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Sex determination in nematode germ cells

Ronald E. Ellis

Department of Molecular Biology, Rowan University SOM, Stratford, USA

Abstract

Animal germ cells differentiate as sperm or as oocytes. These sexual fates are controlled by complex regulatory pathways, to ensure that the proper gametes are made at the appropriate times. Nematodes like *C. elegans* and its close relatives are ideal models for studying how this regulation works, because the *XX* animals are self-fertile hermaphrodites that produce both sperm and oocytes. In these worms, germ cells use the same signal-transduction pathway that functions in somatic cells. This pathway determines the activity of the transcription factor TRA-1, a Gli protein that can repress male genes. However, the pathway is extensively modified in germ cells, largely by the action of translational regulators like the PUF proteins. Many of these modifications play critical roles in allowing the *XX* hermaphrodites to make sperm in an otherwise female body. Finally, TRA-1 cooperates with chromatin regulators in the germ line to control the activity of *fog-1* and *fog-3,* which are essential for spermatogenesis. FOG-1 and FOG-3 work together to determine germ cell fates by blocking the translation of oogenic transcripts. Although there is great diversity in how germ cell fates are controlled in other animals, many of the key nematode genes are conserved, and the critical role of translational regulators may be universal.

Keywords

sex determination; germ cell; nematode; *C. elegans* ; reproduction

1. The animal germ line

The world teems with multicellular life, and even today new forms are evolving from unicellular ancestors [Kirk, 2005]. A critical trait shared by almost all of these groups is the use of dimorphic gametes. These differ dramatically in size and function: small ones like sperm are adapted for dispersal, and large ones like eggs for nurturing the new zygote [Parker et al., 1972]. Besides animals, this type of dimorphism is seen in many plants [Pereira et al., 2014], fungi [Nagy et al., 2018], and even in very simple creatures like Volvox [Geng et al., 2014].

Although complex life has originated many times, it seems likely that *multicellular animals* evolved only once [Cavalier-Smith, 2017]. Analyses of sponges and cnidarians, which

Corresponding Author: Ronald E Ellis, Department of Molecular Biology, Rowan University SOM, B303 Science Center, 2 Medical Center Drive, Stratford, NJ 08084, (856) 566-2768, ellisre@rowan.edu.

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diverged very early from bilaterian animals, suggest that our common ancestor had a simple organization and lacked most of the tissues found in modern animals. However, from the beginning, the ability to generate germ cells has formed a central part of the animal body plan. In some animals, like nematodes and fruit flies, the germ line is set aside from the rest of the organism early in development. By contrast, in mammals and many other animals the germ line is specified later [Extavour and Akam, 2003]. Indeed, in flatworms, it can even be re-established during tissue regeneration [Newmark et al., 2008].

Whatever approach is adopted to form the germ line, it has to produce cells that have exceptionally stable genomes and are capable of undergoing meiosis to produce haploid gametes. The meiotic program is sexually specialized in animals, with germ cells in males undergoing spermatogenesis to yield four spermatids, and those in females undergoing oogenesis to yield a single mature oocyte and polar bodies.

The sexual fate of germ cells determines the expression of thousands of genes

As with other animals, mature gametes from nematodes like *C. elegans* differ dramatically in size. Their internal makeup is also dimorphic, since spermatids are specialized for crawling, with extremely compact DNA, specialized organelles that control sperm activation, and a unique cytoskeleton [reviewed by Ellis and Stanfield, 2014]. By contrast, the oocyte is a large cell that carries the information and nutrients needed to nurture the embryo. It arrests in prophase of meiosis I and matures just before fertilization [reviewed by Huelgas-Morales and Greenstein, 2018]. These differences are reflected in highly divergent programs of gene expression, with more than a thousand genes specific to each sexual fate [Reinke et al., 2000; Reinke et al., 2004; Ortiz et al., 2014; Tzur et al., 2018]. Nematode sperm and oocytes can therefore be considered representative of the dimorphism found in gametes throughout the animal phylogeny. This review will consider how so many genes can be so accurately controlled.

2. Nematodes are ideal for studying the germ line

Sydney Brenner originally chose the nematode *C. elegans* as a model for studying the nervous system [Brenner, 1974; White, 2020], but it has several advantages that quickly made it a leading system for studying sex determination and the germ line. First, the two sexes are hermaphrodites and males [Maupas, 1900], which dramatically simplifies isolating mutations that cause sterility when homozygous [Brenner, 1974]. Second, these hermaphrodites are essentially modified females that produce sperm at the end of larval development, which allows the study of both male and female gametes in a single individual. Third, hermaphrodite development in *C. elegans* is specified by two *X* chromosomes *(XX)*, and male development by a single one *(XO)* [Nigon, 1949]. The lack of a *Y* chromosome simplifies the study of sex determination in germ cells, because the *Y* tends to carry genes needed for male reproduction [Bachtrog, 2013]. Fourth, these worms are transparent, which allows both gonad and germ cell development to be observed in living animals [Kimble and Hirsh, 1979]. Fifth, there have been important changes in mating systems during the recent evolution of *Caenorhabditis,* with at least three independent transitions to species with

self-fertile hermaphrodites [Kiontke et al., 2011]; these species provide a window into how germ cell fates change under selection.

The germ line is the largest tissue in *Caenohrabditis* nematodes, which devote most of their resources to producing hundreds of offspring in just a few days. As with other animals, nematode germ cells have only a few decisions to make during development. The first is whether to proliferate by mitosis or enter meiosis and differentiate [reviewed by Kimble and Crittenden, 2007]. The next is sexual, whether to undergo spermatogenesis or oogenesis, which we discuss here. The last, which only occurs within the pool of female germ cells, is whether to complete oogenesis or undergo programmed cell death [reviewed by Gartner et al., 2008]. And if conditions are poor, larvae arrest in diapause and their germ cells enter quiescence, whereas adult germlines first shrink and then quiesce [Rashid et al., 2021].

All germ cells in males eventually undergo spermatogenesis, but in hermaphrodites only those differentiating early do so, whereas those differentiating during adulthood undergo oogenesis. Each spermatocyte produces four haploid spermatids; these are round cells that will extend a pseudopod when activated, so they can crawl towards oocytes. By contrast, each surviving oocyte is a large cell that arrests in meiosis I, and only completes meiosis after fertilization.

The gonads are designed to nurture and regulate germ cells

In hermaphrodites, the gonad consists of two U-shaped tubes that meet in a central uterus (Fig. 1A,C). The distal end of each tube is organized by a single large cell, the distal tip cell, forming a niche where germline stem cells can proliferate [Kimble and White, 1981]. This niche ends where the fingers of the distal tip cell intercalate with those of adjacent gonad sheath cells [Gordon et al., 2020]. These sheath cells help the distal tip cell regulate when germ cells enter meiosis. They also influence the sex of germ cells, since ablation of sheath and spermathecal cells blocks the male fate of spermatogenesis [McCarter et al., 1997]. Finally, more proximal sheath cells contract to force mature oocytes into the spermatheca, a flexible tube with numerous internal folds where sperm are stored. Fertilized oocytes then pass through the spermathecal/uterine valve into the uterus, and soon afterwards the new embryos are laid.

In males, the gonad forms a single J-shaped tube (Fig. 1D,F). At one end, the two distal tip cells organize the stem cell niche. The testis reflexes, with spermatogenesis occurring past the bend, and mature spermatids being stored at the end. The testis abuts the seminal vesicle and vas deferens, through which sperm pass during mating, so they can exit from the cloaca into the hermaphrodite vulva. Cells in the vas deferens secrete components of the seminal fluid, which activate sperm and aid in mating.

In both sexes, the gonad occupies much of the center of the body. Nematodes have a simple body plan, with the hypodermis, nerves and muscle forming a hollow tube. This tube is filled with fluid (the pseudocoelom) in which the gonad and intestine are located (Fig. 1B,E). This central location makes it easy for the gonad to communicate with other tissues and receive the yolk and other nutrients needed to produce eggs or sperm.

3. A core sex-determination pathway controls both germline and somatic fates

In nematodes, sex-determination is controlled by a core signal transduction pathway that coordinates development throughout the body [Fig. 2A, reviewed by Zarkower, 2006]. Although many features of this pathway are unique to worms, the TRA-2 receptor is distantly related to Patched [Kuwabara et al., 1992], and the TRA-1 transcription factor is a homolog of Cubitus interruptus and the Gli proteins [Zarkower and Hodgkin, 1992]. Thus, worm sex determination might share its origins with the Hedgehog pathway [Kuwabara et al., 1992; reviewed by Burglin and Kuwabara, 2006].

