Comparisons of Sex Determining Pathways Across *Caenorhabditis* Species

by

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Abstract

Biological pathways have the ability to tolerate mutations and evolve to control novel traits. Selection pressures act on these pathways playing a key role in the evolution and divergence of species. Sex determination in *Caenorhabditis* nematodes is a rapidly evolving trait which can provide insights into how biological pathways can be modified from a common set of ancestral genes. *C. elegans* and *C. briggsae* are two of three androdioecious species within the Elegans group of the *Caenorhabditis* genus. These two species are both morphologically and developmentally similar but their genetic control of hermaphroditism is different. XX animals in these species are somatically female but are capable of producing and storing sperm before switching to oocyte production. Genetic screens have been used to isolate mutants which disrupt the *C. briggsae* sex determining pathway, the results of which have identified orthologs of the *C. elegans* sex determining genes. Regulation of spermatogenesis differs in *C. elegans* and *C. briggsae; C. elegans fem* mutants are females whereas *C. briggsae fem* mutants are hermaphrodites. Identification of the molecular lesion in the *fem* mutants isolated from these screens have been useful for determining the functional domains of these important proteins. In the same genetic screen that identified the *Cbr-fem* mutants, three mutants were isolated which appeared to be novel members of the *C. briggsae* sex determining pathway. These mutants display phenotypes not seen in *C. elegans*. Whole genome sequencing followed by mapping has revealed that one of these appears to be a gain of function *tra-1* allele, one of them appears to be a weak hypomorph of *fem-3* and the third one does not contain a mutation in any known sex determining gene. The current candidate for this mutant is the kinase, *pink-1*. *C. briggsae* has a closely related sister species which uses a male/female sex determining system. Comparisons between sex determination gene orthologs in these two species show greater than ninety percent identity at the amino acid level. Sex determining genes that were known to be present in *C. briggsae* but not in *C. elegans* were also found in *C. nigoni* so their presence alone is not enough to facilitate hermaphroditism in *C. briggsae.*

Dedication

For Beth

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List of Abbreviations

ANK……...……...Ankryin repeat domain
BLAST…….……...…….Basic Local Alignment Search Tool
Cbr……...*C. briggsae*
Cby……..…….Chubby phenotype
Ce……...……...*C. elegans*
Cni……...*C. nigoni*
CPEB……..……Cytoplasmic Polyadenylation Element Binding protein
Cre…..……..*C. remanei*
CRISPR…….....Clustered Regularly Interspersed Short Palindromic Repeats
CTD……..…….C-Terminal Domain
cM……centi Morgan
DIC…….…….……Differential Interference Microscopy
DM……..……DSX and MAB-3
EMS…..……..…….Ethyl methyl sulfonate
ENU ……...……N-nitroso-N-ethylurea
ESD……..……...……Environmental Sex Determination
EtOH…….……Ethanol
F1……..……..Filial 1
F2……..……..Filial 2
F3……..……..Filial 3
Fem……..……Feminized phenotype
Fog……...……..……Feminization Of Germline phenotype
g……..……..grams
gf……...…...……Gain of Function
GSD……...……..……..Genetic Sex Determination
Her……..……..Hermaphrodite phenotype
Hox……..……...Homeotic
hr……..……..hour
Hs……..……*H. sapiens*
ic…….……..…….....intracellular
indel……..……insertion-deletion
kbp...……...……..……kilo base pair
L……...……...Litre
L3……...……...……..Larval stage 3
L4……...……Larval stage 4
m…….……milli
M…….…….Molar
mins…….……..minutes
mog……..……...……Masculinization Of Germline
n…….……nano
NGM…….……...……Nematode Growth Media
NP-40……..……..Tergitol-type nonyl phenoxypolyethoxylethanol
NTD…….……..N-Terminal Domain

PCR……Polymerase Chain Reaction
PME……Point Mutation Element
PP2C……Protein Phosphatase type 2C
RAM……..RAy Morphology
RPM……Revolutions Per Minute
SD……Sex Determination
SDC……Sex Determining Complex
SDS……...Sex Determining Systems
SAP……..Simple Allele-discriminating PCR
SMART……...Simple Modular Architecture Research Tool
SNP……...Single Nucleotide Polymorphism
snRNP……small nuclear RiboNucleic Particle
SRS……Substrate Recognition Subunit
Sup……...Suppressor
THP……...Tamm-Horsfall Protein
TM……Trans Membrane loop
Tra……Transformer phenotype
ts……...temperature sensitive
UBL……Ubiquitin Like protein
UTR……UnTranslated Region
VHL……..Von Hippel-Lindau motif
Wnt……int/Wingless protein family
°C……..degree Celsius
µ……micro

1.0 Introduction

1.1 Conservation of biological processes

The diversity of the shape, size, colour, and body plans of metazoans is staggering. Some of these traits have been selected for over millennia. Through generations they have been subjected to selective pressures, becoming more refined to give individuals an advantage or allowing them to carve out a niche in their environment. Novel traits can evolve in this way through small changes in ancestral genes allowing them to be expressed in different tissues at different times or with different proteins. Ancestral genes can be co-opted to perform novel roles leading to the evolution of new traits. Alternatively traits can arise rapidly *de novo*, heralded by large scale genomic changes such as duplication events.

The identification of the *Drosophila* Hox genes in segment pattern formation (Lewis, 1978) and the subsequent discovery of their conservation in mammalian anterior-posterior patterning (Gaunt *et al.*1988; Duboule and Dolle, 1989; Graham *et al.,* 1989; Akam, 1989) led to the revelation that many basic animal developmental processes are conserved and vastly different organisms are, at a basal level, very similar to each other. Since the advent of next generation sequencing technology we have had access to a wider variety and greater number of annotated genomes than ever. Comparisons between multicellular organism genomes have revealed that gene number is not proportional to organismal complexity. Humans are considered one of the most complex organisms on the planet however the estimated number of human genes is ~30,000 (International Human Genome Sequencing Consortium, 2001) which is not much larger than estimates for *Caenorhabditis* (~20,000) (*C. elegans* Sequencing Consortium, 1998), *Drosophila* (~13,000) (Adams *et al.,* 2000) and *Arabidopsis* (~25,000) (*Arabadopsis* Genome Initiative, 2000). While vertebrates do have some unique gene families that plant, fungal and invertebrate genomes lack (e.g. MHC gene family) (Brown, 1999), the evolution of novel traits can also be attributed to novel regulation of ancestral genes.

An important goal in evolutionary developmental biology is to understand how development is regulated and can be modified to enable a novel trait. While studying how a trait or feature develops in an organism is in itself fascinating, the genetic changes which have occurred to allow them to exist at all are remarkable and can provide insight into how evolution occurs at a molecular level. If all animals share a common ancestor they must have all started with the same set of ancestral gene functions like cellular signaling pathways and transcriptional regulators. The plethora of diversity seen in modern animals is due in part to *de novo* appearance of novel genes but also through gradual modification of ancestral pathways in countless ways (Carroll *et al*., 2001). The common ancestral origin of a biological pathway makes it possible to study processes in model organisms and extrapolate and apply findings to a wider range of distantly related organisms, including humans. The Wnt signaling pathway is an excellent example of this and has been extensively studied in *Drosophila*. However not all biological processes are so well conserved. Sex determination is an excellent example of a critically important process but it is not well conserved and is rapidly evolving.

1.2 Sex determining systems are diverse

Sex is a widely used form of reproduction which is almost universally used by eukaryotes (Bell, 1982). Sexual reproduction is achieved by a variety of mechanisms leading to a vast array of sex determining systems (SDS). One of the most fundamental features of sexual reproduction is the use of female and male gametes of different sizes (ansiogamy). This in turn often, but not always, leads to two different sexes with each one exclusively producing one type of gamete (Bull, 1983). This production of a specific type of gamete, whether it be relatively few large ova or many small sperm, is how we define the sex of an organism. Even though development as a male or female is a highly conserved result of SDS the developmental pathways which leads up to sexual fate specification are remarkably diverse.

In organisms that have evolved separate sexes, correct development into fertile males and females is paramount for sexual reproduction and is under strong selection pressures. Despite these evolutionary forces, or perhaps because of them, diverse mechanisms are used to determine sex (Bell, 1982). Broadly speaking, SDS can be divided into two categories; environmental sex determination (ESD) and genetic sex determination (GSD). ESD encompasses many methods for sex determination (SD). All crocodiles, most turtles (Bull, 1980) and some fish (Ospina-Álvarez and Piferrer, 2008) use temperature to specify sexual fate. Marine worms in the genus *Bonellia* become males only if they encounter a female (Berec *et al*., 2005). Many arthropod species can be infected by *Wolbachia*, a bacteria that overrides existing SDS and forces a shift towards female development as this provides a reproductive advantage for the bacteria (Hurst *et al*., 1999). Many animal and plant species can also shift sex part way through their lives in response to environmental cues (reviewed in Valenzula and Lance, 2004).

GSD occurs when genetic elements determine whether an individual develops as a male or female. The most well-known example of this are the XY chromosomes in humans. XX individuals develop as females whereas XY individuals develop as males. Perhaps less well known is that this paradigm of male heterogamy is used by almost all mammals and beetles, many flies and some fish. Female heterogamy (ZW chromosomes) is common in birds, snakes, butterflies and some fish. GSD is often not as simple has having a single master regulator SD locus but can be a polygenic trait. Zebrafish, a popular model organism, determine sex over multiple genomic regions where a quantitative threshold controls male or female development (Liew *et al.*, 2012). In many animals SD requires the entire genome. Haplodiploidy is used by all ant, wasp and bee species (approximately 12% of all animal species). Males develop from unfertilized haploid eggs while females develop from fertilized diploid eggs.

Of course, like most processes in biology, many species do not fall into one of these discreet categories of either GSD or ESD. An example of this is the

snow skink, which has a different SDS depending on its environment. At low altitudes the variance in temperature is low, resulting in earlier births which is an advantage for females. At higher altitudes there is more variance in temperature and the selective advantage for females no longer exists. In this instance the skinks revert to a GSD to avoid unfavorable skews in a population's sex ratio.

In contrast to the myriad of different primary sex signals, key types of regulatory genes appear to be consistently involved in SD. The double sex-mab-3 (DM) family of transcription factors are specifically expressed in the gonads of invertebrates (*Drosophila*, *Caenorhabditis,* crustaceans and mollusks) and vertebrates (mammals, birds, reptiles and fish) (reviewed in Bachtrog *et al*., 2014). This is consistent with the bottom up hypothesis of SD pathway evolution (Wilkins, 1995). This hypothesis predicts that the most downstream members of a pathway are the most ancient, and therefore the most conserved, and that additional steps of regulation are built up over time until the gene switch that controls sexual fate becomes fixed, leading to the evolution of sex chromosomes. This is one explanation as to how primary sex signals can be so diverse, but further downstream members of SD pathways appear more closely related.

To understand the diversity seen in sex determining systems, it is necessary to compare the differences between closely related species. Looking at closely related species allows us to examine small scale molecular changes which lead to large changes in sex determination before they diverge too much and these differences become obscured (Haag and Doty, 2005). *Caenorhabditis* nematodes provide an excellent system for studying convergent evolution of sex determining pathways. *C. elegans* and *C. briggsae* share a gonochoristic (male/female) common ancestor approximately 80-100 million years ago (Kiontke *et al.,* 2004). Since these two species diverged from their common ancestor they have both evolved hermaphroditism independently from each other (Hill *et al*., 2006). These species started with the same conserved set of sex determining genes and both of them evolved hermaphroditism. Because the molecular mechanism controlling

this hermaphroditic trait is different in these species, it makes them the ideal model for studying how this novel trait evolved.

