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Caenorhabditis Nematodes as a Model for the Adaptive Evolution of Germ Cells

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Abstract

A number of major adaptations in animals have been mediated by alteration of germ cells and their immediate derivatives, the gametes. Here, several such cases are discussed, including examples from echinoderms, vertebrates, insects, and nematodes. A feature of germ cells that make their development (and hence evolution) distinct from the soma is the prominent role played by post-transcriptional controls of mRNA translation in the regulation of proliferation and differentiation. This presents a number of special challenges for investigation of the evolution of germline development. *Caenorhabditis* nematodes represent a particularly favorable system for addressing these challenges, both because of technical advantages and (most importantly) because of natural variation in mating system that is rooted in alterations of germline sex determination. Recent studies that employ comparative genetic methods in this rapidly maturing system are discussed, and likely areas for future progress are identified.

Introduction

Beginning in the early 1980's, developmental biology was transformed by two nearly simultaneous revolutions, namely the advent of molecular-level developmental genetics and the rebirth of evolutionary developmental biology. These two revolutions were linked from the beginning, and were often furthered by the same researchers (*e.g.* see Bonner, 1981). Since its early days, a central goal of evolutionary developmental biology has been to understand how development is modified to enable major adaptations. However, the bulk of the animal adaptations that have been scrutinized developmentally are somatic attributes of larvae or adults, such as pigmentation, skeletal and exoskeletal morphologies, etc. This article is generally concerned with a less-appreciated type of developmental evolution, in which reproductive adaptations are mediated wholly or in part by changes in germ cells and their derivatives, the gametes. After an overview, recent studies using the model nematode genus *Caenorhabditis* are reviewed and synthesized.

Germ Cell Adaptation and the Evolution of New Life Histories

Examples of germ line-mediated adaptations of great ecological significance include both everyday and more obscure organisms. Every time someone cracks a hen's egg into a bowl, they are holding in their hands one of the most spectacular of these adaptations: the amniote oocyte and surrounding albumen (the "white") and shell (formed around the oocyte by the shell gland). The amniote lineage has been so successful in large part because of the derived properties of this egg and its associated coverings (Packard and Seymour, 1997; Stewart, 1997). First, the dessication-resistant shell allowed them to commit to a fully terrestrial life cycle, while the extra-embryonic membranes evolved to facilitate gas exchange, waste sequestration, and (in the archosaurs) calcium absorption required for direct development of a

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bony skeleton. Second, the enormous yolk reserves of the oocyte proper allows direct development of the embryo into a miniature adult, eliminating the larval phase of amphibian tetrapods. These traits likely first appeared in the Pennsylvanian epoch of the Carboniferous era, roughly 300 million years ago, although they are inferred indirectly from the features of fossil adults (Clack, 2002).

Perhaps less familiar to many are examples of the relatively large eggs of some animals, generally associated with major shifts in lifestyle and reproductive strategy. One example is direct development in anuran amphibians, which is associated with terrestrial or arboreal life. A well-studied case is *Eleutherodactylis coqui*, native to Puerto Rico. Although embryonic development is radically altered to allow the development of a miniature frog at hatching, it all begins with a large (3.5 mm diameter) egg (*e.g*. Callery et al., 2001; Elinson and Beckham, 2002). *E. coqui* also eggs differ from those of tadpole-forming species in tolerating some polyspermy (Elinson, 1987).

Another extreme case of egg enlargement is associated with the evolution of direct development in some echinoderms. A model system here is the Australian echinoid *Heliocidaris erythrogramma*, whose eggs are 100 times the volume of their indirect-developing sister species *H. tuberculata* (Figure 1A). In terms of bulk constituents, a significant portion of the increase in cytoplasmic volume can be attributed to large lipid droplets, initially described by Williams and Anderson (1975) as “vesicular yolk.” These droplets are later secreted into the blastocoel to form an acellular nutritive deposit (Henry et al., 1991) that persists through metamorphosis (Haag et al., 1999). Similar lipid droplets have evolved independently in different lineages with large eggs and direct development (Villinski et al., 2002). They were inferred by thin layer chromatography to be composed largely of waxy esters by Villinski et al. (2002), but other studies using other methods have failed to support this diagnosis (Byrne et al., in press; Prowse et al., 2008). Whatever their precise composition, the droplets are deposited during oogenesis after a conserved, early phase of yolk production is completed (Byrne et al., 1999). This post-vitellogenic phase thus represents a novel aspect of direct-developing oogenesis. Surprisingly, this lipid is not necessary for the completion of metamorphosis, but is required for survival of the non-feeding juvenile stage that occurs between metamorphosis and eruption of the mouth (Emlet and Hoegh-Guldberg, 1996; Williams and Anderson, 1975). With respect to developmental patterning, embryological experiments indicate that the egg of the direct-developing *Heliocidaris erythrogramma* incorporates axial patterning cues that are specified only after first cleavage in its indirect-developing relatives (Henry and Raff, 1990; Henry et al., 1990).

In addition to size, germ cells also mediate extreme shifts in reproductive mode by facilitating the loss of obligate mating. An example from the vertebrates is parthenogenesis, seen both in lizards and salamanders. This is invariably associated with hybrid species with cytogenetically distinguishable karyotypes, and includes both allo-diploids and allo-triploids, the latter presumably formed by fertilization of a diploid oocyte of one species by a sperm of another (Uzzell, 1970). Parthenogenic species may, in principle, use several genetic mechanisms to produce oocytes with the same ploidy as their somatic cells (Uzzell, 1970). In both the related salamanders *Ambystoma platineum* and *Ambystoma jeffersonianum* (Macgregor and Uzzell, 1964), and in the whiptail lizard *Cnemidophorus uniparens* (Cuellar, 1971; Figure 1B), it appears that triploid primary oocytes undergo one round of mitosis without cytokinesis. This allows formation of a set of pseudo-bivalents composed of pairs of newly replicated daughter chromosomes. As pairing is always between chromosomes of the same hybrid parent species, the two karyotypes present in the hybrid genome undergo no effective recombination. It is unclear how rapidly this sort of pseudo-meiosis can evolve, but it is possible that it represents a latent capacity of oocytes that cannot produce interspecies bivalents. If so, this trait would appear as soon as a hybrid lineage forms, though selection may further enhance its reliability.