The initial cue that determines sex is the ratio of *X* chromosomes to autosomes [Madl and Herman, 1979], which regulates the activity of the *xol-1* gene [Farboud et al., 2013]. As a result, *XO* animals have high levels of XOL-1 protein, and *XX* animals have low levels. In *XO* worms XOL-1 prevents the SDC complex from repressing the transcription of *her-1* [Chu et al., 2002], allowing it to produce a small, secreted protein [Trent et al., 1991]. Genetic mosaic experiments showed that this secreted protein, HER-1, is a sex hormone that promotes male development throughout the body [Hunter and Wood, 1992]. Furthermore, they revealed that HER-1 also controls germ cell fates, causing them to differentiate as sperm. Thus, a signal from the soma plays a major role in determining the sexual fate of nematode germ cells.

How does HER-1 do this? Its target, TRA-2, is a transmembrane protein [Kuwabara et al., 1992] that is required for hermaphrodite development [Hodgkin and Brenner, 1977]. Mutations that prevent HER-1 from binding TRA-2 cause *XO* animals to become hermaphrodites [Kuwabara, 1996a], as expected if HER-1 represses TRA-2 (Fig. 2B,C). Their physical interaction appears to prevent TRA-2 itself from being cleaved by the calpain protease TRA-3 [Barnes and Hodgkin, 1996; Sokol and Kuwabara, 2000], which would release a key intracellular fragment called TRA-2^{ic}. *C. elegans* germ cells produce a second *tra-2* transcript that directly codes for this intracellular fragment [Kuwabara and Kimble, 1995; Kuwabara et al., 1998], providing an alternative source in the germ line. However, this smaller transcript is not found in the related nematode *C. briggsae* [Kuwabara, 1996b], so it is not fundamental to how the pathway works.

The ultimate target of TRA-2^{ic} is TRA-1, a Gli transcription factor [Zarkower and Hodgkin, 1992] that controls all sexual fates in both the soma and germ line. Mutations that result in constitutive TRA-1 activity cause *XX* and *XO* animals to develop as females and make oocytes [Hodgkin, 1980; Hodgkin, 1987; de Bono et al., 1995], whereas mutations that inactivate *tra-1* cause them to develop as males [Hodgkin and Brenner, 1977; Hodgkin, 1987]. Surprisingly, these *tra-1* mutant males produce sperm early in life but oocytes in old age, which reveals a secondary role in sustaining spermatogenesis [Hodgkin, 1987; Schedl et al., 1989]. Thus TRA-1 controls germ cell fates but is not absolutely necessary for either spermatogenesis or oogenesis to occur. Like other Gli proteins, TRA-1 is cleaved to form a repressor [Schvarzstein and Spence, 2006], which turns off many males genes [Berkseth et al., 2013]. These include *fog-3* [Chen and Ellis, 2000] and *fog-1* [Jin et al., 2001b], which directly specify male germ cell fates.

TRA-1 is degraded in *XO* animals to prevent female development [Schvarzstein and Spence, 2006]. This process is controlled by three *fem* genes, which act downstream of TRA-2^{ic} to promote male development and spermatogenesis [Kimble et al., 1984; Hodgkin, 1986]. The FEM-1 protein contains ankyrin repeats [Spence et al., 1990], FEM-2 is a phosphatase [Chin-Sang and Spence, 1996] and FEM-3 is novel [Ahringer et al., 1992]. Together, they form a complex with CUL-2 that ubiquitinates TRA-1 [Starostina et al., 2007]. The CDC-48 proteins [Sasagawa et al., 2009] and three of their UBX co-factors aid in this process [Sasagawa et al., 2010]. By contrast, in *XX* animals TRA-2^{ic} is active and binds FEM-3 [Mehra et al., 1999], which protects TRA-1 from the FEM complex and prevents its degradation.

TRA-1 degradation is carried out by the proteosome, which contains both a catalytic unit and a regulatory particle. Studies in yeast revealed that some components of the regulatory particle are not essential [van Nocker et al., 1996]. Surprisingly, mutations in the *C. elegans* homolog of one of these components, *rpn-10,* are viable and cause all germ cells to differentiate as oocytes [Shimada et al., 2006]. A similar result was obtained for another component of the regulatory particle, *rpn-12* [Fernando et al., 2021]. Although knocking down *tra-1* can restore self-fertility to mutants of either gene, knocking down *tra-2* is most effective at suppressing *rpn-10*. Thus, mutations in these two genes might reveal different aspects of proteosomal regulation. RPN-12, at least, appears to be a good candidate for directly promoting TRA-1 degradation.

TRA-2 not only regulates the FEM complex, but also directly binds TRA-1, in an interaction that is blocked by several *mixomorphic tra-2* alleles [Lum et al., 2000; Wang and Kimble, 2001]. These mutations cause all hermaphrodite germ cells to become oocytes rather than sperm but have only minor effects on somatic tissues [Doniach, 1986; Kuwabara et al., 1998]. Thus, it appears that TRA-2^{ic} interacts with TRA-1 to promote spermatogenesis. This surprising result, along with the role of TRA-1 in both turning off and sustaining spermatogenesis, highlights the frequency of dual functions among sex-determination genes. Since the mixomorphic alleles do not affect male germ cell fates, the interaction between TRA-2^{ic} and TRA-1 might occur only in *XX* animals, or it might not be strong enough to perturb germ cell fates in males.

Chromatin regulators cooperate with TRA-1 to control cell fates

Like most transcription factors, TRA-1 cooperates with chromatin regulators to specify germ cell fates. The Nucleosome Remodeling Factor (or NURF) complex is needed during larval development to promote spermatogenesis in *C. briggsae* males and hermaphrodites [Chen et al., 2014], where it acts independently of TRA-1 to promote the expression of *fog-1* and *fog-3*. The simplest model is that the NURF complex opens the chromatin so that it is accessible to TRA-1. However, the *nurf-1* locus is complex, and in *C. elegans* one isoform promotes spermatogenesis, whereas another favors oogenesis [Xu et al., 2019]. Thus, a balancing act between different products of this complex locus seem to be critical for specifying germ cell fates.

In addition, TRR-1, a homolog of TRRAP, works with the Tip60 Histone Acetyl Transferase complex to promote spermatogenesis [Guo et al., 2013]. Although *trr-1* null alleles are

sickly and sterile, weak alleles cause animals of both sexes to make oocytes instead of sperm. Surprisingly, this phenotype is suppressed by *tra-1* mutations. One possible explanation is that full-length TRA-1, like full-length Cubitus interruptus, is an activator of transcription. If so, it could work with TRR-1 and the Tip60 complex to turn on sperm genes [Guo et al., 2013].

Finally, the WDR-5 proteins act as part of the COMPASS complex in nematodes to regulate many aspects of germline development, by methylating lysine 4 of histone 3 [Li and Kelly, 2011]. Surprisingly, inactivation of two of the three *wdr-5* genes causes hermaphrodites to make sperm throughout their lives [Li and Kelly, 2014]. However, this effect does not involve other members of the COMPASS complex. Instead, these WDR-5 proteins are needed for TRA-1 repressor to associate with the chromatin and block transcription of *fog-3*.

4. The translational regulators FOG-1 and FOG-3 specify germ cell fates

In nematodes, two genes directly control germ cell fates — *fog-1* and *fog-3*. We know they are essential for spermatogenesis but not other sexual fates since mutations in either gene cause germ cells to differentiate as oocytes instead of sperm, but don't affect other tissues [Barton and Kimble, 1990; Ellis and Kimble, 1995]. In addition, double mutants with *tra-1* or other sex-determination genes confirm that *fog-1* and *fog-3* act at the end of the pathway. Finally, *fog-1* and *fog-3* are directly regulated by TRA-1, with multiple Gli binding sites in each promoter [Chen and Ellis, 2000; Jin et al., 2001b]. Thus, these two genes act in response to the global regulatory pathway to choose between spermatogenesis and oogenesis.

The only qualification to this model is that mutations in *fshr-1,* which encodes a glycopeptide hormone receptor that acts in somatic tissues, can work in combination with translational regulatory mutations to suppress *fog-1* or *fog-3* [Cho et al. 2007]. This trait is not yet understood.