1.3 Sex determination in *Caenorhabditis*

Caenorhabditis is a genus of free-living nematodes which can be found naturally in soil and rotting fruit, feeding on bacteria. Its life cycle is comprises an embryonic stage, four larval stages (l-4) and an adult stage with a generation time of about 3 days (Byerly *et al.,* 1976). At the end of their second larval stage animals can enter a developmentally static period called dauer. This typically occurs if the environment is unsuitable for further growth e.g. lack of food, high population density or high temperature (Wood, 1988). A dauer larva can survive for around four months after which time it can reenter its developmental cycle as an L4 worm.

Within the Elegans group of Rhabiditidae, *C. elegans* and *C. briggsae* are two of the three known species that have an androdiocious (hermaphrodite/male) sex determining system whereas all the other species have a gonochoristic (female/male) sex determining system, (Kiontke *et al*., 2004; Felix *et al*., 2014) (figure 1). While X-chromosome dosage determines cell somatic fate (Nigon, 1951; Madl and Herman, 1979), the temporal control of germline fate provides a female worm with a limited number of sperm cells used for self-fertilization in androdiocious species. In the laboratory, populations of these species consist mainly of hermaphrodite (XX) worms while males (XO) are generally rare. The unusual protandrous (production of male gametes followed by production of female gametes in an individual) mating strategy of *C. elegans* and *C. briggsae* is possible due to the presence of an ovo-testis in XX worms that produces both types of gametes during non-overlapping developmental stages. The hermaphrodite germline first differentiates into around 300 sperm cells during the L4 larval period before irreversibly switching to oocyte production in the adult (Wood, 1988). Because male structures needed for mating do not develop in a

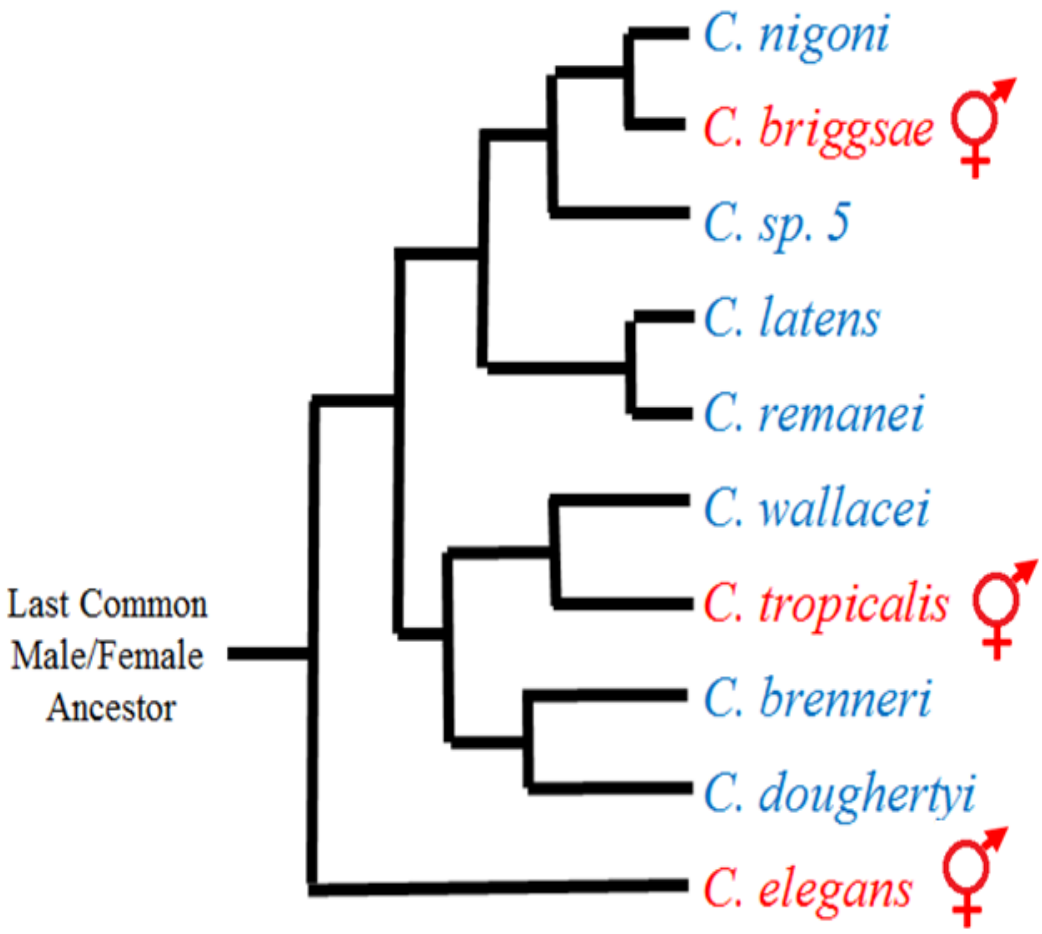


Figure 1. Phylogeny of the Elegans group of the *Caenorhabditis* genus. Androdiocious species are shown in red, gonochoristic species are shown in blue. These species diverged from a common ancestor approximately 80-100 million years ago (MYA). This common ancestor most likely used a male/female sex determining system (Felix *et al.,* 2014).

female soma, cross fertilization among hermaphrodites cannot occur. Furthermore, the commitment of the hermaphrodite germline to produce both gametes comes at the expense of lower fertility and self-progeny invariably results in lower brood sizes if compared to cross progeny (Hodgkin and Barnes, 1991).

1.4 *C. elegans* somatic sex determining pathway

C. elegans was first isolated and described in 1900 by Emile Maupas (Maupas, 1900). Its potential as a genetic model organism was first recognized in 1948 by Dougherty, who noted its simple structure, for a multicellular organism, and its invariant cell lineage (Dougherty and Calhoun, 1948). However, it was not until 1963 that Sydney Brenner proposed to use *C. elegans* as a genetic model for investigating development, particularly development of the nervous system. In 1974 Brenner published a paper describing the isolation, characterization and mapping of approximately three hundred EMS-induced mutants (Brenner, 1974). One of the first aspects of *C. elegans* biology to be systematically studied with genetic analysis was sex determination (Hodgkin and Brenner, 1977). It had been known for several decades that *C. elegans* used an XX/XO sex chromosome system (Nigon, 1949) but it was not till 1979 that it was confirmed that the ratio of X chromosomes: autosomes (X:A) determined sex rather than the total number of X chromosomes (Madl and Herman, 1979).

The sex determining pathway is a series of inhibitory interactions between male- and female-promoting genes and has been well studied in *C. elegans* (figure 2). The initial signal for *C. elegans* sex determination is the X: A ratio. XX animals develop as hermaphrodites while XO animals develop as males (figure 3) (Madl and Herman, 1979). Ce-XOL-1 (*XO lethal*) is a kinase whose activity is controlled by the X: A ratio and controls both sex determination and dosage compensation. High levels of Ce-XOL-1 promote male development while low

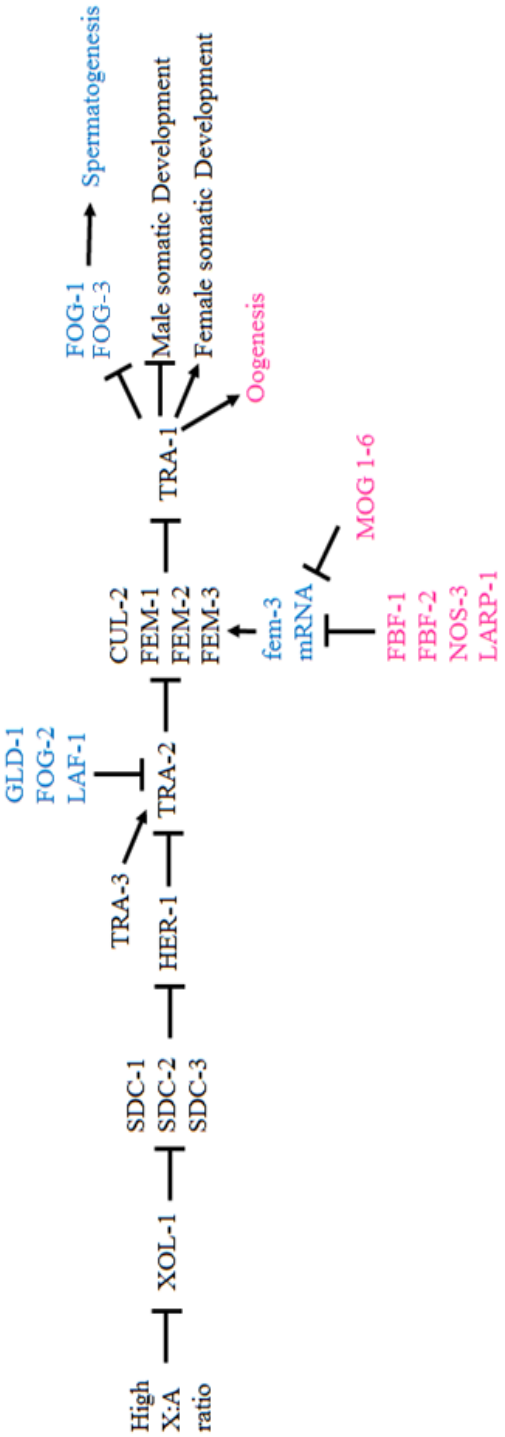


Figure 2. Schematic of the *C. elegans* sex determining pathway. The global pathway is shown in black. The germline specific pathway for control of spermatogenesis is shown in blue and the germline control of oogenesis is shown in pink (Dewar, 2011).

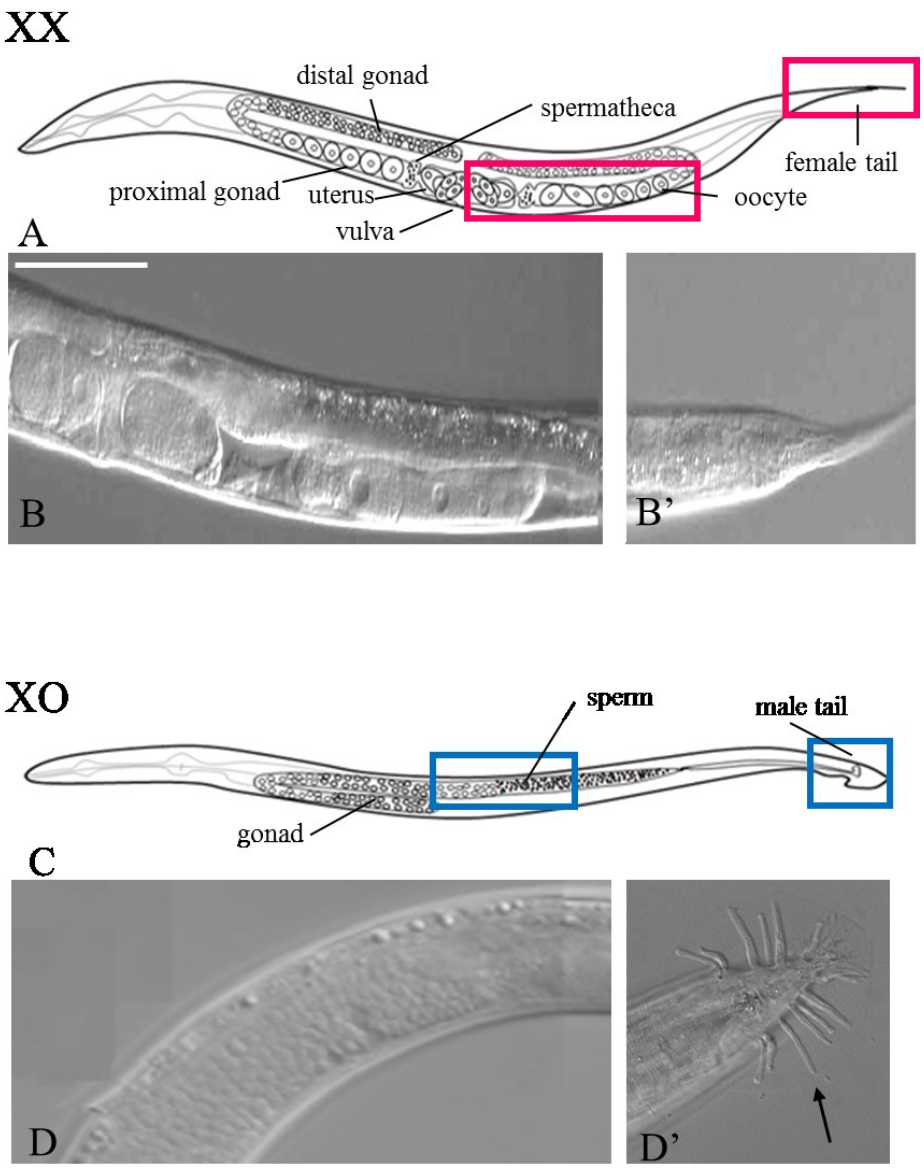


Figure 3. Anatomy of adult hermaphrodite (XX) and male (XO) *Caenorhabditis*. Hermaphrodites are typically larger than males. They have a double armed somatic gonad which is the site of sperm and oocyte production (B). The long slender tail is indicative of a female soma. In the distal gonad, germ cell nuclei are present in a syncytium and as they move toward the proximal gonad individual nuclei become cellularized. Scale bar = 50µm. Fully formed oocytes pass through the spermatheca, an organ for storing sperm. Stored sperm can be the hermaphrodites own sperm or can be sperm from a mating event with a male. As the oocytes pass through the spermatheca they are fertilized and are then passed into the uterus where they undergo the first few cellular divisions before being laid. Males (C) have a single armed gonad which exclusively produces sperm (D). They have a characteristic blunt ended tail (D') that has tail rays projecting from it (arrow) used for finding a mate and copulation.

