1* strains reproduce as XX females and XO males, which suggested that each gene might

be the key factor responsible for self-fertility in its respective species. However, we have shown here that haploinsufficiency for *tra-2* does not cause spermatogenesis in *C. nigoni XX* animals, whereas it does so in *C. elegans fog-2* strains and *C. briggsae she-1* strains. Hence, we propose that *tra-2* activity is much higher relative to that of its competitors in *C. nigoni* germ cells than in either hermaphroditic species, which implies that lowering *tra-2* activity might have been crucial for allowing genes like *fog-2* and *she-1* to evolve and function. Consistent with this model, levels of the TRA-2 protein are so low in *C. elegans* germ cells as to be almost undetectable (Hu *et al.* 2019).

tra-1 plays a conserved role in gonad development that depends on genetic background

In *C. nigoni*, *tra-1 XX* null mutants have an unexpected phenotype —gonad development is severely compromised. Although *C. elegans tra-1 XX* mutants often make fully functional males with normal gonads, they sometimes produce defective gonads, and the *tra-1 XO* mutants occasionally make defective gonads too (Hodgkin 1987). These defects stem from problems with the positioning of the somatic gonad precursors within the gonad primordium of young larvae, as well as defects in the polarity of the Z1 cell division (Mathies *et al.* 2004). In *C. briggsae*, the strong *tra-1* mutant *nm2* produces *XX* males with normal somatic gonads, although they initially have defects in polarity of the Z1 cell division (Kelleher *et al.* 2008). The *XO* phenotype has not been studied.

By contrast, our results reveal a more severe *tra-1* gonadal defect than seen in either *C. elegans* or *C. briggsae*. However, they also show strong dependence on karyotype—the *XX* animals all have defective gonads, whereas the *XO* animals are normal males, which we have used successfully in crosses. This result implies that the *X:Autosome* ratio controls gonadal sex determination in 2 ways: by acting directly through the sex determination pathway to control *tra-1* activity and by acting in parallel to this pathway on gonad development. For example, *C. nigoni tra-1* might act in conjunction with another gene to control early development of the gonad in *XX* animals. One possibility for future exploration is *fkh-6*, which works with *tra-1* to control male gonad development in *C. elegans* (Chang *et al.* 2004) and which has orthologs in other *Caenorhabditis* species.

The *X:Autosome* ratio can influence sex outside of the normal sex determination pathway

These *tra-1* results establish two roles for the *X:Autosome* ratio in *C. nigoni* sexual development. One study in *C. elegans* also raised the possibility of split regulatory processes: the *xol-1* gene, which directly interprets the *X:Autosome* ratio, acts both upstream and downstream of *tra-2* to regulate male tail development (Miller *et al.* 1988). In these 2 cases, these effects might represent distinct gene regulatory pathways, or the influence of changes in dosage compensation on particularly sensitive genes in the sex determination pathway.

Hybrid mutant analysis facilitates the study of rapidly evolving traits

To date, most work on interspecies hybrids has focused on the genes responsible for incompatibility. These studies are most advanced in *Drosophila* (reviewed by Presgraves and Meiklejohn 2021), but have made significant progress in *Caenorhabditis* as well (Bi *et al.* 2019; Xie *et al.* 2022). However, speciation involves not only barriers to hybridization, but also rapid changes in traits critical for each new species to adapt to its environment. We have used mutants in hybrid nematodes to elucidate key changes in sex determination during recent evolution and anticipate that a similar use of hybrids will be fruitful for many additional studies, now that the necessary mutations can be produced by gene editing. Furthermore, genetic studies like we present here can be combined with the analysis of hybrid transcriptomes (Sánchez-Ramírez *et al.* 2021) to deepen our understanding of regulatory networks.