Although FEM-1, FEM-2 and FEM-3 are also needed for spermatogenesis in *C. elegans* [Kimble et al., 1984; Hodgkin, 1986], they are dispensable in *C. briggsae* [Hill et al., 2006], despite the fact that FEM-3 does play a small role downstream of TRA-1 [Hill and Haag, 2009]. These results suggest that the *fem* genes are not central to this cell fate decision. Instead, they are likely to help regulate FOG-1, FOG-3 or an associated protein in *C. elegans*, in addition to ubiquitinating TRA-1.

How do FOG-1 and FOG-3 control germ cell fates? FOG-1 is a Cytoplasmic Polyadenylation Element Binding (CPEB) protein [Luitjens et al., 2000; Jin et al., 2001b]. It has two RNA recognition motifs and a zinc-finger domain, all of which are required for function. Moreover, FOG-1 binds RNA *in vitro* [Jin et al., 2001a]. During development, FOG-1 can be detected in the germ line of L3 larvae around the time that sex determination is beginning and it is expressed before the earliest sperm proteins [Lamont and Kimble, 2007]. These results support the idea that FOG-1 specifies male germ cell fates.

FOG-3 is a member of the Tob /BTG family of proteins [Chen et al., 2000]. Mammalian homologs interact with CPEB proteins and regulate mRNA de-adenylation by the CCR4-

CAF1-NOT complex [reviewed by Mauxion et al., 2009]. The members of this complex are expressed at high levels in the nematode germline, but their mutant phenotypes are broad and complex [Molin and Puisieux, 2005; Nousch et al., 2013], which has obscured any role they might play with FOG-3 in sex determination.

Like other Tob proteins, FOG-3 is regulated by its phosphorylation state [Lee et al., 2011]. Lack of phosphorylation is critical for the initiation of spermatogenesis in both sexes, whereas phosphorylated T108 and S260 are needed to maintain spermatogenesis in adult males. Although Map Kinase activity promotes spermatogenesis in worms [Lee et al., 2007b], it is unlikely to control these target sites, since unphosphorylated FOG-3 is needed to initiate spermatogenesis. Thus, the kinase that modifies FOG-3 remains unknown.

FOG-1 and FOG-3 co-immunoprecipitate and are both required for male germ cell fates, so they appear to form a complex [Noble et al., 2016]. Moreover, FOG-3 can dimerize, and its dimers assemble into large arrays [Aoki et al., 2018]. Thus, the full structure of this regulatory complex could be very large. Although studies of mammalian Tob proteins suggest that CCR4 and CAF1 homologs could also be part of this complex, current data do not seem to support this hypothesis [Aoki et al., 2018].

The mRNAs that are isolated from FOG-1 or FOG-3 precipitates include a large set of overlapping targets [Noble et al., 2016]. Furthermore, these common targets are mostly oogenic messages, which suggests that FOG-1 and FOG-3 might work by blocking their translation [Noble et al., 2016; Aoki et al., 2018]. Thus, the simplest model is that FOG-1 and FOG-3 select male germ cell fates by blocking the translation of mRNAs that encode oogenic proteins. This model fits nicely with a study showing that the expression patterns of most genes that act in oogenesis are primarily regulated by their 3'-UTRs [Merritt et al., 2008].

Competing polyA polymerases help determine germ cell fates

Since the FOG-3/FOG-1 complex blocks translation of messenger RNAs, does it do so through de-adenylation of the targets? Observation of key mRNAs supports the idea that polyadenylation state is important. For example, *fem-3* messages have longer polyA tails in germ lines where FEM-3 is actively specifying male fates [Ahringer and Kimble, 1991; Ahringer et al., 1992]. However, two polyA polymerases also play a major role in regulating the sexual fates of germ cells, which complicates the resolution of this question.

These two polymerases include the same core subunit — GLD-2, a regulatory polyA polymerase [Wang et al., 2002]. GLD-2 controls many aspects of germ cell development [Kadyk and Kimble, 1998]. When bound to GLD-3, it promotes entry into meiosis [Eckmann et al., 2004] and spermatogenesis [Eckmann et al., 2002]. GLD-2 can also bind RNP-8, forming a complex that promotes oogenesis [Kim et al., 2009]. These two polyA polymerases, GLD-2/GLD-3 and GLD-2/RNP-8, compete with each other to specify germ cell fates. However, the effects of mutations in *gld-3* or *rnp-8* are small in isolation, and only become dramatic when combined with other mutations that influence sex. These results suggest that these alternative polyA polymerases might reinforce male or female germ cell fates by forming part of two larger competitive regulatory systems.

A transcriptional network operates downstream of FOG-1 and FOG-3

These studies show that FOG-1 and FOG-3 use a post-transcriptional mechanism to control the sperm fate decision, and that this mechanism probably involves the control of translation. Despite this, subsequent spermatogenesis relies heavily on transcriptional regulation [Merritt et al., 2008]. There does not seem to be a single master transcriptional regulator for this fate, but instead several transcription factors act together to control sperm differentiation. For example, the transcription factor SPE-44 promotes the expression of a large number of sperm genes and is required for germ cells to complete spermatogenesis [Kulkarni et al., 2012]. One of its targets is the GATA transcription factor ELT-1, which controls the expression of many of the Major Sperm Protein (*msp*) genes [Shim, 1999; del Castillo-Olivares et al., 2009], and also regulates development in other tissues. Finally, the nuclear hormone receptor NHR-23 controls a third set of sperm genes [Ragle et al., 2020]. Since some sperm genes are not regulated by any of these factors, additional ones probably remain to be discovered.

As one might expect if FOG-1 and FOG-3 are broad regulators of translation in the germ line, they also influence the decision of cells to proliferate or enter meiosis [Thompson et al., 2005; Snow et al., 2013]. To do this, they cooperate with the PUF proteins FBF-1 and FBF-2, and the precise dose of both FOG-1 and of FOG-3 plays a critical role in determining which outcome occurs. Thus, they resemble several other translational regulators (to be discussed below) that also control both proliferation and germ cell fates. The broad overlap in function of these regulators suggests that the control of sexual fates and cell division programs are inextricably linked in the germ line [Kimble and Page, 2007].

5. The sex-determination pathway is modified in hermaphrodite germ cells

Although the core pathway regulates sexual fates in both the soma and the germ line, it has to be modified in hermaphrodite germ cells. These hermaphrodites face the complex task of producing male germ cells in a female gonad, and then producing female germ cells from the same population of stem cells. Thus, the somatic HER-1 signal is not sufficient on its own. As a result, sex determination differs extensively between soma and germ line in species like *C. elegans,* but there are often important modifications in male/female species as well, as first observed in the fruit fly *Drosophila melanogaster*.

GLD-1 and FOG-2 regulate *tra-2* messages to promote *XX* spermatogenesis

In *C. elegans,* hermaphrodite spermatogenesis depends on regulatory steps that lower the expression of TRA-2 in *XX* germ cells. The first evidence for this system was the identification of mutations in *tra-2* that cause a gain-of-function (*gf*) in the hermaphrodite germ line, resulting in *XX* animals that only make oocytes [Doniach, 1986]. These mutations don't alter the TRA-2 protein, but instead map to the 3'-UTR [Okkema and Kimble, 1991; Kuwabara et al., 1992], where they affect one or both copies of a repeated element, altering translation of the *tra-2* mRNA [Goodwin et al., 1993]. Five of these *gf* mutations affect only the *XX* germ line but the sixth, *e2020,* also weakly affects the *XO* germ line and some aspects of somatic development [Doniach, 1986]. The *e2020* mutation

is a large deletion that removes both repeats entirely, plus flanking DNA [Kuwabara et al., 1992], which could explain its more severe phenotype.

Regulation of *tra-2* through these direct repeats is controlled by two genes, *fog-2* and *gld-1*. Loss-of-function mutations in *fog-2* are recessive and result in the same phenotype as the gain-of-function mutations in *tra-2*. [Schedl and Kimble, 1988]. Thus, FOG-2 is required for *XX* animals to make sperm, but not for *XO* animals. The FOG-2 protein contains an F box, but it is not known if it functions as part of an E3 ubiquitin ligase complex [Clifford et al., 2000]. FOG-2 is otherwise unique, evolved recently and exists only in *C. elegans* [Nayak et al., 2005].