levels promote female development (Miller *et al.,* 1988; Rhind *et al*., 1995). Mutations in *Ce-xol-1* result in feminization of XO animals and cause their deaths due to disruption of dosage compensation. Immediately downstream of *Ce-xol-1* are the female promoting factors *Ce-sdc-1*, *Ce-sdc-2* and *Ce-sdc-3 (*sex determination and dosage compensation defective). These factors work together as an SDC complex that binds to the X-chromosome and regulates dosage compensation. Ce-XOL-1 propagates its male specifying signal via negative regulation of *Ce-sdc-2* but exactly how Ce-XOL-1 is doing this is unknown. The SDC complex is thought to promote the female developmental signal by blocking transcription of *Ce-her-1*.

Ce-HER-1 is a secreted signaling molecule derived from male-specific transcription. It acts cell non-autonomously to promote a male fate by binding and inhibiting Ce-TRA-2 activity (Perry *et al.*, 1993; Hamoka *et al.,* 2004). Ce-HER-1 is dispensable in XX animals but XO *Ce-her-1* mutants are feminized while retaining the ability to produce sperm; they are hermaphrodites. Ce-TRA-2 is a transmembrane receptor with two conformational states. When Ce-HER-1 is bound to Ce-TRA-2, its intracellular domain cannot interact with the Ce-FEM/CUL-2 complex (figure 4A), however when unbound, Ce-TRA-2 is capable of repressing the function of Ce-FEM/CUL-2 by binding to Ce-FEM-3 (figure 4B) (Mehra *et al.*, 1999) Ce-TRA-2's repression of Ce-FEM/CUL-2 is enhanced by Ce-TRA-3, a calpain protease. When there is no Ce-HER-1 bound to Ce-TRA-2, Ce-TRA-3 can cleave the intracellular (ic) domain from Ce-TRA-2, which is necessary for Ce-FEM/CUL-2 repression (Barnes and Hodgkin, 1996; Sokol and Kuwabara, 2000). The intracellular domain of Ce-TRA-2 can also be transcribed and translated without the transmembrane loop domains.

The proteins Ce-FEM-1, Ce-FEM-2 and Ce-FEM-3 along with Ce-CUL-2 compose a complex that is necessary for male somatic development (Doniach and Hodgkin, 1984; Rosenquist and Kimble, 1988; Chin-Sang and Spence, 1996; Starostina *et al.,* 2007). Ce-FEM-1's function is not entirely known; however it contains ankyrin repeat regions (Li *et al*., 2006) which are common motifs in

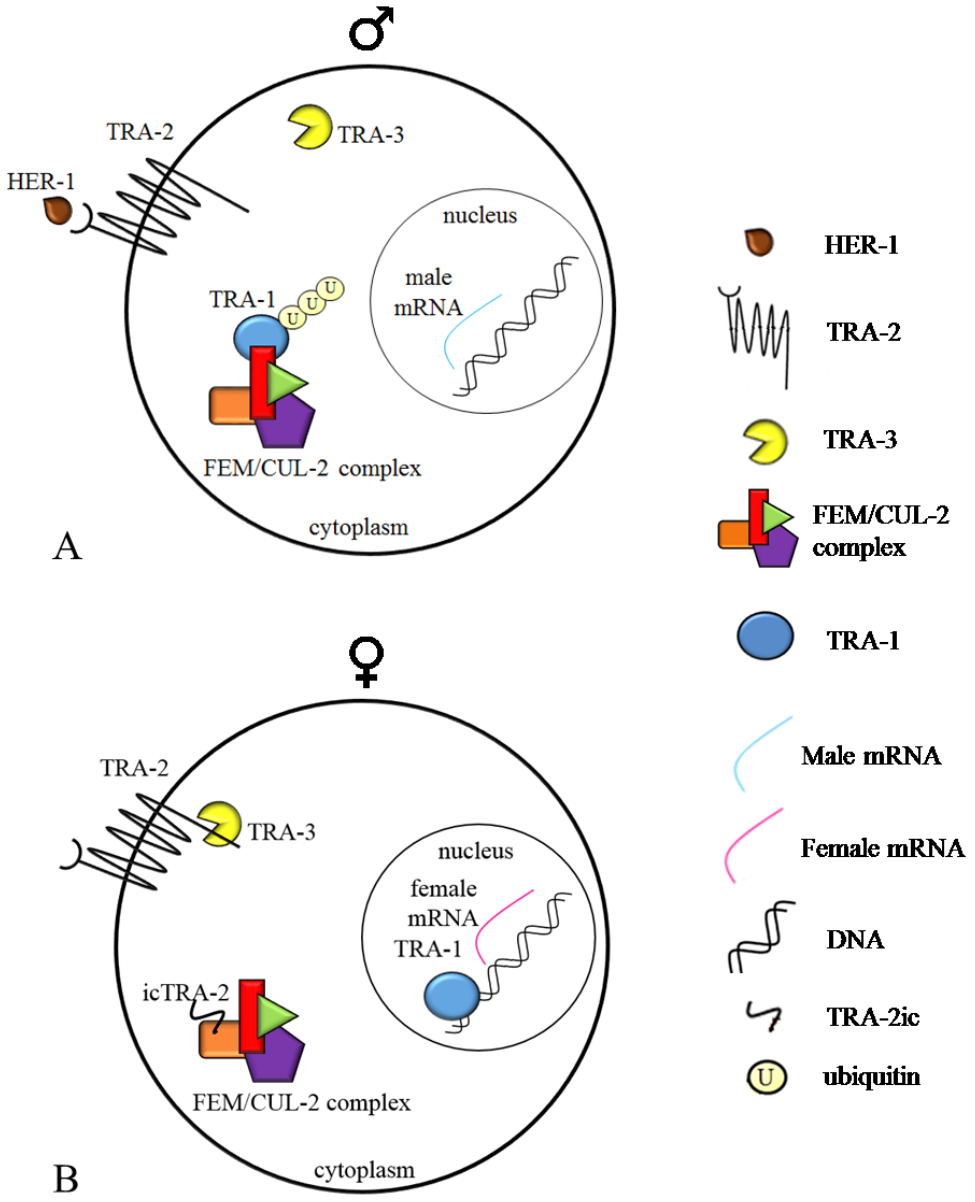


Figure 4. Molecular mechanism of male (A) and female (B) development in *C. elegans* somatic cells. In male cells HER-1 is bound to TRA-2 which stops TRA-3 being able to cleave the TRA-2 intracellular domain. The FEM/CUL-2 complex is then able to bind to TRA-1 via FEM-1 and tag it with ubiquitin for degradation. The absence of TRA-1 allows male specific genes to be expressed while female specific genes are not turned on. In female cells there is no HER-1 to bind to TRA-2. This allows TRA-3 to cleave the intracellular domain of TRA-2. TRA-2ic then inhibits the FEM/CUL-2 complex via direct interaction with FEM-3. This inhibition allows TRA-1 to localize to the nucleus where it actively represses male promoting genes and induces female promoting genes.

protein-protein interactions. Ce-FEM-1 has been shown to bind directly to Ce-CUL-2, an ubiquitin ligase, and serves as the Ce-FEM/CUL-2 complex binding domain for Ce-TRA-1 (Starostina *et al.,* 2007). Ce-FEM-2 is a member of the PP2C serine/threonine phosphatase family (Pilgrim *et al.,* 1995; Chin-Sang and Spence, 1996); however its substrates and corresponding kinases have not been identified. Ce-FEM-3 contains no conserved motifs (Arhinger *et al.,* 1992). Ce-FEM-3 has been shown to bind the cytoplasmic domain of Ce-TRA-2 *in vivo,* suggesting that Ce-FEM-3 acts as the regulatory subunit for the Ce-FEM/CUL-2 complex that Ce-TRA-2 acts upon. The Ce-FEM/CUL-2 complex acts to bind to Ce-TRA-1 and sequester it in the cytoplasm, where it is unable to regulate transcription (Hodgkin and Brenner, 1977; Hodgkin, 1987; Hunter and Wood, 1990; Starostina *et al.,* 2007).

Ce-tra-1 encodes a transcription factor that acts as the terminus for the sex determining pathway (Hodgkin and Brenner, 1977; Hodgkin 1987; Schedl *et al.*, 1989). It functions to actively promote female development and repress male development in both the germline and the soma (figure 4). Ce-TRA-1 is negatively regulated by the Ce-FEM/CUL-2 complex and specifically interacts with Ce-FEM-1 (Starostina *et al*., 2007). Ce-TRA-1 contains an N-terminal region, known as the gain-of-function (gf) region, to which Ce-FEM-1 binds (de Bono *et al*., 1994; de Bono and Hodgkin, 1996). Mutations in the gf region result in a loss of inhibition due to Ce-FEM-1's inability to bind and leads to feminization of XX and XO animals. Ce-TRA-1 also contains a C-terminal domain (CTD), to which the intracellular domain of Ce-TRA-2 can bind to (Wang and Kimble, 2001). This interaction promotes Ce-TRA-1 activity by localizing Ce-TRA-1 to the nucleus, allowing it to carry out its transcription factor duties. This interaction occurs specifically in the germline, as small levels of Ce-TRA-1 are necessary for sperm production. *Ce-tra-1* can also be transcribed and translated as a peptide that contains the Ce-FEM-1 binding region but lacks the ability to function as a transcription factor, called Ce-TRA-1B. Ce-TRA-1A and Ce-TRA-1B compete to bind to the Ce-FEM/CUL-2 complex, which results in some Ce-TRA-1A reaching the nucleus to carry out its functions.

