From "the Worm" to "the Worms" and Back Again: The Evolutionary Developmental Biology of Nematodes

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ABSTRACT Since the earliest days of research on nematodes, scientists have noted the developmental and morphological variation that exists within and between species. As various cellular and developmental processes were revealed through intense focus on *Caenorhabditis elegans*, these comparative studies have expanded. Within the genus *Caenorhabditis*, they include characterization of intraspecific polymorphisms and comparisons of distinct species, all generally amenable to the same laboratory culture methods and supported by robust genomic and experimental tools. The *C. elegans* paradigm has also motivated studies with more distantly related nematodes and animals. Combined with improved phylogenies, this work has led to important insights about the evolution of nematode development. First, while many aspects of *C. elegans* development are representative of *Caenorhabditis*, and of terrestrial nematodes more generally, others vary in ways both obvious and cryptic. Second, the system has revealed several clear examples of developmental flexibility in achieving a particular trait. This includes developmental system drift, in which the developmental control of homologous traits has diverged in different lineages, and cases of convergent evolution. Overall, the wealth of information and experimental techniques developed in *C. elegans* is being leveraged to make nematodes a powerful system for evolutionary cellular and developmental biology.

KEYWORDS *C. elegans*; connectome; developmental systems drift; embryo; evolution; gene regulatory network; sex determination; sperm; vulva; WormBook

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THE small, laboratory-friendly nematodes of the genus *Caenorhabditis* were first developed as a system for genetic analysis of animal development by a few early champions. One of the first experimental studies on *C. elegans* was performed by Japanese American Hikokuro Honda, who found that sperm determine the sex of progeny, and discovered that oocyte meiosis is not completed until after fertilization (Honda 1925). Two decades later, the French biologist Victor Nigon and his American colleague Ellsworth Dougherty greatly extended this work (Nigon 1943; Dougherty and Nigon 1949; Ferris and Hieb 2015; Nigon and Félix 2017), aided by improvements in culture methodology by Briggs (1946). These workers set the stage for Sydney Brenner's breakthroughs with *C. elegans* (Brenner 1974, 2009). Along with French biologist Emile Maupas, who first described *C. elegans* (Maupas 1900), all of these early researchers were struck by the fact that, within a stereotypical body form, evolutionary variation in habitat choice, feeding strategy, reproductive mode, behavior, and anatomical details are rampant. Thus, research focusing on *C. elegans* was always complemented by the work of other nematologists working in other groups, such as other nematodes in the order Rhabditida (Figure 1) (Sudhaus 1976). It can therefore be fairly said that questions of biodiversity, the evolution of developmental processes, and their connections to ecology were very much lingering over the field even in the earliest days. The authors of this review represent examples of contemporary biologists who share their predecessors' fascination with the evolution of nematode development. Trained in the *C. elegans* paradigm, we and others take particular delight in gazing outward across the phylogeny, always on the lookout for new phenomena and explanations for how they evolved.

Unique Attributes of the Caenorhabditis System

Caenorhabditis offers an attractive set of attributes for evolutionary developmental biology (EDB, or "evo-devo"). First, it presents a highly simplified and stereotyped developmental system. Worms are transparent and have a small number of somatic cells formed by a predictable lineage (Sulston and Horvitz 1977; Kimble and Hirsh 1979; Sulston *et al.* 1983). This allows one to homologize and compare developmental processes at the resolution of individual cells (Zhao *et al.* 2008). Nevertheless, the major tissues of larger, more complex animals (*e.g.*, muscles, integument, nerves, sensory cells, renal, digestive and reproductive organs, and immune cells) are present (see www.wormatlas.org). While zoologists of the past believed the simple anatomy of nematodes represented a primitive state, molecular phylogenetics (Figure 1) have generally supported the membership of the phylum Nematoda in the Ecdysozoan superphylum of protostomes (Giribet and Edgecombe 2017). This implies that nematodes' often miniature bodies are actually highly derived and highly specialized. An alternative interpretation to the *C. elegans* body, therefore, is that it is a sophisticated, "microchip animal" that evolved from a larger progenitor. Along the way, some ancestral regulators of animal development have been shed or modified. For example, *Caenorhabditis* have fewer Hox genes than other nematodes or more distantly related animals (Aboobaker and Blaxter 2003), and the *hedgehog* signaling pathway has both diverged in its roles (Bürglin and Kuwabara 2006; Soloviev *et al.* 2011) and been co-opted to form the core of the global sex determination pathway (Zarkower 2006). Simultaneously, proteins

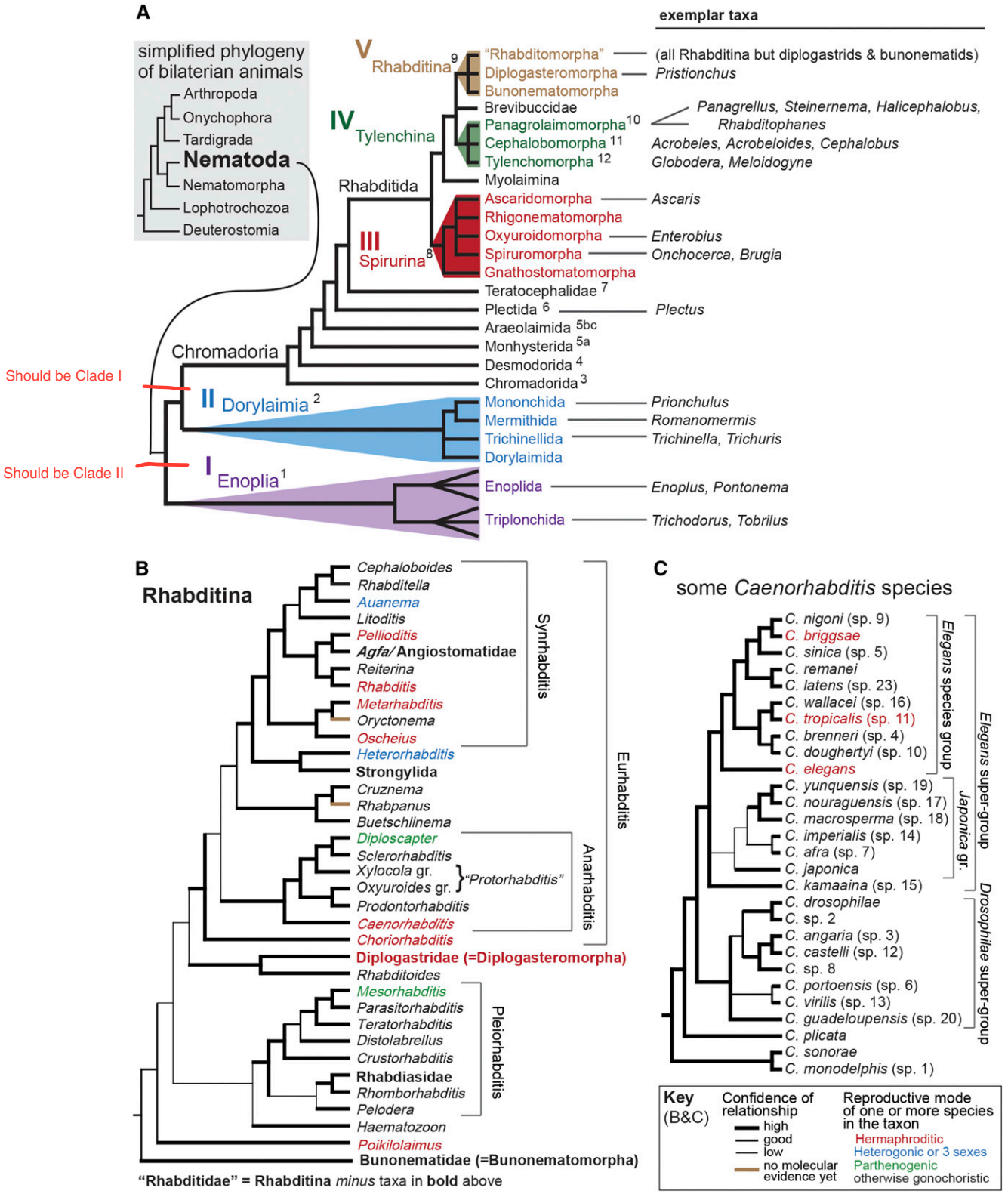

Figure 1 Phylogenies of phylum Nematoda, suborder Rhabditina, and genus *Caenorhabditis*, based on molecular data. (A) Inset shows the phylogenetic position of Nematoda within a very simplified phylogeny of bilaterian animals. Recent molecular studies place Nematoda together with its sister group Nematomorpha as the closest relatives of Panarthopoda (Arthropoda, Onychophora, Tardigrada) in a clade often called Ecdysozoa (Giribet 2016; Giribet and Edgecombe 2017). The phylogeny of Nematoda has been derived mainly from ribosomal RNA (rRNA) genes and contains several well-defined clades: clades I–V (De Ley and Blaxter 2004; De Ley 2006) designated in like-colored roman numerals, taxon names, and polygons; and clades 1–12 designated in black superscripts to corresponding taxon names (Holterman *et al.* 2008; van Megen *et al.* 2009). Some taxa have been left out here for

implicated in chemosensation, such as rhodopsin-related G protein-coupled receptors, have been amplified and diversified (Bargmann 2006).

A striking variable distinguishing some nematodes, such as *Caenorhabditis*, *Pristionchus*, and some other clade V taxa relates to sexual mode. Although the ancestral gonochoristic (male-female), obligately outcrossing mode is retained by most species, several have evolved a self-fertile hermaphrodite (Kiontke *et al.* 2011) (Figure 1). Males persist at greatly reduced frequencies, creating an androdioecious mating system. Androdioecy is rare in both animals and plants (Pannell 2002; Weeks *et al.* 2006), but because it makes genetic manipulations simpler and faster, selfing species like *C. elegans* and *C. briggsae* (and peas!) have always been favored by experimental biologists. A major area of research reviewed below involves comparisons between close relatives with different sexual modes.

Despite its fame for exhibiting "invariant" development, *C. elegans* also offers one of the best-characterized examples of an adaptive phenotypic plasticity: the formation of the dauer larva. This resistant variant of the third larval stage is triggered by crowding or starvation in the previous stage (Albert *et al.* 1981), which, in turn, alters pheromones and nutritional status. These cues are then translated into differential states of signaling pathways and circulating hormones (Fielenbach and Antebi 2008). Because the dauer larva appears to be a universal dispersal form for both free-living and parasitic terrestrial nematodes (Crook 2014), the cues that induce its development and the attributes it possesses are likely to vary with ecological niche. Some first examples of this variation are reviewed below.

C. elegans has also enjoyed early and intense attention to the characterization of its genome and its relation to various processes. It was the first animal species to have a complete sequence assembly (*C. elegans* Sequencing Consortium 1998), and this quickly became a handmaiden to gene-focused EDB (*e.g.*, Kuwabara and Shah 1994; Haag and Kimble 2000). Interest in examining interspecies variation led to a collection of genome assemblies from other *Caenorhabditis* species (Stein *et al.*, 2003; Hillier *et al.* 2007; Ross *et al.* 2011; Fierst *et al.* 2015; see also http://www.nematodes.org/nematodegenomes/index.php/Main_Page). This work is ongoing on an ever-larger scale, driven by both the discovery of many new species (Kiontke *et al.* 2011; Barrière and Félix 2014; Huang *et al.* 2014; Ferrari *et al.* 2017; Slos *et al.* 2017) and advances in sequencing technology (see caenorhabditis.org). Note that genome sequencing and annotation have been completed, or are in progress, for all of the *Caenorhabditis* species shown in Figure 1C except for *C. sonorae*, which has been refractory to reisolation. Within species, the genomes of many genetically distinct isolates from around the world are also being characterized (Cutter *et al.* 2006; Rockman and Kruglyak 2009; Dey *et al.* 2012; Thomas *et al.* 2015; Cook *et al.* 2016). This presents a rich resource with which to examine standing variation in molecules and processes (*e.g.*, Cook *et al.* 2017).

Perhaps not surprisingly, evolutionary studies of *Caenorhabditis* grew as comparative offshoots of the major topics of *C. elegans* research. Essentially, once an aspect of the development of *C. elegans* came to be understood in some detail, several obvious questions followed quickly: Is that general? If it is general, can it help us understand natural variation in form? If it is not general, how did it evolve? Sometimes the reverse line of questioning, starting with an appreciation of variation in a particular feature, has also sparked more in-depth work in *C. elegans* itself. In this fashion, EDB using nematodes has focused on these topics:

zygotic mitosis and founder cell specification.
embryonic cell lineage.
developmental regulation of gene expression.
neuroanatomy.
sex determination.
germ cell development.
spermatogenesis and sexual behavior.
vulva and somatic gonad development.
nongonadal somatic sexual dimorphism and male development, *e.g.*, the tail.
dauer formation.

simplicity. Taxa other than Rhabditina that are mentioned in this review are listed at the right. Adapted with permission from Blaxter (2011) and Kiontke and Fitch (2013). Taxa in quotation marks are paraphyletic: "Rhabditomorpha" includes all Rhabditina except Diplogasteromorpha and Bunonematomorpha. (B) Phylogeny of Rhabditina (clade V), almost entirely based on molecular data from rRNA and other loci (Kiontke *et al.* 2007; Ross *et al.* 2010; Kanzaki *et al.* 2017). Thickness of the lineages, as indicated in the key at lower right, indicates the approximate level of confidence estimated from statistical tests. The systematics of "Rhabditidae" was recently revised (Sudhaus 2011) based almost entirely on the molecular phylogeny (Kiontke *et al.* 2007) with some consideration of morphological characters to place taxa only known from literature descriptions (brown lineages). A few, mostly monotypic taxa of uncertain position are not shown. Four named suprageneric clades are shown with brackets. Despite being paraphyletic, "Rhabditidae" is a useful taxon because it includes many free-living (rarely parasitic) species with fairly similar Bauplan and excludes three specialized parasitic taxa (Angiostomatidae/*Agfa*, Strongylida, Rhabdiasidae) and Diplogastridae, a clade of species morphologically distinguished from "Rhabditidae" that have undergone an extensive adaptive radiation. *Pristionchus pacificus* and its relatives are included in the Diplogastridae. The "Rhabditidae" sister taxa to each of these special groups provide important resources for investigating the evolutionary origins of parasitism and other specializations that have resulted in adaptive radiations. Colored fonts indicate taxa in which reproductive mode has evolved from gonochorism to hermaphroditism, heterogonism or parthenogenesis (see key at lower right). Taxon names in bold font are at higher levels than the genera otherwise depicted. For more complete information, see RhabditinaDB at rhabditina.org. (C) Phylogeny for some *Caenorhabditis* species as inferred by molecular data from rRNA and several other loci (Kiontke *et al.* 2011). Due to the rapid rate of discovery, species are provisionally designated with numbers (sp. *n*) until names can be attached to these species units (Félix *et al.* 2014). Only 28 of the ~50 known species are shown here; however, this phylogeny shows all the major known clades (demarcated here as "species groups"). Several *Caenorhabditis* species are only known from morphological descriptions and not included here. Hermaphroditic species are indicated in red font; other species are gonochoristic.

Below, we summarize key findings from *C. elegans* research on the above developmental processes, and discuss the evolutionary studies that they have enabled. While the latter would, in principle, include many studies of deeply diverged nematodes and other phyla, we emphasize here the more recent evolution revealed by comparisons within Rhabditida (Clade V, Figure 1). Finally, we attempt to distill the major insights that have emerged from nematode EDB.

Findings

Zygotic mitosis

The first embryonic divisions of the *C. elegans* embryo have been extensively described (Rose and Gönczy 2014) (Figure 2). Briefly, oocytes are blocked in prophase of meiosis I and unpolarized. At fertilization, the sperm brings in the paternal DNA and a pair of centrioles. These centrioles rapidly recruit pericentriolar material, which locally destabilizes the cortical actomyosin contractility, leading to the asymmetric repartition of the PAR polarity proteins. At fertilization, the anteroposterior (AP) axis of the cell is thus established, and the sperm entry site defines the posterior side of the cell. In response to the PAR polarity, cytoplasmic proteins localize asymmetrically in the cell, and the mitotic spindle that is initially centrally located becomes posteriorly positioned along the AP axis during anaphase (Figure 2). This asymmetric displacement comes with impressive transverse oscillations of the spindle—the manifestation of excess forces pulling on posterior astral microtubules. Because the cell cleavage plane is perpendicular to the spindle, two daughter cells of unequal size and asymmetric fate are formed, the posterior cell P1 being smaller than the anterior cell AB. This stereotyped asymmetric division has become a model to study oriented cell division because of the exquisite spatiotemporal resolution of events during this first cell cycle and because of the strong conservation of molecules involved across phyla (Neumuller and Knoblich 2009). At each subsequent division, a similar asymmetric cell division is reproduced in the P lineage, ultimately giving birth to the founder cell of the germline, the P4 cell.

At the second cell cycle, while the founder cell AB divides symmetrically to generate ABa and ABp, P1 gives rise to the small P2 cell and EMS. During this division, the mitotic spindle of P1 rotates along the AP axis of the embryo and becomes perpendicular to the spindle of AB. This leads to a rhomboid organization of the four first blastomeres, which is essential for the subsequent cellular interactions (Figure 2). Indeed, at the four-cell stage, P2 sends a Wnt signal to EMS, which then divides asymmetrically to give rise to the founder cell of the intestine (the E cell) and the founder cell of the mesoderm (MS). In the absence of P2 or Wnt signaling, EMS gives rise to two MS cells. Through Notch/Delta signaling, P2 also induces different fate acquisition in ABp compared to ABa. P2 next divides to give the founder cell C and P3, which divides again to give the founder cells D and P4. These cell divisions thus rapidly produce the six key founder cells of *C. elegans* embryos (Sulston *et al.* 1983).