Model for the evolution of self-fertile hermaphrodites

Putting our current results in the context of previous work in our field, we propose that self-fertility involved a series of regulatory changes. (1) One of the two male sperm activation programs could have been activated in otherwise female animals (Baldi *et al.* 2009; Wei *et al.* 2014b). (2) Mutations in some sex determination genes might have compromised the robustness of the process in germ cells, without themselves creating *XX* hermaphrodites. Such changes would create the ground state needed for co-option of male programs to occur. Kitano (2004) notes that robustness is facilitated by redundancy. Because redundant interactions can be shed or augmented in different ways without altering the phenotype, robustness could facilitate the evolution of the system (Haag 2007). Such cryptic evolution has been dubbed developmental systems drift (True and Haag 2001). Sex determination systems, which evolve to produce exactly two alternative outcomes, may be especially prone to this type of developmental system drift. (3) A precipitating mutation could then have tilted the regulatory balance so that *XX* animals made some sperm as well as oocytes. (4) Finally, multiple mutations would refine this process, optimizing its efficiency and the number of self-sperm.

Once self-fertile hermaphrodites existed, they might have spread easily because of their natural efficiency at colonization (Pannell 2015). At the same time, the new population would have gone through inbreeding crises before eventually stabilizing, once many harmful recessive mutations were purged (Dolgin *et al.* 2007). It would eventually reach a point where outcrossing might even be detrimental (Gimond *et al.* 2013). Meanwhile, a host of associated changes in male traits and genome size would occur as the change in mating systems altered the way natural selection affected the 2 sexes (Yin *et al.* 2018). Given the richness and scope of this model for evolutionary change and the numerous developments in experimental evolution in nematodes (Cutter *et al.* 2019), we anticipate the repetition of these natural experiments in laboratory simulations, where every detail can be monitored.

Data availability

For the *C. nigoni* CP168 genome, its raw sequencing reads, genome assembly, and protein-coding gene annotations are available under GenBank BioProject accession number PRJNA1143586. These data are also archived in the Open Science Framework (https://osf.io/qzupj).

Supplemental material available at GENETICS online.

Acknowledgments

Some strains were provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440).

Funding

The authors thank National Institutes of Health grants R01GM118836 and R01GM121688 for funding to REE and the American Cancer Society for a Postdoctoral Award to support S-YL (126627-PF-15-228-01-DDC).

Conflicts of interest

The author(s) declare no conflict of interest.

Literature cited

Ahringer J, Kimble J. 1991. Control of the sperm-oocyte switch in *Caenorhabditis elegans* hermaphrodites by the *fem-3* 3′ untranslated region. Nature. 349(6307):346–348. https://doi.org/10.1038/349346a0.

Alonge M, Lebeigle L, Kirsche M, Jenike K, Ou S, Aganezov S, Wang X, Lippman ZB, Schatz MC, Soyk S. 2022. Automated assembly scaffolding using RagTag elevates a new tomato system for high-throughput genome editing. Genome Biol. 23(1):258. https://doi.org/10.1186/s13059-022-02823-7.

Baldi C, Cho S, Ellis RE. 2009. Mutations in two independent pathways are sufficient to create hermaphroditic nematodes. Science. 326(5955):1002–1005. https://doi.org/10.1126/science.1176013.

Barton MK, Schedl TB, Kimble J. 1987. Gain-of-function mutations of *fem-3*, a sex-determination gene in *Caenorhabditis elegans*. Genetics. 115(1):107–119. https://doi.org/10.1093/genetics/115.1.107.

Berkseth M, Ikegami K, Arur S, Lieb JD, Zarkower D. 2013. TRA-1 ChIP-seq reveals regulators of sexual differentiation and multilevel feedback in nematode sex determination. Proc Natl Acad Sci U S A. 110(40):16033–16038. https://doi.org/10.1073/pnas.1312087110.

Bernstein D, Hook B, Hajarnavis A, Opperman L, Wickens M. 2005. Binding specificity and mRNA targets of a *C. elegans* PUF protein, FBF-1. RNA. 11(4):447–458. https://doi.org/10.1261/rna.7255805.

Bi Y, Ren X, Li R, Ding Q, Xie D, Zhao Z. 2019. Specific interactions between autosome and X chromosomes cause hybrid male sterility in *Caenorhabditis* species. Genetics. 212(3):801–813. https://doi.org/10.1534/genetics.119.302202.

Brenner S. 1974. The genetics of *Caenorhabditis elegans*. Genetics. 77(1):71–94. https://doi.org/10.1093/genetics/77.1.71.