1.5 *C. elegans* somatic sex determining pathway mutants

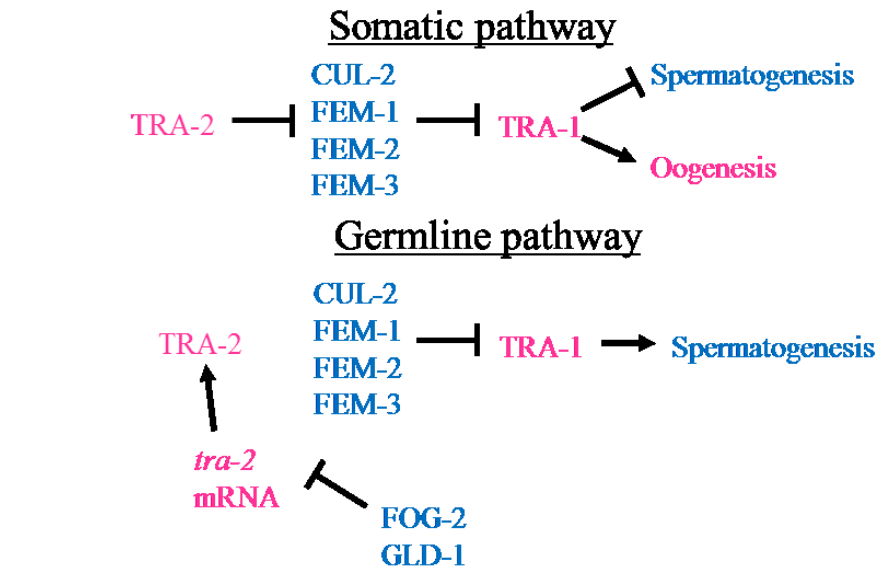
The systematic characterization of the *C. elegans* sex determining pathway began with a characterization of SD mutants using mutagenesis screens. Broadly speaking genes in the SD pathway can be divided into two categories, those which promote a male fate and those which promote a female fate. When female promoting genes are mutated in worms they exhibit a transformer (Tra) phenotype (Hodgkin and Brenner, 1977). Worms with a Tra phenotype have a masculinized germline and soma; they develop a single armed gonad which can only produce sperm and a male tail. However, they are not completely masculinized as they show abnormalities in tail ray development (Hodgkin and Brenner, 1977). Mutations in *Ce-tra-1*, *Ce-tra-2* and *Ce-tra-3* result in a Tra phenotype (Hodgkin and Brenner, 1977). Mutations in male promoting genes result in worms with a feminization (Fem) phenotype (Hodgkin, 1986). Worms with a Fem phenotype have a feminized germline and soma. XX and XO *fem* mutants look identical; they have long narrow tails develop double armed gonads, and exclusively produce oocytes. Mutations in *Ce-fem-1*, *Ce-fem-2* and *Ce-fem-3* result in a Fem phenotype (Hodgkin, 1986). These *fem* mutants are fertile and are capable of successful outcrosses with males. Due to the series of inhibitory interactions which make up the pathway, suppressor analysis has been instrumental in determining the epistatic relationship between members and in establishing each members place in the pathway.

1.6 *C. elegans* germline sex determining pathway

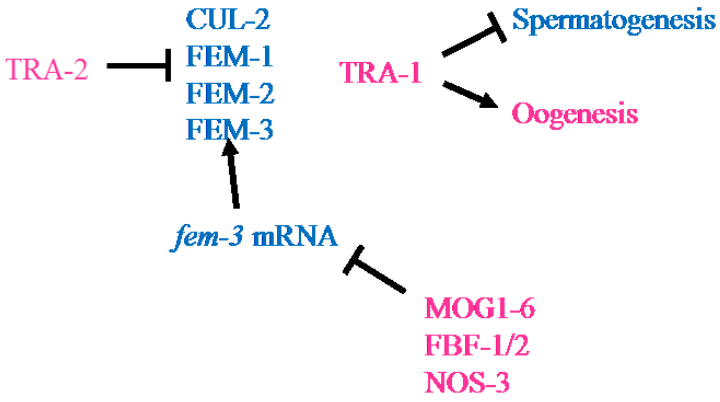
In *C. elegans* hermaphrodite sperm production is facilitated by post-transcriptional down regulation of Ce-TRA-2 activity in the germline of L4 worms (Kuwabara *et al*., 1998). Development of male gametes in a female gonad means that germ cells must specify a male fate cell autonomously without Ce-HER-1 inhibiting Ce-TRA-2. A germ cell's decision to become a sperm or oocyte is directly related to the balance between Ce-TRA-2 activity (promotes oocyte

development) and Ce-FEM-3 activity (promotes sperm development) (Doniach, 1986; Kuwabara and Perry, 2001). In *C. elegans* the Ce-GLD-1/FOG-2 complex targets the 3'UTR of *Ce-tra-2* mRNA, blocking its translation and removing Ce-TRA-2 activity in the cell (Goodwin *et al*., 1993; Goodwin *et al*., 1997). This allows for a transient up-regulation of Ce-FEM activity to repress Ce-TRA-1 (Francis *et al.,* 1995a; Francis *et al*., 1995b) (figure 5). Removal of Ce-TRA-1 inhibition on *Ce-fog-1* and *Ce-fog-3* results in sperm production. Ce-LAF-1 is also involved in translational repression of *Ce-tra-2* during male fate specification (Jan *et al*., 1999). The Ce-LAF-1 and Ce-GLD-1/FOG-2 mechanisms are independent and necessary for spermatogenesis. Ce-FOG-1 and Ce-FOG-3 are the terminal members of the male germline sex determining pathway (Barton and Kimble, 1990; Ellis and Kimble, 1995; Chen and Ellis, 2000). They play an important role in the initiation and maintenance of spermatogenesis. Ce-FOG-1 is a member of the CPEB (cytoplasmic polyadenylation element binding) protein family and is necessary for the decision of a germ cell to become a sperm rather than an oocyte (Barton and Kimble, 1990; Lamout and Kimble, 2007). Ce-FOG-3 is a member of the Tob family of proteins (Chen e*t al.,* 2000) which functions to promote the commitment of germ cells to meiosis (Ellis and Kimble, 1995).

As larval development finishes the hermaphrodite must end its sperm production and switch to oocyte production for the rest of its life. This is facilitated through the down-regulation of *Ce-fem-3* mRNA and disassembly of the *Ce-tra-2* inhibitory complexes (figure 5) (Ahringer and Kimble, 1991). *C. elegans* Ce-FBF-1, Ce-FBF-2 and Ce-NOS-3 are homologues of the *Drosophila* RNA-binding Pumilio and Nanos proteins, respectively. They regulate *Ce-fem-3* translation by binding to its 3' UTR PME element (Kraemer *et al*., 1999). Further *Ce-fem-3* regulation is provided by Ce-CBP-1, a homologue of *Xenopus* CPEB protein (Jin *et al*., 2001) and six Ce-MOG proteins to promote oocyte development. *Ce-fem-3* regulation by the proteins occurs at the post-transcriptional level. Three of the MOGs (Ce-MOG-1, Ce-MOG-4 and Ce-MOG-5) are DEAH-box proteins that function as RNA helicases (Puoti *et al*., 1999; Sanjuan and Marin, 2001). *Ce-mog-2* encodes a snRNP (small nuclear ribonucleic



A. Switching on spermatogenesis



B. Switching off spermatogenesis and switching on oogenesis

Figure 5. Interactions that facilitate sperm production in the *C. elegans* hermaphrodite germline. To switch on spermatogenesis female promoting genes need to be transiently down-regulated. This is achieved by male-promoting factors inhibiting the translation of *tra-2* mRNA thereby relieving the inhibition on the FEM/CUL-2 complex. After the hermaphrodite becomes an adult it turns off spermatogenesis and switches exclusively to oogenesis for the remainder of its life. This is done by female-promoting factors down regulating FEM activity by targeting *fem-3* mRNA and preventing it from being translated. This relieves the FEM/CUL-2 inhibition on TRA-1 which re-establishes the female promoting pathway.

particle) that is required for *Ce-fem-3* inhibition via its 3' UTR (Graham *et al.,* 1993; Gallegos *et al.,* 1998; Zanetti *et al.,* 2011). *Ce-mog-3* and *Ce-mog-6* encode conserved nuclear proteins which are also required to repress *Ce-fem-3* translation (Katsuri *et al.,* 2010; Belfiore *et al.*, 2004). Further control of the switch from spermatogenesis to oogenesis is provided by Ce-FBF-1 and Ce-FBF-2 negatively regulating *Ce-fog-1* at the post-transcriptional level (Thompson *et al.,* 2005).

1.7 *C. elegans* germline sex determining pathway mutants

Mutations in the germline-specific members of the SD pathway result in characteristic phenotypes which specifically skew the outcome of gametogenesis to exclusive production of either sperm or oocytes. A Fog (feminization of germline) phenotype is seen when XX and XO animals remain somatically normal but only produce oocytes (Schedl and Kimble, 1988; Barton and Kimble, 1990; Ellis and Kimble, 1995). Mutations in any of the three *Ce-fog* genes is sufficient to cause a Fog phenotype. A Mog (masculinization of germline) phenotype is seen in XX animals which exclusively produce sperm in their double armed gonad (Graham and Kimble, 1993; Graham *et al.,* 1993). Mutations in any of the *Ce-mog* genes can cause a Mog phenotype.

1.8 Conservation of the *Caenorhabditis* somatic sex determining pathway

Comparisons between the SD systems of distantly related species have not yielded many insights into how SD pathways evolve. For example most genes involved in *Drosophila* and *C. elegans* SD appear to be species-specific (Raymond *et al*., 1998; Raymond *et al.,* 2000). Due to the rapidly evolving nature of SD pathways any differences which may have been informative in interphyla comparisons of two species may have been obscured by the overall divergence of the two species. However the rapid evolution of SD pathways means that comparisons between

two closely related species are likely to show informative differences in pathway modifications that have led to hermaphroditism (Haag and Doty, 2005).

The nematodes *C. elegans, C. briggsae* and *C. remanei* diverged from a common ancestor approximately 80-100 million years ago (Stein *et al*, 2003). These species are closely related and share a large degree of conservation between non-sex determining pathway proteins (e.g. UNC-45, 90% amino acid identity) (Hillier *et al.*, 2007). Nearly all of the proteins present in the *C. elegans* somatic sex determination pathway have orthologs in other species including *C. briggsae* (table 1), as well as the gonochoristic species *C. remanei*. While orthologs exist in all three species, they have varying degrees of similarity (figures 6-11) (Haag, 2005; Nayak *et al.,* 2005). This is not surprising as genes involved in reproductive success are often plastic and evolve rapidly. Previous work has shown that despite the varying degrees of structural conservation the function of the sex determining genes seem to be generally conserved between *C. elegans, C. briggsae* and *C. remanei,* however there are some significant differences (Kuwabara and Kimble, 1995; Kuwabara, 1996; Barnes and Hodgkin, 1996; Starostina, *et al*., 2007; Spence *et al*., 1990; Pilgrim *et al*., 1995; Hansen and Pilgrim, 1998; Haag *et al*., 2002; Zarkower and Hodgkin, 1993; de Bono and Hodgkin, 1996).

While sequence comparisons are useful, they are not the only indicator of evolution. Interspecific hybrids are viable but not fertile and often show sexual transformations. This indicates that while orthologous SD genes are inherited from each parent the proteins may not interact, which could be a result of divergence in functional domains or an incorrect expression pattern (Baird, 2002; Stothard and Pilgrim, 2003). It appears that specific interactions between members of the somatic SD pathway have been conserved throughout *Caenorhabditis* evolution but the interacting proteins themselves have diverged enough that the interactions have been preserved in a species specific manner (Stothard and Pilgrim, 2003). This functional conservation may extend even

Table 1. *C. elegans* and *C. briggsae* sex determining protein sequence conservation (modified from Haag, 2005). Proteins are arranged in the order in which they are found in the pathway. Somatic pathway genes are placed before germline specific genes. The median amino acid identity between *C. elegans* and *C. briggsae* orthologs is 80%.