The embryos of most nematodes, in particular free-living forms, can easily develop ex-utero. The first cell divisions are easy to monitor under slide and coverslip because cells are large and transparent and the pace of cell divisions is relatively fast. These properties allowed the analysis of the early steps of embryogenesis in very diverse nematode species, starting with the founding work of T. Boveri on *Ascaris megalocephala* (=*Parascaris equorum*) (Maderspacher 2008) and followed by Nigon and others in the early twentieth century (Nigon and Félix 2017).

Among the long list of free-living and parasitic species that have been observed since then, only species from Enoplia (Clade I, Figure 1A) undergo a series of symmetric embryonic first divisions, with late specification of cellular identity (Malakhov 1994; Schulze and Schierenberg 2008, 2009, 2011). In all the other Chromadoria species so far observed, the first embryonic division is asymmetric, giving rise to two unequally sized, asymmetrically fated daughter cells (Brauchle *et al.* 2009; Schulze and Schierenberg 2011; Landmann *et al.* 2014; Calderón-Urrea *et al.* 2016). Thus, as in *C. elegans*, the polarity of the embryo is already established during the first mitosis in all these species. However, in *Acrobeloides* sp. PS1146 (Cephalobomorpha), the sperm entry site does not correlate with the posterior side of the embryo, in contrast to *C. elegans* (Goldstein *et al.* 1998). There is also an absence of cytoplasmic movements toward the site of sperm entry, further suggesting that the sperm is not the polarity cue in this species. In a study of 16 other free-living and parasitic nematodes, a clade that includes *Acrobeloides* does not show any sign of cytoplasmic flow, while the other groups resemble *C. elegans* (Goldstein *et al.* 1998) (Figure 2). Thus, embryonic early polarity can be established independently of the sperm centrosomes in many nematode species. Parthenogenesis has emerged several times in the group of species for which polarity is independent of the sperm, leading to the hypothesis that the ability to polarize the embryo independently of sperm might have been a preadaptation to the emergence of parthenogenesis (Goldstein *et al.* 1998). Such a transition state—to sperm-independent polarization in gonochoristic species—is, however, not a prerequisite for the emergence of parthenogenesis, because parthenogenesis is also found in the *Diploscapter* genus within the "Rhabditidae" (Figure 1B) (Fradin *et al.* 2017)—a paraphyletic family composed mainly of gonochoristic species that use sperm as a polarity cue.

The origin of the polarity cue in the absence of sperm has been investigated (Lahl *et al.* 2006). Because, in some experimental conditions, the female meiotic spindle can promote PAR asymmetric localization in *C. elegans* embryos (Wallenfang and Seydoux 2000), one tempting hypothesis is that the female meiotic spindle becomes the polarity cue in parthenogenetic species. However, this hypothesis can be ruled out, as the position of the posterior pole does not correlate with the position of the polar bodies in *Acrobeloides nanus* and in *Diploscapter coronatus* (Lahl *et al.* 2009). In *Acrobeloides*, the anterior side of the

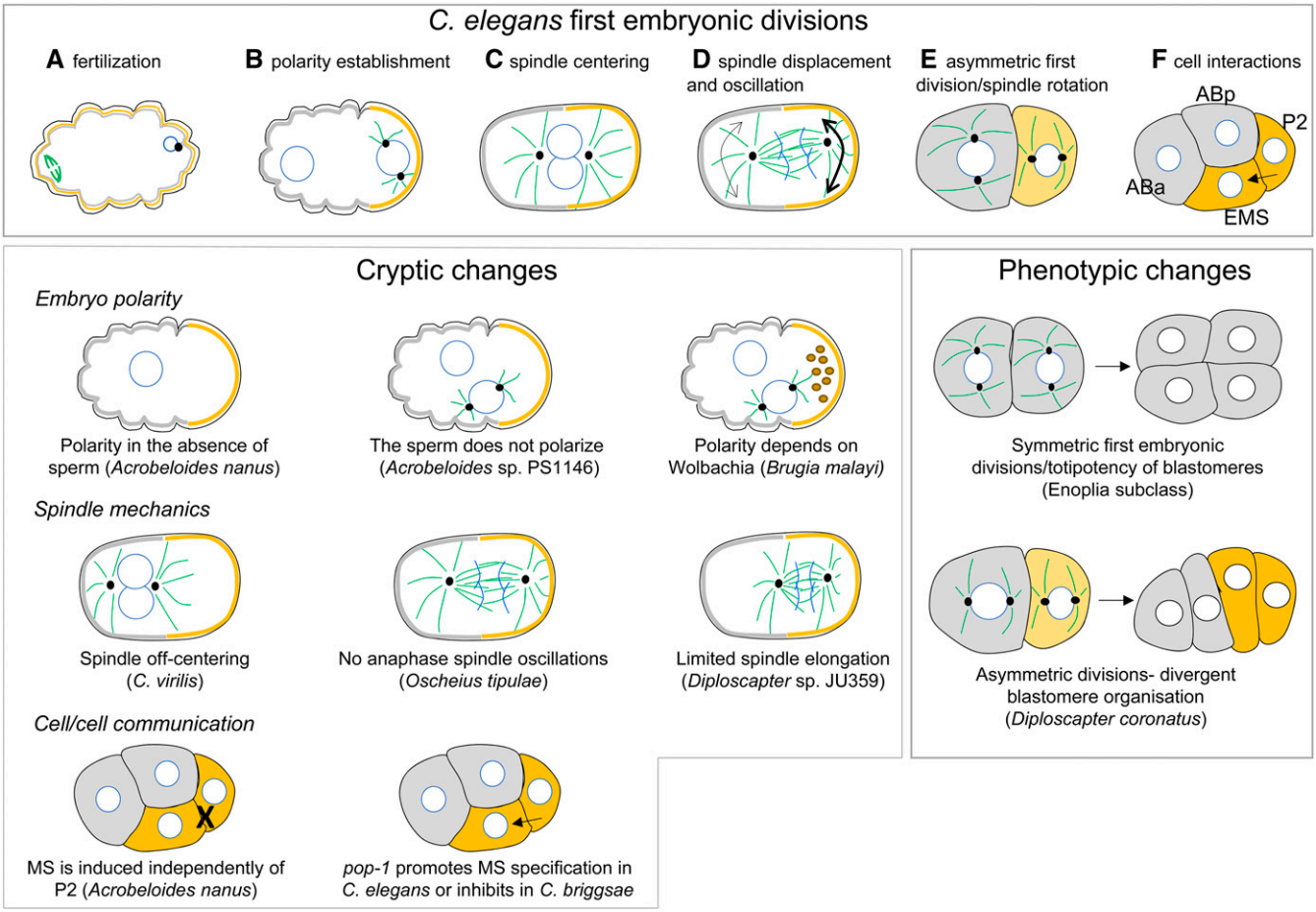

Figure 2 First embryonic cell divisions in *C. elegans* and variations in other species. Top panel (A–F) schematic representation of the two first cell division of the *C. elegans* embryo. Microtubules are shown in green, centrosomes are represented by black dots and nuclei by white circles. Polarity proteins are shown in gray and yellow. (A) Initially, the oocyte is unpolarized. After the sperm entry (on the right), female meiosis resumes (spindle on the left). (B) After fertilization, polarity proteins are asymmetrically localized and the sperm entry site defines the posterior pole of the cell on the right (B). In response to polarity, the mitotic spindle (D) and cell fate determinants (E) are asymmetrically localized. During the second cell division, spindle orientation is different between the two cells (E), giving rise to a rhomboid organization of blastomeres at the four-cell stage (F). At this stage, the P2 cell sends a Wnt signal to EMS. Phenotypic changes: timing of cell divisions and cell orientations can vary between species leading to different cellular contacts and blastomeres organization. Cryptic changes: among species that have similar embryonic cell divisions than *C. elegans*, evolutionary changes are found in the polarization of the embryo, the positioning of the first mitotic spindle or in cell/cell communication.

embryos always faces the vulva, suggesting that the orientation of the oocytes within the gonadal tract provides a polarity cue. In contrast, there is no correlation between embryo orientation within the uterus and the position of the posterior pole in *Diploscapter*, suggesting that polarity in these species is established randomly. Because polarization relies on the local destablilization of the actomyosin network in *C. elegans*, one could imagine that spontaneous self-organization of the actomyosin cortex triggers symmetry breaking to define the anterior–posterior axis of the embryo in *Diploscapter*.

Early embryo polarization is also observed in the parasitic nematode *Brugia malayi* (Spirurina) (Landmann *et al.* 2014). In this species, a microtubule-organizing center is found in oocytes prior to fertilization at the future posterior side of the cell, opposite to the location of the female meiotic spindle, suggesting a microtubule-based mechanism of polarization from a maternal origin. *Wolbachia* endosymbionts are found enriched at the posterior side of the one-cell embryo and in the P1 cell after the first division in this species; their removal leads to polarity defects in two-cell embryos (Landmann *et al.* 2014). Whether *Wolbachia* are required for the initiation of polarity or its maintenance remains to be determined, but this example nicely illustrates the diversity of mechanisms that exist to establish the first embryonic polarity axis of nematode embryos during the first cell cycle.

In *C. elegans*, the early polarization of the embryo after fertilization can be easily scored by a series of cortical contractions following the reorganization of the actomyosin network. Similarly, in response to the asymmetric localization of PAR proteins, microtubule force generators produce movements of the nuclei and the spindle that are extremely

stereotyped. In one study, the first two embryonic divisions of 34 rhabditids were scored, uncovering a large degree of variability in these subcellular phenomena (Brauchle *et al.* 2009). Farhadifar *et al.* (2015) analyzed the first embryonic cell division of 42 different rhabditid species and of natural isolates and mutation accumulation lines of *C. elegans*. Spindle length appears to be constrained by stabilizing selection on cell and embryo size, with the two linked in *C. elegans* by a linear scaling relationship. However, the observed variations in spindle movements could not be explained by evolutionary changes in cell size between species (Valfort *et al.* 2018). Moreover, traits associated with spindle movements combined in ways contrasting with the expectation based on *C. elegans* studies, suggesting that mechanical optimization of the mitotic spindle differs between species despite a conserved output phenotype: the asymmetry of division.

The origin of differences in spindle positioning between *C. elegans* and its congener *C. briggsae* have been explored (Riche *et al.* 2013). In *C. briggsae*, at the onset of mitosis, the spindle is anteriorly shifted in contrast to a central position found in *C. elegans*. During anaphase, the spindle is pulled posteriorly in both species. However, this movement is accompanied by much-reduced transverse spindle oscillations in *C. briggsae* compared to *C. elegans*. These phenotypes were attributable to the GPR-1/2 proteins—components of the cortical force generator complex. While two recently duplicated genes *gpr-1* and *gpr-2* are found in the genome of *C. elegans*, *C. briggsae* has only one *gpr-2* gene. This difference in gene copy number correlated with a lower expression level in *C. briggsae* compared to *C. elegans* but also with a different spatio-temporal regulation.. Thus, the processes that produce a conserved and essential cellular feature, asymmetric spindle position, are distinct. This represents a case of what has been dubbed developmental system drift (DSD; True and Haag 2001) or phenogenetic drift (Weiss and Fullerton 2000) at the earliest stages of embryonic development.

Postzygotic cell lineage and founder cell specification

Although descriptions of early embryogenesis in Enoplia and Dorylaimia (Clades I and II; Figure 1) remain scarce because species of these clades are difficult to maintain in laboratory conditions (Schulze and Schierenberg 2011), what is known suggests that a striking diversity of mechanisms for early-development evolved early in the phylum. In *Enoplus brevis* (Enoplia, Clade I) the first embryonic divisions are symmetric and body axes are not specified during the first cell divisions. Moreover, except for the endoderm (E) lineage, no founder cells are identified and cells become determined later "*en bloc*" (Schulze and Schierenberg 2011). In *Pontonema vulgare*, the spatial arrangements of the blast cells producing specific lineages can also vary substantially among embryos (Malakhov 1994; Voronov 1999), also suggestive of "regulative" development. In another representative of Enoplia, *Tobrilus*, a blastocoel is even observed with a canonical gastrulation—a feature that was unexpected in this phylum of pseudocoelomate worms. However, anteroposterior polarity is established at the four-cell stage, and three founder cells for the germline, the pharynx, and the intestine are found (Schierenberg 2005; Schulze and Schierenberg 2011). Results obtained in *Prionchulus punctatus* (Mononchida) are contradictory. On the one hand, laser ablation of half of the embryo does not prevent the development of a normal fertile adult (Borgonie *et al.* 2000). On the other hand, there are five founder cells (E, pharynx, D, C, and P), suggesting an early specification of cellular identities (Schulze and Schierenberg 2011). While *Romanomermis culicivorax* (Dorylaimia, a.k.a. Clade II) has six founder cells like *C. elegans*, tissues are formed by rings of cells, reminiscent of a segmentation process (Schulze and Schierenberg 2008, 2009). These species present extremely divergent early embryonic development, making it difficult to infer the ancestral pattern of development in nematodes. Nevertheless, because it is shared with outgroup phyla, the absence of deterministic lineage was most likely an ancestral character associated with slow embryogenesis.

On the other hand, the more derived Chromadoria are characterized by a fast embryonic development with largely deterministic lineages (Malakhov 1994; Schulze and Schierenberg 2011). *Plectus* species (Plectida) seem to have an intermediate way to specify cell types between Enoplia (no early founder cells) and Rhabditina (six founder cells established by the 16-cell stage): while the P lineage is clearly specified, the AB lineage is highly variable, leading to variable cell–cell contacts from one embryo to the other (Schulze *et al.* 2012). Within the Chromadoria, the early lineages of many species resemble *C. elegans*. Early examples came from work with the Clade IV species *Panagrellus redivivus*, *Turbatrix aceti*, and *Aphelencoides blastophthorus* imbedded in the seminal description of the *C. elegans* embryonic lineages (Sulston *et al.* 1983). Later studies examined fellow Clade V taxa, such as *C. briggsae* (Zhao *et al.* 2008), *Litoditis marina* (a.k.a. *Pellioditis marina*) (Houthoofd *et al.* 2003), *Pristionchus pacificus* (Vangestel *et al.* 2008), and *Oscheius shamimi* (Tahseen and Nisa 2006). Other species within the Tylenchina a.k.a. Clade IV have also been described, such as *Rhabditophanes* (Houthoofd *et al.* 2008), *Halicephalobus* (Borgonie *et al.* 2000), as well as from the more distantly related Spirurina a.k.a. Clade III (*Ascaris*; Boveri 1899). Nevertheless, in a detailed analysis of 70 different species from 19 different nematode families within Chromadoria, differences were found in the spatial and temporal organization of the founder cells (Dolinski *et al.* 2001). First, AB and P1 divide at the same rate as *C. elegans* (synchrony), or at different rates (asynchrony) as in *Acrobeloides*, in which all the P divisions take place before the first division of AB. It has been previously proposed that such timely separation of soma and germline divisions would ensure proper germline identity (Schlicht and Schierenberg 1991). Yet, in species for which AB and P1 divide in the same generation, a delay in cell divisions can exist, such as in *C. elegans*, where AB divides 2 min before P1, or in *Diploscapter* and *Poikilolaimus oxycercus*, where P1

divides first (Brauchle *et al.* 2009). Moreover, species have either a rhomboid organization of blastomeres as in *C. elegans*, or a linear arrangement at the four-cell stage when both AB and P1 spindles rotate to align along the AP axis. Such linear organization is found in *Diploscapter* and some "*Protorhabditis*" species (Dolinski *et al.* 2001; Brauchle *et al.* 2009; Lahl *et al.* 2009; Fradin *et al.* 2017) or in *Meloidogyne* (Dolinski *et al.* 2001; Calderón-Urrea *et al.* 2016) (Figure 2).

In species with linear arrangement of the early blastomeres, the question of lineage specification remains open. In *C. elegans*, ABp fate is induced by P2 via Notch signaling (Mello *et al.* 1994; Mickey *et al.* 1996). The linear arrangement in the four-cell embryo means that this signaling must occur in a different way, if it occurs at all (Brauchle *et al.* 2009). Also, in *Diploscapter coronatus*, and some other species of the "*Protorhabditis*" group, P2 has already divided into C and P3 at the time of EMS and ABp division (Lahl *et al.* 2009; Fradin *et al.* 2017). Moreover, the orientation of C and P3 is random, at least in *Diploscapter coronatus*. Thus, in only 50% of embryos does ABp contact C while EMS contacts P3. Despite these random contacts, ABp and ABa have a distinct lineage, suggesting that ABp specification is independent of an induction by either C or P3. Whether EMS requires an inductive signal by a neighboring cell or is cell-autonomous remains to be determined. Importantly, removal of EMS leads to an absence of intestinal cells, demonstrating an absence of multipotency, as in *C. elegans* (Lahl *et al.* 2009). In striking contrast, in *Acrobeloides nanus*, where cellular contacts at the four-cell stage are similar to *C. elegans*, the absence of P2 does not prevent gut specification (Figure 2). Rather, any cell at the three-cell stage can give rise to intestinal cells after ablation of the others. Similarly, if AB is ablated, EMS takes over and C becomes EMS. Thus, in this species, multipotency and hierarchy of transformations is observed, despite an early segregation of the lineage in wild-type embryos (Wiegner and Schierenberg 1998, 1999). Unexpectedly, in the distantly related *Plectus*, the situation resembles *C. elegans*, since an induction of EMS by P2 is necessary to specify the intestine (Schulze *et al.* 2012). Therefore, many different solutions and reversals are found over the course of nematode evolution to specify cellular identities during early embryogenesis.