Chandley AC. 1998. Chromosome anomalies and Y chromosome microdeletions as causal factors in male infertility. Hum Reprod. 13(Suppl 1):45–50. https://doi.org/10.1093/humrep/13.suppl_1.45.

Chang W, Tilmann C, Thoemke K, Markussen FH, Mathies LD, Kimble J, Zarkower D. 2004. A forkhead protein controls sexual identity of the *C. elegans* male somatic gonad. Development. 131(6):1425–1436. https://doi.org/10.1242/dev.01012.

Chen S. 2023. Ultrafast one-pass FASTQ data preprocessing, quality control, and deduplication using fastp. Imeta. 2(2):e107. https://doi.org/10.1002/imt2.107.

Chen PJ, Cho S, Jin SW, Ellis RE. 2001. Specification of germ cell fates by FOG-3 has been conserved during nematode evolution. Genetics. 158(4):1513–1525. https://doi.org/10.1093/genetics/158.4.1513.

Chen PJ, Ellis RE. 2000. TRA-1A regulates transcription of *fog-3*, which controls germ cell fate in *C. elegans*. Development. 127(14):3119–3129. https://doi.org/10.1242/dev.127.14.3119.

Clifford R, Lee MH, Nayak S, Ohmachi M, Giorgini F, Schedl T. 2000. FOG-2, a novel F-box containing protein, associates with the GLD-1 RNA binding protein and directs male sex determination in the *C. elegans* hermaphrodite germline. Development. 127(24):5265–5276. https://doi.org/10.1242/dev.127.24.5265.

Cutter AD, Morran LT, Phillips PC. 2019. Males, outcrossing, and sexual selection in *Caenorhabditis* nematodes. Genetics. 213(1):27–57. https://doi.org/10.1534/genetics.119.300244.

Dey A, Chan CK, Thomas CG, Cutter AD. 2013. Molecular hyperdiversity defines populations of the nematode *Caenorhabditis brenneri*. Proc Natl Acad Sci U S A. 110(27):11056–11060. https://doi.org/10.1073/pnas.1303057110.

Dolgin ES, Charlesworth B, Baird SE, Cutter AD. 2007. Inbreeding and outbreeding depression in *Caenorhabditis* nematodes. Evolution. 61(6):1339–1352. https://doi.org/10.1111/j.1558-5646.2007.00118.x.

Doniach T. 1986. Activity of the sex-determining gene *tra-2* is modulated to allow spermatogenesis in the *C. elegans* hermaphrodite. Genetics. 114(1):53–76. https://doi.org/10.1093/genetics/114.1.53.

Ellis RE. 2017. The persistence of memory - hermaphroditism in nematodes. Mol Reprod Dev. 84(2):144–157.

Ellis RE. 2022. Sex determination in nematode germ cells. Sex Dev. 16(5-6):305–322. https://doi.org/10.1159/000520872.

Ellis RE, Lin SY. 2014. The evolutionary origins and consequences of self-fertility in nematodes. F1000Prime Rep. 6:62. https://doi.org/10.12703/P6-62.

Fay D, Bender A. 2008. SNPs: introduction and two-point mapping. WormBook. https://doi.org/10.1895/wormbook.1.93.2.

Félix MA, Barkoulas M. 2015. Pervasive robustness in biological systems. Nat Rev Genet. 16(8):483–496. https://doi.org/10.1038/nrg3949.

Félix MA, Braendle C, Cutter AD. 2014. A streamlined system for species diagnosis in *Caenorhabditis* (Nematoda: Rhabditidae) with name designations for 15 distinct biological species. PLoS One. 9(4):e94723. https://doi.org/10.1371/journal.pone.0094723.

Fierst JL, Willis JH, Thomas CG, Wang W, Reynolds RM, Ahearne TE, Cutter AD, Phillips PC. 2015. Reproductive mode and the evolution of genome size and structure in *Caenorhabditis* nematodes. PLoS Genet. 11(6):e1005323. https://doi.org/10.1371/journal.pgen.1005323.