The best clue to FOG-2's function is that it binds GLD-1 [Clifford et al., 2000]. GLD-1 is also required for spermatogenesis in hermaphrodites but not in males [Francis et al., 1995a]. However, *gld-1* mutations cause a complex array of additional phenotypes like tumorigenesis, because they also affect other aspects of germline development [Francis et al., 1995b]. The GLD-1 protein contains a KH domain that binds RNA [Jones and Schedl, 1995], and GLD-1 precipitates contain many mRNAs involved in oogenesis [Lee and Schedl, 2001]. Furthermore, GLD-1 binds *tra-2* directly, in an interaction that depends on the repeat elements in the 3'-UTR [Jan et al., 1999]. Thus, the simplest model is that a GLD-1/FOG-2 complex controls sexual fate in germ cells by repressing the translation of *tra-2* messenger RNAs. This repression should result in less TRA-2, allowing spermatogenesis in young hermaphrodites.

In *XX* hermaphrodites, the FOG-2 protein is expressed in mitotic germ cells, and in the early stages of meiosis [Clifford et al., 2000], where it would be needed to control sexual fates. GLD-1 is also expressed in these regions, but the protein remains detectable throughout the pachytene stage of meiosis I, as expected from its broad role in regulating oocyte development [Jones et al., 1996]. Although both proteins are expressed in males, they have no known functions in them.

A few alleles of *gld-1* highlight its critical role in sex determination. The mutation *gld-1(q126)* causes hermaphrodites to make oocytes instead of sperm, but otherwise has little effect [Francis et al., 1995a]. This allele changes a single amino acid near the end of the GLD-1/Quaking conserved region [Jones and Schedl, 1995]. Although it does not affect GLD-1's general ability to bind RNA, it strongly decreases binding to *tra-2* messages, which could explain the Fog phenotype [Lee and Schedl, 2001]. By contrast, missense mutations like *gld-1(q93)* cause hermaphrodites to make only sperm [Francis et al., 1995a]. These mutations map to two amino acids just outside the KH domain [Jones and Schedl, 1995], and decrease the ability of GLD-1 to bind *glp-1* messages [Hu et al., 2019]. It is not clear how they affect sex determination.

In fact, the overall mechanism by which FOG-2 and GLD-1 work remains mysterious. For example, GLD-1 represses the translation of a large number of targets (e.g. Lee and Schedl, 2004; Scheckel et al., 2012). Furthermore the binding sites in these target mRNAs contain the same UACU(C/A)A sequence element found in *tra-2* [Ryder et al., 2004]. Thus, it is not clear why FOG-2 should be needed for GLD-1 to repress *tra-2* mRNA translation, when

FOG-2 is not needed for other GLD-1 targets. One possibility is that GLD-1 binds both the 5' and 3'-ends of the *rme-2* message, but only the 3'-end of *tra-2,* a difference that might be remedied by FOG-2 activity [Lee and Schedl, 2001]. An additional puzzle is the fact that *fog-2* mutations can still influence germ cell fate in *tra-2(e2020gf)* animals, even though *e2020* completely removes the known GLD-1 binding sites from the transcript [Hu et al., 2019]. Analyses of the DEAD box helicase SACY-1 also reveal differences between mutations in FOG-2 and in the *tra-2* target site [Kim et al., 2012]. Knocking down SACY-1 suppresses *fog-2* mutants but not *tra-2(e2020)* mutants. Thus, both of these studies suggest FOG-2 does not function *exclusively* through the known GLD-1 target sequences in the 3'-UTR of *tra-2*.

TRA-2 is regulated by a different mechanism in *C. briggsae* germ cells

Although *C. briggsae* lacks a *fog-2* gene [Nayak et al., 2005], its *XX* animals nonetheless undergo hermaphrodite development. To do this they utilize SHE-1, a novel F-box protein that is unrelated to FOG-2 [Guo et al., 2009]. SHE-1 can bind SKR-1, a critical component of the SCF ubiquitin ligase complex, and mutations that alter its F box prevent SHE-1 from working. Thus, SHE-1 might control the stability of a target protein. Although this target has not been identified, analysis of double mutants suggests that SHE-1 acts upstream of TRA-2 [Guo et al., 2009].

Despite the superficial similarity between SHE-1 and FOG-2, SHE-1 does not bind *C. briggsae* GLD-1 [Guo et al., 2009], and Cbr-GLD-1 does not seem to promote spermatogenesis [Nayak et al., 2005; Beadell et al., 2011]. Indeed, knocking down *Cbr-gld-1* by RNA interference causes spermatogenesis! Instead, Cbr-GLD-1 regulates the expression of PUF-8 [Beadell et al., 2011], and Cbr-GLD-1 expression is itself controlled by PUF-2 and PUF-1.2 [Liu et al., 2012]. These PUF proteins are related to Pumilio, and form part of a conserved family that regulates cell fates in both the germ line and soma [reviewed by Wang and Voronina, 2020]. How this translational module impinges on the sex-determination pathway in *C. briggsae* remains unknown.

PUF proteins turn off spermatogenesis by repressing *fem-3* in *C. elegans* hermaphrodites

Just as a complex system for regulating TRA-2 activity in hermaphrodites was revealed by Fog mutations, which feminize the germ line, a system for controlling FEM-3 was discovered through Mog mutations, which masculinize the germline. These mutations in *fem-3* cause *XX* animals to make sperm throughout their lives, without affecting other sexual decisions [Barton et al., 1987]. Each of them alters a regulatory site in the 3-UTR of the message [Ahringer and Kimble, 1991], and this site is a target for repression by the PUF proteins FBF-1 and FBF-2 [Zhang et al., 1997]. When both FBF proteins are inactivated by RNA interference, the double mutants make only sperm, just like the *fem-3(gf)* mutants. Thus, this regulatory system is crucial for allowing *C. elegans* hermaphrodites to switch from spermatogenesis to oogenesis.

At least one nanos gene in *C. elegans* cooperates in this regulatory step [Kraemer et al., 1999]. The NOS-3 protein physically interacts with FBF to regulate the expression of *fem-3* in germ cells. Similar studies in *Drosophila* found that Pumilio interacts with Nanos to

control their *hunchback* mRNA target [Sonoda and Wharton, 1999]. However, mutations in *nos-3* have a weak effect on their own, probably because *nos-1* and *nos-2* help turn off *fem-3*. Surprisingly, neither NOS-1 nor NOS-2 seems to bind the FBF proteins directly [Kraemer et al., 1999].

Finally, LARP-1 contains an LA motif and binds RNA [Nykamp et al., 2008]. Like FBF, it is also redundant with NOS-3 in the control of germ cell fates, and the majority of *nos-3; larp-1* double mutants are Mog [Zanin et al., 2010]. However, *larp-1* mutations appear to act by increasing the levels of *fem-3* transcripts in the germ line, rather than by altering their translation. Thus, there might be multiple levels of regulation controlling these *fem-3* transcripts.

PUF proteins regulate germ cell fates at many decision points

The ancient PUF family of proteins not only regulates FEM-3 but also plays a central role in coordinating many other aspects of germ cell development and sex determination.

First, the two FBF proteins also bind to and block the translation of both *fog-1* [Thompson et al., 2005] and *fog-3* messages [Thompson et al., 2005; Kershner and Kimble, 2010; Snow et al., 2013]. Furthermore, *fbf-1* and *fbf-2* also interact with *fog-1* and *fog-3* to regulate cell proliferation, one of the other key choices facing germ cells [reviewed by Kimble and Crittenden, 2007].

Second, the PUF proteins play critical roles in the regulation of MAP kinase. In nematodes, genes of the MAP kinase (or ERK) pathway control many aspects of germline development [Church et al., 1995; Lee et al., 2007b]. One of these functions is promoting differentiation as spermatocytes rather than as oocytes [Lee et al., 2007b]. For example, some weak mutations in the MAP kinase gene *mpk-1,* or in other members of the pathway, lead all germ cells to differentiate as oocytes. Furthermore, strong mutations in these genes cause germ cells to arrest in the pachytene stage of meiosis I, and the arrested cells express oocyte transcripts, even in males. Finally, treating *puf-8; lip-1* mutants or *fbf-1; lip-1* mutants with a MAP kinase inhibitor causes germ cells to develop as oocytes rather than as sperm [Morgan et al., 2010; Sorokin et al., 2014]. Genetic tests imply that MAP kinase acts near the end of the pathway to control sexual fates, but its targets are not known. However, the *mpk-1* transcript itself has binding sites for FBF-1 and FBF-2 [Lee et al., 2007a], and the analysis of double mutants revealed that MAP kinase activity is redundantly controlled by these FBF proteins and the phosphatase LIP-1. Finally, MAP kinase targets the FBF partner NOS-3 in a feedback loop [Arur et al., 2011].