Interestingly, even within *Caenorhabditis*, differences in gut specification have been revealed at the molecular level, despite conservation of cellular interactions and blastomere specification (Coroian *et al.* 2006; Lin *et al.* 2009). Upon Wnt signaling by P2, the transcription factors SKN-1 and POP-1 act to specify E and MS identity. While POP-1 has a positive contribution to MS specification in *C. elegans*, it represses the MS fate in *C. briggsae*. In an interesting twist to the story, MED-1,2, two GATA transcription factors that act downstream of SKN-1, evolved in the lineage to *C. elegans* and are not present in *C. briggsae*. One model for the co-option of these new factors is via a transitional feed-forward architecture in which SKN-1 acts both through and independently of MED-1,2 (Maduro 2009). Given the highly conserved cell lineages in the two species (Zhao *et al.* 2008), such an opposite role for a key signaling pathway is an unexpected case of DSD.

The above results demonstrate that—despite a very constrained body plan—early steps of embryogenesis vary considerably between nematodes. The molecular signature of such diversity in the early steps of embryogenesis was explored in five different species within *Caenorhabditis* (Levin *et al.* 2012). Embryos from 10 different morphological stages were collected, from four-cell stage embryos to L1 larvae, and their transcriptomes were analyzed. Despite species-specific developmental timing, embryos from specific stages showed a similar pattern of gene expression across species, suggesting the existence of conserved "milestones" in development. Importantly, at midembryogenesis, corresponding to ventral enclosure, transcriptomes from different species were the least divergent. Moreover, genes that were activated at this stage showed enrichment in crucial functions such as patterning by Hox genes or locomotion. These results led to the proposition (Levin *et al.* 2012) that for nematodes, ventral enclosure represents a key, body plan-defining point in development, the so-called phylotypic stage (Slack *et al.* 1993; Richardson *et al.* 1998). Transcriptome profiles throughout embryonic development were also performed in 20 mutation accumulation lines of *C. elegans*, in which the effect of selection is largely abolished. For all developmental stages, except ventral enclosure, variation in gene expression was much higher in the MA lines. This result strongly suggests that gene expression during ventral enclosure is highly conserved because of stabilizing selection (Zalts and Yanai 2017). Regardless of whether or not there is a phylotypic stage, these results do support an hourglass model (Raff 1996), in which nematode development shows the greatest diversity prior to or after a conserved point midway through embryogenesis.

Developmental regulation of gene expression

The variation in global embryonic gene expression described above indicates that the transcriptional controls acting on each gene evolve readily. Several studies have examined this process at the level of individual genes. One early focus was on *lin-48*, which encodes a transcription factor related to *Drosophila ovo*. *lin-48* is expressed in the developing excretory duct cell in *C. elegans*, but is not in *C. briggsae*. Using reporter transgenes, Wang and Chamberlin (2002) found that only the combination of *C. elegans* regulatory sequences with a *C. elegans* host supported *lin-48* excretory cell expression, suggesting the difference between species was due to changes in both *cis*-regulatory sequences and *trans*-acting factors. At least four *C. elegans*-specific sequences contribute to the former. Further, the absence of *lin-48* expression in *C. briggsae* correlates with a more anterior location of the excretory duct cell—a shift also seen in *lin-48* loss-of-function mutants in *C. elegans*. A subsequent study (Wang and Chamberlin 2004) found that *C. elegans lin-48* recently gained a binding site for the bZip transcription factor CES-2 that is necessary in *C. elegans* for both strong excretory cell

expression and anterior excretory duct cell location. Forcing expression of LIN-48 in the *C. briggsae* excretory duct cell is sufficient for anterior location. Thus, the gain of a novel regulatory linkage during evolution altered both *lin-48* expression and morphology. In addition, enhancers that mediate the conserved hindgut expression of *lin-48*, which are bound by EGL-38, have diverged between *C. elegans* and *C. briggsae* (Wang *et al.* 2004).

Gene regulatory evolution has also been examined in subsets of homologous neurons conserved across *Caenorhabditis*. Barrière *et al.* (2012) focused on the GABAergic cell marker *unc-47*. Though expressed in identical ways in *C. elegans* and *C. briggsae*, cross-species reporter transgenes produced additional, ectopic sites of expression. Further experiments revealed that coordinated evolution between *cis* and *trans* factors has occurred in each lineage. A subsequent study (Barrière and Ruvinsky 2014) expanded the neuronal genes analyzed to seven (*unc-25*, *unc-46*, *unc-47*, *oig-1*, *acr-14*, *gpa-5*, and *mod-5*) and the species to five (*C. elegans*, *C. briggsae*, *C. remanei*, *C. brenneri*, and *C. japonica*). Again, while regulatory regions from non-*elegans* species generally drive expression in the expected *C. elegans* cells, ectopic expression and/or cell-specific lack of expression is seen in nearly all cases. Interestingly, ectopic expression of cross-species transgenes is much more common, suggesting that the repressive mode of regulation evolves faster than the activating mode. Similar reporters based on homologs from the much more distantly related parasites *Meloidogyne*, *Brugia*, and *Trichinella* (Figure 1) showed that conserved patterns of expression can be driven by sequences that are essentially unalignable (Gordon *et al.* 2015).

The above studies show that changes in *cis*-regulatory sequences evolve rapidly. They can sometimes have developmental effects, but more often remain phenotypically cryptic. This is likely due to the action of stabilizing selection, which mandates an outcome, but not a mechanism. This allows compensatory evolution (or apparently compensatory, see Haag 2007) to proceed unchecked, accelerated by directional selection on other loci that share *trans*-regulators (Johnson and Porter 2007). Over time complex dependencies between distinct promoter regions form (Ludwig *et al.* 2000).

Neuronal development

Of the 957 somatic cells of the *C. elegans* hermaphrodite, 302 are neurons, with another 56 providing support (Chalfie and White 1988). Males have over 100 additional neurons and glia, mostly with mating-related roles. Pioneering work of John White and his colleagues determined the full connectome of the hermaphrodite (White *et al.* 1986), and 25 years later a full description of the male posterior nervous system completed the picture (Jarrell *et al.* 2012). A large body of literature has described normal and perturbed nervous system development in *C. elegans* as well (Hobert 2010; Cherra and Jin 2015; Shaham 2015). Such a wealth of information about this one species, as with other topics explored here, begs the question of conservation. Are all *Caenorhabditis* nematodes put together this way? How about more distantly related nematodes? The earliest comparisons were with the larger, distantly related parasite, *Ascaris* (*e.g.*, Sulston *et al.* 1975; Walrond *et al.* 1985; Niebur and Erdos 1993; Holden-Dye and Walker 1994), and revealed a surprising fine-scale congruence of neurons over a large evolutionary distance (Schafer 2016).

Perhaps not surprising given their overtly similar anatomy, homologous neurons are produced in *C. briggsae* from a congruent embryonic cell lineage (Zhao *et al.* 2008). The more distantly related *P. pacificus* shares all 20 of the pharyngeal neurons, despite substantial divergence in feeding strategies (Bumbarger *et al.* 2013). Interestingly, however, these homologous pharyngeal neurons are connected in substantially different ways. The cell lineages producing them have yet to be determined in *P. pacificus*, but even if they differ somewhat, the nervous system appears to evolve novel connections far faster than novel neurons. That finding presents an interesting parallel to work on the evolution of gene regulatory networks (GRNs; Peter and Davidson 2011). In both cases, homologous components (either neurons or genes) evolve distinct regulatory connections to other components. How neural development is modified to produce novel connections is an important area for future research.

Sex determination

Sex determination was one of the first aspects of *C. elegans* development to be tackled using forward genetic approaches (Hodgkin and Brenner 1977; Hodgkin 2002). X chromosome dosage had long been known to be the ultimate regulator of sexual fate (Nigon 1951). The discovery of a genetic pathway linking X dosage to cell fate (Hodgkin 1986) was subsequently confirmed by molecular cloning of the genes (reviewed by Zarkower 2006). It soon became apparent, however, that this pathway did not resemble those that link chromosomes to sexual fate in *Drosophila* or mammals (Cline and Meyer 1996; Eggers *et al.* 2014). The cloning of *C. elegans mab-3* revealed the first widely conserved sex-specifier, the DM family of transcription factors (Raymond *et al.* 1998; Zarkower 2001). Thus, the disparity in sex determination mechanisms among different phyla is not due to wholly independent origins of sexual dimorphism, but rather to rapid divergence of sex determination pathways, most likely upstream and downstream of conserved DM factors (Haag and Doty 2005; Kopp 2012). This realization provided further motivation to examine the evolution of sex determination over shorter time scales.

The first comparisons of sex determination genes within *Caenorhabditis* focused on the "core pathway" that regulates dimorphism body-wide (Figure 3), starting with the identification of *C. briggsae* homologs of the genes *tra-2* (Kuwabara and Shah 1994; Kuwabara 1996) and *tra-1* (de Bono and Hodgkin 1996). These early studies revealed rapid sequence evolution but conserved functions in the promotion of female somatic development. Similar results were subsequently reported for the male-promoting *xol-1*, *her-1*, *fem-2*, and *fem-3* (Hansen and Pilgrim 1998; Streit *et al.* 1999; Haag *et al.*

2002; Luz *et al.* 2003) and the male-promoting *tra-3* (Kelleher *et al.* 2008). XOL-1 and FEM-3 are particularly divergent, with only 22 and 38% amino acid sequence identity between their *C. elegans* and *C. briggsae* orthologs, respectively. For FEM-3, its overall rapid divergence is mirrored by the region of the C-terminal domain of TRA-2 with which it interacts (Haag and Kimble 2000). In three species tested, the interaction between conspecific TRA-2 and FEM-3 partners was conserved, but interspecies pairings invariably failed (Haag *et al.* 2002). Less complete interspecies incompatibility was observed for the FEM-2-FEM-3 interaction (Stothard and Pilgrim 2006). Another interaction, between a C-terminal domain of TRA-2 and TRA-1, has been documented in both *C. elegans* and *C. briggsae* (Lum *et al.* 2000; Wang and Kimble 2001). These results indicate that, contrary to the conventional wisdom of molecular biology, even protein domains of critical importance can evolve rapidly. This may be especially true if the only role of a sequence is to interact with one other partner (*i.e.*, there is no pleiotropy at the molecular level). Abundant polymorphisms that do not disrupt interaction are observed in *C. remanei* TRA-2 and FEM-3 (Haag and Ackerman 2005). A population model suggests such variants can allow rapid coevolution by reducing the deleterious effects of other changes that would reduce fitness on their own (Haag and Molla 2005).

In addition to rapid ortholog sequence evolution, *C. briggsae* is apparently lacking a clear ortholog of *sea-1*, an autosomal regulator of *xol-1*, the upstream-most "master regulator" of sexual fate (E.S.H., unpublished data). Thus, over the roughly 20 MY since *C. elegans* and *C. briggsae* diverged (Cutter 2008), their global sex determination pathways have undergone rapid sequence evolution and coevolution of conserved genes, and have begun to exhibit gene-level pathway incongruence. The existence of a highly diverged *tra-1* homolog in the more distantly related *P. pacificus* (Pires-daSilva and Sommer 2004) suggests that key aspects of the core sex determination pathway nevertheless remain after substantially longer periods of divergence.

Self-fertile hermaphrodites have evolved at least three times within *Caenorhabditis* (Kiontke *et al.* 2004, 2011) (Figure 1 and Figure 3). This novel strategy is enabled by production of sperm in the XX ovary, making germline sex determination an obvious topic of interest for EDB. Before examining that, however, it is worth noting that, unlike *Drosophila* and mammals, the somatic niches for germline stem cells are very similar (if not identical) in male and female *Caenorhabditis* (Kimble and Hirsh 1979; Kimble and White 1981; Milloz *et al.* 2008), and a male somatic gonad is not required to support the differentiation of spermatocytes (Graham and Kimble 1993; Graham *et al.* 1993). Further, the *C. elegans* hermaphrodite does not express HER-1, a secreted protein that specifies male fate in XO animals, even in the L4 stage when sperm are produced (Trent *et al.* 1991; Perry *et al.* 1993). Self-fertility thus represents a cell-autonomous change in sexual fate. Extensive mutagenesis screens for XX animals with germline-specific sexual transformations (*e.g.*, the masculinization of germline, or Mog, and feminization of germline, or Fog phenotypes) have identified *cis*-regulatory elements in core sex-determination gene mRNAs that are sites of negative regulation by germline RNA-binding proteins [RBPs, reviewed by Zanetti and Puoti (2013)]. The reconfiguration of RBP-target mRNA networks thus appears to be the key to XX spermatogenesis, distinguishing it from other phenotypic novelties that are rooted in changes in transcription factors and their target genes (Carroll 2008).

What were the changes that allowed XX spermatogenesis to evolve, and how similar are they in selfing species that evolved convergently? Examination of conserved global sex-determiners in the hermaphroditic *C. briggsae* and the outcrossing *C. remanei* revealed identical roles for the female-promoting *tra-1*, *tra-2*, and *tra-3* (de Bono and Hodgkin 1996; Kuwabara 1996; Haag and Kimble 2000; Kelleher *et al.* 2008), and the male-promoting *her-1* (Streit *et al.* 1999). In contrast, while RNAi knockdown of *Cbr-fem-2* and *Cbr-fem-3* function could feminize the germ cells of *C. briggsae* males, it had no effect on hermaphrodites (Haag *et al.* 2002; Stothard *et al.* 2002). The dispensability of the *C. briggsae fem* genes for hermaphrodite spermatogenesis was subsequently confirmed by deletion mutations and exhaustive *tra-2*(*ts*) suppressor screens (Hill *et al.* 2006). These results suggested that regulatory mechanisms that allow *C. briggsae* spermatogenesis act downstream of the *fem* genes. *Cbr-fem-3*; *Cbr-tra-1* double mutants have the perfect male soma characteristic of *Cbr-tra-1* mutants, but a well-regulated hermaphrodite germline, as in *Cbr-fem-3* mutants (Hill and Haag 2009). This indicates that, as in *C. elegans* (Hodgkin 1986), the *fem* mutations are epistatic to *tra-1* in the germ line. Interestingly, in both species *fog-3* expression, which is controlled by *tra-1*, and thus by the *fem* genes, remains high in *tra-1* mutants even when the germline is feminized by simultaneous loss of one or more *fem* genes (Chen and Ellis 2000; Hill and Haag 2009). This indicates that the *fem* genes act in multiple places near the terminus of the germline sex determination pathway. The degree of identity of these sites of control between the two selfing species, and the extent to which they were present in their gonochoristic ancestors, remains to be determined.

How conserved are the germline-specific sex determination factors known from *C. elegans*? The promoter of sperm fate *fog-3* is conserved and plays a similar role across the genus (Chen *et al.* 2001). Clear orthologs of *fog-1* also exist in all *Caenorhabditis* species (Cho *et al.* 2004), but their loss-of-function phenotypes have not yet been reported. GLD-1, the RBP that binds the *tra-2* 3′ UTR (Jan *et al.* 1999), is also conserved across species (Nayak *et al.* 2005; Beadell and Haag 2014). However, *C. briggsae* GLD-1 is a repressor of sperm fate, rather than an enabler, *Cbr-tra-2* lacks the duplicated motifs that recruit GLD-1 in *C. elegans*, and *gld-1* has no apparent role in sex determination in any male-female species (Nayak *et al.* 2005; Beadell *et al.* 2011). GLD-1 thus appears to have been co-opted into sex determination independently, and to opposite effect, in *C. elegans* and

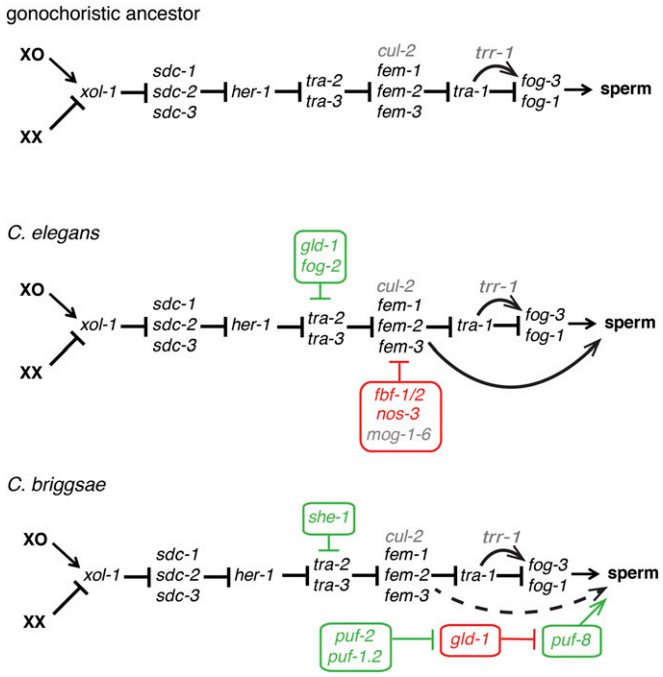

Figure 3 Convergent evolution of self-fertility via distinct changes alterations of germline sex determination. The core body-wide sex determination pathway (black), which acts in all dimorphic tissues, is shared with outcrossing relatives (top). Upstream factors that sense X dosage and regulate both sex determination and dosage compensation (*xol-1* and the *sdc* genes), are not depicted here for simplicity. The XX hermaphrodites of *C. elegans* (middle) and *C. briggsae* (bottom) both produce sperm in an otherwise female body by germline-specific modification of sex determination. Germline-specific factors that promote sperm production in each are indicated in green, while those limiting it are in red. Note that in the *C. briggsae* case, the influence of *she-1* on *tra-2* is indirect, and the action of the pathway consisting of *puf-2*, *puf-1.2*, *gld-1*, and *puf-8* has not yet been placed along the global pathway, and is thus conservatively depicted as a parallel pathway. Pleiotropic accessory factors with important roles in sexual fate are indicated in gray. The alternative functions of homologous genes and the role of species-specific genes in both hermaphrodites are particularly noteworthy. The arrows connecting *fem* genes directly to sperm fate in *C. elegans* depicts how loss of any of the *fem* genes phenotypically feminizes *tra-1* germ cells without loss of *fog-3* expression (Chen and Ellis 2000). In *C. briggsae*, a similar result is found for *fem-3*, but not *fem-2*, and the effect is to convert the mostly male *tra-1* germ line to a consistent hermaphroditic (rather than female) pattern (Hill and Haag 2009). For this reason, the equivalent arrow is dashed.