Fodor A, Riddle DL, Nelson FK, Golden JW. 1983. Comparison of a new wild-type *Caenorhabditis briggsae* with laboratory strains of *C. briggsae* and *C. elegans*. Nematologica. 29(2):203–217. https://doi.org/10.1163/187529283X00456.

Gimond C, Jovelin R, Han S, Ferrari C, Cutter AD, Braendle C. 2013. Outbreeding depression with low genetic variation in selfing *Caenorhabditis* nematodes. Evolution. 67(11):3087–3101. https://doi.org/10.1111/evo.12203.

Goodwin EB, Okkema PG, Evans TC, Kimble J. 1993. Translational regulation of *tra-2* by its 3'-untranslated region controls sexual identity in *C. elegans*. Cell. 75(2):329–339. https://doi.org/10.1016/0092-8674(93)80074-O.

Guo Y, Lang S, Ellis RE. 2009. Independent recruitment of F box genes to regulate hermaphrodite development during nematode evolution. Curr Biol. 19(21):1853–1860. https://doi.org/10.1016/j.cub.2009.09.042.

Haag ES. 2007. Compensatory vs. pseudocompensatory evolution in molecular and developmental interactions. Genetica. 129(1):45–55. https://doi.org/10.1007/s10709-006-0032-3.

Haag ES, Kimble J. 2000. Regulatory elements required for development of *Caenorhabditis elegans* hermaphrodites are conserved in the *tra-2* homologue of *C. remanei*, a male/female sister species. Genetics. 155(1):105–116. https://doi.org/10.1093/genetics/155.1.105.

Hall DH, Hedgecock EM. 1991. Kinesin-related gene unc-104 is required for axonal transport of synaptic vesicles in *C. elegans*. Cell. 65(5):837–847. https://doi.org/10.1016/0092-8674(91)90391-B.

Harbin JP, Ellis RE. 2023. Efficient production of CRISPR/Cas9 gene knockouts in the male/female nematode *Caenorhabditis nigoni*. MicroPubl Biol. 2023:10.17912/micropub.biology.000968. https://doi.org/10.17912/micropub.biology.000968.

Hennig W, Brand RC, Hackstein J, Hochstenbach R, Kremer H, Lankenau DH, Lankenau S, Miedema K, Pötgens A. 1989. Y chromosomal fertility genes of Drosophila: a new type of eukaryotic genes. Genome. 31(2):561–571. https://doi.org/10.1139/g89-105.

Hill RC, De Carvalho CE, Salogiannis J, Schlager B, Pilgrim D, Haag ES. 2006. Genetic flexibility in the convergent evolution of hermaphroditism in *Caenorhabditis* nematodes. Dev Cell. 10(4):531–538. https://doi.org/10.1016/j.devcel.2006.02.002.

Hill RC, Haag ES. 2009. A sensitized genetic background reveals evolution near the terminus of the *Caenorhabditis* germline sex determination pathway. Evol Dev. 11(4):333–342. https://doi.org/10.1111/j.1525-142X.2009.00340.x.

Hodgkin J. 1980. More sex-determination mutants of *Caenorhabditis elegans*. Genetics. 96(3):649–664. https://doi.org/10.1093/genetics/96.3.649.

Hodgkin J. 1986. Sex determination in the nematode *C. elegans:* analysis of *tra-3* suppressors and characterization of *fem* genes. Genetics. 114(1):15–52. https://doi.org/10.1093/genetics/114.1.15.

Hodgkin J. 1987. A genetic analysis of the sex-determining gene, *tra-1*, in the nematode *Caenorhabditis elegans*. Genes Dev. 1(7):731–745. https://doi.org/10.1101/gad.1.7.731.

Hodgkin JA, Brenner S. 1977. Mutations causing transformation of sexual phenotype in the nematode *Caenorhabditis elegans*. Genetics. 86(2):275–287. https://doi.org/10.1093/genetics/86.2.275.

Hong L, Dumond M, Tsugawa S, Sapala A, Routier-Kierzkowska AL, Zhou Y, Chen C, Kiss A, Zhu M, Hamant O, *et al.* 2016. Variable cell growth yields reproducible OrganDevelopment through spatiotemporal averaging. Dev Cell. 38(1):15–32. https://doi.org/10.1016/j.devcel.2016.06.016.