Gene	Role in Sex Determination	Protein Motifs	Amino Acid Identity	References
xol-1	integrator of X and autosomal dosage (male-promoting)	GHMP kinase-related	22%	Luz *et al.*, 2003
sdc-1	X dosage compensation complex component, *her-1* transcriptional repressor (female-promoting)	Zn-finger TF	39%	Nonet and Meyer, 1991
sdc-2	X dosage compensation complex component, *her-1* transcriptional repressor (female-promoting)	novel, with coiled-coil	32%	Dawes *et al.*, 1999
sdc-3	X dosage compensation complex component, *her-1* transcriptional repressor (female-promoting)	Zn-fingers, myosin-like ATPase	28%	Klein and Meyer, 1993
her-1	secreted signal coordinating gender (male-promoting)	No motifs, signal peptide	57%	Streit *et al.*, 1999; Hamaoka *et al.*, 2004; Perry *et al.*, 1993
tra-2	receptor for HER-1, repressor of *fem* genes (female-promoting)	integral membrane protein	43%	Kuwabara and Kimble, 1995; Kuwabara, 1996
tra-3	positive regulator of TRA-2 processing (female promoting)	calpain-related protease	91%	Barnes and Hodgkin, 1996
fem-1	cytoplasmic responder to TRA-2 activity (male-promoting)	ankyrin repeats	72%	Spence *et al.*, 1990
fem-2	cytoplasmic responder to TRA-2 activity (male-promoting)	protein phosphatase 2C	63%	Pilgrim *et al.*, 1995; Hansen and Pilgrim, 1998
fem-3	cytoplasmic responder to TRA-2 activity (male-promoting)	None	38%	Haag *et al.*, 2002
cul-2	cytoplasmic responder to TRA-2 activity (male-promoting)	ubiquitin ligase	83%	Starostina, *et al.*, 2007
tra-1	global regulator of sex-specific transcription (female-promoting)	Zn-finger TF	44%	Zarkower and Hodgkin, 1993; de Bono and Hodgkin, 1996
laf-1	somatic repressor of *tra-2* translation (male-promoting)	noncoding RNA	N/A	Goodwin, pers. comm.
gld-1	germline translational repressor of *tra-2* (male-promoting)	KH RNA-binding protein	83%	Jones and Schedl, 1995
fog-2	germline cofactor of GLD-1, repressor of *tra-2* translation (male-promoting)	F-box	No ortholog	Clifford *et al.*, 2000
nos-3	germline cofactor of FBF-1/2, repressor of *fem-3* translation (female-promoting)	*nanos*-related RNA-binding domain	51%	Haag, 2005
mog-1	global repressor of *fem-3* translation (female-promoting)	DEAH RNA helicase	92%	Puoti and Kimble, 1999
mog-4	global repressor of *fem-3* translation (female-promoting)	DEAH RNA helicase	90%	Puoti and Kimble, 2000
mog-5	global repressor of *fem-3* translation (female-promoting)	DEAH RNA helicase	92%	Puoti and Kimble, 2000
mog-6	global repressor of *fem-3* translation (female-promoting)	divergent cyclophilin	92%	Belfiore *et al.*, 2004
fog-1	promoter of spermatogenesis (male-promoting)	CPEB	60%	Jin *et al.*, 2001; Luitjens *et al.*, 2000
fog-3	promoter of spermatogenesis (male-promoting)	TOB family	56%	Chen *et al.*, 2000; Chen *et al.*, 2001

Figure 6. Alignment between *C. elegans* (Y47D3A.6a), *C. briggsae* (CBG13188a) and *C. remanei* (CRE04883) TRA-1 orthologs. * indicates identical amino acids, : indicates highly conserved amino acids, . indicates mildly conserved amino acids. Alignments were made using ClustalW.

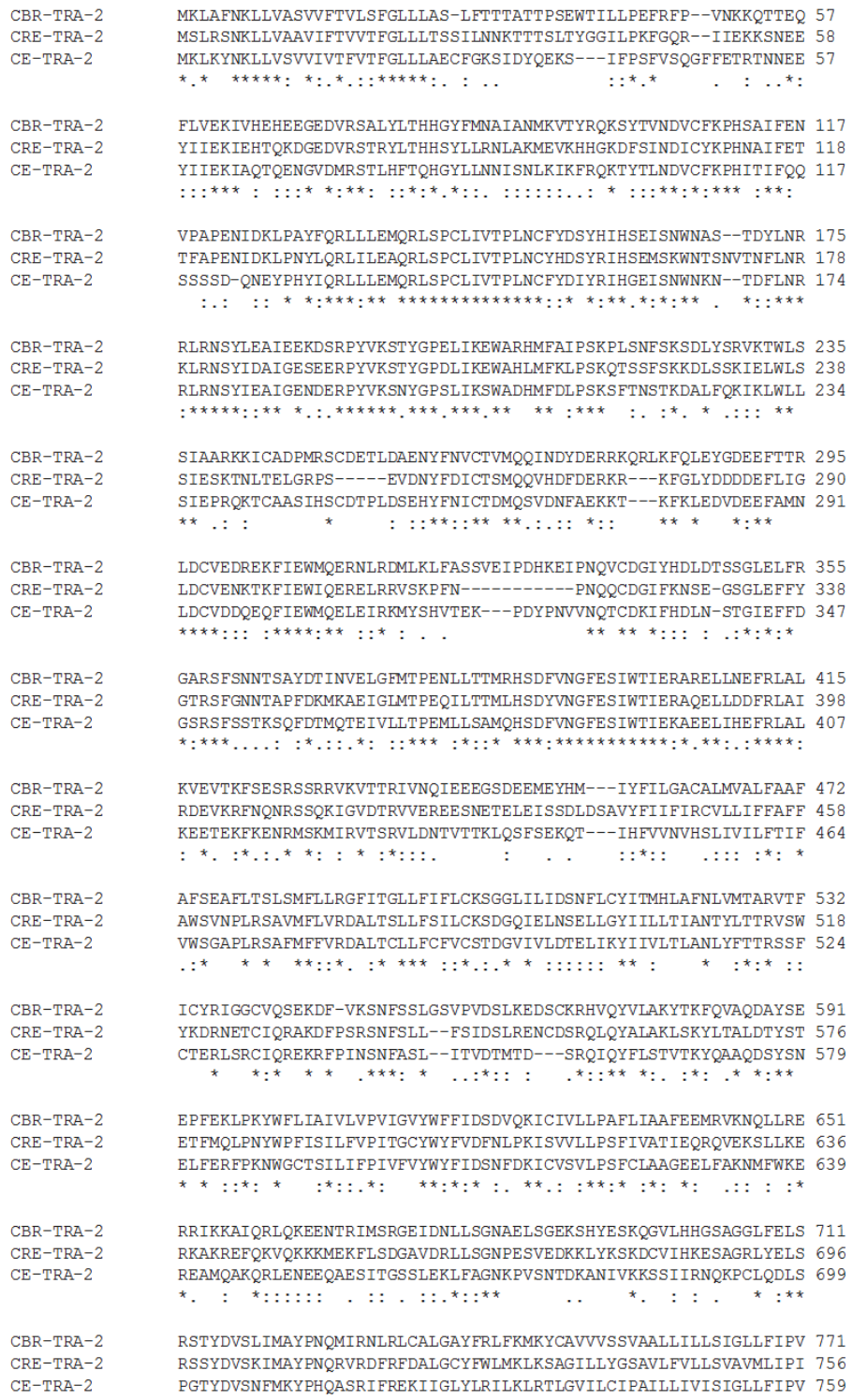


Figure 7. Alignment between *C. elegans* (C15F1.3a), *C. briggsae* (CBG11193a) and *C. remanei* (CRE11406) TRA-2 orthologs. * indicates identical amino acids, : indicates highly conserved amino acids, . indicates mildly conserved amino acids. Alignments were made using ClustalW.

Figure 8. Alignment between *C. elegans* (LLC1.1)*, C. briggsae* (CBG21580) and *C. remanei* (CRE11406) TRA-3 orthologs. * indicates identical amino acids, : indicates highly conserved amino acids, . indicates mildly conserved amino acids. Alignments were made using ClustalW.

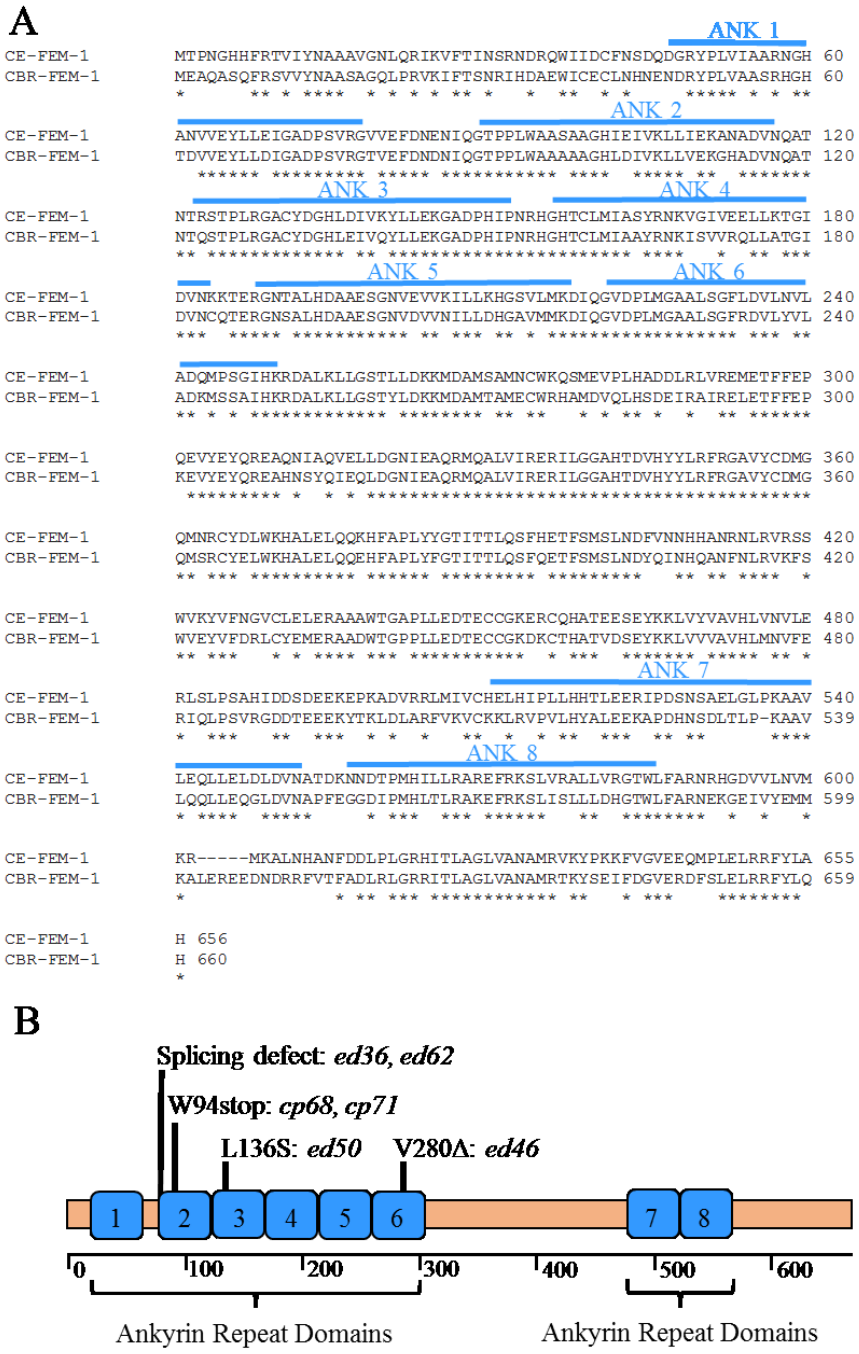


Figure 9. A. Alignment between *C. elegans* (F35D6.1) and *C. briggsae* (CBG19924) FEM-1 orthologs. The *C. remanei* FEM-1 sequence is not available. * indicates identical amino acids, : indicates highly conserved amino acids, . indicates mildly conserved amino acids. Functional domains are underlined in blue. B. Location of all the mutations found in *C. briggsae* FEM-1. Numbered boxes represent the ankyrin repeat domains. Alignment was made using ClustalW.