C. briggsae. This may have occurred because of its simple RNA target motif (Ryder *et al.* 2004) and conserved expression in early meiotic germ cells (Jones *et al.* 1996; Nayak *et al.* 2005). FOG-2, an F-box protein cofactor for GLD-1 in *C. elegans* that is essential for XX (but not male) spermatogenesis (Schedl and Kimble 1988; Clifford *et al.* 2000) is a recent gene duplicate that is found only in this species (Nayak *et al.* 2005).

The *gld-1* mRNA is itself subject to translational repression via its own 3′ UTR by the PUF family RBP FBF (Crittenden *et al.* 2006). The PUF family is somewhat dynamic in *Caenorhabditis*, such that in *C. briggsae* there are not strict orthologs of FBF. However, both biochemical and genetic studies indicate that the three paralogs of the PUF-2 subfamily (*Cbr-puf-2*, *Cbr-puf-1.1*, and *Cbr-puf-1.2*) represent the *C. briggsae* equivalents (Liu *et al.* 2012). Given the opposite roles of *C. briggsae* and *C. elegans gld-1* in sex determination, it is not surprising that simultaneous RNAi knockdown of *Cbr-puf-2 and Cbr-puf-1.2* function feminizes the germ line, rather than masculinizes as does *C. elegans fbf*(*RNAi*). Surprisingly, however, complete elimination of *Cbr-puf-2* activity alone (via a deletion mutation) leads to a fully penetrant larval arrest. Subsequent studies revealed this was due to a defect in pharyngeal development, apparently related to the brief expression of *Cbr-puf-2* in three pharyngeal muscle cells (Liu and Haag 2014). This suggests that the PUF protein family may spin off paralogs as they acquire novel roles outside of the germ line, an example of the neofunctionalization process thought to favor retention of otherwise redundant gene copies (Lynch *et al.* 2001).

The above studies revealed evolutionary variation through reverse-genetic targeting of conserved genes. Another fruitful approach has been to conduct unbiased forward screens for germline-specific feminizers in *C. briggsae* (reviewed by Ellis 2017). For example, alleles of *Cbr-gld-1* emerged from screens for Mog hermaphrodites (Beadell *et al.* 2011). Similarly, screens for *fog-2*-like mutations conferring hermaphrodite-specific germline feminization led to the discovery of *she-1* (Guo *et al.* 2009). Like FOG-2, SHE-1 is an F-box protein that depends upon *tra-2* for its function. However, there is no indication that it directly regulates *tra-2*, nor that it interacts with GLD-1. Its exact role in enabling XX spermatogenesis thus remains a subject for future work.

Another novel factor required for sperm development of both sexes of *C. briggsae* is encoded by *trr-1* (Guo *et al.* 2013). This component of the Tip60 histone acetyl transferase complex is conserved across *Caenorhabditis*, but loss of *trr-1* alone is incapable of causing similar feminization of the *C. elegans* germ line. *Cbr-trr-1* mutations enhance the incomplete somatic masculinization of *Cbr-tra-2*, and, in the germ line, help activate *fog-3* expression, suggesting that TRR-1 promotes male development. However, the effect on *fog-3* is dependent upon the presence of *tra-1*. This suggests that, as for Gli and its other *hedgehog* pathway transcription factor homologs, TRA-1 has both activating and repressing effects on target genes, with TRR-1 being important for the former. A previously unknown role of the *C. elegans trr-1* ortholog in promoting male development can be revealed through enhancement of weak *fem* alleles (Guo *et al.* 2013). These results are consistent with existence of separate and conserved *tra-2/fem* (repressor) and *tra-1/trr-1* (activator) branches of the sex determination pathway. Though apparently conserved in both *C. elegans* and *C. briggsae*, their relative importance is reversed. The case of *trr-1* also shows how use of a second "satellite model" organism can shed important light on cryptic evolution underlying conserved phenotypes of the more widely studied species.

The impact of *trr-1* described above, as well as related work in *C. elegans* (Grote and Conradt 2006) suggest that

chromatin regulators may be frequent contributors to sexual regulation via modulation of TRA-1 function. Chen *et al.* (2014) thus pursued possible roles for the nucleosome remodeling factor (NURF) complex in *C. briggsae*. Using the TALEN-based genome editing methods they had developed (Wei *et al.* 2014a), they discovered that, while complete loss of *Cbr-isw-1* and *Cbr-nurf-1* were sterile, hypomorphic mutations were sometimes Fog, and RNAi knockdown of either gene increased the penetrance of this. Surprisingly, however, the feminizing impact is not observed in *C. elegans*, or in the outcrossing *C. nigoni* and *C. remanei*. The NURF complex thus appears to be uniquely important in *C. briggsae*, and likely represents another component of the species-specific regulation that each hermaphrodite evolved to produce sperm transiently.

Germ cell proliferation

The proliferation of germ cells at the distal tip of the *C. elegans* gonad is directed by a somatic niche, comprised of the many finger-like projections of a single distal tip cell (DTC, Hall *et al.* 1999; Byrd *et al.* 2014). The DTC stimulates mitotic proliferation of germline stem cells via Notch signaling (Kimble and Hirsh 1979; Kimble and White 1981; Austin and Kimble 1987; Cinquin *et al.* 2010). As proliferation pushes stem cells out of the DTC niche, they undergo a final mitosis and then enter meiosis. No further mitoses are normally observed in either sex, and there is no evidence for a mostly quiescent, or "label-retaining" subpopulation of stem cells (Crittenden *et al.* 2006). In addition, for all *Caenorhabditis* species that are self-fertile, spermatocytes are found only during the L4 larval stage and (depending on species) the first few hours of adulthood as defined by the final molt. Thus, sperm are of a finite number established prior to ovulation, and when sperm are exhausted reproduction ceases unless mating with a male occurs. Recent studies in other nematode groups have revealed significant deviations from these aspects of *Caenorhabditis* germ cell proliferation.

The recently described genus *Auanema* (Kanzaki *et al.* 2017) has presented several unexpected aspects of germline development. Though similar to *Caenorhabditis* in overall form and habitat, and within the same family, "Rhabditidae" (Kiontke and Fitch 2005), at least three *Auanema* species (*A. rhodensis*, *A. freiburgenesis*, and *A. viguieri*) exhibit a reproductive polyphenism in the development of XX individuals, such that those that develop directly via a normal L3 larva mature into females, while those produced from dauer larvae (L3d) develop as selfing hermaphrodites (Félix 2004; Kanzaki *et al.* 2017). This presents another convergently evolved self-fertile taxon, which has now been examined in some detail. Among their unexpected features, hermaphrodite spermatocytes are not specified briefly in the L4 stage, as in *Caenorhabditis*, but instead are continuously replenished via coherent populations of spermatagonia (McCaig *et al.* 2017). These form elongated cysts that proliferate mitotically far from the distal stem cell niche, and undergo meiosis and spermatogenesis adjacent to oocytes. Other surprising features of *Auanema* germline biology are described below.

More distant relatives of *Caenorhabditis* are the mammalian filarial parasites (onchocercids, Spiruromorpha, Figure 1), such as *Brugia malayi*, the causative agent of human filariasis. These parasites have a radically different life history from the bacteriovores in "Rhabditidae" discussed thus far. A female *Brugia* adult can lay over 1000 embryos per day, and sustain this rate for over 5 years (Taylor *et al.* 2010)—a reproductive output three orders of magnitude greater than that of *C. elegans*. In addition, they and many of their relatives have harbored *Wolbachia* bacteria as obligatory symbionts for millions of years (McLaren *et al.* 1975; Bandi *et al.* 1998; Taylor *et al.* 1999). Importantly, curing these nematodes of *Wolbachia* with antibiotics adversely affects them without harming their mammalian host (Bosshardt *et al.* 1993). In *Onchocerca ochengi*, a parasite of livestock, tetracycline treatment kills adults (Langworthy *et al.* 2000). In cured *Brugia malayi* and *B. pahangi*, females produce inviable embryos that die via extensive apoptosis, while males retain normal fertility (Bandi *et al.* 1999; Landmann *et al.* 2011). This inviability is likely caused by the requirement for *Wolbachia* in proper polarization of the first zygotic cell division, as noted earlier (see section *Zygotic mitosis*). A subsequent study (Foray *et al.* 2018) revealed that the *Wolbachia* symbiont and the *Brugia* female have coevolved to jointly support oocyte proliferation. The dynamics of this proliferation differ markedly from that of *Caenorhabditis*, in that it occurs predominantly in a zone proximal to the distal stem cell niche, with the most distal cells represent a quiescent population (Foray *et al.* 2018). Loss of *Wolbachia* stimulates ectopic proliferation in the distal zone, with the effect of exhausting the quiescent pool. It thus appears that *Wolbachia* has become such an integral part of the female germline development that the nematodes can no longer prosper without it. What, if anything, the nematode hosts derive from the symbiosis is another mystery.

In addition to the presence of the *Wolbachia* symbiont, the somatic niche for germline stem cells differs between *Caenorhabditis* and *Brugia* (Foray *et al.* 2018). Ablation of the DTC in *Brugia* is not sufficient to eliminate germline proliferation, as it is in *Caenorhabditis*. Nevertheless, broad treatment with inhibitors of Notch signaling reduce proliferation. These results suggest that the somatic niche in *Brugia* is similar to that of *Caenorhabditis*, but on a larger scale. This finding is consistent with the ongoing anatomical (Rundell and Leander 2010) and genomic (Aboobaker and Blaxter 2003) miniaturization of nematodes that accompanied their invasion of tiny meiofaunal habitats.

Spermatogenesis

Compared to the wild variety seen in other phyla (Lüpold and Pitnick 2018), the peculiar amoeboid sperm of nematodes are notably constant in their major sperm protein (MSP)-based motility and overall shape. However, this outward constancy masks tremendous variation that impacts organismally important traits, such as sex ratio, self-fertility, sexual selection, and resistance to cross-species mating. Some of these variables are described below.

Just within *Caenorhabditis*, sperm can differ in volume as much as 50-fold between species (Vielle *et al.* 2016). Sperm size is correlated with competitive ability within species (LaMunyon and Ward 1998). In selfing species, male sperm are consistently larger than those of hermaphrodites, in part because of somatic gonad effects (Baldi *et al.* 2011). However, male sperm of outcrossing species are generally larger than those of males from selfing species (LaMunyon and Ward 1999; Hill and L'Hernault 2001). Further, conditions that select for the most competitive sperm also increase sperm size (LaMunyon and Ward 2002). These correlations indicate that postcopulatory sexual selection and its relaxation in selfing species is a major force that shapes sperm development. They also suggest a simple effect of sperm size on competitive ability, yet interspecies matings reveal a more complex relationship. Males of outcrossing species frequently suppress self-fertility in hermaphrodites, and tend to have larger sperm. However, across a matrix of many pair-wise crosses, the extent of this effect is not correlated with difference in the sperm size of the two species (Ting *et al.* 2014). This suggests that other factors contribute to competitiveness. A likely candidate is the sperm proteome, which can be much larger in outcrossing species (Thomas *et al.* 2012b; Yin *et al.* 2018).

Several lines of evidence have revealed that male-expressed genes are disproportionately lost as part of widespread genome shrinkage in self-fertile lineages (Thomas *et al.* 2012b; Fierst *et al.* 2015). One case that has been investigated functionally is that of the MSS family of sperm surface glycoproteins. Yin *et al.* (2018) found that *mss* genes are found in nearly all outcrossing *Caenorhabditis*, but are missing in all self-fertile species. MSS proteins are both necessary (in *C. remanei*) and sufficient (when restored to *C. briggsae*) for optimal sperm competition. The increased success in siring cross-progeny that an *mss*+ transgene confers to *C. briggsae* males (Yin *et al.* 2018) may provide an important clue about its independent loss. With greater suppression of selfing comes a greater fraction of male progeny. The reproductive assurance and lack of inbreeding depression of selfing species (Dolgin *et al.* 2008), combined with the small, transient habitats they favor likely create conditions that select for lower male frequency via interdemic selection. This is reminiscent of the local mate competition scenario of Hamilton (1967). Loss of *mss* may provide a way to reduce male frequency without complete loss of outcrossing, which is likely needed at some level (Morran *et al.* 2009a,b).

Beyond competiveness, the sperm of some nematodes exhibit oddities that lead to unexpected sex ratios, as in the heterogonic sheep parasite *Strongyloides papillosus*. Adults in a host are always parthenogenic females. Many of their XX progeny develop directly into infective larvae, creating a simple asexual life cycle. However, females can also produce sexual XX female and XO male progeny, which mate outside the host and produce outcrossed infective larvae. How does a parthenogenic XX female produce a male without mating? Albertson *et al.* (1979) had suggested that one X chromosome (present as part of an X-autosome fusion in this species) may be lost in some diploid oocytes via chromosomal diminution. Using molecular markers and heroic crosses through sheep, Nemetschke *et al.* (2010) found clear support for this hypothesis. Which of the two X chromosomes is lost appears to be random, but some mechanism must prevent both from being lost.

Auanema rhodensis presents another interesting sperm-mediated sex ratio anomaly. Though males are XO and females XX, cross progeny are <2% male (Félix 2004). Examination of male spermatogenesis provided an explanation (Shakes *et al.* 2011). As spermatocytes proceed through meiosis I, the two X chromosomes are not paired, as in *C. elegans*. This produces secondary spermatocytes with one X chromatid. When these divide, the spermatid possessing the X attracts nearly all of the organelles required for sperm function (mitochondria, membranous organelles, and MSP), while the nullo-X chromosome set ends up in a residual body incapable of supporting spermiogenesis. As a result, nearly all spermatozoa capable of fertilizating an oocyte are X-bearing, which, in turn, produces extremely female-biased broods. Interestingly, matings between male and free-living female *Strongyloides papilillosus* also produce all-female broods, but it is not yet known whether the mechanism is the same as that described for *A. rhodensis* (Streit *et al.* 1999). *A. rhodensis* hermaphrodite morphs employ yet another non-Mendelian mechanism of X chromosome segregation during spermatogenesis, as the functional self-sperm contain two X chromosomes (Tandonnet *et al.* 2018). This is coupled with loss of both oocyte X chromosomes to the first polar body. As a result, self-progeny are always XX, but crosses between XX hermaphrodites and males yield exclusively male progeny. These dynamics are another strong indication that selection on sex ratio can push the evolution of sperm attributes, in this case via unexpected meiotic novelties.

Though it is obvious that self-fertility depends upon XX spermatogenesis, the final step of sperm development—spermiogenesis or activation—plays another important role in its evolution. In male nematodes, spermatids are stored in an inactive state in the seminal vesicle, and are not activated to become motile spermatozoa until exposure to factors during their passage through the vas deferens activates two parallel pathways (Ellis and Stanfield 2014). One of these pathways is composed of SPE-8 and associated sperm proteins, which responds to a signal from the vas deferens (Nishimura and L'Hernault 2010) that may be zinc cations (Liu *et al.* 2013). The other is mediated by the seminal protease TRY-5 and its inhibitor, SWM-1 (Stanfield and Villeneuve 2006; Smith and Stanfield 2011). The requirement for activators expressed in the male somatic gonad presents a problem for would-be selfing hermaphrodites, which must evolve male-independent sperm auto-activation.

C. elegans spe-8 group mutants exhibit hermaphrodite-specific activation defects, suggesting that only the *spe-8* pathway is used to achieve auto-activation. The independent origins of selfing in *C. briggsae* and *C. tropicalis* raise the question of whether convergently evolved hermaphrodites

used the identical means to achieve sperm auto-activation. Wei *et al.* (2014b) found that genes of both male sperm activation pathways are conserved across the genus. Knockout mutants in multiple *spe-8* group genes cause self-sterility in *C. briggsae* hermaphrodites (but not males), suggesting parallel co-options of the same sperm activation pathway. However, loss of *spe-8* group homologs had no effect on *C. tropicalis* hermaphrodites, but *try-5* mutant hermaphrodites were self-sterile. This indicates that, in the *C. tropicalis* lineage, the alternative pathway evolved to enable auto-activation (*i.e.*, convergence). Surprisingly, *C. tropicalis* males are also rendered sterile upon loss of only *try-5*, indicating that the two pathways are no longer redundant in this species.