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A second example of germ cell changes that decouple mating and reproduction is the evolution of self-fertile hermaphroditism in nematodes of the family Rhabditidae, including the model species *Caenorhabditis elegans* and its close relative, *C. briggsae*. This trait has evolved repeatedly from the ancestral male/female (gonochoristic) condition in soil nematodes, and even the two *Caenorhabditis* cases are likely cases of convergent evolution (Kiontke and Fitch, 2005; Kiontke et al., 2004; Kiontke and Sudhaus, 2006). In all cases, self-fertility is mediated by the evolution of limited spermatogenesis in the XX (female) sex (Figure 1C). This situation may evolve repeatedly instead of the sperm-swapping hermaphroditism seen in other protostomes (*e.g.* gastropod molluscs; Jarne ref) because of the extreme sexual dimorphism and the associated internal fertilization of the gonochoristic ancestors. More specifically, because the entire posterior of the male is specialized for copulation and sperm transfer, an entirely new mechanism would be required to allow hermaphrodites to accomplish the same task.

Though the above examples are all instances where germ cell attributes have been the result of natural selection acting on life history, germ cells are also are involved in extreme examples of sexual selection. For example, in the dipteran insect *Drosophila bifurca*, the sperm are many times longer than the adult male that produces them, and require elaborate coiling in order to fit into the female reproductive tract (Figure 1D). These giant sperm are likely to have evolved from runaway post-copulatory sexual selection imposed by elongation of the female's seminal receptacles (Miller and Pitnick, 2002), and are now so big that in many respects the species is effectively isogamous (Bjork and Pitnick, 2006). All of the above examples make the point that, far from being adaptively inert custodians of the genome, germ cells themselves can rapidly respond to selection to enable important adaptations or extreme sexual traits.

Germ Cell Adaptation: Evo-devo Meets RNA

Because it is a premier model species for developmental genetics, and even more so because germline sex determination has a long history of genetic and molecular research (Wormbook refs, etc.), *Caenorhabditis elegans* and its close relatives make a powerful system for addressing the evolution of hermaphrodite development. The author, along with his students and colleagues, has spent much of the last decade developing tools for non-*elegans* species of *Caenorhabditis* that will enable the realization of the great potential in this area. But to understand the our approach, it is important to first understand some of the ways in which germ cell development is different from that of somatic tissues.

While the question of whether transcriptional regulation or coding sequence changes contribute more to phenotypic evolution has received much recent attention (Carroll, 2008; Hoekstra and Coyne, 2007; Prud'homme et al., 2007), evidence from *C. elegans* and other systems suggests that germ cells often use a third type of regulation to control cell cycle progression and differentiation, that of post-transcriptional control of mRNA translation. Why germ cells rely so heavily upon RNA-level regulation is still unclear, but one idea (Kimble and Crittenden, 2007; Seydoux and Braun, 2006) is that they are poised on the cusp of initiating embryonic differentiation via their diverse maternal mRNAs, but are restrained from doing so prematurely by translational repression via various RNP complexes. We might term this the "frozen almost-embryo" hypothesis, and there is a large body of data supporting it (Evans and Hunter, 205). An alternative idea is that meiosis, which is unique to germ cells, may impose special requirement on gene expression. As the vast majority of adult *C. elegans* germ cells are in various stages of meiosis, which is marked by condensed chromatin, it may be that differentiation and cell cycle control must be handled to a large extent in the absence of new transcription. We could term this the "meiotic transcriptional block" hypothesis, and a number of studies in *C. elegans* have indeed suggested that transcription in germ cells is generally

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repressed by chromatin modifications (Kelly and Fire, 1998; Schaner and Kelly, 2006). Of course, these two hypotheses are not mutually exclusive, and both are probably relevant.

Though the evolution of RNA-level controls are only just beginning to be investigated, they do bear some similarity to the more familiar cis-regulatory control of transcription by DNA-binding transcription factors. For example, they are generally mediated by *cis* elements, typically in 3′ untranslated regions (UTR) of mRNA. These UTR elements serve as specific docking sites for various RNA-binding proteins (RBPs) that, like transcription factors, are often combinatorial in their effects on a single target and highly pleiotropic in that they bind many different mRNAs (Jin et al., 2001; Lee and Schedl, 2001; Luitjens et al., 2000; Piqué et al., 2008; Standart and Minshall, 2008; Wickens et al., 2002). To understand how adaptive evolution works in germ cells, then, it is important to develop methods that allow the discovery and functional perturbation of potentially complex regulatory networks in multiple species. Such studies would necessarily address both target mRNAs and the RBPs that regulate them. As the most obvious adaptation in hermaphroditic *Caenorhabditis* is a change in sexual fate of germ cells from oocytes to sperm, the target mRNAs we focus upon are those encoding components of the sex determination pathway.

Overview of C. elegans sex determination

In *C. elegans*, germ cell sex is controlled by same pathway of negative regulation that governs sex in the rest of the body (the core pathway; Figure 2). At the simplest level, this pathway links the ratio of X chromosomes to autosomes to the activity state of the terminal global regulator, the transcription factor TRA-1 (Zarkower and Hodgkin, 1992). TRA-1 exists at high levels in XX hermaphrodites as a proteolytically processed form (TRA-1^{100}) that represses male development (Schvarzstein and Spence, 2006). An unprocessed form of TRA-1 is present at much lower levels in both sexes (Schvarzstein and Spence, 2006). As complete loss of TRA-1 via mutations converts XX animals into near-perfect males that can sire progeny (Hodgkin, 1987;Hodgkin and Brenner, 1977), most of *tra-1*'s activity can be ascribed to repression of male fates by TRA-1^{100}.