Hu S, Skelly LE, Kaymak E, Freeberg L, Lo TW, Kuersten S, Ryder SP, Haag ES. 2019. Multi-modal regulation of *C. elegans* hermaphrodite spermatogenesis by the GLD-1-FOG-2 complex. Dev Biol. 446(2):193–205. https://doi.org/10.1016/j.ydbio.2018.11.024.

Hunter CP, Wood WB. 1992. Evidence from mosaic analysis of the masculinizing gene *her-1* for cell interactions in *C. elegans* sex determination. Nature. 355(6360):551–555. https://doi.org/10.1038/355551a0.

Kagawa H, Shino Y, Kobayashi D, Demizu S, Shimada M, Ariga H, Kawahara H. 2011. A novel signaling pathway mediated by the nuclear targeting of C-terminal fragments of mammalian patched 1. PLoS One. 6(4):e18638. https://doi.org/10.1371/journal.pone.0018638.

Katsanos D, Koneru SL, Mestek Boukhibar L, Gritti N, Ghose R, Appleford PJ, Doitsidou M, Woollard A, Van Zon JS, Poole RJ, *et al.* 2017. Stochastic loss and gain of symmetric divisions in the *C. elegans* epidermis perturbs robustness of stem cell number. PLoS Biol. 15(11):e2002429. https://doi.org/10.1371/journal.pbio.2002429.

Kelleher DF, De Carvalho CE, Doty AV, Layton M, Cheng AT, Mathies LD, Pilgrim D, Haag ES. 2008. Comparative genetics of sex determination: masculinizing mutations in *Caenorhabditis briggsae*. Genetics. 178(3):1415–1429. https://doi.org/10.1534/genetics.107.073668.

Kiontke KC, Félix MA, Ailion M, Rockman MV, Braendle C, Pénigault JB, Fitch DH. 2011. A phylogeny and molecular barcodes for *Caenorhabditis*, with numerous new species from rotting fruits. BMC Evol Biol. 11(1):339. https://doi.org/10.1186/1471-2148-11-339.

Kitano H. 2004. Biological robustness. Nat Rev Genet. 5(11):826–837. https://doi.org/10.1038/nrg1471.

Kuwabara PE, Okkema PG, Kimble J. 1992. *tra-2* encodes a membrane protein and may mediate cell communication in the *Caenorhabditis elegans* sex determination pathway. Mol Biol Cell. 3(4):461–473. https://doi.org/10.1091/mbc.3.4.461.

Lum DH, Kuwabara PE, Zarkower D, Spence AM. 2000. Direct protein-protein interaction between the intracellular domain of TRA-2 and the transcription factor TRA-1A modulates feminizing activity in *C. elegans*. Genes Dev. 14(24):3153–3165. https://doi.org/10.1101/gad.853700.

Ly K, Reid SJ, Snell RG. 2015. Rapid RNA analysis of individual *Caenorhabditis elegans*. MethodsX. 2:59–63. https://doi.org/10.1016/j.mex.2015.02.002.

Mathies LD, Schvarzstein M, Morphy KM, Blelloch R, Spence AM, Kimble J. 2004. TRA-1/GLI controls development of somatic gonadal precursors in *C. elegans*. Development. 131(17):4333–4343. https://doi.org/10.1242/dev.01288.

Meyer BJ. 2022. The X chromosome in *C. elegans* sex determination and dosage compensation. Curr Opin Genet Dev. 74:101912. https://doi.org/10.1016/j.gde.2022.101912.

Miller LM, Plenefisch JD, Casson LP, Meyer BJ. 1988. xol-1: a gene that controls the male modes of both sex determination and X chromosome dosage compensation in C. elegans. Cell. 55(1):167–183. https://doi.org/10.1016/0092-8674(88)90019-0.

Nayak S, Goree J, Schedl T. 2005. *fog-2* and the evolution of self-fertile hermaphroditism in *Caenorhabditis*. PLoS Biol. 3(1):e6. https://doi.org/10.1371/journal.pbio.0030006.