Given the need for both XX spermatogenesis and sperm auto-activation, how did self-fertility ever evolve? In an elegant experiment, Baldi *et al.* (2009) simulated this transformation using the gonochoristic *C. remanei*. Partial loss of *Cre-tra-2* function with RNA interference creates XX pseudohermaphrodites that produce sperm (Haag and Kimble 2000), but these sperm are not active and the animals are not self-fertile. However, mating with males is sufficient to activate these sperm and allow production of selfed progeny. Moreover, when *Cre-swm-1* is also knocked down, pseudohermaphrodite self sperm spontaneously activate and sire self-progeny (Baldi *et al.* 2009). Because there is no known role for TRY-5 protease in females, this ability of *Cre-swm-1* (*RNAi*) to activate these XX sperm is surprising. Examination of sex-specific transcriptome data (Thomas *et al.* 2012a) reveals that *C. remanei swm-1* is abundantly and comparably expressed in both females and males, while *try-5* is highly male-biased. One possibility is that the low level of TRY-5 expression in *C. remanei* females is sufficient to activate self sperm when SWM-1 is eliminated. Alternatively, knockdown of *tra-2* may elevate TRY-5 levels to a point that potentiates loss of *swm-1*. In either case, simultaneous modification of sex determination and sperm activation factors is sufficient to allow rudimentary selfing.

The above experiments suggest a two-step model for the evolution of self-fertility (Figure 4). In the first phase, a germline-specific change in the regulation of the sex determination (discussed above) could have produced a small population of XX spermatids. By virtue of developing in a female body, these were initially inactive, and also smaller than male sperm (Baldi *et al.* 2011). However, their *trans*-activation could be achieved via seminal fluid from conspecific (or closely related) males, as shown by Baldi *et al.* (2009). Evolving in a population of gonochoristic conspecifics, such mates would likely be readily available to the incipient selfer. This would produce a mixed-paternity brood with an XX-skewed sex ratio, which has two potential consequences. First, selfed progeny would retain the maternal genotype that promotes XX spermatogenesis, which is likely a recessive trait (Woodruff *et al.* 2010). Second, the XX-biased broods may be adaptive if population sizes shrink and local mate competition conditions set in (see above). Such partial selfing would have the further benefit of allowing recessive deleterious mutations to be gradually purged (Garcia-Dorado 2012). Eventually, a greater degree of selfing would become well tolerated. In the final phase, hermaphrodites could have evolved sperm auto-activation by upregulating expression of one of the spermiogenesis signals in their otherwise female reproductive tract. Baldi *et al.* (2009) also suggested that the capacity for XX sperm autoactivation may have evolved as a neutral polymorphism first, allowing subsequent changes in sex determination that enable spermatogenesis to achieve great impact without mating. In either case, an autonomous selfer would enjoy reproductive assurance at low density without accompanying inbreeding depression, allowing them access to habitats that would be marginal for obligate outcrossers.

Vulva development

The vulva is an opening in the center of the *C. elegans* hermaphrodite (but can be in different positions in other taxa) that serves for copulation and egg laying through its direct connection with the uterus. Because of extensive work on the vulva in *C. elegans*, it has also become an important evo-devo model and is a primary exemplar of DSD. The vulva is a simple organ that originates from a handful of ventral epidermal cells during the larval stages (Figure 5). Cellular division and organogenesis can be tracked by differential interference contrast (DIC) microscopy. Moreover, *C. elegans* mutants with abnormal vulvae remain fertile, which has allowed vulva development to be explored in exquisite detail (Sternberg 2005; Gupta *et al.* 2012). While the morphology of the adult vulva can be a slit or a round pore depending on the species (Kiontke *et al.* 2007), the fate patterns of the vulva precursor cells (VPCs) are quite conserved between species. Are the VPCs specified similarly in all species? Where does the induction signal come from, and is it always the same molecular signal? Studies that have posed these questions have revealed an impressive diversity of cryptic changes (*i.e.*, changes in mechanism without changes in phenotype), between both closely and distantly related species, and even strains of the same species. The field has also benefited from the establishment of other selfing species, *P. pacificus* and *Oscheius tipulae*, as genetically tractable systems to explore changes in vulval development over large evolutionary distances (Félix 2006; Sommer 2006). Cryptic genetic changes have been deciphered further by exploring different species of the same genus and even different natural isolates of the same species in the three genera, *Caenorhabditis*, *Oscheius* and *Pristionchus*.

Twelve ventral epidermal cells (P1.p to P12.p) originate during the L1 stage (Figure 5): P1.p being the most anterior cell and P12.p the most posterior cell (Sulston and Horvitz 1977). In *C. elegans*, during the L3 stage, and upon signaling from a specialized cell of the uterus called the anchor cell (AC), the central cells P5.p to P7.p divide to give rise to 22 cells that will fuse in late L4 in concentric circles to form the vulva. The pattern of division of each cell is highly reproducible and reflects the fate of the cells. While the most central cell P6.p adopts the 1° fate ("inner" vulval cells that will detach from the cuticle and involute), P5.p and P7.p adopt the 2° fate ("outer" vulval cells that remain connected

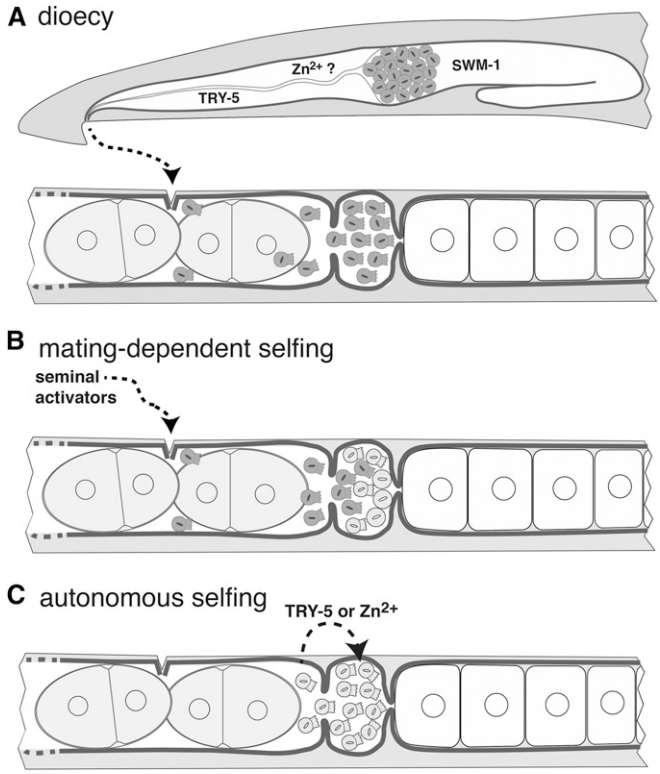

Figure 4 Scenario for the evolution of self-fertility in *Caenorhabditis*. (A) In the gonochoristic/dioecious *Caenorhabditis* ancestor, males (top) store gametes as inactive spermatids in the seminal vesicle, maintained in this state by the protease inhibitor SWM-1. Upon mating and ejaculation, male spermatids (gray) pass through the glandular vas deferens, where they encounter active TRY-5 protease and the signal for the *spe-8* pathway, which may be zinc ions. Once inside the female (bottom), they are activated and migrate from the uterus to the spermatheca, where they await ovulation and a chance to fertilize an egg. (B) A hypothetical first step to self-fertility is a change in germline sex determination that allows the production of some self-spermatids (light circles). These cannot activate on their own, but, after mating and transfer of some male seminal fluid, they are activated in *trans* (light spermatozoa). (C) In the second step, hermaphrodites evolve the ability to activate self-spermatids autonomously, by increasing the level of active TRY-5 protease (as in *C. tropicalis*) or the signal for the *spe-8* pathway (in *C. elegans* and *C. briggsae*).

to the cuticle to anchor the vulva). P3.p, P4.p and P8.p daughter cells adopt a 3° (nonvulval) fate, because they do not form the vulva in wild type animals. All of these P(5–7).p cells form a vulval competence group, as any of these cells can replace an ablated cell and become vulval. All the other Pn.p cells are incapable of forming the vulva, even upon ablation of P3.p to P8.p (Sternberg and Horvitz 1986). This vulval competence group is defined by expression of two Hox proteins, with LIN-39 in central cells promoting competence and MAB-5 repressing it in more posterior Pn.p cells (Clandinin *et al.* 1997). The AC sends a LIN-3/EGF signal that acts as a morphogen on the VPCs (Hill and Sternberg 1992). Closest proximity to the signal determines the 1° fate (generally P6.p); P5.p and P7.p receive a lower dose and adopt the 2° fate. Activation of the EGF/Ras signaling pathway in P6.p activates the Notch/Delta pathway (Sternberg 1988). This leads to the inhibition of the 1° fate in P5.p and P7.p through lateral inhibition and to the activation of the 2° fate in these same cells. The Wnt pathway is also involved in vulval specification, as loss of negative regulators of Wnt causes mote than three VPCs to be induced (Gleason *et al.* 2002). Conversely, VPCs adopt a 3° fate or fuse with the hypodermis in the absence of positive regulators of the Wnt pathway (Eisenmann and Kim 2000).

Variation in the position of the vulva: *C. elegans* has two gonadal arms extending from the center of the animal, with a central uterus and vulva derived from the central epidermal Pn.p cells. Some species have a single gonadal arm (monodelphy), which extends anteriorly. The evolution of monodelphy *per se* will not be covered here (but see Félix 1999). In most cases, monodelphy is accompanied by a posterior shift of the uterus and the vulva. In the monodelphic species *P. redivivus*, the vulva forms at 60% of body length because of a posterior displacement of the central Pn.p cells and because the vulva is centered in between P6.p and P7.p (Sternberg and Horvitz 1982). Within "Rhabditidae," monodelphy and a posterior vulva are derived and evolved several times (Kiontke *et al.* 2007). In the three posterior-vulva species of *Cruznema*, *Mesorhabditis*, and *Teratorhabditis* that have been analyzed, again only the central Pn.p cells (P5.p to P7.p) are competent, and they migrate posteriorly (Sommer and Sternberg 1994). However, mechanisms of vulva induction differ between species. The developing gonad induces the VPCs in *Cruznema*, but is not required to induce the VPCs in *Mesorhabditis* and *Teratorhabditis*. Establishment of the competence group by LIN-39 is conserved in *P. pacificus* (Eizinger and Sommer 1997) and *O. tipulae* (Louvet-Vallee *et al.* 2003). HOX specification represents a constraint on specifying which cells can form the vulva; to make a posterior vulva, this constraint has been overcome in at least four independent lineages in Rhabditida by a similar mechanism, *i.e.*, posterior migration of the vulva cells (Kiontke *et al.* 2007).

Variation in the number and fate of VPCs: Large variations are found in the size of the competence group, in the number of divisions of competent cells, as well as in the fate of the noncompetent Pn.p cells (for an evolutionary synthesis of most of these differences among species in Rhabditida, see Kiontke *et al.* 2007). While the competence group includes P3.p up to P10.p in *P. redivivus* (Sternberg and Horvitz 1982), much larger than the number of cells that are induced, in *Rhabditophanes* and *Strongyloides ratti* the competence group is restricted to the cells that form the vulva (Félix *et al.* 2000a). The size of the competence group can also vary between closely related species. For instance, P3.p is competent in *C. elegans* but not in *C. briggsae* or in some other *Caenorhabditis* species (Pénigault and Félix 2011). Overexpression of Wnt in *C. briggsae* is sufficient to induce the division of P3.p, while downregulation in *C. elegans* prevents the division of P3.p (Pénigault and Félix 2011). Because several Wnt ligands

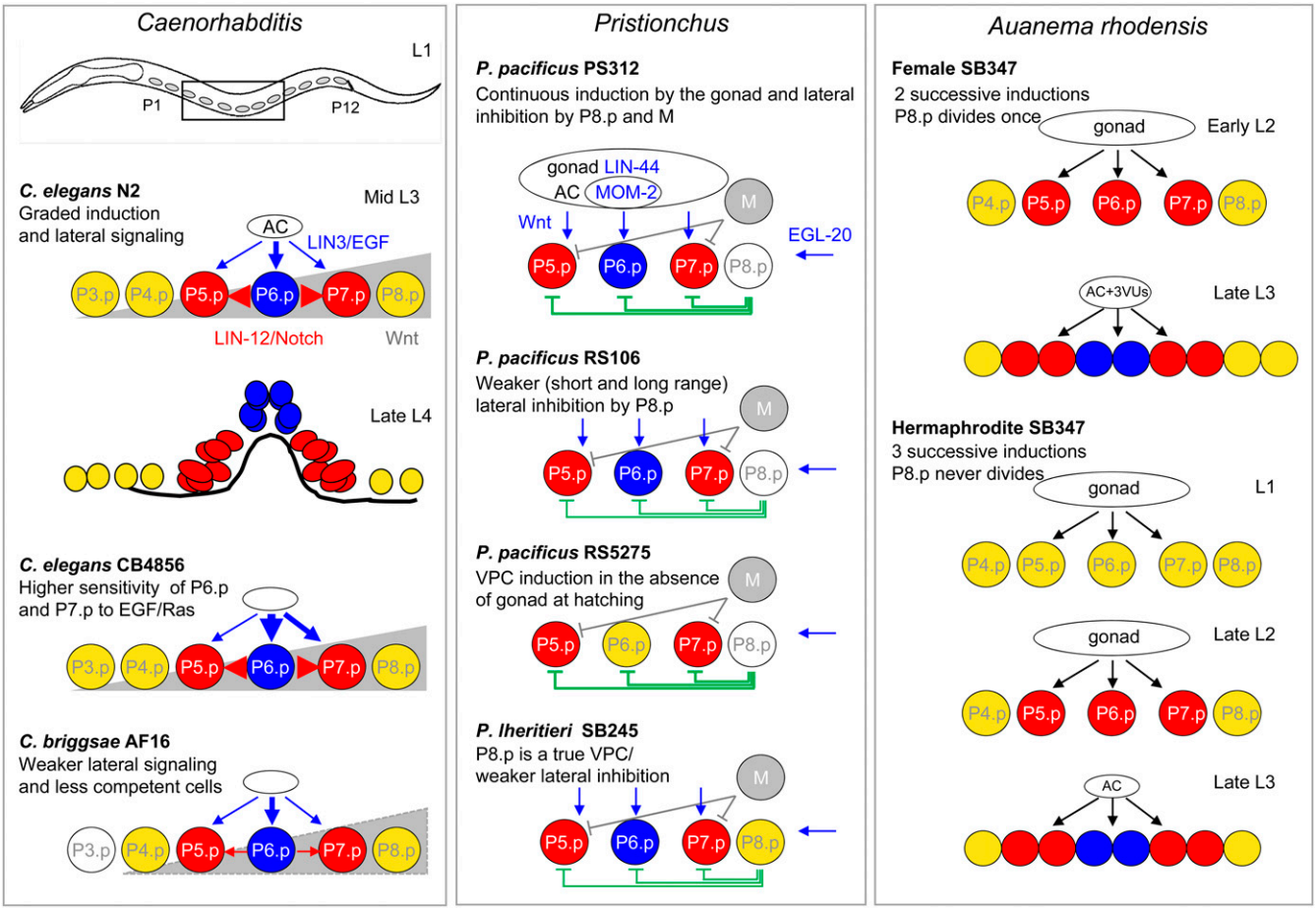

Figure 5 Variations in vulval development in *Caenorhabditis*, *Pristionchus*, and *Auanema*. Left panel: schematic representation of vulva development in *C. elegans*, and some cryptic variations found within *Caenorhabditis*. From ventral epidermal cells, six competent cells, P3.p to P8.p are defined in *C. elegans*. During the L3 larval stage, VPCs are specified and induced by the combined action of a graded EGF signal from the anchor cell (AC), a lateral Notch signal between the most central cells and a Wnt gradient emanating from the posterior of the body (gray wedge). Blue cells adopt a primary fate and divide to form the center of the vulva in late L4. Red cells have a secondary fate and form the lateral part of the vulva. Yellow cells form the vulva only if blue or red cells are absent. The respective contributions of the EGF and Notch pathways vary quantitatively (shown by arrows of different size) among *Caenorhabditis* species and even among strains of the same species. In *C. briggsae*, reduction in Wnt signaling (compared to *C. elegans*) is responsible for the lack of competency of P3.p. This could be due to truncation of the Wnt gradient (depicted here), or because of reduced sensitivity of P3.p to an identical gradient. Middle panel: Schematic representation of vulva development in *P. pacificus*, and variations found within *Pristionchus*. In *P. pacificus*, the VPCs are induced by redundant Wnt signaling signals sent by the gonad and the AC. The M cell, as well as the P8.p cell (which is only partially competent), send lateral inhibitory signals to prevent the adoption of 1° Cell fate by P5.p and P7.p. Within *Pristionchus*, cryptic quantitative changes in the signaling pathways are observed. In particular, the extent of lateral inhibition by P8.p varies frequently between and within species. Right panel: example of changes in vulva development between morphs of the same species is shown for *Auanema rhodensis* SB347. In this species, three sexes coexist because female larvae that go through the dauer stage become self-fertile hermaphrodite adults. This plasticity is accompanied by changes in vulva formation between females and hermaphrodites, in the number of inductive signaling steps from the gonad that are required to specify the Pn.p cells, as well as in the number of divisions of P8.p.

are expressed in a gradient from posterior to anterior in the *C. elegans* body (Gleason *et al.* 2006), one possible explanation is that the lack of competency of P3.p in *C. briggsae* could be due to a shorter Wnt gradient in *C. briggsae* compared to *C. elegans* (Figure 5; Pénigault and Félix 2011). It is also possible that, in *C. briggsae*, P3.p is less sensitive to Wnt signals. Within *Pristionchus*, P8.p is partially competent in *P. pacificus* (Sommer 1997), but is a true VPC in *P. lheritieri* (Srinivasan *et al.* 2001).