Third, the two FBF proteins bind to and regulate *gld-1* at its 3'-UTR [Crittenden et al., 2002]. This repression prevents GLD-1 from promoting entry into meiosis and from promoting male germ cell fates in developing hermaphrodites. Furthermore, the PUF proteins FBF-1 and PUF-8 regulate the expression of FOG-2, the partner of GLD-1 in activating hermaphrodite spermatogenesis [Bachorik and Kimble, 2005], although direct binding has not been shown.

Fourth, the FBF proteins also target one of the transcripts for *gld-3,* which encodes a polyA polymerase subunit that promotes male germ cell fates [Eckmann et al., 2004]. Surprisingly, the GLD-3 and FBF proteins can bind each other [Eckmann et al., 2002], but the manner in which this interaction affects FBF activity remains unclear [Eckmann et al., 2002; Wu et al., 2013].

Thus, the FBF proteins, assisted at times by partners like PUF-8 or NOS-3, block the translation of many genes needed for male germ cell fates in *C. elegans* (Fig. 3). However, they also regulate other aspects of germline development, and interact with more than 1000 known targets [Kershner and Kimble, 2010; Porter et al., 2019]. Their antagonism towards hermaphrodite spermatogenesis is not common to all nematode species, since some PUF proteins in *C. briggsae* promote spermatogenesis, instead of preventing it [Liu et al., 2012]. Instead, it seems likely that PUF proteins are central to germline development in all animals and have been recruited into the sperm/oocyte decision independently in different hermaphroditic species.

Translational regulation maintains a balance between TRA-2 and FEM-3 in *C. elegans*

As these results show, mutations that alter the expression of either *fem-3* or *tra-2* have dramatic effects on the hermaphrodite germ line. For example, *fem-3(gf)* animals make only sperm, whereas *tra-2(gf)* animals make only oocytes. Furthermore, *tra-2(gf); fem-3(gf)* double mutants are restored to normal hermaphrodite development [Barton et al., 1987]. Thus, the balance between FEM-3 and TRA-2 levels might be critical for determining *C. elegans* germ cell fates.

Surprisingly, careful studies of TRA-2 protein expression show that the levels in the germ line are so low as to be barely detectable, despite the critical role that TRA-2 plays in this tissue [Hu et al., 2019]. Perhaps the evolution of hermaphrodites requires TRA-2 and FEM-3 expression to be reduced to low but similar levels, which can easily be pushed towards spermatogenesis or oogenesis by small changes in their relative levels.

Although this hypothesis can explain *C. elegans* germ cell fates, things must be different in *C. briggsae,* where *fem-3* is not needed for spermatogenesis, and *tra-2(null); fem-3(null)* double mutants develop as normal hermaphrodites [Hill et al., 2006].

6. GENES THAT CONTROL RNA PROCESSING AND METABOLISM INFLUENCE GERM CELL FATES

Although translational regulators like the PUF proteins and GLD-1 play critical roles in determining germ cell fates in *C. elegans*, many other genes that control RNA processing, localization or function also strongly influence the sperm/oocyte decision.

Many putative splicing factors influence the expression of *fem-3*

The genes *mog-1* through *mog-6* were identified through mutations that cause *XX* animals to make only sperm, but don't affect *XO* animals [Graham and Kimble, 1993; Graham et al., 1993]. Although this phenotype is superficially similar to that of the *fem-3(gf)* mutants,

there are a few important differences. First, the germ lines of these *XX* mutants appear less healthy than those of *fem-3(gf)* animals and contain fewer sperm. Second, if these mutants produce oocytes because of suppression by another sex-determination gene, the progeny all die, revealing a maternal effect for embryonic viability. Mutations in any of these six *mog* genes strongly affect the expression of a reporter gene carrying the *fem-3* 3'-UTR in somatic tissues, which implies that they might encode components of the general translational machinery that are not restricted to the germ line [Gallegos et al., 1998]. Surprisingly, the *fem-3(gf)* mutations, which only affect germ cell fates, strongly upregulate this reporter in somatic tissues [Gallegos et al., 1998].

Molecular studies show that these MOG proteins are involved in RNA regulation. MOG-1 contains a DEAH box and is homologous to PRP16 [Puoti and Kimble, 1999]. MOG-4 and MOG-5 are also DEAH-box proteins, related to PRP2 and PRP22 respectively [Puoti and Kimble, 2000]. All three PRP yeast proteins are involved in splicing. Furthermore, MOG-2 is a homolog of the spliceosomal protein U2A' and binds other components of the spliceosome [Zanetti et al., 2011]. MOG-3 is also homologous to spliceosome components [Kasturi et al., 2010]. And MOG-6 is a cyclophilin [Belfiore et al., 2004]. Finally, yeast two-hybrid studies identified MEP-1 as a zinc-finger protein that binds MOG-1, MOG-3, MOG-4, MOG-5, and MOG-6, as well as RNA [Belfiore et al., 2002; Belfiore et al., 2004; Kasturi et al., 2010], and studies using a reporter gene show that *mep-1* is required for repression of the *fem-3* 3'-UTR [Belfiore et al., 2002]. Thus, these MOG proteins might work in a complex to regulate *fem-3*.

Over the years, many other genes have been found with this combination of phenotypes. For example, MAG-1 is homologous to *Drosophila* Mago Nashi, and *mag-1(RNAi)* mutants make only sperm, but produce dead embryos if a suppressor allows oogenesis [Li et al., 2000]. In addition, mutations in *atx-2,* the homolog of human ataxin-2, cause a Mog phenotype — many hermaphrodites make only sperm, but other germline processes are also affected, and embryos die [Maine et al., 2004]. ATX-2 binds the Poly A Binding protein and regulates translation in the germ line [Ciosk et al., 2004]. Furthermore, the *trd-1* gene, which encodes a tetratricopeptide-repeat protein, also has the classic Mog phenotype, as well as specific defects in larval development [Hughes et al., 2014]. And *prp-17* mutants show all the classic Mog phenotypes [Kerins et al., 2010]. Since PRP-17 is a component of the splicing machinery, Kerins *et al* [2010] did a general screen of splicing factors and found several additional ones that control the sperm/oocyte decision. Although homology suggests these *mog* genes should regulate splicing, there is no evidence of general splicing defects in *mog-1* or *mog-6* mutants [Puoti and Kimble, 1999; Belfiore et al., 2004]. However, these general splicing assays are difficult in nematodes.

Finally, quantitative analyses of *fem-3* transcripts suggest that the *fem-3(gf)* alleles do not alter the efficiency of translation, but instead increase the levels of transcripts that contain the *gf* mutations [Zanetti et al., 2012]. Taking all these studies together, the PUF proteins might cooperate with a broad range of factors involved in RNA splicing to regulate mRNA stability. These splicing factors might directly participate in ribonucleoprotein complexes that regulate the stability or translation of *fem-3* transcripts. Alternatively, they might control

the splicing of a sex-determination transcript that is very sensitive to small changes in efficiency.

LAF-1 and other P granule components regulate *tra-2* expression

Mutations in *laf-1* show a complex set of phenotypes — they are dominant suppressors of the *fem-3(gf)* mutations but lethal when homozygous [Goodwin et al., 1997]. Furthermore, their effects on sex determination are not limited to germ cells but extend to somatic tissues in *XO* animals. All of these phenotypes are suppressed by *tra-2* mutations, which implies that LAF-1 acts upstream of *tra-2*. Furthermore, *laf-1* heterozygotes show elevated expression of reporter genes that contain the *tra-2* 3'-UTR [Goodwin et al., 1997; Jan et al., 1997] and a mild increase in the levels of the TRA-2^{ic} protein itself [Hubert and Anderson, 2009]. Thus, factors like LAF-1 might regulate *tra-2* messages through their 3'-UTRs, in addition to the FOG-2/GLD-1 complex.