We can examine the molecular logic that underlies the diagram shown in Figure 2 by backing up from *tra-1*. The sex difference in TRA-1^{100} abundance is due to male-specific ubiquitination and proteolysis, which is mediated by the three cytoplasmic FEM proteins acting in a complex (Chin-Sang and Spence, 1996;Starostina et al., 2007;Tan et al., 2001). In XX animals the FEM proteins are prevented from targeting TRA-1 for degradation by an interaction between FEM-3 and the membrane protein TRA-2 (Mehra et al., 1999). *tra-2* function also requires that TRA-2 be cleaved by the calpain protease TRA-3 (Hodgkin and Brenner, 1977;Sokol and Kuwabara, 2000), indicating that repression of the FEM proteins by TRA-2 may actually be accomplished by a cytoplasmic C-terminal fragment rather than the intact transmembrane protein. The TRA-2-FEM interaction, in turn, is prevented in XO males by the secreted protein HER-1, which interacts with the extracellular domain of TRA-2 (Hamaoka et al., 2004). In keeping with the cell non-autonomy implied by HER-1 secretion, germ cell sex can be influenced by surrounding somatic tissues (Cho et al., 2007;Hunter and Wood, 1992;McCarter et al., 1997).

Continuing upstream, HER-1 levels are regulated at the transcription level by the SDC proteins, which also mediate dosage compensation of the X chromosomes (Chu et al., 2002). This dual function of the SDC proteins ensures that transcription of both *her-1* and most X-linked genes are repressed in XX cells. Finally, the *sdc* genes are regulated by *xol-1*, which sits atop the signaling cascade and whose transcription is directly controlled by the relative levels of X-linked and autosomal factors (Meyer, 2005).

With the above pathway in mind, we now return to the subject of the derived germ cell differentiation of hermaphrodites. It is crucial to note that although hermaphroditic

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Caenorhabditis, such as *C. elegans* and *C. briggsae*, make sperm, they do so without expressing HER-1 (Trent et al., 1991). Therefore, they must set the downstream part of the sex determination pathway in male mode without HER-1, and only in the germ line. A large body of genetic and molecular work has revealed that this feat requires the activity of a number of germline-specific factors. Two, the cytoplasmic polyadenylation element-binding (CPEB) protein homologue FOG-1 and the TOB domain protein FOG-3, act downstream of TRA-1, with *fog-3* being a direct transcriptional target (Barton and Kimble, 1990; Chen and Ellis, 2000; Ellis and Kimble, 1995). Another group of RBPs affect sex determination upstream of *tra-1*, and several have been shown to directly regulate sex determination mRNAs. In particular, the KH-domain RBP GLD-1 (Francis et al., 1995a; Francis et al., 1995b; Jones and Schedl, 1995), its cofactor, FOG-2 (Clifford et al., 2000; Schedl and Kimble, 1988), and the RNA helicase LAF-1 (Goodwin et al., 1997; A. Hubert, MS submitted) are all required to allow initiation of XX spermatogenesis. All of these factors are directly or indirectly involved in regulating the translation of *tra-2* mRNA, which harbors an essential GLD-1-binding site in its 3′ UTR (Goodwin et al., 1993; Jan et al., 1999; Lee and Schedl, 2001). This has led to model in which XX spermatogenesis requires, and may be specifically activated by, repression of *tra-2* translation, which mimics HER-1 inhibition of TRA-2 activity in the XO male (Figure 2).

Cessation of spermatogenesis, the "sperm-to-oocyte switch," is also a crucial step in hermaphrodite development. Again, a large body of work has implicated RBPs in the translational control of a second sex determination gene, the male-promoting *fem-3*. As with *tra-2*, *fem-3* contains a crucial binding site for an RBP complex (Ahringer and Kimble, 1991; Barton et al., 1987), which is composed of the PUF family members FBF-1 and FBF-2 and their cofactor, the *Nanos* homologue NOS-3 (Kraemer et al., 1999; Zhang et al., 1997). The translational repression of *fem-3* also requires the six *mog* genes (Gallegos et al., 1998), at least three of which encode homologues of mRNA splicing factors and as well as a cyclophillin-related protein (Belfiore et al., 2004; Puoti and Kimble, 1999; Puoti and Kimble, 2000). Finally, the RBP DAZ-1 appears to promote the sperm-oocyte switch by stimulating translation of the *fbf-1* and *fbf-2* mRNA (Otori et al., 2006).

While the above two paragraphs catalog an impressive array of discoveries in the area of germline sex determination, a cautionary note is appropriate. While many factors are necessary for proper execution of the sperm-then-oocyte pattern of hermaphrodite germ line development, the identity of the sex determination pathway component(s) whose activity is differentially modulated under natural physiological conditions to effect the switch represented by the arrowhead in Figure 1C is still not known. To underscore this point, when the *tra-2* and *fem-3* translational controls described above are both abrogated through mutations that eliminate their translational control elements, self-fertile hermaphrodites are produced at high frequencies (Barton et al., 1987; Schedl and Kimble, 1988). Whichever factor serves as the natural switch element, the distal expression of *rme-2* mRNA (encoding an egg-specific yolk receptor) in the last larval (L4) stage implies that oocyte fate is specified in, or soon after cells exit from, the distal mitotic stem cell zone (Ellis and Schedl, 2007).