Within the competence group, the pattern of cell division is also variable. The numbers of cells that form the vulva vary between 16 cells in *O. tipulae*, to 34 cells in *Rhabditoides regina* (Sommer and Sternberg 1995). Within *Caenorhabditis*, 22 cells form the vulva in all species that have been observed (Félix 2007). However, the division pattern of the competent cell P3.p is highly variable, between and within *Caenorhabditis* species (Delattre and Félix 2001; Félix 2007; Pénigault and Félix 2011). Similarly, the pattern of P4.p and P8.p division shows a high degree of intraspecies and interspecies variation in *Oscheius*. The fate of the cells that are not competent to form the vulva also vary. Pn.p cells that do not express LIN-39 fuse with the hypodermis in *C. elegans* and in *O. tipulae*

(Louvet-Vallee *et al.* 2003), while the expression of LIN-39 prevents cell death in *P. pacificus* (Eizinger and Sommer 1997). The restriction of the competence group by cell death is a derived character that is observed several times in the phylogeny, yet reversals of this restriction are very rare, constituting an interesting evolutionary bias (Sommer and Sternberg 1996; Kiontke *et al.* 2007). In *Turbatrix aceti*, as in three other Panagrolaimomorpha species, the survival of the VPCs depends on a signal emanating from the gonad during the L2 stage, in contrast to *P. pacificus* (Sternberg and Horvitz 1982; Félix and Sternberg 1997, 1998).

Variation in the mechanisms of VPC induction: Laser ablation of the AC or the gonad has been performed in a wide range of species. These experiments have revealed an impressive diversity of induction mechanisms, apparently evolved from an ancestral two-step induction signal from the gonad (Kiontke *et al.* 2007). Most surprisingly, systematic characterization of *Caenorhabditis* and *Pristionchus* has uncovered cryptic genetic changes (*e.g.*, changes in the contributions of different signaling mechanisms, in competence level and even genetic variation affecting the requirement for induction) between closely related species and even between strains of the same species (Srinivasan *et al.* 2001; Félix 2007; Zauner and Sommer 2007; Milloz *et al.* 2008; Kienle and Sommer 2013).

In Clades IV and V (Figure 1), the VPCs can be induced independently of the gonad, as in *Brevibucca*, or require continuous or possibly consecutive signals from the gonad, as in *Halicephalobus* sp. (Félix *et al.* 2000a). As shown above, *Mesorhabditis* and *Teratorhabditis* also do not rely on the gonad for induction (Sommer and Sternberg 1994), while two consecutive signals from the AC are required in *O. tipulae* and *Rhabditella axei* (Félix and Sternberg 1997). In *O. tipulae*, the early and late induction signals depend on the activity of MEK kinase—a component of the Ras pathway involved in *C. elegans* late-only induction (Dichtel-Danjoy and Félix 2004b). One possible evolutionary scenario is that a heterochronic shift occurred in the *Caenorhabditis* lineage with regard to both the requirement for, and expression of, the homologous induction event (Kiontke *et al.* 2007).

In *P. pacificus*, a continuous 10-hr induction from several cells of the somatic gonad is required to induce the VPCs (Sigrist and Sommer 1999), seemingly comparable to the two-step induction of *O. tipulae* (Kiontke *et al.* 2007). As in *C. elegans* (Gleason *et al.* 2006), simultaneous inactivation of several Wnt ligands and receptors leads to Vulvaless phenotypes in *P. pacificus* (Zheng *et al.* 2005; Tian *et al.* 2008; Wang and Sommer 2011). However, after inactivation of *Ppa-bar-1*/β-catenin (the Wnt signal transducer), VPCs do not die of apoptosis but adopt a 3° fate, similar to the phenotype obtained after gonad ablation (Tian *et al.* 2008). Further, Wnt ligands MOM-2 and LIN-44 are expressed in the AC before the division of VPCs and in the central cells of the somatic gonad, respectively (Tian *et al.* 2008). This suggests that Wnt signals comprise the gonadal signal that induces formation of the vulva in *P. pacificus* (Figure 5), whereas they are primarily involved in establishing VPC competence to respond to that signal in *Caenorhabditis*. The involvement of LIN-3 and its downstream cascade has not been demonstrated in *P. pacificus* vulval induction, raising the possibility that a secondary, largely redundant Wnt pathway in one species (*C. elegans*) could be central to the homologous process in another (*P. pacificus*). Interestingly, P8.p and the mesoblast M cell are both responsible for the lateral inhibition that prevents too many VPCs from adopting a 1° fate (Jungblut and Sommer 2000). Thus, P8.p inhibits the induction of VPCs even though it is not a VPC itself. Of note, Wnt signaling is also used in *P. pacificus* to shape the distinct "pretzel" morphology of the somatic gonad (Rudel *et al.* 2008). Selection on either gonad shape or vulva induction would thus target a pleiotropic Wnt module, with potential consequences (overt or cryptic) for the other trait.

Variation of the induction mechanism of VPCs is also found between species belonging to the same genus or even between strains of the same species. For instance, a single late induction from the AC is required for VPC divisions in *Panagrolaimus* sp. PS1579 as in *C. elegans*, while early and continuous (or possibly two consecutive) signals from the gonad are necessary in another *Panagrolaimus* species, *P.* sp. PS1732 (Félix *et al.* 2000). Within *Pristionchus*, the system of induction found in the laboratory strain *P. pacificus* PS312 is not widely conserved. For instance, in *P. lheritieri* and *P. maupasi* and even different strains of *P. pacificus*, some VPCs are induced even when the gonad is ablated just after hatching, and the extent of the lateral inhibition exerted by P8.p on the VPCs can vary (Srinivasan *et al.* 2001; Zauner and Sommer 2007). Mapping of the quantitative trait locus (QTL) responsible for the differences in the gonad-independent induction of VPCs between *P. pacificus* strains revealed a new role for the Notch ligand *apx-1/Delta* (Kienle and Sommer 2013). In many wild strains, absence of a binding site for the HAIRY transcription factor in the *cis*-regulatory region of *apx*-1 leads to its expression in P6.p and confers a gonad-independent induction of this cell, while in the laboratory strain PS312, *apx-1* is not expressed in the VPCs, which thus require the gonad for induction (Kienle and Sommer 2013).

While the pattern of division of the VPCs is very conserved among *Caenorhabditis* species, cryptic changes in the mechanism of induction were revealed by ablation of the AC or overexpression of the LIN-3/EGF inductive signal (Félix 2007). Early ablation of the AC leads to adoption of the 3° fate for all VPCs in all species. However, ablation of the AC during patterning, *i.e.*, mid-L3 stage, has different outcomes depending on the species or strain within a species (Félix 2007; Milloz *et al.* 2008). For instance, in *C. remanei*, the VPCs adopt a 2°3°2° pattern, suggesting that, in contrast to *C. elegans*, a low level of induction from the AC is sufficient for P6.p to induce its neighboring cells, but not enough for its own fate acquisition. In *C. briggsae*, the same experiment leads to a 2°2°2° pattern. Moreover, mild overexpression of LIN-3 in *C. briggsae* generates adjacent 1° cells, a phenotype that is obtained in *C. elegans* only after strong overexpression

of LIN-3 (Katz *et al.* 1995). Thus, lateral inhibition from P6.p on adjacent Pn.p cells can be overcome easily in *C. briggsae* and less so in *C. elegans*. Nevertheless, the LIN-12/Notch pathway is still involved in lateral inhibition in *C. briggsae* and LIN-3/EGF acts in a dose-dependent manner on VPC fate specification (Félix 2007). However, inactivation of genes of the vulva specification pathways in *C. elegans* and *C. briggsae* often leads to different phenotypes, revealing a difference in the respective contributions of these pathways (Rudel and Kimble 2001; Sharanya *et al.* 2012, 2015; Mahalak *et al.* 2017). Thus, evolutionary changes in the patterning of the vulva are not necessarily due to rewiring of the signaling pathways, but can also be attributed to quantitative changes of the same network of signaling pathways (Haag and True 2007). Modeling of the vulva induction pathways confirms that quantitative tuning of the same network parameters could account for the different vulva patterning obtained experimentally among *Caenorhabditis* species (Hoyos *et al.* 2011).

Other experiments provided indirect evidence of cryptic genetic changes between species or between strains of the same species. One approach is mutagenesis screens for vulva defects. These yielded a different spectrum of mutations depending on the species. In *O. tipulae*, although 50,000 gametes were mutagenized, only a handful of hypo- and hyper-induction mutants were isolated. The far larger category was represented by mutations that affect the number of cell division of VPCs, but not their specification, while this category of mutants was very rarely found in *C. elegans*. These results may indicate a rewiring of the system, depending on the species. Alternatively, higher pleiotropy of the genes involved in vulval patterning in *O. tipulae* could lead to embryonic death or sterility, thus preventing the detection of specific classes of mutants (Dichtel *et al.* 2001; Louvet-Vallee *et al.* 2003; Dichtel-Danjoy and Félix 2004a). Similarly, screens in *C. briggsae* (Sharanya *et al.* 2012, 2015) failed to identify mutants lacking VPC induction, and mapping of mutants indicates there are novel players relative to the *C. elegans* paradigm.

An alternative approach is to characterize the spectrum of vulval defects in mutation accumulation lines. Phenotypes observed in *C. elegans* are quantitatively different from those obtained in *C. briggsae* (Braendle *et al.* 2010). Although the division of the VPCs is highly reproducible within a strain, developmental errors arise at low frequency (~1%; Braendle and Félix 2008). Interestingly, the frequency and type of errors differ between closely related species or strains of the same species (Zauner and Sommer 2007; Braendle and Félix 2008). Introgression of a mutant allele in different strains of *C. elegans* also revealed intraspecific cryptic changes. For instance, the impact of different alleles of the Ras pathway on vulva induction vary, depending on the genetic background (Milloz *et al.* 2008). The background factors that distinguish *C. elegans* natural isolates were next explored by introgressing an allele of the EGF receptor *let-23* and performing QTL mapping. This revealed that *C. elegans* N2 harbors a mutation in the conserved acetyltransferase NATH-10 that is mainly responsible for the difference in expressivity of the *let-23* allele. Because this *nath-10* allele also confers high fitness on the laboratory strain N2 compared to others, this experiment demonstrated that cryptic genetic changes can accumulate in the genomes by indirect selection and pleiotropic effects (Duveau and Félix 2012). Even seemingly constant features of the signaling network are subject to DSD at the molecular level. For example, while expression of *lin-3*/EGF remained constant between *Caenorhabditis* species and *O. tipulae*, the *cis*-regulatory elements that underlie it have been substantially reconfigured, with elements required for expression in one species completely missing in the other (Barkoulas *et al.* 2016).

Last, but not least, the development of the vulva has been shown to vary between female and hermaphrodite morphs of *A. rhodensis* (Félix 2004; Kanzaki *et al.* 2017). While both females and hermaphrodites have a competence group formed by P(4-8).p, P8.p divides in females but not in hermaphrodites. Most strikingly, while three successive gonadal inductions are necessary to form a vulva in hermaphrodites, two rounds of induction are sufficient in females. Although the molecular basis of such a switch remains unknown, this example illustrates that vulva induction can go through different routes even for animals from the same genotype.

The comparative work on vulva development reviewed above has initiated a virtuous cycle, in which interesting differences between *C. elegans* and its relatives have been appreciated directly, and also motivated further research in *C. elegans*. For example, the observation of an intrinsic difference among Pn.p cells in *Mesorhabditis* (Sommer and Sternberg 1994) led to re-evaluation of the differential competence of cells of the "equivalence" group in *C. elegans* (Clandinin *et al.* 1997). Similarly, *C. briggsae pry-1* mutants are multi-vulva (like their *C. elegans* counterparts), but also frequently show a failure of P7.p induction. This led to the discovery of a similar, albeit weakly penetrant, defect in *C. elegans pry-1* mutants (Seetharaman *et al.* 2010).

Male tail

Outwardly at least, nematodes appear to vary little with regard to morphology, especially compared to animals with appendages, like arthropods. One clear exception is the male copulatory apparatus, or "male tail." Because of the abundant variation in male tail morphology, it has long been used as an important tool (along with the feeding apparatus) for nematode morphological systematics (Chitwood and Chitwood 1974; Sudhaus 1976; Andrássy 1983, 1984; Fagerholm 1991; Sudhaus and Fitch 2001; Sudhaus and Fürst von Lieven 2003; Sudhaus 2011; Ragsdale *et al.* 2015). In the context of the *C. elegans* model system, additional "satellite" model species and the phylogeny of related species, male tail morphological variation also provides much material for studying the developmental-genetic basis of morphological evolution.

Much of the evolutionary developmental work on the male tail has involved rhabditid nematodes, on which this review is primarily focused. Mapping male tail character states onto the rhabditid phylogeny shows that some characters have evolved uniquely, or nearly uniquely, in some clades (*i.e.*, are "apomorphic") and are thus important for systematics (Sudhaus 2011). On the other hand, several characters have evolved repeatedly (*i.e.*, are "homoplastic"). In many ways, these latter characters are the more interesting for EDB, since such repeated evolution provides the potential to address questions about bias or constraints of the developmental-genetic system on evolutionary trajectories.

These male tail structures all play some role in the series of stereotypical behaviors involved in copulation (Sudhaus 1976; Loer and Kenyon 1993; Liu and Sternberg 1995; Barr and Garcia 2006; Koo *et al.* 2011; Sherlekar and Lints 2014). External structures that help the male sense contact with a hermaphrodite and determine correct orientation and body position include the genital papillae. In many species, these sensilla are arrayed within a cuticular "bursa velum" or "fan," which in different species exist in different sizes and shapes or can be absent altogether. The mechanosensory genital papillae (called "rays" when they form finger-like extensions in the fan) occur in different positional patterns in different species. Other structures include the precloacal papilla and any associated structure (*e.g.*, the "hook" in *C. elegans*), the chemosensory phasmids (which exist in both sexes but with some sexual dimorphism, and are found in different positions relative to the rays in different species), and the tail tip (which undergoes male-specific morphogenesis in some species, like *C. elegans*, but is sexually monomorphic in other species). Internal structures include the sclerotic spicules and the gubernaculum. The spicules are inserted into the vulva, providing a means of anchorage and sperm delivery; the gubernaculum covers the roof of the proctodeum and provides a shield that guides the spicules during their protraction. Both of these structures also show marked morphological variation among different groups of rhabditid species (Sudhaus and Fitch 2001; Kiontke *et al.* 2011).

Evolutionary developmental studies have focused primarily on variation in the patterning of genital papillae/rays and tail tip morphogenesis (Sternberg and Horvitz 1982; Fitch and Emmons 1995; Fitch 1997, 2000; Baird 2001; Sudhaus and Fitch 2001; Sudhaus and Fürst von Lieven 2003; Baird *et al.* 2005; Kiontke and Fitch 2005). For *C. elegans*, much progress has been made in elucidating the ultrastructural anatomy, neural connectivity, development, and genetics underlying these structures (Emmons 2005, 2014). Briefly, the overall form of the male tail is a result of morphogenetic "retractions" that begin in the second half of the L4 stage (Figure 6). The first indication of this process occurs as the tail tip cells detach from the L4 cuticle, become rounded, fuse, and migrate a short distance anteriorly. This is followed by the retraction of more anterior hypodermal cells. Because the inner and outer layers of the adult cuticle in the area of the fan are not connected, the outer layer folds and flattens in the wake of the retractions of the cells that are covered by the inner cuticle layer. As the tips of the rays have fixed points of attachment to the outer cuticle, they are drawn out into finger-like projections during the retractions of surrounding tissue, and sandwiched between the dorsal and ventral layers of outer cuticle that form the fan.

The cell lineages that produce the nine rays on each side originate from left-right pairs of the three most posterior blast cells of the lateral "seam:" V5, V6 and T (Figure 6) (Sulston *et al.* 1980; Emmons 2014). The posterior branch of the T blast cell lineage produces phasmid socket cells that hold the phasmid neurons in place; the anterior branch gives rise to the three most posterior rays. V5 gives rise to the most anterior ray and V6 produces the lineages of the other five rays. Each sublineage that produces a ray is stereotypic: for each ray r*n* ($n = 1$–9) an R*n* blast cell divides at the end of L3 to produce a posterior R*n*.p hypodermal or "tail seam" cell and an anterior blast cell. Then, at the beginning of the L4 stage, this blast cell produces four granddaughters, one of which dies and three of which become the ray components, the R*n*A and R*n*B neurons and the glial-like R*n*st "structural cell." The structural cell holds the ray in place and forms a clearly visible papilla on the surface of the tail before the morphogenetic retractions reveal the rays.