Orr TJ, Brennan PL. 2015. Sperm storage: distinguishing selective processes and evaluating criteria. Trends Ecol Evol. 30(5):261–272. https://doi.org/10.1016/j.tree.2015.03.006.

Pannell JR. 2015. Evolution of the mating system in colonizing plants. Mol Ecol. 24(9):2018–2037. https://doi.org/10.1111/mec.13087.

Perry MD, Li W, Trent C, Robertson B, Fire A, Hageman JM, Wood WB. 1993. Molecular characterization of the *her-1* gene suggests a direct role in cell signaling during *Caenorhabditis elegans* sex determination. Genes Dev. 7(2):216–228. https://doi.org/10.1101/gad.7.2.216.

Pertea G, Pertea M. 2020. GFF utilities: GffRead and GffCompare. F1000Res. 9:ISCB Comm J-304. https://doi.org/10.12688/f1000research.23297.1.

Presgraves DC, Meiklejohn CD. 2021. Hybrid sterility, genetic conflict and complex speciation: lessons from the *Drosophila simulans* clade species. Front Genet. 12:669045. https://doi.org/10.3389/fgene.2021.669045.

Prjibelski A, Antipov D, Meleshko D, Lapidus A, Korobeynikov A. 2020. Using SPAdes de novo assembler. Curr Protoc Bioinformatics. 70(1):e102. https://doi.org/10.1002/cpbi.102.

Sánchez-Ramírez S, Weiss JG, Thomas CG, Cutter AD. 2021. Widespread misregulation of inter-species hybrid transcriptomes due to sex-specific and sex-chromosome regulatory evolution. PLoS Genet. 17(3):e1009409. https://doi.org/10.1371/journal.pgen.1009409.

Schedl T, Graham PL, Barton MK, Kimble J. 1989. Analysis of the role of *tra-1* in germline sex determination in the nematode *Caenorhabditis elegans*. Genetics. 123(4):755–769. https://doi.org/10.1093/genetics/123.4.755.

Schedl T, Kimble J. 1988. *fog-2*, a germ-line-specific sex determination gene required for hermaphrodite spermatogenesis in *Caenorhabditis elegans*. Genetics. 119(1):43–61. https://doi.org/10.1093/genetics/119.1.43.

Schvarzstein M, Spence AM. 2006. The *C. elegans* sex-determining GLI protein TRA-1A is regulated by sex-specific proteolysis. Dev Cell. 11(5):733–740. https://doi.org/10.1016/j.devcel.2006.09.017.

Schwander T, Marais G, Roze D. 2014. Sex uncovered: the evolutionary biology of reproductive systems. J Evol Biol. 27(7):1287–1291. https://doi.org/10.1111/jeb.12424.

Shen Y, Lin SY, Harbin J, Amin R, Vassalotti A, Romanowski J, Schmidt E, Tierney A, Ellis RE. 2024a. Rewiring the sex-determination pathway during the evolution of self-fertility. Mol Biol Evol. 41(6): msae101. https://doi.org/10.1093/molbev/msae101.

Shen Y, Lin SY, Guo Y, Imtiaz J, Corley K, Ellis RE. 2024b. The Caenorhabditis Gli protein TRA-1 makes a transcriptional activator that promotes spermatogenesis. [Manuscript submitted for publication].

Shumate A, Salzberg SL. 2021. Liftoff: accurate mapping of gene annotations. Bioinformatics. 37(12):1639–1643. https://doi.org/10.1093/bioinformatics/btaa1016.

Starostina NG, Lim JM, Schvarzstein M, Wells L, Spence AM, Kipreos ET. 2007. A CUL-2 ubiquitin ligase containing three FEM proteins degrades TRA-1 to regulate *C. elegans* sex determination. Dev Cell. 13(1):127–139. https://doi.org/10.1016/j.devcel.2007.05.008.

Strome S, Kelly WG, Ercan S, Lieb JD. 2014. Regulation of the X chromosomes in *Caenorhabditis elegans*. Cold Spring Harb Perspect Biol. 6(3): a018366. https://doi.org/10.1101/cshperspect.a018366.