There are additional complications that make germline sex determination different from that seen in the soma. One is that while XX *tra-1* loss-of-function mutants are transformed into mating males, they usually have intersexual germ line development, rather than the full maleness seen in the soma (Hodgkin, 1987). This suggests that, unlike in the soma, the repression of maleness is not TRA-1's only function in germ cells. As XO *tra-1(lf)* mutants also suffer germline feminization, it is likely that this phenotype results from a germline-specific requirement for the full-length (unprocessed) form of TRA-1 in reliable specification of the sperm fate. Thus, *tra-1* may have both repressive and activating roles in male development, which would be reminiscent of the similar dual roles of its homologues, the

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hedgehog pathway effectors *Cubitus interruptus* (in *Drosophila*) and Gli (in vertebrates; reviewed by Østerlund and Kogerman, 2006).

A second complication comes from double mutant analyses. The core sex determination pathway shown in Figure 2 indicates that the sole purpose of the FEM proteins is to regulate TRA-1 activity. In the soma this seems to hold up well, as the three possible *fem; tra-1* double mutants all have the same completely male anatomy and behavior found in true XO males (Doniach and Hodgkin, 1984;Hodgkin, 1986). However, the germline phenotype of these double mutants is complete feminization. This unexpected result suggests that the FEM proteins may promote sperm fate independently of their action on TRA-1, such that the already partially feminized germ line of *tra-1(lf)* mutants is pushed into completely female territory when they are compromised. A more specific variation on this is that TRA-1 transcriptionally represses the *fem* genes in the germline as part of its general male-repressing function. Under this model, loss of *tra-1* produces a partly masculinized germ line because of upregulation of *fem* transcription, which in turn promotes spermatogenesis. Mutations in *fem* genes thus reverse this phenotype by preventing them from responding to reduced TRA-1.

Caenorhabditis: a Window on the World of Germline Adaptation

As we have seen, germ cell biology is marked by a strong reliance upon RNA-protein complexes, many of which serve to regulate mRNA translation, and sex determination in *C. elegans* is no exception. Germ cell translational control is mediated by a number of widely conserved, often germ line-specific proteins. Since choosing between oocyte and spermatocyte fate is the main task that a nematode germ cell must accomplish prior to fertilization, perturbations of many translational regulators produces sexual phenotypes. This may be further exaggerated by the existence of reinforcement, feedback, and threshhold controls that are normally in place to prevent intersexuality. Such controls would be expected to create sharp phenotypic transitions upon experimental perturbation. *Caenorhabditis* gives us a system to explore how these post-transcriptional controls are modified to produce an ecologically important adaptation—XX spermatogeneis. Two main approaches we have used are:

1. Evaluation of candidate translational controls in gonochoristic species
2. Genetic and molecular comparison of sex determination in convergently evolved hermaphrodites (Figure 3).

Below, recent results from both areas are summarized.

What makes a Female Different from a Hermaphrodite?

In the simplest possible model, the translational controls that regulate *tra-2* and *fem-3* levels in *C. elegans* are the essence of hermaphrodite development, and evolved specifically for this purpose. Motivated by this hypothesis, Haag and Kimble (2000) characterized the first sex determination gene from a gonochoristic nematode, the ortholog of *tra-2* in *C. remanei*. RNAi interference experiments showed that TRA-2 promotes female fates in both the soma and germ line, as in *C. elegans*. The study also revealed two surprising aspects of *tra-2* evolution. First, though *C. remanei* females never initiate spermatogenesis, the 3′ UTR of *Cr-tra-2* nevertheless bound a factor in extracts that had properties similar to DRF, the GLD-1-containing translational repressor. This suggested that it was not the evolution of translational control *per se* that enabled hermaphrodite spermatogenesis, and that perhaps more subtle modulation of preexisting controls was closer to the truth. Second, though TRA-2 was overall rather divergent, as expected from earlier work on the *C. briggsae* homologue (Kuwabara, 1996), the C-terminal cytoplasmic domain shown to bind FEM-3 was hypervariable—so much so that there are essentially no conserved residues in a three-way alignment. Given the essential nature of the TRA-2-FEM-3 interaction, this lack of sequence constraint was wholly unexpected.

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Given the results for *Cr-tra-2*, it became important to also examine *Cr-fem-3*. Previous attempts to clone homologues of *fem-3* from other *Caenorhabditis* species by low-stringency nucleic acid hybridization failed, presumably due to unusually low sequence conservation (J. Kimble, pers. comm.). Using the synteny-based strategy pioneered by Kuwabara and Shah (1994), Haag et al. (2002) identified phage and fosmid clones from *C. remanei* and *C. briggsae* (respectively) that contained both the conserved copine gene used to identify the clones as well as highly diverged orthologs of *fem-3*. As with the domain of TRA-2 with which it interacts, conservation of FEM-3 as a whole is remarkably poor, with pairwise identities ranging from 31–38% and only very short motifs conserved in all three homologues. Despite this rapid sequence evolution, however, in all three species the C-terminus of TRA-2 interacts strongly with the conspecific FEM-3 homologue in yeast two-hybrid assays (Haag et al., 2002). That none of the mixed-species pairings did suggested that rapid coevolution was occurring, prompting the author to examine both the theoretical and empirical population genetics of this phenomenon (Haag, 2007; Haag and Ackerman, 2005; Haag and Molla, 2005).

Functional assays also support a conserved interaction between *fem*-3 and *tra-2* products. *fem-3 (RNAi)* feminizes the soma of XO animals of both *C. remanei* and *C. briggsae*. Importantly, knocking down both *Cr-fem-3* and *Cr-tra-2* reversed the somatic masculinization of *Cr-tra-2 (RNAi)* alone, indicating that despite their molecular divergence they perform similar roles and have similar epistatic relationships that are independent of reproductive mode. However, the one tissue in XO males that was not feminized by *Cr-fem-3(RNAi)* was the germ line. Further, *Cr-fem-3(RNAi)* could not suppress the masculinized germ line of XX *Cr-tra-2(RNAi)* animals, even though it did reverse somatic phenotypes. Taken together, these results indicated that *C. remanei fem-3* is important for male somatic development, but is not used to regulate germ cell fates.