The ray cell sublineages are determined by the proneural *lin-32/atonal* gene, which also acts in combination with the *lin-44/Wnt* pathway to pattern asymmetry within the sublineage (Zhao and Emmons 1995; Portman and Emmons 2000, 2004). Male-specificity provided via DM-domain genes *mab-3*, *mab-23*, and *dmd-3* (homologous to *Drosophila dsx* and human *DMRT*) is required for these lineages as well (Shen and Hodgkin 1988; Yi *et al.* 2000; Lints and Emmons 2002; Ross *et al.* 2005; Siehr *et al.* 2011). Despite identical sublineage patterns, each bilateral pair of rays has a different identity from every other pair in terms of its AP position, whether its terminus opens on the dorsal or ventral surface of the fan, and what type of neurotransmitters, connectivities, and behavioral proclivities are associated with it (Loer and Kenyon 1993; Chow and Emmons 1994; Liu and Sternberg 1995; Chamberlin and Thomas 2000; Lints *et al.* 2004; Sherlekar and Lints 2014; Serrano-Saiz *et al.* 2017b). These identities are patterned along the AP axis by HOX genes (*mab-5/Antp* and *egl-5/AbdB*) and their *Polycomb*- and *trithorax*-group regulators (*sop-2*, *lin-49*, *lin-59*) (Chow and Emmons 1994; Salser and Kenyon 1996; Chamberlin and Thomas 2000; Lints *et al.* 2004; Zhang *et al.* 2004). Identity of the dopaminergic rays that open dorsally on the fan requires signaling by a TGFβ morphogen, DBL-1 (Savage *et al.* 1996; Krishna *et al.* 1999; Lints and Emmons 1999; Morita *et al.* 1999; Suzuki *et al.* 1999; Wong *et al.* 2010; Siehr *et al.* 2011). Further differentiation among ray identities is provided by temporal differences in HOX expression during the ray lineages (Ferreira *et al.* 1999) and by additional factors, including *VAB-3*/Pax6 (Baird *et al.* 1991; Zhang and Emmons 1995), ephrins, and semaphorins (Roy *et al.* 2000; Hahn and Emmons 2003), and the probable chromatin

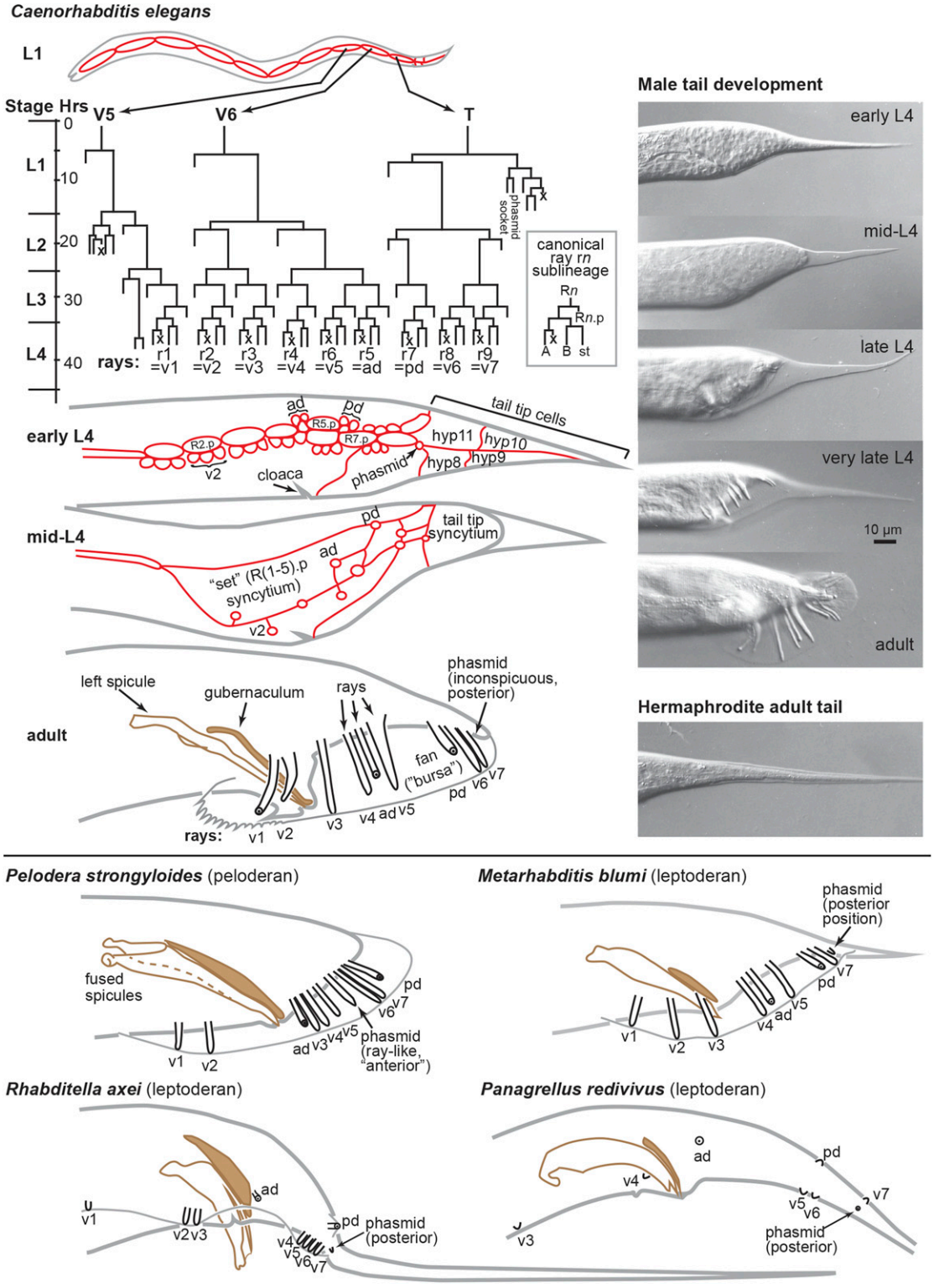

Figure 6 Male tail development in *C. elegans* and male tails of some other species. Top left: the left-side cell lineages giving rise to the R*n*.p "tail seam" hypodermal cell, the three cells of each ray *rn* (with designations v1–v7, ad and pd used for comparing ray homologs across species), and the phasmid socket cells (see text). These lineages are produced from bilateral pairs of V5, V6 and T blast cells, shown in the L1 larva (Sulston *et al.* 1980). Red lines represent apical boundaries of cells as would be visualized by immunostaining with MH27 or AJM-1::GFP. Inset: canonical ray sublineage in which an R*n* neuroblast produces an R*n*.p hypodermal cell (part of the "tail seam"), two ray neurons R*n*A and R*n*B, a ray structural cell R*n*st and a programmed cell death ("x"). Below the cell lineage: arrangements of these cells in the left lateral hypodermis right after their origins at early L4, and at mid-L4 after the R*n*A and R*n*B neurons have sunk a little below the surface. Tail tip cells hyp(8–11) and phasmid socket are also depicted. At the mid-L4 stage, the tail tip cells fuse and some of the R*n*.p cells fuse together (leading to absence of adherens junctions separating those cells) and begin to change shape (Fitch and Emmons 1995).

modifier VAB-3 (Chow *et al.* 1995; Ho *et al.* 2001). These patterning systems act combinatorially to establish individual ray identities (Lints *et al.* 2004); perturbations to these systems result in ray losses/gains or changes in ray positions, including complete homeotic transformations—different rays adopting the same identity cluster together and can fuse into a single ray (Baird *et al.* 1991; Chow and Emmons 1994).

Evolution of ray pattern: Rays provide a good example of how developmental analysis can provide information about organ homologies between different species, a fundamental step to any evolutionary reconstruction. In *P. redivivus*—a member of suborder Tylenchina and thus an outgroup representative relative to the rhabditids—the genital papillae are generated by R*n* sublineages identical to those in *C. elegans* (Figure 6) (Sternberg and Horvitz 1982). The main difference is that V5 does not produce a ray lineage, and V6 only produces rays homologous to *C. elegans* rays r3–r6; the T lineage produces the same three rays as in *C. elegans*. The spatial pattern of ray cell origins in the L4 hypodermis (Figure 6) is also highly conserved, allowing the ray homologies among species to be traced (Fitch 1997, 2000). Some rules have emerged, allowing ray homologies to be inferred without having to follow development in each species: (1) the rays homologous to rays r5 and r7 of *C. elegans* are always dorsal (labeled "ad" and "pd," the anterior and posterior dorsal rays respectively; Figure 6), and (2) the seven other rays homologous to *C. elegans* r1–r4, r6, r8 and r9 (relabeled "v1-v7"; Figure 6) are almost always arranged in that order ventral to the two dorsal rays (Fitch 1997, 2000; Sudhaus and Fitch 2001; Sudhaus and Fürst von Lieven 2003).

Whereas the pattern of ray and R*n*.p cell origins in the L4 hypodermis is highly conserved across Clade V (and likely even Clade IV, to which *Panagrellus* belongs), the ray structural cell tips then migrate to species-specific positions, tending to be at junctions between R*n*.p cells (Fitch and Emmons 1995). This planar array of structural cells prefigures the species-specific pattern of rays in the adult tail. The two dorsal rays can thus be in very different positions relative to the ventral (v1–v7) rays, which can also cluster together in different groups in different species. The ability to identify the ray homologies allows evolutionary changes in ray positioning to be reconstructed on the phylogeny (Fitch 1997).

The ability to homologize rays also allows identification of which rays are missing in species with fewer than nine ray pairs. For example, the R8 cell stopped dividing in the lineage leading to the *Metarhabditis* clade, resulting in a loss of v6 (homologous to *C. elegans* ray r8) (Figure 6) (Fitch and Emmons 1995; Fitch 1997). It has been noted that this ray is particularly susceptible to loss in mutants with altered activity of the proneural factor LIN-32/Achaete-Scute (Zhao and Emmons 1995; Fitch 1997). Thus, *lin-32* or its regulatory pathway are good candidate loci in which variation could cause such an evolutionary change in development and morphology and *Metarhabditis* would be a good group in which to test this hypothesis in future work.

Other *C. elegans* male tail mutants suggest candidate loci for evolutionary change. For example, in *C. elegans* and its close relatives, the first two rays are located anterior of the cloaca, ray v3 is positioned at the cloaca and the other rays are clustered in triplets posterior of the cloaca. In *C. briggsae*, ray v3 moved posterior of the cloaca and is frequently found to be fused with ray v4 (Nigon and Dougherty 1949; Friedman *et al.* 1977), as if the v3 identity were transformed partially or fully to a v4 identity (Fitch 1997; Baird 2001; Baird *et al.* 2005). This phenotype is mimicked by several *C. elegans* mutations, including mutations in HOX genes *mab-5/Antp* and *egl-5/AbdB* or in genes that regulate HOX genes (Chow and Emmons 1994; Chamberlin and Thomas 2000; Toker *et al.* 2003; Lints *et al.* 2004; Zhang *et al.* 2004; Baird *et al.* 2005). The AP position of ray v3 in the fan coincides with the border between *mab-5* and *egl-5* expression domains (Ferreira *et al.* 1999). Thus, variation in HOX genes or their regulators are likely to underlie evolutionary changes in ray pattern, a hypothesis that could be tested further, *e.g.*, by CRISPR editing experiments in future research.

Although the posterior placement of ray v3 is canonical for *C. briggsae*, there is considerable strain-specific variation with respect to the frequency of the derived *vs.* ancestral v3 positions (Baird 2001; Baird *et al.* 2005). Using recombinant inbred lines (RILs) between these different strains, it has been shown that as few as two loci—the *C. briggsae* HOX genes *mab-5* and *egl-5* or closely linked loci—are sufficient to explain the posterior v3 localization, but that at least two additional loci are involved, one of which only affects the frequency of v3+v4 fusion (Baird *et al.* 2005). A similar RIL approach revealed cryptic genetic variation, *i.e.*, transgressive variation in v3 position, likely due to epistatic interactions between different alleles at different loci from different *C. elegans* strains (Guess 2008). Such intraspecific genetic variation has the potential for leading to the types of

DIC photomicrographs at the right: tail morphogenesis, left side view. The first visual sign of morphogenesis occurs when the tail tip cells separate from the pointed L4 cuticle, round up and retract anteriorly at mid-L4. At late L4, because the tips of the rays are attached to the adult outer cuticle (beneath the L4 cuticle), the rays are formed as the rest of the body retracts and the fan folds flat around them. The fully formed adult emerges after the pointed-tailed L4 cuticle is molted off. These events do not occur in hermaphrodites/females, which retain the pointed shape of the larval tails. Bottom: left side views of adult male tails of *C. elegans* and four other species: *Pelodera strongyloides*, *Metarhabditis blumi*, *Rhabditella axei*, and *Panagrellus redivivus*. Outlines of the body and fan (if any) are depicted as gray lines, the internal left spicule (or fused left-right spicule in *P. strongyloides*) and gubernaculum are in brown, and the rays are outlined in black and labeled using the ray homolog designations. Also shown is the position of the phasmid. The tail tip cells retract to make the independently evolved peloderan tails of *C. elegans* and *P. strongyloides*, and do not retract in *R. axei* and *M. blumi* (derived, "apomorphic" leptoderan) and *P. redivivus* (ancestrally, "plesiomorphic" leptoderan) (see references cited in text).

evolutionary differences in ray pattern that we now observe between species.

Evolution of phasmid position: Several rhabditid species have been described as having ten bilateral pairs of rays, but one of these is actually the phasmid, which is positioned anterior (instead of posterior) to the posterior-most three rays (Fitch and Emmons 1995; Kiontke and Sudhaus 2000; Sudhaus and Fitch 2001). Because the phasmid tip is usually attached to the outer cuticle that makes the fan, the phasmid is drawn out like the rays, and was often mistaken as a 10th ray in original species descriptions. Evolutionary switches between "anterior" and "posterior" positions of phasmids occurred independently in at least three lineages in Clade V: in the clade including *Haematozoon* + Pleiorhabditis (*e.g.*, *Pelodera strongyloides*; Figure 6), in the lineage to *Cruznema*, and in Diplogastridae; Figure 1 and Figure 6; Fitch and Emmons 1995; Kiontke and Sudhaus 2000). These apparently "saltational" changes in phasmid positions have been hypothesized to be due to a simple developmental change: *i.e.*, a reversal in T blast cell division polarity (Fitch 1997; Kiontke and Sudhaus 2000). In *C. elegans*, the three most posterior rays derive from T.a and the phasmid sockets derive from T.p, the anterior and posterior daughters of the T blast cell, respectively (Figure 6) (Sulston and Horvitz 1977; Sulston *et al.* 1980). Reversal of the T division, as occurs in mutants of the *lin-44/Wnt* signaling pathway (Herman and Horvitz 1994; Herman *et al.* 1995), would place phasmids in the anterior position. Similar cell division polarity differences have been observed in other nematode species comparisons (Sternberg and Horvitz 1981, 1982). However, an alternative hypothesis is that the polarity of the T division has not changed and there have been subsequent migrations of ray or phasmid precursors along the AP axis. These hypotheses are currently being tested.

Male-specific tail tip morphogenesis and its evolution: In *C. elegans*, the four cells (hyp8-11) that constitute the tip of the tail in both sexes originate during embryogenesis and form their tapered, pointed shape during elongation (Hall and Altun 2008). This pointed shape is maintained throughout development in both sexes and into the adult stage of the hermaphrodite. In L4 males, however, these cells fuse, round up, and migrate inwardly and anteriorly (Nguyen *et al.* 1999). An associated sex-shared neuron is also extensively remodeled (Serrano-Saiz *et al.* 2017a). As a result of these processes, the tail tip of the adult male is rounded, or "peloderan" (Gk. "bowl" + "skin"). It is noteworthy that this is a case of sexual dimorphism at the level of homologous, sex-shared cells.

Tail tip morphogenesis has changed repeatedly during the evolution of rhabditid nematodes (Sudhaus and Fitch 2001; Kiontke and Fitch 2005). Besides peloderan species like *C. elegans* (and *M. blumi* and *P. strongyloides*; Figure 6), there are "leptoderan" (Gk. "narrow" + "skin") species in which tail tip retraction does not occur in males, and in which the tail tips (but not the rest of the tails) are thus sexually monomorphic. In leptoderan males, the pointed tail tips nearly always stick out behind the fan (*e.g.*, *Rhabditella axei*; Figure 6). Within rhabditids, species with peloderan tails have evolved from leptoderan ancestors and vice versa several times (Kiontke and Fitch 2005). Such repeated evolution provides an opportunity to explore the extent to which evolutionary trajectories are constrained by genetic architecture, "developmental constraints" or other biases in the production of morphological variation (Funk and Brooks 1990; Harvey and Pagel 1991; Brooks 1996; Kiontke *et al.* 2007; Gompel and Prud'homme 2009).

DMD-3 is a transcription factor required and sufficient for initiation of male tail tip morphogenesis, as well as the remodeling of associated neurons (Mason *et al.* 2008; Serrano-Saiz *et al.* 2017a). Mutants of *dmd-3* generate males with leptoderan tails. DMD-3 appears to be at the center of a "bow-tie" gene-regulatory network, in which it integrates temporal (the heterochronic pathway), spatial (HOX genes), sexual and other cues and coordinates downstream processes associated with cell fusion, vesicular trafficking, and regulation of cytoskeletal architecture (Nelson *et al.* 2011). Such DMRT factors have been repeatedly recruited for the production of male-specific features (Kopp 2012); whether or not DMD-3 has been recruited in the repeated evolution of tail tip sexual dimorphism is a focus of current studies.