The LAF-1 protein is a DEAD-box helicase related to VBH-1 in worms, and Vasa and Belle in fruit flies [Hubert and Anderson, 2009]. VBH-1 works with LAF-1 to regulate germ cell fates, since more *vbh-1(RNAi); laf-1(RNAi)* double mutants are female than occurs for either gene alone [Hubert and Anderson, 2009]. Although LAF-1 is expressed throughout the body and VBH-1 only in the germ line, both proteins localize to P granules [Salinas et al., 2007; Hubert and Anderson, 2009]. These complex RNA/protein structures play a central role in RNA regulation in germ cells [reviewed by Voronina, 2013; Seydoux, 2018]. LAF-1 helps establish the phase separation that is needed to form the P granule 'droplets' within the cell and knocking down *laf-1* by RNA interference eliminates these structures [Elbaum-Garfinkle et al., 2015].

Related components also influence sex determination in germ cells. For example, GLS-1 localizes to P granules, where it can bind GLD-3, releasing FBF and promoting the switch to oogenesis [Rybarska et al., 2009]. In addition, the eIF4E-transporter IFET-1 helps localize the translational regulators CGH-1 and CAR-1 to P granules [Sengupta et al., 2013], and double mutants between *ifet-1* and these genes make only sperm. Similarly, the eIF4E isoform IFE-3 also causes a Mog phenotype and acts upstream of *fem-3,* although IFE-3 localizes to germ granules rather than the related P granules [Huggins et al., 2020]. Finally, MEL-46 influences sperm number, and *mel-46* mutations weakly suppress *fog-2* mutations [Minasaki et al., 2009]. Since MEL-46 is a DEAD-box helicase, it might also localize to P granules.

Thus, P granules play a complex role in the sperm/oocyte decision, since components like LAF-1 promote spermatogenesis, whereas others like IFET-1 promote oogenesis. An additional layer of complexity involves licensing of transcripts by the Argonaute protein CSR-1, which acts in P granules. Mutations in *csr-1* block P granule formation and cause a large increase in the transcription of genes needed for hermaphrodite spermatogenesis [Campbell and Updike, 2015].

MicroRNA regulation of sexual fates

Although microRNAs play critical roles in the regulation of many aspects of development [reviewed by Galagali and Kim, 2020], they have been challenging to study, because they are often redundant and have broad, pleiotropic phenotypes.

The *mir-35–42* microRNA family regulates several aspects of reproduction [McJunkin and Ambros, 2014]. One of these is the specification of sperm in hermaphrodites — a *mir-35–42* deletion mutant makes fewer sperm than a wildtype hermaphrodite. Moreover, this effect is partially suppressed by mutations in *sup-26,* a predicted target of these microRNAs. SUP-26 itself influences sex-determination by regulating the translation of *tra-2* [Manser et al., 2002]. Surprisingly, it works in the soma to block translation of *tra-2* messages, in a process that depends on the same target sequence that GLD-1 binds in the germ line [Mapes et al., 2010]. The *mir-35–42* microRNAs also influence sex-determination by regulating the DEAD-box helicase NHL-2 [McJunkin and Ambros, 2017]. Whether these genes also act in the germ line, or influence germ cell fates purely through their function in the soma, is unknown.

The *mir-44* and *mir-45* microRNAs belong to a different family that also controls hermaphrodite fertility [Maniates et al., 2021]. As with *mir-35–42,* the mutants make fewer sperm than normal. However, a *mir-45* reporter is expressed in the hermaphrodite gonad where germ cells are deciding their fates, but not in the male gonad. Furthermore, mutations in *mir-44* and *mir-45* interact with other germline sex-determination mutations to control the number of sperm. Thus, this family is likely to act in germ cells themselves to control the sperm/oocyte decision.

Intriguingly, mutations in the PcG gene *sop-2* cause hermaphrodites to make only sperm, and probably act by increasing the effectiveness of microRNA-mediated repression [Cai et al., 2008]. However, we don't know if this effect is mediated by the *mir-35* or *mir-44* families. And we don't yet know if the *mir-35* family controls the sperm/oocyte decision by acting in germ cells, or what messages *mir-45* targets in the germ line. Thus, the full story of microRNA regulation of germ cell fates is still being written.

Germ cell fates are extremely sensitive to changes in RNA processing and metabolism

Thus, many genes that influence RNA metabolism affect sexual fates in hermaphrodite germ cells. Three factors probably make hermaphrodites sensitive to changes in RNA metabolism. First, they face the complex task of switching quickly and accurately from making sperm to making oocytes. Second, they use several translational regulators, which act at many different regulatory points, to control this cell fate decision. Third, there is a competitive balance between regulators promoting male germ cell fates and those promoting female fates, such as FEM-3 *versus* TRA-2 and GLD-2/GLD-3 *versus* GLD-2/RPN-8. Thus, it is not surprising that this decision is exquisitely sensitive to regulatory changes in general RNA processing and control.

7. Germ cell fate regulation changes quickly during evolution

To date, most studies of how sex-determination has evolved feature the related nematode *C. briggsae,* which also shares the advantages of hermaphrodite reproduction. Fortunately, the two species developed self-fertility independently from a common male/female ancestor [Cho et al., 2004; Kiontke et al., 2004; Kiontke et al., 2011]. However, the components of the core sex-determination pathway remain the same in both, such as *tra-1* [de Bono and Hodgkin, 1996; Kelleher et al., 2008], *tra-2* [Kuwabara, 1996b; Kelleher et al., 2008], the *fem* genes [Hansen and Pilgrim, 1998; Hill et al., 2006] and *fog-3* [Chen et al., 2001]. Although many of these proteins are evolving rapidly in sequence, they have preserved the same interaction partners, as seen for TRA-2/FEM-3 [Haag et al., 2002] and TRA-2/TRA-1 [Wang and Kimble, 2001].

However, the role of the *fem* genes differs significantly, since they are required for hermaphrodite spermatogenesis in *C. elegans* but not in *C. briggsae* [Hill et al., 2006]. The role of the TRR-1/Tip60 complex differs as well, since weak mutations prevent spermatogenesis in *C. briggsae* but not in *C. elegans* [Guo et al., 2013]. Furthermore, it appears that some translational modifiers of the pathway were recruited independently in these two species, and as a result play very different roles [Beadell et al., 2011; Liu et al., 2012]. Finally, novel genes have been recruited to the pathway in each species [Nayak et al., 2005; Guo et al., 2009].

How have these changes come about? Nematodes appear to have certain features that simplify the evolution of self-fertile hermaphrodites [reviewed by Ellis and Lin, 2014]. These include an *XX/XO* chromosomal system of sex determination and a conveniently located sperm storage organ. As a result, self-fertility can be achieved with only a small number of genetic changes [Baldi et al., 2009]. Furthermore, nematodes are capable of passing through intermediate stages in which they produce three distinct sexes — *XX* females, *XX* hermaphrodites and *XO* males — as observed in the new model worm *Auanema rhodensis* [Felix, 2004; Chaudhuri et al., 2011].

In *Auanema,* environmental cues not only control the pattern of larval development, but also determine whether *XX* animals will develop female or hermaphrodite germ lines [Chaudhuri et al., 2011]. Animals raised in optimal conditions develop rapidly into females, whereas those that are starving develop *via* the dispersive dauer larval stage and become hermaphrodites. Hence, nutrition and other environmental cues can affect the sperm/oocyte decision in this species. Surprisingly, meiosis in *Auanema* hermaphrodites has been modified to alter sex ratios in crosses with males [Tandonnet et al., 2018]. Thus, this species shows many differences not found in *Caenorhabditis,* highlighting the great plasticity of developmental regulation in germ cells.

8. Conclusions

Sex determination needs to be modified to meet the needs of developing germ cells

Although nematodes use the same regulatory pathway to control sex determination in the soma and germ line, this pathway is highly modified in germ cells. These changes are

critical because the germ line has unique functions. (1) Unlike the soma, germ cells need to decide between mitotic and meiotic patterns of cell division. Many of the regulators that influence this choice also play key roles in sex determination, like *fbf-1, fbf-2, gld-1, fog-1* or *fog-3*. Thus, the two decisions might have to be linked [reviewed by Kimble and Page, 2007]. (2) *XX* animals need to provision oocytes with nutrients and messenger RNAs for the young embryo, which requires the extensive use of translational regulators and special structures like P granules to manage the transcripts. (3) Fidelity is critical, both to prevent errors in replication, and to avoid the activation of transposons or genetic distorter elements. These needs require special systems for handling DNA and licensing gene expression. And (4) the core pathway needs to be modified in self-fertile hermaphrodites to allow *XX* animals to make both sperm and oocytes.