Although the above results might suggest that *fem-3* translational control would not occur in *C. remanei*, the 3′ UTR of *Cr-fem-3* nevertheless contains a well-conserved Point Mutation Element (Haag et al., 2002), the short sequence known to bind the FBF-1 and FBF-2 proteins in *C. elegans* (Ahringer and Kimble, 1991; Zhang et al., 1997). Similar to the case with *Cr-tra-2*, then, we see that translational controls *per se* probably preceded the evolution of self fertility, though they may have been modified in hermaphrodite lineages. As translational control of both *tra*-2 and *fem-3* occurs in the *C. elegans* soma (Gallegos et al., 1998; Jan et al., 1997), this may be their original site of action in gonochoristic species.

So, what evidence is there that hermaphrodites do have unique translational controls that act on sex determination genes? The most compelling so far is the case of *fog-2*. Mutant *C. elegans* hermaphrodites lacking *fog-2* activity are converted into true females, yet homozygous males make copious sperm (Schedl and Kimble, 1988). *fog-2* was cloned when its F-box protein product was found as an interactor of the RBP GLD-1 (Clifford et al., 2000). GLD-1 had previously been identified as a major component of DRF, the repressor of *tra-2* translation (Jan et al., 1999). Interestingly, *fog-2* is the recent product of recent tandem duplications. Nayak et al. (2005) expanded on this initial observation by showing that FOG-2 is part of a large family of F-box-containing proteins, and that the entire *C. elegans* gene family coalesces to a common ancestral gene that is younger than the time at which *C. elegans* split from the lineage it shared with *C. briggsae*. Further, Nayak et al. demonstrated that only FOG-2, and not its paralogs, has the C-terminal sequences necessary to mediate an interaction with GLD-1. Taken together, *fog-2* is a lineage-specific gene with a new function in germline sex that is required to make a hermaphrodite a hermaphrodite. It is therefore likely that the evolution of *fog-2* was a key step in the evolution of XX spermatogenesis in the *C. elegans* lineage.

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Are There Really 50 Ways to Leave Your Lover?

Another asset of the *Caenorhabditis* system is the existence of at least two outwardly similar hermaphroditic species, *C. elegans* and *C. briggsae*, which are inferred from phylogenies to be independently evolved (Cho et al., 2004; Kiontke and Fitch, 2005; Kiontke et al., 2004; Figure 3). This enables us to examine how reproducible the evolution of XX spermatogenesis is at the level of developmental genetics. Although the convergent acquisition of selfing was not known at the time, some of the earliest gene homologues to be characerized in non-*elegans Caenorhabditis* species were components of the *C. briggsae* sex determination pathway (Chen et al., 2001; de Bono and Hodgkin, 1996; Haag et al., 2002; Hansen and Pilgrim, 1998; Kuwabara, 1996; Streit et al., 1999). These studies found that sequence conservation was generally lower than for typical orthologous pairs (Stein and others, 2003), ranging from roughly one to two-thirds amino acid identity (summarized by Haag, 2005b; Nayak et al., 2005). Nevertheless, using cross-species transgenic rescue assays and RNA interference methods, these studies generally found that sex determination functions were conserved. A notable exception, however, was seen in the *Cb-fem-2* and *Cb-fem-3* genes, which could not be implicated in germline sex determination using these assays (see also Stothard et al., 2002). These results are reminiscent of those for *C. remanei* described above, in that the germline function of the *fem* genes emerges as an exception to more general conservation.

Though considered cutting-edge at the time, neither RNAi nor cross-species transgenes produce completely penetrant phenotypes. As a result, doubt remained whether the unexpected results for the *C. briggsae fem* homologues were due to true functional differences or to technical limitations of the method. To provide the same standard of proof used in *C. elegans*, the author and his coworkers have developed mutational methods in *C. briggsae* (see Table 1 for summary). We began by following the historically successful approach (Hodgkin and Brenner, 1977) of screening for masculinized (Tra) mutants among mutagenized *C. briggsae* animals. This work identified multiple mutant alleles of the homologues of the three known *tra* loci, *Cb-tra-1, Cb-tra-2,* and *Cb-tra-3*, including conditional alleles of the latter two (Kelleher et al., 2008). The phenotypes of these mutants are generally congruent with those of their *C. elegans* equivalents, and specifically they cause complete germline masculinization.

As noted above, the *Cb-fem* genes were the ones that showed unexpected germline phenotypes in knockdown and rescue experiments. To identify true mutations in these genes, we took two approaches. One was to screen for suppressors of the Tra phenotype of *Cb-tra-2(ts)* and *Cb-tra-3(ts)* at nonpermissive temperature, similar to earlier work in *C. elegans* (Hodgkin, 1986). Using two different alleles of *Cb-tra-2*, 75 different alleles were isolated that reversed the somatic masculinization of XX *Cb-tra-2(ts)* mutations (Hill et al., 2006). Interestingly, none of these mutations produced true females, as their *C. elegans* equivalents would, but instead converted the Tra pseudomales into self-fertile hermaphrodites. However, as provocative as these results were, the identities of the suppressors and the nature of their molecular lesions remained unknown.