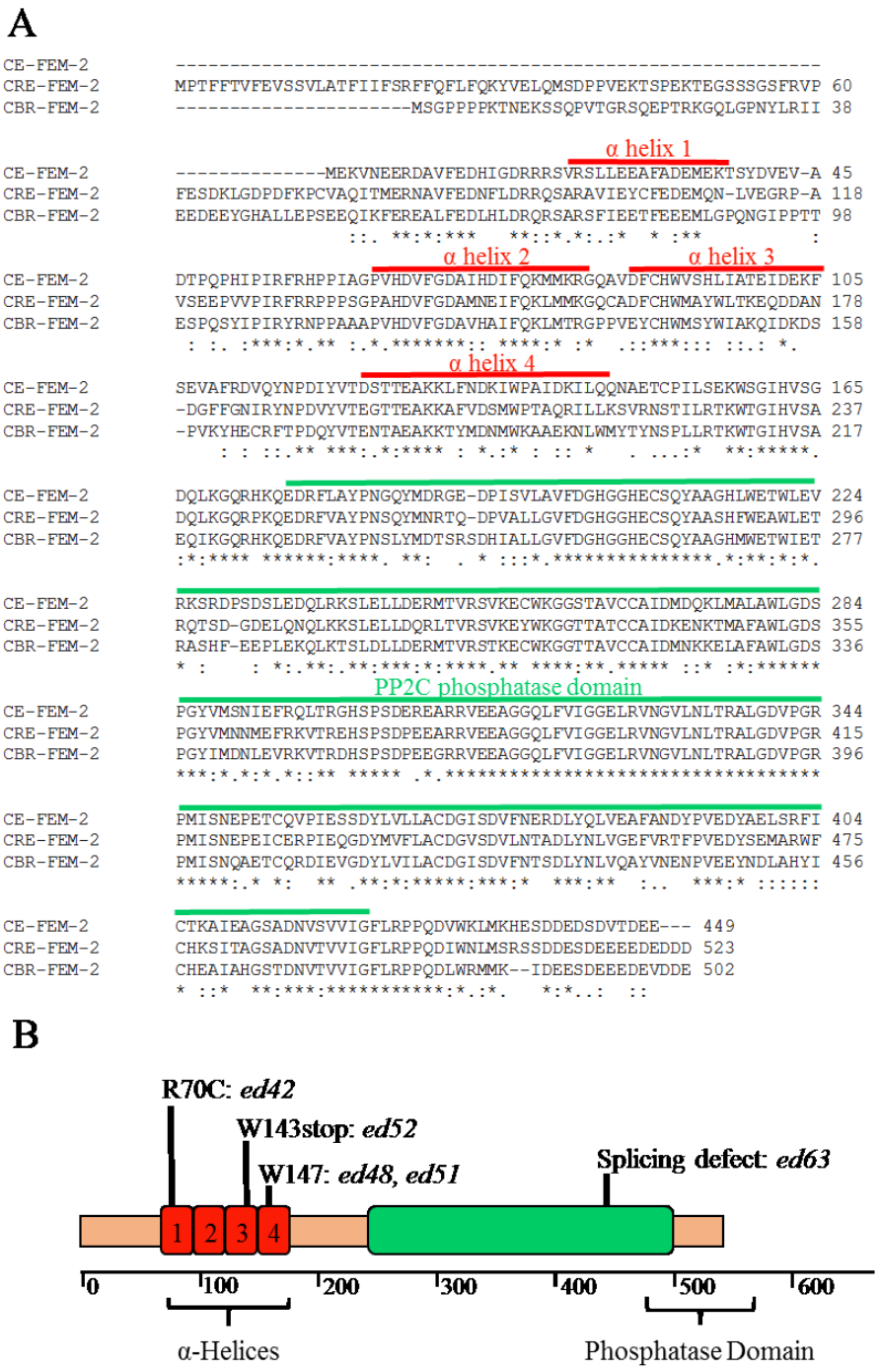


Figure 10. A. Alignment between *C. elegans* (T19C3.8), *C. briggsae* (CBG15267) and *C. remanei* (CRE25665) FEM-2 orthologs. * indicates identical amino acids, : indicates highly conserved amino acids, . indicates mildly conserved amino acids. A-helix domains are underlined in red, phosphatase domain is underlined in green. B. Location of all the mutations found in *C. briggsae* FEM-2. FEM-1/FEM-3 binding domains are shown in red. Phosphatase domain is shown in green. Alignments were made using ClustalW.

A

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CE-FEM-3        ------------------MEVDPG-------SDDVEADRETRAQK--------------- 20
CBR-FEM-3       ----------MVPDDVEPMEVDDDGALIVDLNETVEEDEETKKEK--------------- 35
CRE-FEM-3       MGAMKMTCDDFRAEDEQPIEVDERRRRLKRKANDDDDDDETVRERVDDAESSMEVFDAE 60
                          :***          :  : * :   .:

CE-FEM-3        --------LKLKRNVKFRAQMRRFDEYCGVTNLTVDDLNWPLISGIPLQRQRLTGATYYD 72
CBR-FEM-3       --------KRRKR---FREKLKRFDHYSQFSGISIAQIDWPLIQGRSLQRSPLTGQSFNA 84
CRE-FEM-3       YPPILDPLQDQREAKYFRERMQRFDLYSRTTGLSVDDIDWPLIRGRSLQKGRVAGISFVY 120
                           :.   ** :::*** *.  :.::: :::**** * .**:  ::* ::

CE-FEM-3        DSLLDQNPWDEFSIDRFLEITSIQLITAGAGYERNDEITRFVFQRTMKTIVTYCNFMYDL 132
CBR-FEM-3       DENIFR--IDEWPRETFLQITSTLTFCAGAALLSNEKITLFVFQRTMKTLVAYCNFMYHR 142
CRE-FEM-3       DDTRYP--INRFSDTWLLCVTKQKLFSFGAGCVEDLNITSFVLRRTMKVLSTYCNWLFEA 178
                *.       :.:.   :* :*.   :  **.   : :** **::****.: :***:::.

CE-FEM-3        ARRNGKVQITRFELQDLIHRDEFRFYMYFRQFLPNPDPNCTAFSNHYTSLLHTLYFNIPG 192
CBR-FEM-3       AITHNRRQINRIDVHELISRNPLRFHMFLQKFLPHPDINRTHFNNEFLYYFHNLYFQDET 202
CRE-FEM-3       AKRNNRRHITHKEIQELINRDGFRFHQYLQKFLIGRGMEYTEYNNRFFKYLHEEYNKNPG 238
                *  :.: :*.: ::::** *: :**: ::::**   . : * :.*.:   :*  * :

CE-FEM-3        MPQFWNNSQMYN-YAATRGQRLVQNIAAFYPPEYFWNEDESKYHTTFVVPRGTEFSKFYA 251
CBR-FEM-3       CRLLYHDVARYSPIINQQGTRMSLQHQIYYPDVMRNPAFDALWFTSFINPSGYSFSRFHA 262
CRE-FEM-3       GLETIYSNQDFIAKETAQANYIYATVRAKYGGIEELPLFRHALKISFTQPGEHYFSRFYA 298
                      .   :      :.  :       *               :*  *    **:*:*

CE-FEM-3        RRFHEALGMPPLENEIITVLDWLAKLCILEIVYHTTIWCDITGFGG-LPRIEHYRLAMEN 310
CBR-FEM-3       YRFHEALGMPPLESELIIVLDWLAKLIICDIGYKVLAWRDARGFQG-LPDLLSFQMAMLE 321
CRE-FEM-3       KRFHEALGWPPLDSEIIMILDWFGVLIMNQIAYKTIRWHEEEYNDGSFPVLDSYHKALAD 358
                 ******* ***:.*:* :***:. * : :* *:.  * :     * :* :  :: *: :

CE-FEM-3        V---------------EDIIFDLAIDDFS-ISRLQLQISPFEIS-RYSP---LDVS---- 346
CBR-FEM-3       E---------------GDPLFDLDIDYTAPPTRLFSEPTRFQTYPKFQPRRRIDFPSRFD 366
CRE-FEM-3       ESKCPKACLISISLFPDDPLFDLDIDYGTNPPPVNTAYQKVRRTPRDPT----------- 407
                             * :*** **  :  . :      ..   :  .

CE-FEM-3        GYYETIKRKKDIEEYQNRFYEVHY-SDDVRIMNVYATDCSRKR 388
CBR-FEM-3       GFYKKRRLERGLEEIQESFIMNHFPTKPLRTVYVYTHPEERRR 409
CRE-FEM-3       ----PFYRLMEFEDYKSCLVKMHFNFSELTGEWLRKICARGR- 445
                          :*: :. :   *:  . :    :       :
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B

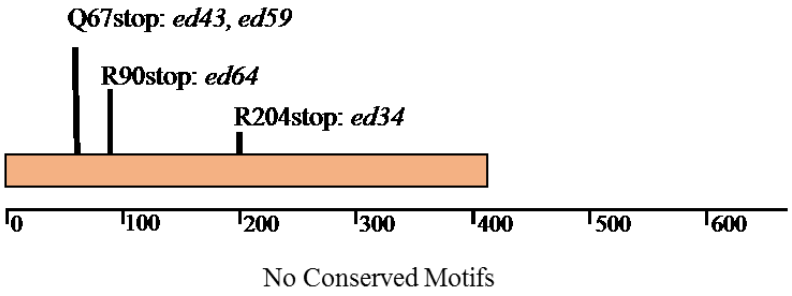

Figure 11. A. Alignment between *C. elegans* (C01F6.4), *C. briggsae* (CBG21774) and *C. remanei* (CRE04410) FEM-3 orthologs. * indicates identical amino acids, : indicates highly conserved amino acids, . indicates mildly conserved amino acids. B. Location of all the mutations found in *C. briggsae* FEM-3. All mutations found in *fem-3* are nonsense mutations which occur close to the N-terminus of the protein. Neither the glutamine nor the arginine residue are conserved between *C. elegans* and *C. briggsae*. Alignments were made using ClustalW.

further as an ortholog of *Caenorhabditis tra-1* regulates sexual development in the distantly related species *Pristionchus pacificus* (Pires-daSilva and Sommer, 2004).

Cbr-TRA-1, Cbr-TRA-2 and Cbr-TRA-3 have female promoting roles in *C. briggsae*. As in *C. elegans* mutations in any of these *Cbr-tra* genes results in masculinization of the soma and germline (figure 12) (Hodgkin, 1987; Kelleher *et al.*, 2008). Cbr-TRA-1 acts as the terminal regulator of the somatic pathway and is functionally conserved in *C. elegans.* Unlike many of the other SD ortholgs *Cbr-tra-1* is able to partially rescue *Ce-tra-1* mutants. The rescue is restricted to the restoration of the hermaphrodite tail but the gonad remains male (de Bono and Hodgkin, 1996). TRA-2 is one of the more rapidly evolving members of the *Caenorhabditis* SD pathway with only 43% amino acid identity between *C. elegans* and *C. briggsae.* Despite its low sequence identity it still acts as a transmembrane receptor in *C. briggsae* and negatively regulates the Cbr-FEM/CUL-2 complex to specify a female fate. The interaction between Cbr-TRA-1B and Cbr-TRA-2 appears to be maintained in *C. briggsae in vitro* (Wang and Kimble, 2001). However while the Cbr-TRA-1B isoform retains the Cbr-TRA-2ic binding domain it lacks the N-terminal Cbr-FEM-1 binding domain (Wang and Kimble, 2001). It has never been shown *in vivo* that Cbr-TRA-2 and Cbr-TRA-1 directly interact or whether there is a functional difference between Ce-TRA-1B and Cbr-TRA-1B. While this interaction appears to be evolutionary conserved it has been shown to be species-specific in *C. elegans*, *C. briggsae* and *C. remanei*. (Wang and Kimble, 2001). Cbr-TRA-3 is structurally and functionally conserved and one of the least divergent of the SD pathway members however inter-species rescues have not been performed. The positions of the TRA genes in the pathway are also conserved.