An obvious question with regard to male tail variation is whether or not a particular feature of an organism is an adaptation crafted by natural selection. Though fundamental, this can be difficult to test. The "comparative method" tests for phylogenetic correlations between traits that indicate if one trait is dependent on another; repeated, homoplastic evolutionary events provide the power to test such correlations (Funk and Brooks 1990; Harvey and Pagel 1991; Brooks 1996). One hypothesis is that the shape of the male tail is an adaptation to mating behavior or mating position (Sudhaus 1976; Fitch 2000). For example, according to the most parsimonious reconstruction of trait evolution, ancestral rhabditids had no fan and were leptoderan. A fan then arose independently in the Pleiorhabditis and Eurhabditis clades (also in diplogastrids) and was subsequently lost or greatly reduced several times independently (Fitch 2000; Sudhaus and Fitch 2001; Sudhaus and Fürst von Lieven 2003). Males with broad fans (often but not always peloderan) tend to use their tails sort of like suction cups and mate in a "parallel" body position relative to the female, whereas leptoderan males with reduced or no fans mate in a "spiral" fashion, wrapping around the female's body (Sudhaus 1976). Consistent with selection imposing interdependency on these characters, there is a significant correlation between retaining a fan and retaining parallel mating (Fitch 2000). It is conceivable that some mating positions may be more favorable in some ecological environments than others; *e.g.*, spiral mating may provide stability in fluid environments, whereas parallel positions may be more efficient on solid substrates. Essentially nothing is known about the

natural conditions of living for most of these species—an open field for future investigations.

Although this selectionist explanation seems reasonable for overall morphological differences in male tails, other aspects of male tail variation may have alternative explanations. For example, there is considerable redundancy among different rays regarding their functions in mating behavior, probably to ensure robustness and efficiency of mating success (Liu and Sternberg 1995; Koo *et al.* 2011; Sherlekar and Lints 2014). Variations in ray position might therefore contribute little, if any, advantage to mating success, but might instead be due to pleiotropic effects of selection on AP patterning of other parts of the body. Alternatively, variation in ray position may have little to do with selection at all, and instead be due to the fixation of particular variants by genetic drift. Whatever the ultimate cause of this variation, the male tail holds great promise to uncover proximate mechanisms underlying developmental changes important for morphological evolution.

Dauer formation and phenotypic plasticity

Though *C. elegans* is famous for having invariant embryonic development, it also presents one of the best characterized examples of developmental plasticity. In response to crowding (via a pheromone), starvation (via reduction of insulin and TGF-β signaling), and/or heat stress during the second larval stage (L2), an alternative form of the L3 larva develops that is highly resistant to subsequent stresses. The dauer is crucial across nematode diversity for dispersal and survival of adverse conditions (Perry and Wharton 2011), and, in *Caenorhabditis*, differs from the reproductive L3 in many ways (Androwski *et al.* 2017). We refer readers interested in the details of dauer regulation in *C. elegans* to other reviews (Hu 2007; Fielenbach and Antebi 2008), and focus here on conservation and variation across the nematodes.

A key role of insulin signaling in the transition from dauer to postdauer reproductive development is conserved in diverse nematodes. For example, pharmacological inhibition of PI3 kinase (AGE-1 in *C. elegans*) blocks dauer exit in the hookworm *Ancyclostoma* (Brand and Hawdon 2004) and the related strongylid *Nippostrongylus* (Huang *et al.* 2010). Similarly, loss-of-function mutations in the DAF-16/FOXO homolog of *Pristionchus pacificus* block dauer entry (Ogawa *et al.* 2011). It is thus likely that a canonical insulin pathway regulated the entry and exit from the dauer, including its infective variant that enables parasitic life cycles (Crook 2014), in an ancestor to Rhabditida (Sudhaus 2010).

Beyond serving as a trait that may facilitate parasitism, dauer formation is tied to other variable phenotypes. As noted above, *Auanema* XX larvae develop into either selfing hermaphrodites or obligately outcrossing females, depending upon whether they pass through dauer or not. Because application of dafachronic acid blocks both dauer formation and hermaphrodite development (Chaudhuri *et al.* 2011), it appears that control of this sexual mode polyphenism is downstream of the same DAF-12 nuclear hormone receptor that integrates various sensory inputs to the dauer decision. Thus, a pre-existing switch mechanism has been co-opted to regulate a new trait: gonad development. A similar co-option appears to have evolved with regard to the mouth form of *Pristionchus pacificus*. This species exhibits a polyphenism, in which adults show either a narrow-mouthed (stenostomatous) bacteriovore or a wide-mouthed (eurystomatous) predator-omnivore morphology. The latter is likely to be adaptive when bacteria become limiting (Serobyan *et al.* 2014), and thus would be expected to form under dauer-inducing conditions. Indeed, starvation or application of crowding pheromones greatly increase the frequency of the eurystomatous form (Bento *et al.* 2010). Consistent with dauer signaling co-option, loss of *P. pacificus daf-12* activity via mutations or application of exogenous dafachronic acid ligands greatly reduces formation of both dauer larvae and the eurystomatous form. Interestingly, though these perturbations block dauer formation (Ogawa *et al.* 2009), they do not eliminate the eurystomatous form completely, suggesting that other factors also play a role. In line with this, forward genetic screens have identified other genes that have no obvious relationship to *C. elegans* dauer formation with stronger effects (Ragsdale *et al.* 2013; Kieninger *et al.* 2016).

Discussion

The findings above concern a wide range of developmental processes, from the earliest embryonic divisions to adult reproduction, and were produced by applying the developmental genetic tools of C. elegans to a growing list of other nematodes (Table 1). From the details of these processes and how they vary, several principles can be inferred that are likely to be general for the evolution of animal development. Some of these ideas are incorporated into the review above, but some of the most salient are the following:

An accurate, well-resolved phylogeny is essential for EDB

A phylogeny provides the framework for interspecific comparisons. For example, it is required for testing if compared genes, cells, developmental processes or traits in different species are homologous or independently evolved (nonhomologous). It allows directionality of evolutionary changes to be inferred (what was ancestral or derived), and how many times such changes occurred repeatedly. A phylogeny is required for tests of correlated evolution in different traits. Finally, a phylogeny allows informed selection of species for further research that are representative of the phylogenetic diversity. A good phylogenetic framework for nematodes, particularly for Rhabditida, and, especially, for *Caenorhabditis*, is now available, although improvements to resolution (*e.g.*, using multilocus or whole-genome data) and species representation continue.

Table 1 Status of methods for developmental genetics in various nematode species

C. elegans method	*C. briggsae*	*C. nigoni*	*C. remanei*	*C. brenneri*	*C. tropicalis*	*O. tipulae*	*P. pacificus*	Other Rhabditida
Forward mutagenesis: Spontaneous	Nigon and Dougherty (1950)							
Forward mutagenesis: Chemical	Hill *et al.* (2006)		[a]		Le *et al.* (2017)	Félix *et al.* (2000b)	Sommer and Sternberg (1996)	In *Auanema rhodensis* Tandonnet *et al.* 2018)
Forward mutagenesis: Insertional	Winter *et al.* (2007)							
Positional cloning of a novel mutation	Guo *et al.* (2009)					Besnard *et al.* (2017)	Eizinger and Sommer (1997)	
RNA interference	Kuwabara (1996)	Ting *et al.* (2014)	Haag and Kimble (2000)	Winston *et al.* (2007)[b]	Nuez and Félix (2012)		Cinkornpumin and Hong (2011)	In *Diploscapter* species Fradin *et al.* (2017)
Nontargeted gene deletion	Hill *et al.* (2006)						Gutierrez and Sommer (2007)	
Genome editing: TALENs	Wei *et al.* (2014a)	Lo *et al.* (2013)			Wei *et al.* (2014a)		Lo *et al.* (2013)	
Genome editing: Zinc finger nucleases	Wood *et al.* (2011)							
Genome editing: CRISPR			Yin *et al.* (2018)			Vargas-Velazquez *et al.* (2018)	Witte *et al.* (2015)	
Transgenesis: Injection	Félix (2007)	Nuez and Félix (2012)	Nuez and Félix (2012)	Nuez and Félix (2012)	Nuez and Félix (2012)		Schlager *et al.* (2009)	
Transgenesis: Bombardment	Zhao *et al.* (2010), Semple *et al.* (2010)		Semple *et al.* (2012)	Semple *et al.* (2012)			Namai and Sugimoto (2018)	
mRNA *in situ* hybridization	Lin *et al.* (2009)		Coroian *et al.* (2006)			Barkoulas *et al.* (2016)	Rudel *et al.* (2008)	
Immunohistochemistry	Dufourcq *et al.* (1999)		Geldziler *et al.* (2006)	Jud *et al.* (2007)		Louvet-Vallee *et al.* (2003)	Jungblut and Sommer (2000)	Several species Fitch and Emmons (1995)

Methods for perturbing or measuring gene activity in *C. elegans* (left column) have been employed successfully in a number of other free-living nematodes. The reference given is generally the first published example, but others often exist.

[a] No published studies, but *C. remanei dpy* and *unc* mutants (isolated by K.L. Chow lab at Hong Kong University of Science and Technology) are available from the *Caenorhabditis* Genetics Center (https://cgc.umn.edu).

[b] Susceptible only via gonadal injection.

Many genes, pathways, and cells are highly conserved

Though variation was emphasized here, the very ability to describe the nature of that variation hinges upon recognition of homologous genes, pathways, and cells (which hinges upon a good phylogeny). For example, we are only able to say that *gld-1* has opposite roles in hermaphrodite spermatogenesis, because unambiguous *gld-1* orthologs exist in the species compared. Similarly, conserved developmental origins provide evidence for the homology of VPC and R*n* cells and early blastomeres, supporting the many inferences of evolutionary changes made in the development of the vulva, male tail, and embryo.

Early events are surprisingly evolvable

Though the final outcome of nematode embryogenesis is predictably vermiform, there appear to be many ways to begin that all lead to this shape. Early variation appears to funnel into a more constrained "phylotypic stage," with relatively constant morphogenetic processes and gene expression. This early variation is often apparent in the zygote and first embryonic cell cycle, indicating that the evolution of development can emerge from cell-level behaviors (polarity, lineage, signaling, *etc.*). Nematodes thus represent an excellent system for evolutionary cell biology and its interface with development. Similar variation in the earliest postfertilization events is seen

in insects (El-Sherif *et al.* 2012) and vertebrates (Hasley *et al.* 2017). The same principle applies to the cellular differences underlying the evolution of sex determination, such as meiotic modifications that produce a nullo-X germ cell or an XO parthenogenic male via chromosome diminution.

Gene duplication allows partition and gain of gene functions

Gene duplication and divergence has long been recognized as an important phenomenon in evolution (Ono 1972), and nematodes provide excellent examples. Both *fog-2* and *she-1*, lineage-specific genes that allow hermaphrodite spermatogenesis discussed above, are duplicated and diverged members of the large F-box protein family. Novel features crucial to the role of *she-1* have yet to be determined, but for *fog-2* it appears that acquisition of a C-terminal GLD-1-binding domain was a key event that allowed its co-option into the sex determination pathway (Nayak *et al.* 2005). Gene duplication and divergence can also underlie DSD, as exemplified by *C. briggsae puf-2*. Though Cbr-PUF-2 shares the same RNA-binding properties as its closest paralogs, it alone is required for pharyngeal development (Liu and Haag 2014). This is true even as the pharynx itself has not changed in any obvious way.

Genetic network architecture may influence evolutionary trajectories

Certain nodes in the GRNs, such as central nodes of "bow-tie" or "hour-glass" networks (Nelson *et al.* 2011), may be optimally positioned for evolutionary changes to produce targeted effects on particular traits (Kopp 2009). Examples include the DM-domain genes that coordinate the production of many male-specific traits in nematodes, flies, and other animals (Kopp 2012). Regulators of such genes (*e.g.*, signaling or HOX patterning modules) as well as effectors (*e.g.*, cytoskeletal components and other machinery involved in cellular morphogenesis) may themselves be too pleiotropic (and thus constrained) to be effective targets for specific evolutionary change. Regulatory elements of DM-domain genes themselves, however, could conceivably be more pliable and effective agents of evolutionary change (Kopp 2012). GLD-1 may represent a similar "sweet spot" or "hot-spot," as indicated by its independent co-option in germline sex determination. As another example, the HOX specification of VPC fates appears to constrain which Pn.p cells can become vulval precursors; this constraint is overcome in posterior-vulva species by posteriad migration of the VPCs.

On the other hand, EDB research on the vulva has demonstrated that quantitative changes to the regulation of even very pleiotropic genes, such as those comprising the Wnt and Ras signaling modules, are surprisingly evolvable. There seem to be near-infinite combinations of signaling factors, targets, and quantitative variants in the strength of their interaction that can all produce a functional vulva. This has some bearing on the oft-discussed role of constraints in the evolution of development (Brakefield 2006; Vermeij 2015). Nematode EDB shows that for many traits we do not yet know how many paths within developmental network space are available to build a phenotype, and thus how many potential routes there may be to an adaptive variant. As a result, even factors that appear to constitute constraints at present may instead reflect insufficient sampling.

Pervasive DSD and its implications for research

Another major theme emerging from the above studies is the astonishing ubiquity of DSD. At the level of developmental gene regulation, rapid evolution of *cis*-regulatory sequences occurs, but often remains cryptic due to the action of stabilizing selection. Because stabilizing selection mandates an outcome, but not a mechanism, compensatory evolution [or apparently compensatory, see Haag (2007)] proceeds unchecked. This process can be accelerated by directional selection on other loci that share *trans*-regulators (Johnson and Porter 2007), and generates complex dependencies between distinct promoter regions (Ludwig *et al.* 2000). The facilitation of DSD by pleiotropy is expected at higher levels of organization; selection on one output of a pleiotropic locus is likely to also change its (cryptic) contribution to a second output not under selection. Here, we have seen such examples of DSD as the different contributions of different signaling modules to vulval induction, the conservation of core components and interactions in the sex determination pathway despite rapid evolution at protein–protein recognition domains, and transgressive variation underlying conserved ray positions in the male tail. Other studies have provided further evidence of DSD from genome-scale comparisons of gene function. For example, systematic RNAi knockdown of essential genes in wild isolates of *C. elegans* (Paaby *et al.* 2015), and of their *C. briggsae* orthologs (Verster *et al.* 2014) revealed many instances of distinct phenotypes in both cases that could not be explained by knockdown efficacy. Cryptic genetic changes underlying canalized developmental processes are thus apparently rampant.

In hindsight, the choice of species for initial characterization of a developmental process has a large influence on the path of research. For example, selection of *P. pacificus* for the first genetic analyses of vulva development would have led to focus on the Wnt pathway rather than Notch and EGF, while choosing *Diploscapter* for the oocyte-to-embryo transition would have led to a complete different picture of cellular interactions and embryo polarization.

Microevolution is reflected in macroevolution

Cryptic genetic variation in the development of a conserved phenotypic output abounds in nematodes, and variation between species is mirrored by variation within them. This is true both qualitatively and quantitatively, providing strong support for the existence of biases in the introduction of variation (mutation and its impact on networks), and their influence on interspecies divergence. The interaction between these biases and natural selection offer a more complete view of evolutionary causation (Stoltzfus 2006). Intraspecific variation

also suggests that using a "representative strain" may sometimes lead to a false sense of species divergence. This is particularly true concerning the laboratory strain of *C. elegans*, which has adapted to laboratory conditions and fixed mutations that improve fitness but indirectly affect developmental processes (McGrath *et al.* 2009, 2011; Duveau and Félix 2012; Andersen *et al.* 2014).

Nematodes like *Caenorhabditis* offer the ability to connect specific developmental processes with the general phenomenon of cryptic variation. Beyond being interesting, such connections are fundamental to understanding the genetic architecture of non-Mendelian disease, the response to selection in agriculture, the resistance of pathogens and cancer cells to drugs, and the mechanisms that underlie cellular and organismal homeostasis (Gibson and Dworkin 2004). For example, susceptibility to the topoisomerase-targeting chemotherapy agent etoposide varies among *C. elegans* isolates, and much of this can be explained by a single amino acid polymorphism that appears to be neutral in the absence of the drug (Zdraljevic *et al.* 2017). Amazingly, the same polymorphism distinguishes topoisomerase paralogs in humans as well.

Repeated evolution involves both reproducible co-options and idiosyncratic components

As pointed out by others (Kopp 2009), repeated evolution (phylogenetic replication) in a clade of experimentally tractable species (a "metamodel") provides the opportunity to look for more general principles in the evolution of developmental-genetic systems. The research summarized above abundantly demonstrates the utility of nematodes in such a research program. For example, the convergent evolution of self-fertility has shown how specific conserved genes (*e.g.*, *tra-2* and *gld-1*) or the same gene classes (*e.g.*, those encoding F-box proteins like *fog-2* and *she-1*) are repeatedly co-opted (parallel evolution). Another example is the involvement of *spe-8* in the activation of hermaphrodite sperm in two independent lineages of *Caenorhabditis*. At the same time, repeated evolution can involve idiosyncratic solutions (convergent evolution). Sticking with self-fertility, we see that the sperm activation program deployed in hermaphrodites can vary, as does the precise role of GLD-1 in germline sex determination. Evolutionary biases may also be revealed; *e.g.*, once programmed cell death evolves as the mechanism to restrict the VPC group, reversal is rare. Given the advantages of nematodes for EDB, we can look forward to many more discoveries.

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