In a more direct approach, PCR-based screens for deletion mutations were used to isolate null alleles of *Cb-fem-2* and *Cb-fem-3* (Hill et al., 2006). Confirming previous RNAi studies, both of these mutants had no effect on XX hermaphrodites. Further, XO homozygotes are converted into self-fertile hermaphrodites. In contrast, in *C. elegans* both XX and XO *fem* homozygotes are converted into true females. Thus, while both males and hermaphrodites require the *fem* genes for spermatogenesis in *C. elegans*, in *C. briggsae* the only germline function of the *fem* genes appears to be to prevent males from switching to oogenesis. Overall, the ability to produce mutations in *C. briggsae* sex determination genes delivers a new level of precision to the analysis of developmental evolution *Caenorhabditis*. They enable us to infer with considerable confidence that the genetic control of hermaphrodite germ line development is

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fundamentally different in *C. elegans* and *C. briggsae*, and more specifically that the locus of regulation of XX spermatogenesis in *C. briggsae* probably lies downstream of the *Cb-fem* genes. In combination with the parsimonious reading of current phylogenies (Cho et al., 2004; Kiontke et al., 2004; Fig. 3), these results further indicate that nearly identical germline phenotypes have evolved using distinct genetic paths. The general lesson here is that within the general constraints imposed by the sex determination pathway, considerable flexibility exists in how adaptation can occur.

Evolutionary Dynamics of Germline RNA-binding Proteins

The above synopsis makes clear that the global *Caenorhabditis* sex pathway, while subject to rapid sequence evolution, is generally intact in all species examined thus far. With the exception of *fog*-2, however, little has been said about the germline-specific regulators shown in Figure 2. Although less is known here, it already appears that germline-specific sex determination genes are often well-conserved at the protein level, yet have evolutionary dynamics that go beyond point mutation, including duplication, divergence in functional domains, and cooption into new roles. FOG-2 has all of these attributes, and an RNAi study of its binding partner, GLD-1, suggest that it, too presents surprises (Nayak et al., 2005). While *C. briggsae* GLD-1 is very similar at the amino acid level to its *C. elegans* homologue (Haag, 2005a), *Cb-gld-1 (RNAi)* has a phenotype that is opposite. Specifically, while reduction in *C. elegans gld-1* causes loss of XX spermatogenesis (presumably because of failure to translationally repress *tra-2* translation), *Cb-gld-1(RNAi)* causes germline masculinization (Nayak et al., 2005). Aided by this result, two strong loss-of-function alleles of *Cb-gld-1* have been identified in forward screens for *C. briggsae* Mog mutants (A. Doty and ESH, unpublished data). This confirms the different roles of *gld-1* in germline sex determination of *C. elegans* and *C. briggsae.*

fog-2 is not the only germline sex determination gene that is the product of lineage-specific gene duplication. In *C. elegans*, FBF is encoded by two nearly identical genes that are the product of a recent duplication (Zhang et al., 1997). In *C. briggsae,* the closest PUF family relatives of FBF are encoded by a three-member clade of similarly duplicated genes (Lamont et al., 2004; discussed in Haag, 2005). Recent work in the author's lab suggests that these genes also have unexpected functions in germ cell sex determination, as well as in other processes (Q. Liu, ESH, unpublished data).

Challenges and Future Directions

This article has demonstrated that the bulk of functional divergence in the *Caenorhabditis* sex determination pathway lies in the germ line. As this is the same tissue that undergoes the most dramatic phenotypic evolution, this is perhaps not surprising. However, much of this divergence may be due to inherently dynamic evolution of germ line regulators, and not be specifically related to adaptive shifts in phenotype (see True and Haag, 2001 for further discussion). To identify the subset of changes responsible for germline sex determination adaptation, we must first recognize several challenges. First, RBPs are often pleiotropic and have many targets, with the result that their loss-of-function phenotypes are often complex. For example, most of the germline-specific sex regulators discussed in this paper have other phenotypes when inactivated, such as cell cycle defects and embryonic lethality (*e.g.* Crittenden et al., 2002; Francis et al., 1995a; Graham et al., 1993). For translation-regulating RBPs, this may be the manifestation of a large number of target mRNAs. Second, these sexual regulators are often encoded by members of gene families, in which members may have either similar or dissimilar functions. Therefore both redundancy and unexpectedly paralog-specific phenotypes could emerge, and we see evidence for both in our ongoing studies. Third, *in vivo* assays for translational control are technically more difficult than those for transcriptional control, and are even harder in germline due to transgene silencing.

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While the above challenges are indeed rather daunting, we can still make progress. For example, it is likely that the different phenotypes of otherwise conserved RBPs is due to evolutionary changes in target mRNAs. By extending the same sort of systematic characterization of RBP target mRNAs that has been done in *C. elegans* (e.g. Lee and Schedl, 2001) to other species, species-specific targets could be discovered. With respect to redundancy, we do appear to be fortunate in that RNAi by injection produces fairly reliable germline phenotypes in *C. briggsae* (*e.g.* compare the results of Haag et al., 2002; Stothard et al., 2002 with those of Hill et al., 2006). This allows rapid searches for synthetic phenotypes via double RNAi experiments. Another key method will be production of transgenes that express well in germ cells. The most reliable method currently in *C. elegans* is based on particle bombardment of DNA constructs into an *unc-119* mutant strain (Praitis et al., 2001), and we have recently identified the equivalent mutant in *C. briggsae* (C. Thomas, ESH, unpublished data). Finally, the ongoing discovery of new *Caenorhabditis* species, in particular by M.A. Félix (Institut Jacques Monod, Paris), is opening up the possibility of using hybrids between hermaphroditic and gonochoristic species as a new route to understanding how XX spermatogenesis evolves (M.A. Félix, G. Woodruff, and ESH, unpublished data). Overall, it is fair to say that *Caenorhabditis* is maturing into a sophisticated model meta-system for probing the genetic basic of germ cell adaptations.