FEM-1, FEM-2 and FEM-3 have male promoting roles in *C. briggsae C. remanei* and *C. brenneri* (Stothard and Pilgrim, 2006). Cbr-FEM-1 and Cbr-FEM-2 both show a mild degree of divergence (72% and 63% amino acid identity respectively) to the point that interspecific rescues are unsuccessful. FEM-3 is the most divergent of all known members of the SD pathway with only 38% amino

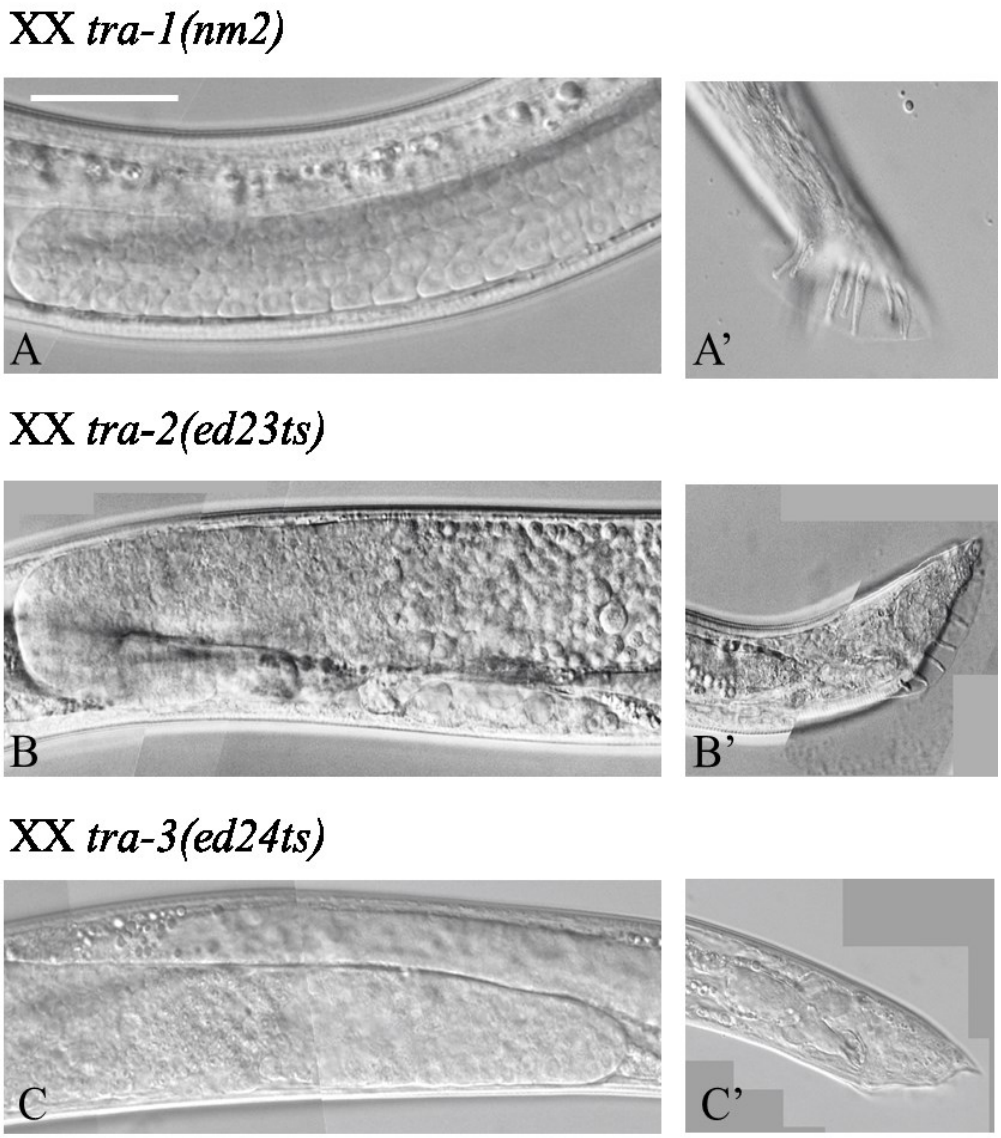

Figure 12. DIC images of *C. briggsae tra-2(ed23ts)* (A, A'), *tra-3(ed24ts)* (B, B'), and *tra-1(nm2)* (C, C') XX animals. Scale bar = 50 μm. *tra-2(ed23ts)* animals have a single armed gonad which only produces sperm (A) and a blunt ended male tail which fails to form tail rays (A'). *tra-3(ed24ts)* animals also have a single armed gonad which only produces sperm (B) and a male tail which fails to develop rays (B'). *tra-1(nm2)* animals have single armed gonads but can produce sperm and poorly formed oocytes (C). *tra-1(nm2)* animals form fully functional male tails with tail rays (C') and the animals are fertile and display male mating behavior (Kelleher *et al*., 2008).

acid identity between *C. elegans* and *C. briggsae.* Despite this low level of conservation the interaction between FEM-3 and TRA-2ic is conserved in a species-specific manner in *C.elegans, C. briggsae* and *C. remanei* (Haag *et al*., 2002). This species specificity in TRA-2/FEM-3 and TRA-2/TRA-1 interactions is indicative of coevolution, further showing that lower percentage amino acid sequence identity does not necessarily lead to a change in function (Haag *et al.*, 2002). What is remarkable about the *Cbr-fem* genes is that when mutated they have a different phenotype than when mutated in *C. elegans*. *Ce-fem* mutants are completely feminized whereas *Cbr-fem* mutants develop as hermaphrodites (the Her phenotype) (figure 13).While *Cbr-fem* genes are required for male somatic development they are dispensable for spermatogenesis as confirmed by RNAi and mutational analysis (Haag *et al.,* 2002; Stothard *et al.,* 2002; Carvalho, 2005; Hill *et al.,* 2006; Dewar, 2011). In *C. elegans*, *Ce-fem-3* plays a vital role in the hermaphrodite gonad as it is down-regulated to stop sperm production and allow the worm to switch to oocyte production for the rest of its life (Ahringer and Kimble, 1991). In *C. briggsae* none of the *Cbr-fem* genes fulfill this role and the molecular mechanism which controls the switch from spermatogenesis to oogenesis is still unknown. Since mutations in the *Cbr-fem* genes result in Her worms it follows that this sperm to oocyte switch would be downstream of the Cbr-FEMs (Haag *et al.,* 2002; Stothard *et al.,* 2002; Carvalho, 2005; Hill *et al.,* 2006; Dewar, 2011). This poses an interesting question of where the switch occurs, because Cbr-TRA-1 is immediately downstream of the Cbr-FEMs and there are no other known male promoting factors before the end of the pathway. Like the *Cbr-tra* genes, the *Cbr-fem* genes retain their position in the somatic sex determining pathway but their role in controlling germ cell fate has been shifted to some other unknown factor. The inability of SD genes to replace their orthologous counterparts is a result of their sequence variation and not necessarily indicative of their biological roles (Stothard and Pilgrim, 2006). To that end it is more informative to make interspecific comparisons between mutants to determine functional conservation of SD orthologs.

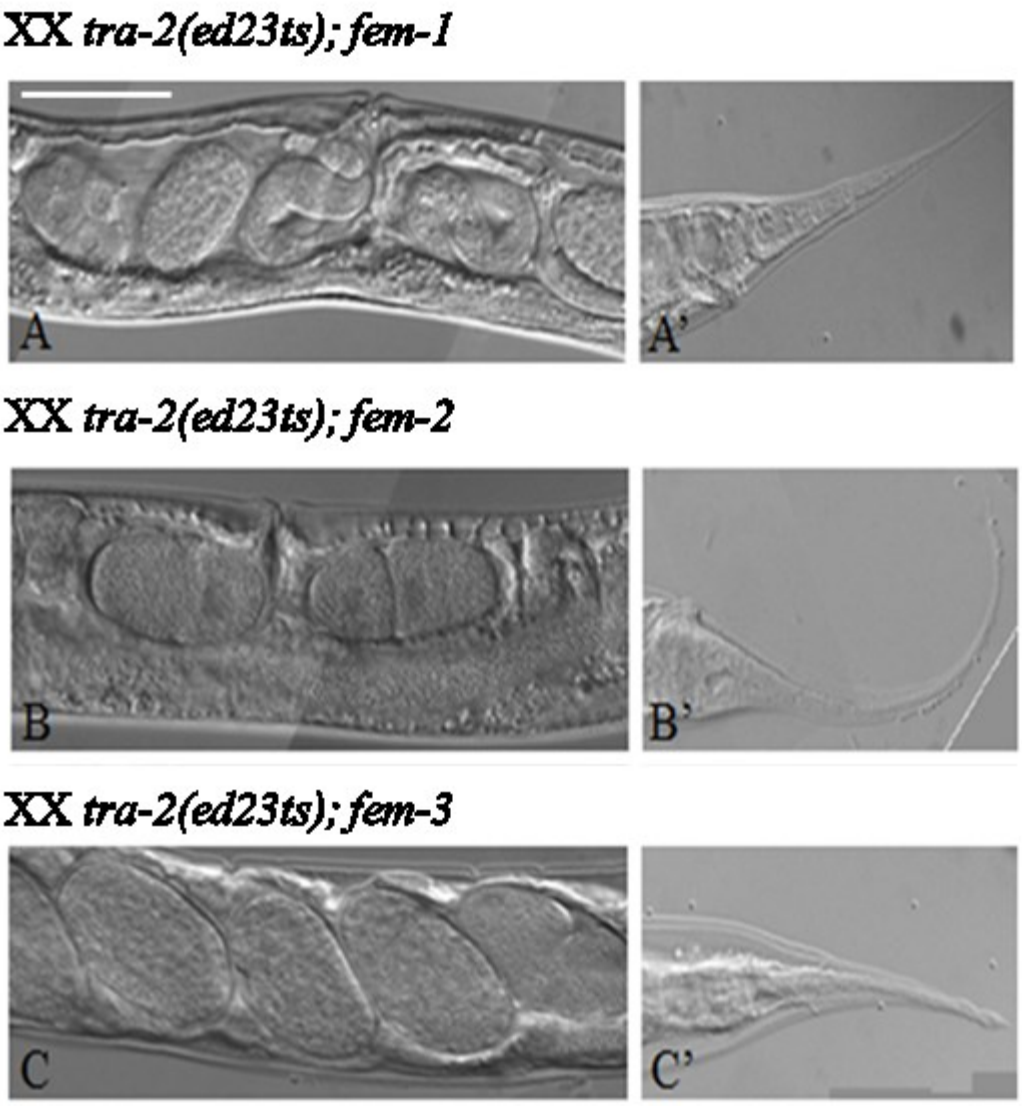

Figure 13. DIC images of *C. briggsae tra-2(ed23ts); fem* mutants. Scale bar = 50μm. Mutations in any of the *fem* genes restores female somatic and germline development in the *tra-2* background, resulting in XX hermaphrodites. There are no distinguishing differences between different *tra-2(ed23ts); fem* mutants. All three *tra-2(ed23ts); fem* mutants have double-armed gonads which produce sperm and eggs, allowing the animals to self-fertilize (A, B, C). They all also have the long, slender tale characteristic of a female soma (A', B', C').

While the overall function of the members of the *Caenorhabditis* SD pathway is known, exactly how each individual member fulfills that function is still being elucidated. This is particularly true for the FEM proteins. While it has been shown that each FEM protein is required for the repression of TRA-1, it is not clear how each individual member contributes to this. For example, FEM-2's phosphatase activity is required for male development but no target has ever been identified in *C. elegans* or *C. briggsae*. Another example is if FEM-3's only role is to be the site for negative regulation of the FEM complex, why do mutations in *fem-3* result in feminization rather than masculinization of the soma and germline in both *C. elegans* and *C. briggsae,* from a loss of repression on FEM-1 and FEM-2?