Trent C, Purnell B, Gavinski S, Hageman J, Chamblin C, Wood WB. 1991. Sex-specific transcriptional regulation of the *C. elegans* sex-determining gene *her-1*. Mech Dev. 34(1):43–55. https://doi.org/10.1016/0925-4773(91)90090-S.

Trochet A, Courtois EA, Stevens VM, Baguette M, Chaine A, Schmeller DS, Clobert J. 2016. Evolution of sex-biased dispersal. Q Rev Biol. 91(3):297–230. https://doi.org/10.1086/688097.

True JR, Haag ES. 2001. Developmental system drift and flexibility in evolutionary trajectories. Evol Dev. 3(2):109–119. https://doi.org/10.1046/j.1525-142x.2001.003002109.x.

Van Goor J, Shakes DC, Haag ES. 2021. Fisher vs. the worms: extraordinary sex ratios in nematodes and the mechanisms that produce them. Cells. 10(7):1793. https://doi.org/10.3390/cells10071793.

Vekemans X, Poux C, Goubet PM, Castric V. 2014. The evolution of selfing from outcrossing ancestors in Brassicaceae: what have we learned from variation at the S-locus. J Evol Biol. 27(7): 1372–1385. https://doi.org/10.1111/jeb.12372.

Wang S, Kimble J. 2001. The TRA-1 transcription factor binds TRA-2 to regulate sexual fates in *Caenorhabditis elegans*. EMBO J. 20(6): 1363–1372. https://doi.org/10.1093/emboj/20.6.1363.

Weeks SC. 2012. The role of androdioecy and gynodioecy in mediating evolutionary transitions between dioecy and hermaphroditism in the animalia. Evolution. 66(12):3670–3686. https://doi.org/10.1111/j.1558-5646.2012.01714.x.

Wei Q, Shen Y, Chen X, Shifman Y, Ellis RE. 2014a. Rapid creation of forward-genetics tools for *C. briggsae* using TALENs: lessons for nonmodel organisms. Mol Biol Evol. 31(2):468–473. https://doi.org/10.1093/molbev/mst213.

Wei Q, Zhao Y, Guo Y, Stomel J, Stires R, Ellis RE. 2014b. Co-option of alternate sperm activation programs in the evolution of self-fertile nematodes. Nat Commun. 5(1):5888. https://doi.org/10.1038/ncomms6888.

Wood WB and the community of *C. elegans researchers*, editors. 1988. *The Nematode C. elegans*. Cold Spring Harbor Laboratory. p.667.

Woodruff GC, Eke O, Baird SE, Félix MA, Haag ES. 2010. Insights into species divergence and the evolution of hermaphroditism from fertile interspecies hybrids of *Caenorhabditis* nematodes. Genetics. 186(3):997–1012. https://doi.org/10.1534/genetics.110.120550.

Xie D, Ye P, Ma Y, Li Y, Liu X, Sarkies P, Zhao Z. 2022. Genetic exchange with an outcrossing sister species causes severe genome-wide dysregulation in a selfing *Caenorhabditis* nematode. Genome Res. 32(11–12):2015–2027. https://doi.org/10.1101/gr.277205.122.

Yin D, Schwarz EM, Thomas CG, Felde RL, Korf IF, Cutter AD, Schartner CM, Ralston EJ, Meyer BJ, Haag ES. 2018. Rapid genome shrinkage in a self-fertile nematode reveals sperm competition proteins. Science. 359(6371):55–61. https://doi.org/10.1126/science.aao0827.

Zarkower D. 2006. Somatic sex determination. WormBook. https://doi.org/10.1895/wormbook.1.84.1.

Zarkower D, Hodgkin J. 1992. Molecular analysis of the *C. elegans* sex-determining gene *tra-1*: a gene encoding two zinc finger proteins. Cell. 70(2):237–249. https://doi.org/10.1016/0092-8674(92)90099-X.

Zhang B, Gallegos M, Puoti A, Durkin E, Fields S, Kimble J, Wickens MP. 1997. A conserved RNA-binding protein that regulates sexual fates in the *C. elegans* hermaphrodite germ line. Nature. 390(6659):477–484. https://doi.org/10.1038/37297.

Editor: B. Conradt