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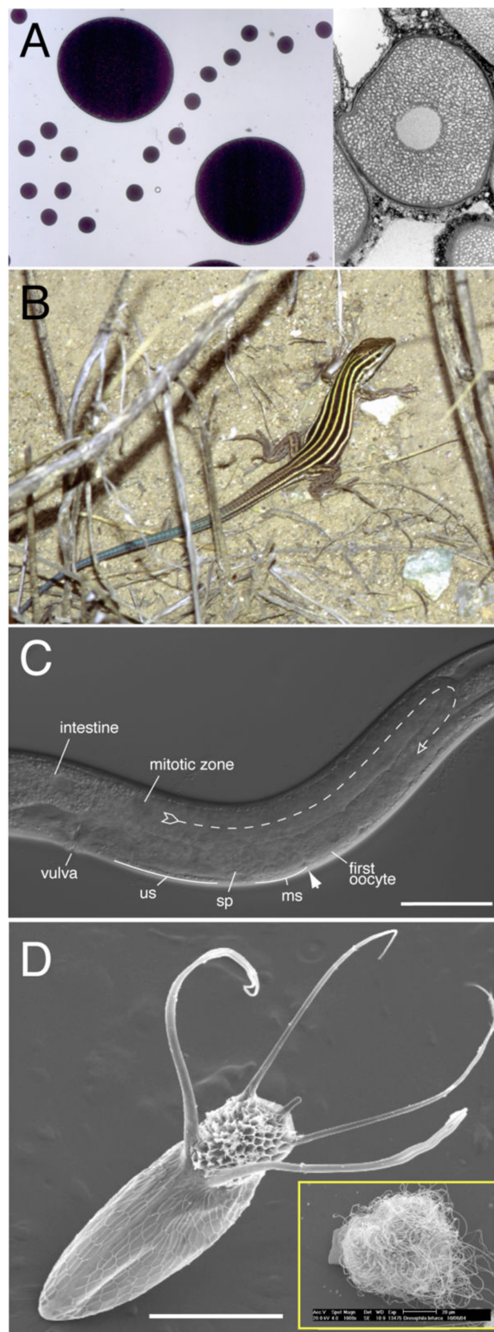

Figure 1.

Examples of extreme germ cell adaptation in four phyla. A. The left panel is a micrograph of a mixture of spawned, mature eggs from the Australian congeneric sea urchins *Heliocidaris tuberculata* (ca. 95 μm dia.) and *H. erythrogramma* (ca. 420 μm dia.). *H. tuberculata* is a typical indirect-developer with a feeding pluteus larva, while *H. erythrogramma* is a lecithotrophic direct-developer. The right panel shows a paraffin section through a maturing *H. erythrogramma* oocyte in the ovary, which reveals abundant cytoplasmic lipid droplets. B. Adult of the all-female parthenogenetic whiptail lizard, *Cnemidophorus uniparens*, in its natural setting in Arizona. C. Differential interference contrast micrograph of the posterior gonad arm of a young adult hermaphrodite *Caenorhabditis briggsae* nematode, showing the

completion of spermatogenesis and initiation of oogenesis in the same germ cell population. In females of gonochoristic *Caenorhabditis* species, spermatocytes are absent. Germ cells move from the distal, mitotic stem cell niche half the length of the gonad arm, at which point the arm reflexes and converges on the uterus and spermatheca (us). The first few hundred germ cells that differentiate produce sperm. Both meiotic spermatocytes (ms) and mature (but inactive) spermatids (sp) can be seen here. Immediately behind the spermatocytes, the first oocytes are starting to differentiate, with an abrupt transition between them (arrowhead). Scale bar is 50 μm. D. Giant sperm in the dipteran insect *Drosophila bifurca*, as seen in scanning electron micrographs. The oocyte, with its elaborate chorion, is shown in the main panel, while a single spermatozoan with its extensively coiled axoneme is shown in the inset panel. Scale bar in the main panel is 200 μm; the sperm image is magnified 2.5× relative to the egg. Image credits: A (left) by Jeff Villinski (courtesy of Rudolf Raff) and A (right) by Maria Byrne; B by Twan Leenders; C by the author; D by Romano Dallai (courtesy of Scott Pitnick).

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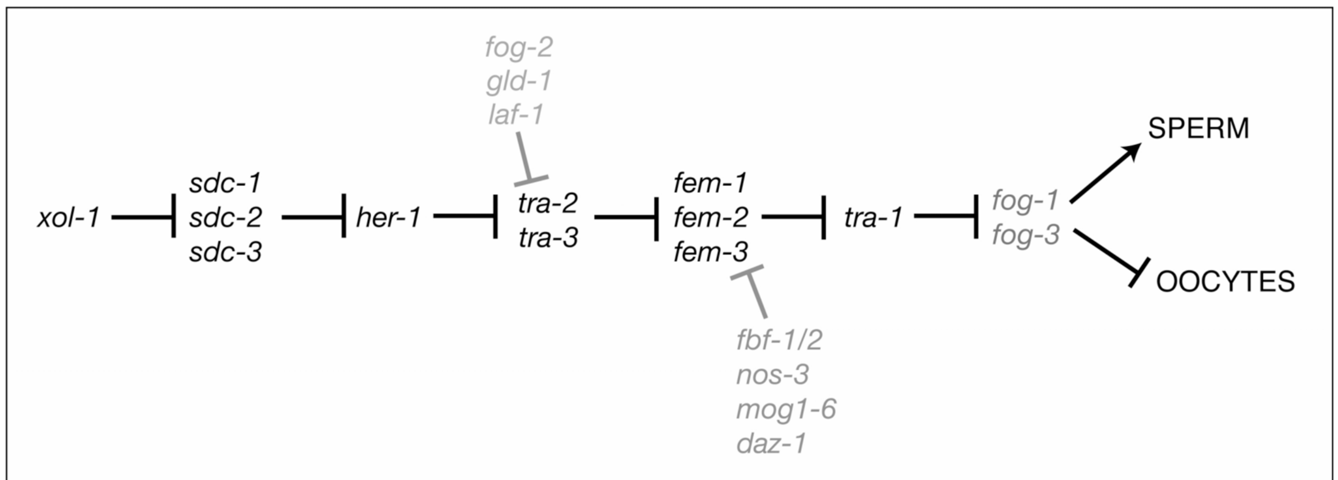

Figure 2.
The *C. elegans* sex determination pathway and its germline-specific modifiers. The "core pathway" acting in all cells is depicted in black typeface, which germline-specific genes are in gray. Germline genes required for the onset of XX spermatogenesis are shown above the horizontal midline, and affect *tra-2*. Genes required for the sperm-oocyte switch are shown below the midline, and affect *fem-3*.