As a result of these factors, the sex-determination process in germ cells shares many components with that in the soma, but also has numerous modifications and unique features. This divergence is even more pronounced in fruit flies than in worms [reviewed by Salz and Erickson, 2010]. Furthermore, several genes that play other roles in the nematode germ line have been recruited to the sex-determination pathway in hermaphroditic species to regulate *XX* spermatogenesis, and thus have different functions in *C. elegans* and *C. briggsae,* which evolved independently.

Germ cell fates in hermaphrodites are sensitive to small changes

The developing germ cells of hermaphrodites are very sensitive to small regulatory changes, so that some differentiating cells can accurately select one sex, while others can select the opposite one. During the fourth larval stage this transition can be seen in a group of cells in the pachytene stage of meiosis I, with some differentiating as spermatocytes, and adjacent ones as oocytes that are expressing yolk receptor mRNAs [Ellis and Schedl, 2006].

A major consequence of this sensitivity is that dozens and dozens of genes have mutations that alter sexual fate in the germ line. Although this profusion of regulators has been a challenge for the field, it also provides enormous opportunities. Identifying male or female germ cells is easy, both by microscopy and by testing for gene products like the major sperm protein in sperm, or yolk receptor in oocytes. Thus, sex-determination in the germ line provides a simple set of assays for studying a broad range of RNA biology and regulation in living animals.

Competitive regulatory systems ensure fidelity and rapid decision making

To ensure rapid and accurate regulatory changes, nematodes use sets of competing regulators in the germ line. In *C. elegans,* these include the closely balanced opposition of the core pathway proteins TRA-2 and FEM-3, as well as the antagonistic relationship between the GLD-2/GLD-3 and GLD-2/RNP-8 polyA polymerases. This regulatory structure is designed to amplify changing signals very rapidly. It should help in the switch from spermatogenesis to oogenesis during hermaphrodite development, and in maintaining stable sexual identity at other times.

Translational regulatory networks play a central role in the germ line

Translational regulators are critical for managing RNAs that will be packaged into oocytes [reviewed by Nousch and Eckmann, 2013]. As a result, they have come to play central roles in the control of all decisions in the germ line, including sex determination. For example, the STAR protein GLD-1, the large family of PUF proteins, their associated Nanos proteins, and the GLD-2 polyA polymerase are all important parts of this process. These networks display extensive cross talk and feedback, which makes traditional pathway diagrams difficult. But they interact to produce self-reinforcing patterns of activity that specify mitosis, spermatogenesis or oogenesis.

Furthermore, the key player in this final decision is the FOG-1/FOG-3 complex, a translational regulator composed of conserved proteins, whose mode of action is still being delineated. CPEB proteins play broad roles in germ cell development throughout the animal kingdom [reviewed by Ivshina et al., 2014]. For example, the fruit fly Orb protein, a CPEB protein like FOG-1, helps specify the oocyte fate, as opposed to the nurse cell fate [Barr et al., 2019]. And Tob/CPEB complexes are broadly conserved as well [White-Grindley et al., 2014]. However, the way the FOG-3/FOG-1 complex works, and the extent to which homologous structures control germ cell fates in other animals, remain to be determined.

Unanswered questions

Despite enormous progress in this field, which has revealed the structure of complex translational networks and identified numerous conserved regulators, much remains to be done. For example, in maturing hermaphrodites, germ cells switch from spermatogenesis to oogenesis while remaining part of a syncytium [Ellis and Schedl, 2006], but the nature of this switch is still a mystery [Ellis 2008]. Furthermore, many of the key mechanistic details of sex-determination are still largely or completely unknown. For example, how does FOG-2 work with the KH protein GLD-1 to regulate translation of *tra-2*? How does the FOG-1/FOG-3 complex block translation of target messages? Indeed, what other proteins act in this complex? And how do components of the splicing machinery influence cell fates through *fem-3*? Fortunately, the recent ability to manipulate genes in their chromosomal context has revolutionized the field, so the next twenty years should be even more exciting than the last.

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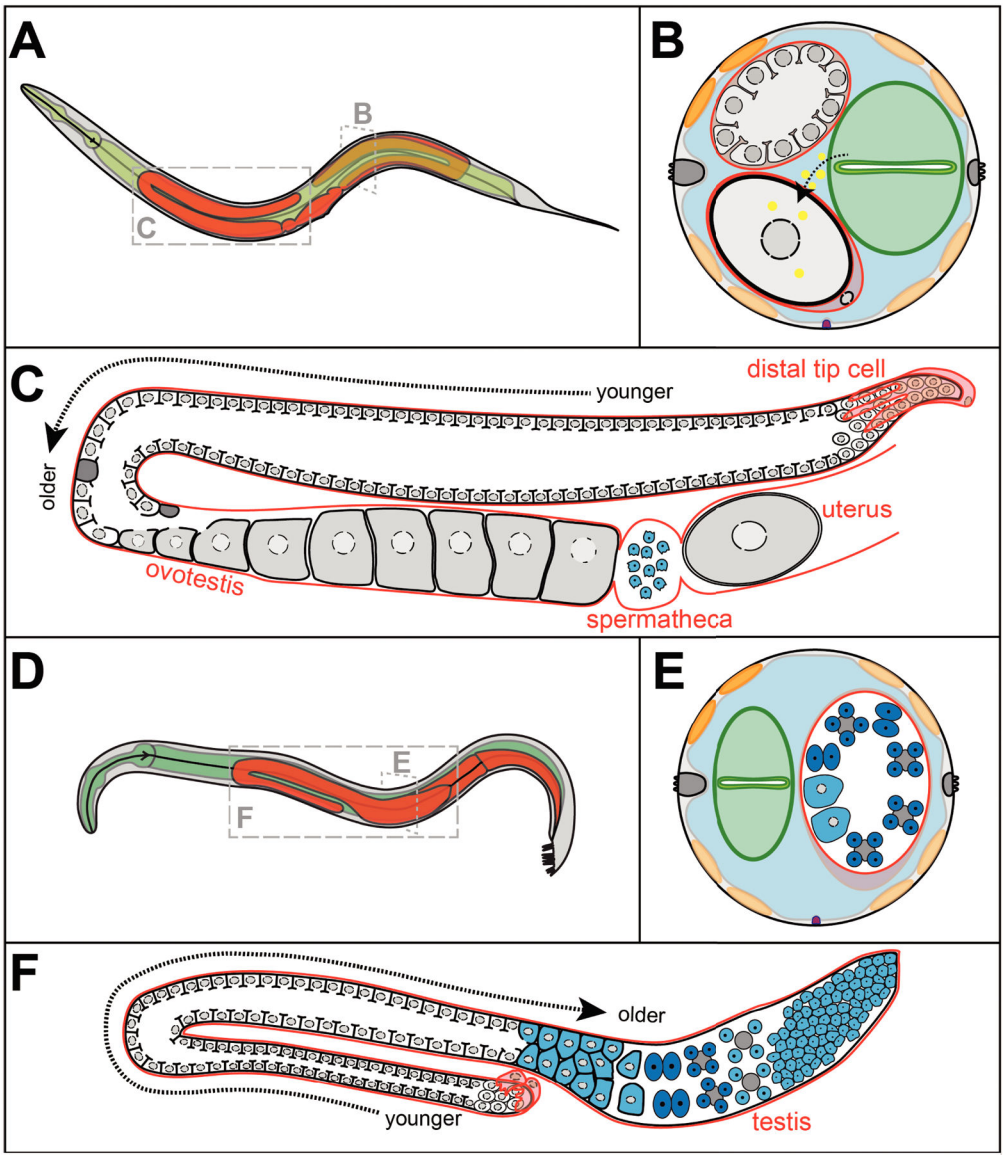

Fig. 1. The *C. elegans* gonad and germline

(a) Diagram of an *XX* hermaphrodite, with the gonad in red and the digestive system in green. Boxes show the location of expanded views. (B) Cross section of an *XX* hermaphrodite. The pseudocoelom is blue, gonad red, intestine green, muscles orange, seam cells dark gray and ventral nerve cord purple. Yolk droplets are yellow and germ cells gray. (C) One-half of the hermaphrodite gonad. Sperm are blue, cell corpses dark gray and other colors as above. (D) Diagram of an *XO* male, colored as in A. (E) Cross section of an *XO* male, as in B. Spermatocytes are blue and residual bodies dark gray. (F) The male testis, as in C and E.