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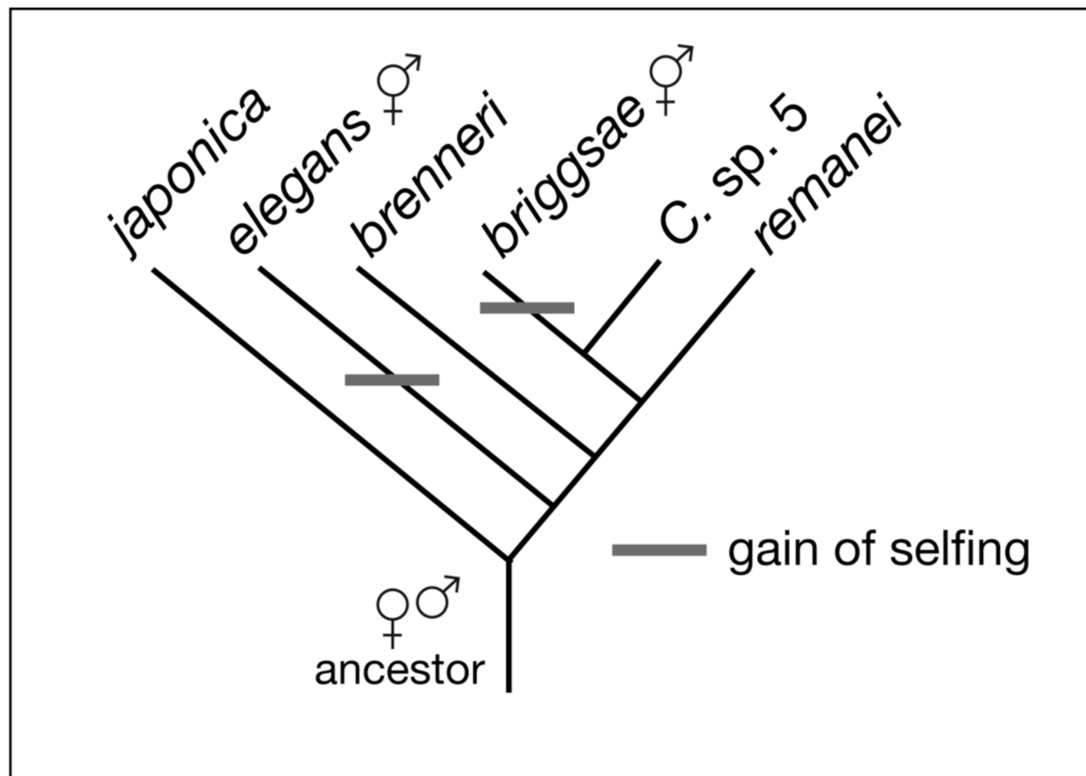

Figure 3.

The current phylogenetic hypothesis for the relationships among *Caenorhabditis* species, with the most parsimonious reconstruction of mating system evolution mapped upon it. This figure synthesizes results of Braendle and Felix (2006), Cho et al. (2004), Hill et al. (2006), Kiontke et al. (2004), Nayak *et al*. (2005), and Sudhaus and Kiontke (2007).

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Table 1

Summary of functional characterization of *C. briggsae* sex determination genes.

gene	*C. elegans* mutant phenotype (lf)	*C. briggsae* RNAi phenotype	*C. briggsae* transgene in *C. elegans*	*C. briggsae* mutant phenotype	References
her-1	XO: Her XX: no effect	XO: weak Her XX: no effect	[*Punc-54::Cb-* HER-1] XX: Tra XO: ND	ND	Hodgkin (1980); Streit *et al.* (1999)
tra-2	XO: no effect XX: imperfect Tra	XO: ND XX: weak Tra	ND	XO: no effect XX: imperfect Tra	Hodgkin and Brenner (1977); Kelleher *et al.* (2008); Kuwabara. (1996)
tra-3	XO: no effect XX: imperfect Tra, maternally rescued	XO: ND XX: no effect	ND	XO: no effect XX: imperfect Tra, maternally rescued	Hodgkin and Brenner (1977); Kelleher *et al.* (2008)
fem-2	XO: Fem XX: Fem	XO: germ line feminized, soma intersex XX: no effect	somatic rescue of Fem phenotype in XO *fem-2(lf)*, no rescue of germline Fem phenotype in XX or XO	XO: Her XX: no effect	Hansen and Pilgrim (1998); Hill *et al.* (2002); Kimble *et al.* (1984); Stothard *et al.* (2002)
fem-3	XO: Fem XX: Fem	XO: weak Fem XX: no effect	ND	XO: Her XX: no effect	Hodgkin, (1986); Haag et al. (2002)
tra-1	XX: Tra soma, intersexual germ line XO: male soma, intersexual germ line	XO: germline Feminization XX: intersex	rescues non-gonadal soma of XX *tra-1* mutants; feminizes wild-type XO animals	XO: intersexual germline XX: Tra soma, intersexual germ line	de Bono and Hodgkin(1996); Hodgkin and Brenner (1977); Kelleher *et al.* (2008)
fog-3	XO: Fog XX: Fog	XO: Fog XX: Fog	rescues Fog	ND	Chen *et al.* (2001); Ellis and Kimble (1995)
gld-1	XO: no effect XX: Fog, tumorous	XO: ND XX: Mog	ND	XO: no effect XX: Mog, tumorous	Francis *et al.* (1995a); Nayak *et al.* (2005); A. Doty and ESH (unpublished)