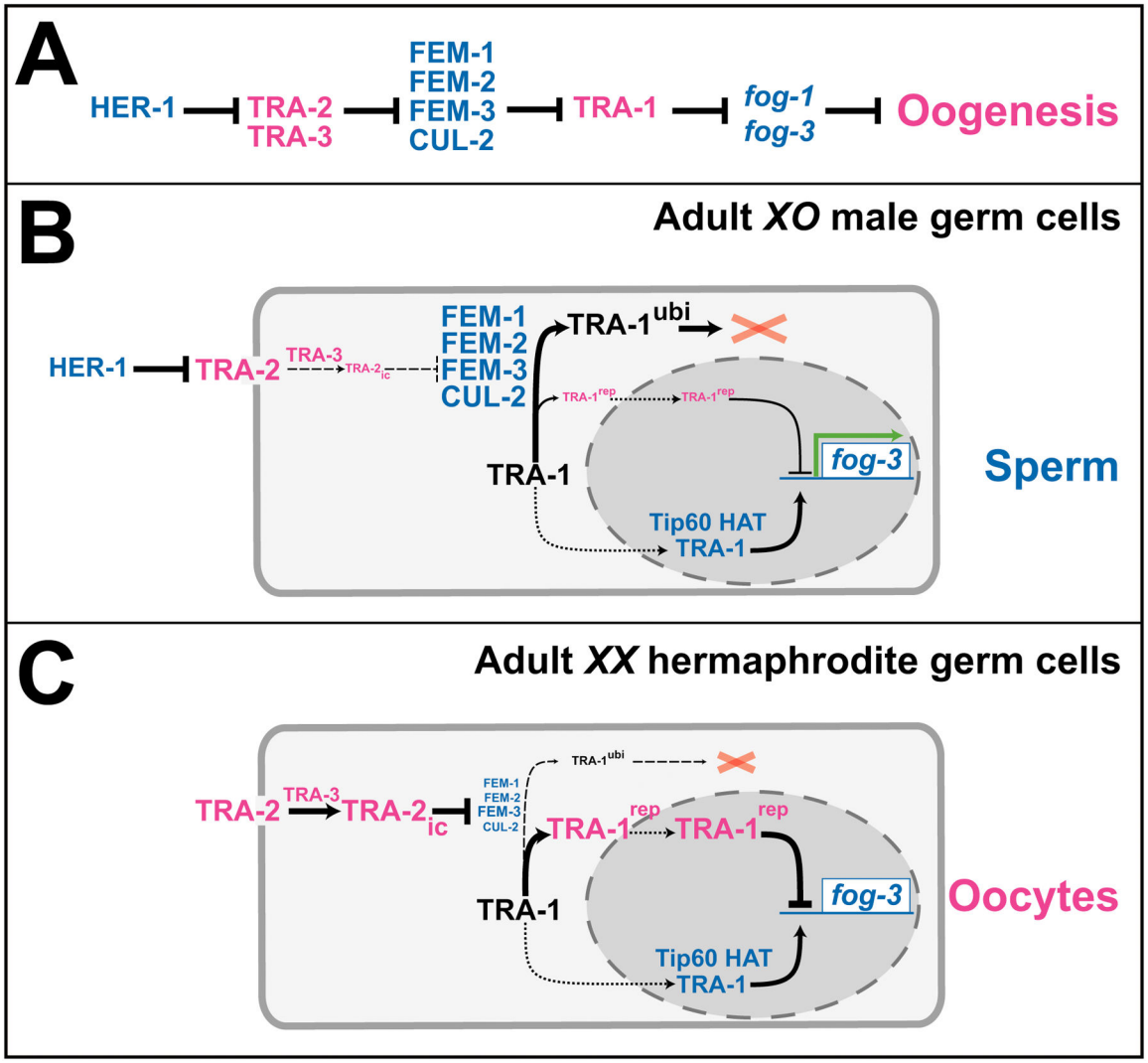

Fig. 2. The core sex-determination pathway for germ cells

Proteins that promote male fates are shaded blue, and those that promote female fates are shaded magenta. A "—|" indicates negative regulation, and an arrow indicates positive regulation. Line thickness denotes the strength of interactions, with dashed lines marking very weak interactions. Dotted lines show import into the nucleus. (A) Outline of the core sex-determination pathway. (B) The regulatory interactions in adult males, where HER-1 inactivates TRA-2, allowing the FEM complex to degrade TRA-1. Some TRA-1 is needed to work with TRR-1 and the Tip60 complex to promote spermatogenesis by inducing transcription of *fog-3*. (C) The regulatory interactions in adult hermaphrodites, where TRA-2 is cleaved to form TRA-2ic, which inactivates FEM-3. This allows TRA-1 to survive and be cleaved to form a Gli repressor that turns off genes like *fog-3*. (See text for details).

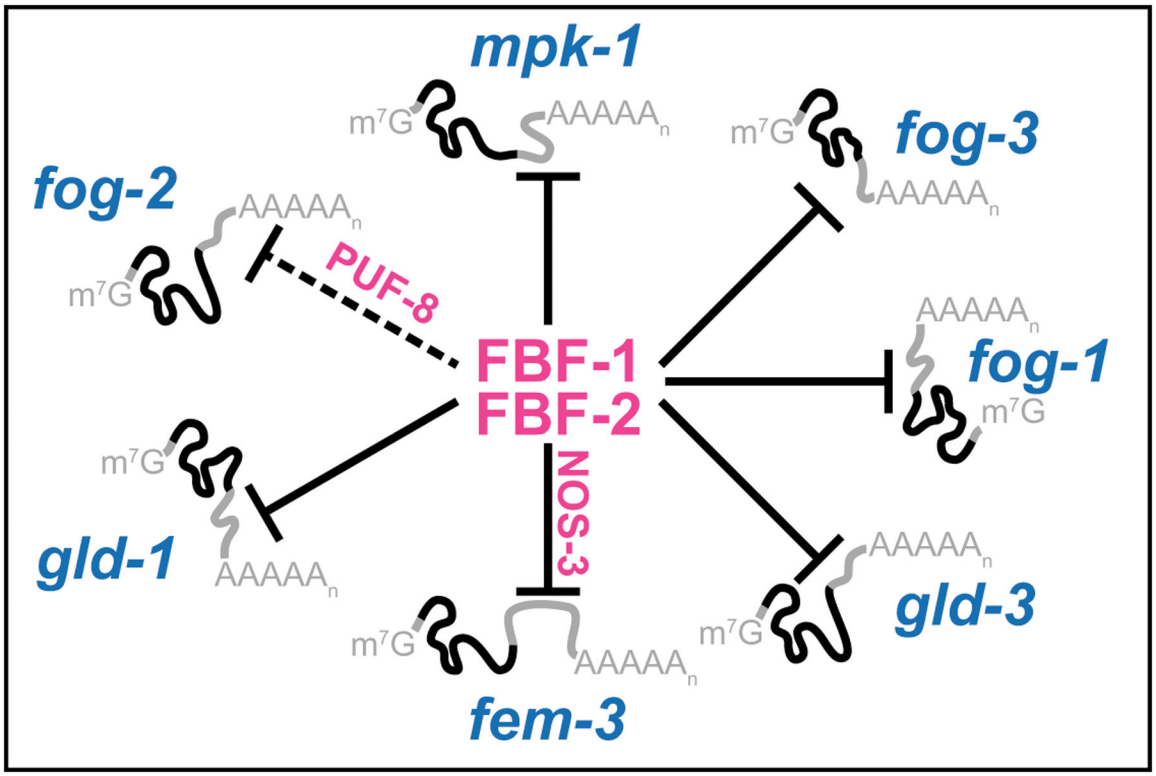

Fig. 3. The FBF proteins oppose male germ cell fates in *C. elegans*.

Messenger RNAs that promote male fates are shaded blue, and proteins that promote female fates are shaded magenta. A "—|" indicates negative regulation, with solid lines connoting direct interaction, and dotted lines connoting unknown interaction.