It is still unknown how spermatogenesis is controlled in *C. briggsae* hermaphrodites. The point of down-regulation of female-promoting genes and up regulation of male-promoting genes, which is facilitated by Ce-TRA-2 and Ce-FEM-3 respectively in *C. elegans*, remains a mystery but it is likely downstream of the *Cbr-fem* genes. If this switch is located downstream of the *Cbr-fem* genes it begs the question what factors of the pathway are involved. Currently the only known core pathway member downstream of the *Cbr-fem* genes is *Cbr-tra-1*. However, *tra-1* mutants appear identical between *C. elegans* and *C. briggsae*. We are already aware of genes that are unique to each species' pathway (e.g. *Cbr-she-1* and *Ce-fog-2*) so the idea of proteins adopting a novel role in the *C. briggsae* SD pathway is quite plausible. What these proteins are and what they are doing is still unknown.

1.9 *C. briggsae* germline sex determining pathway

If the core SD pathway of *Caenorhabditis* is preserved in male/female species, how then can we account for several independent evolutions of hermaphroditism? Characterization of the *C. elegans* pathway has shown that there are no hermaphrodite specific genes but that additional male and female promoting

genes have evolved to regulate the expression of the core pathway genes specifically in the germline. It is not clear how spermatogenesis is regulated in *C. briggsae* hermaphrodites but it is certainly much different than in *C. elegans*. In the *C. briggsae* germline, Cbr-TRA-2 repression is not controlled by Cbr-FOG-2 and Cbr-GLD-1 (Guo *et al.,* 2009; Beadll *et al.,* 2011). *Ce-fog-2* is the result of a recent tandem duplication event and as such no such gene exists in *C. briggsae* (Clifford *et al*., 2000). In *C. briggsae* the role of Ce-FOG-2 is taken over by another F box protein, Cbr-SHE-1. Interestingly Cbr-SHE-1 is unique to *C. briggsae* and is also a result of a tandem duplication event (Guo *et al*., 2009). A *Cbr-gld-1* ortholog does exist in *C. briggsae* but instead of facilitating spermatogenesis it has the opposite role and is required for oogenesis. Mutations in *Cbr-she-1* result in XX animal germline feminization while XO animals remain unaffected indicating that additional factors must be responsible for controlling spermatogenesis in males. The switch to turn off sperm production and turn on oocyte production is also different between *C. briggsae* and *C. elegans. Cbr-fem* mutants are still capable of sperm and oocyte production indicating that the switch from sperm to oocyte production is located downstream of the *Cbr-fem* genes, unlike *C. elegans*. Unsurprisingly *Ce-fbf-1* and *Ce-fbf-2* are missing in *C. briggsae*. Like *Ce-fog-2* the *Ce-fbf* genes arose from a *C. elegans* specific tandem duplication event (Zhang *et al.,* 1997). *Cbr-fog-1* and *Cbr-fog-3* orthologs do exist in *C. briggsae* based on sequence conservation but it has not been confirmed whether or not these two genes retain their roles in spermatogenesis.

1.10 A toolkit for studying sex determining pathways in *C. briggsae*

Based on the success of the forward mutagenesis screens used to originally characterize the *C. elegans* sex determining pathway, a similar experimental approach was taken in *C. briggsae*. Genetic screens to identify masculinizing mutations were performed that determined which facets of *Cbr-tra* function were conserved and provided tools for assaying the genetic interactions and

relationships in the *C. briggsae* SD pathway. The results of these screens included *Cbr-tra-1, Cbr-tra-2* and *Cbr-tra-3* mutants.

To identify downstream components of the *C. briggsae* pathway and potentially identify the molecular regulator of spermatogenesis, a set of suppressor screens was performed on *Cbr-tra-2* temperature-sensitive alleles, similar to the ones conducted in *C. elegans* (Hodgkin, 1987). Two *Cbr-tra-2* alleles were used. *tra-2(ed23ts)* has an aspartic acid to an alanine substitution at residue 587 in the second transmembrane of the protein. *tra-2(nm9ts)* has a proline to leucine substitution at residue 1214, which occurs in the Cbr-FEM-3 binding region of the intracellular part of Cbr-TRA-2 (Hill *et al*., 2006; Kelleher *et al.*, 2008). At the permissive temperature (16°C) XX animals are phenotypically normal but at the restrictive temperature (25°C) XX animals display the Tra phenotype. XO animals at both temperatures appear normal. The *tra-2(ed23ts)* suppressor screen was carried out by members of the Pilgrim lab while the *tra-2(nm9ts)* was carried out by members of the Haag lab. Between both experiments a total of 760,000 haploid genomes were screened resulting in 75 suppressors, of which 54 were found from the *tra-2(ed23ts)* screen and 21 were found from the *tra-2(nm9ts)* screen. The 21 suppressors from the *tra-2(nm9ts)* were sent to the Pilgrim lab for identification and characterization.

Using the same procedure as the *C. elegans* Knockout Consortium (Edgley *et al*., 2002), Hill *et al.,* (2006) screened for deletion mutations in *C. briggsae fem* genes. They found a *Cbr-fem-2* allele that contained a 1.6 kbp deletion that removed the phosphatase domain and the 3' UTR. This allele was named *fem-2(nm27).* This allele is predicted to be a null allele as no *Cbr-fem-2(nm27)* mRNA was detected by high sensitivity in-situ hybridization. They also identified a *Cbr-fem-3* allele, *(nm63),* which contained a 1.1 kbp deletion mutation resulting in the loss of residues 73-227. It is not clear whether *Cbr-fem-3(nm63)* is a null allele however it has been shown that it has a strong loss-of-function phenotype in XO animals and like *Cb-fem-2* it is not necessary for hermaphrodite spermatogenesis (Hill *et al*., 2006). These two *Cbr-fem* alleles were crossed into a *tra-2(ed23ts*)

background along with a phenotypic marker to make strains that could be used to rapidly identify other *Cbr-fem-2* and *Cbr-fem-3* alleles recovered from the *tra-2(ed23ts)* suppressor screen.

One of the suppressors which was found to complement both *Cbr-fem-2* and *Cbr-fem-3* was suppressor *ed36* (Dewar, 2011). *ed36* was mapped to a region of chromosome IV near *Cbr-fem-1*. The *Cbr-fem-1* gene of *ed36* was sequenced and it was found that a substitution of a G to an A had occurred at the first nucleotide of the seventh intron. Examination of the cDNA extracted from homozygous *ed36* animals showed that this induced a splicing defect leading to the expansion of the intron by 25 bp. This caused a frameshift to occur resulting in the deletion of the C-terminus of *fem-1(ed36)* (Dewar, 2011). Like the previously described *tra-2(ed23ts) dpy-15(sy5148); fem-2(nm27)* and *tra-2(ed23ts); cby-1(s1281); fem-3(nm63)* identifier strains, a phenotypic *cby-1(s1281)* marker was introduced into the *tra-2(ed23ts)*; *fem-1 (ed36)* background to allow for rapid identification of other *tra-2(ed23ts)* suppressors (Dewar, 2011).

To locate the position of unknown suppressors within a chromosome, a *C. briggsae* mapping strain was created which contained the *tra-2(ed23ts)* allele in a different *C. briggsae* strain. The common *C. briggsae* laboratory strain is called AF16 which is highly polymorphic to the HK104 strain. *tra-2(ed23ts),* which was made in an AF16 background, was crossed into wild-type HK104 and then backcrossed to HK104 seven times selecting for Tra mutants with theoretically >99% of the final strain comprising the HK104 background.

In addition to the suppressors being mutations in *Cbr-fem-1, Cbr-fem-2* or *Cbr-fem-3,* three other complementation groups of mutants were found. These groups have unique phenotypes which have never before been described in *C. elegans* or *C. briggsae.* It is possible that these mutants represent novel members of the *C. briggsae* SD pathway which were recruited after *C. elegans* and *C. briggsae* diverged. Adopting novel members to the pathway is an important evolutionary event but not unprecedented as evident by the appearance of *Cbr-*

she-1 in the *C. briggsae* germline pathway (Guo *et al*., 2009). What these genes are and how they have integrated into the pathway are important questions to answer to further our understanding of the *C. briggsae* SD pathway and solve the mystery of how it facilitates hermaphroditism at the molecular level.

1.11 Comparisons with *C. briggsae*'s gonochoristic sister species, *C. nigoni*.

C. nigoni is a recently discovered gonochoristic species that is closely related to *C. briggsae* (figure 1) (Cutter *et al*., 2010). It has been found in Kerala, India and the Democratic Republic of Congo (Felix *et al.,* 2014). *C. nigoni* provides an excellent system for determining the differences between a gonochoristic and androdiocious species. Comparisons between *C. nigoni* and *C. briggsae* sex determining genes may elucidate how a female nematode acquired the ability to produce sperm. What is particularly interesting about *C. nigoni* and *C. briggsae* is that they will successfully interbreed (Woodruff *et al*., 2010) which has not been observed in any other pair of *Caenorhabditis* species (Baird *et al*., 1992). The viability of the hybrid offspring is dependent on the mother species. The F1 of a *C. nigoni* mother shows 45% viability in contrast to the F1 of *C. briggsae* mothers which show 30% viability (Woodruff et al., 2010). This difference in viability is accounted for by the absence of F1 males when *C. briggsae* is the mother. All F1 males produced from a *C. nigoni* mother had a developmental delay, gonad defects, were atypically small and never successfully crossed. The presence of selfing hermaphrodites in the F1 crosses was extremely rare and their offspring were not viable (Woodruff *et al.,* 2010). The inability to maintain hermaphroditic hybrids make mapping the genes that gave rise to hermaphroditism impossible.

There are two mutually exclusive scenarios which explain how these two closely related species evolved different reproductive strategies. The first is that the last common ancestor of *C. nigoni* and *C. briggsae* was an inbreeding hermaphrodite species that produced a low frequency of spontaneous males. After the speciation event that separated *C. nigoni* and *C. briggsae*, *C. nigoni*

hermaphrodite worms lost the ability to produce sperm. Alternatively the last common ancestor was a male/female outbreeding species and *C. briggsae* females gained sperm production after they diverged. Given that *C. nigoni's* and *C. briggsae's* closest relatives are male/female species makes the later scenario more convincing. In either scenario, comparing the sequences of the SD genes and their protein products of these two species could provide insights into how hermaphroditism evolved. The limitation to this is that sequence alone does not predict function with certainty. An additional challenge to these comparisons is that *C. nigoni* does not have a fully annotated genome and gene sequences were constructed from a database of contigs. This resulted in *C. nigoni* sequences that contained some gaps, however the majority of the *Cni-tra* and *Cni-fem* gene sequences, as well as *Cni-cul-2*, were found. In addition to the core pathway genes downstream of *Cni-her-1* the two male promoting germline genes, *Cni-she-1* and *Cni-gld-1* were examined as they fulfill a unique role in *C. briggsae* compared to *C. elegans.*

1.12 Thesis Overview

The SD systems of *Caenorhabditis* nematodes are an ideal model for studying how biological pathways evolve. How the molecular mechanisms evolved to facilitate hermaphroditism can be broken down into two fundamental questions; how are genes that specify a female fate and prevent a male fate transiently down regulated to allow sperm production and how are genes that control spermatogenesis temporally up regulated? The mutagenic screens used to characterize the *C. elegans* SD pathway were successful in answering these questions and so similar screens were employed to identify members of the *C. briggsae* SD pathway. The resulting mutants have shown that the mechanisms that control hermaphroditism in *C. elegans* and *C. briggsae* are different. These differences show which parts of the pathway are most susceptible to change and evolution.

Previous work done to characterize the *C. briggsae* SD pathway resulted in *Cbr-tra* and *Cbr-fem* mutants. *tra* mutants show similar phenotypes in *C. elegans* and *C. briggsae* however this is not true for *fem* mutants. *Ce-fem* mutants are female whereas *Cbr-fem* mutants are hermaphrodites. The *Cbr-fem* mutants were isolated from a suppressor screen of two temperature-sensitive *Cbr-tra-2* alleles, *tra-2(ed23ts)* and *tra-2(nm9ts)*. There remained forty five unidentified suppressor which likely represent more *Cbr-fem* mutants. These suppressors can be identified by failing to complement with one of the three *Cbr-fem* genes. Identifying and characterizing the mutations in novel *Cbr-fem* alleles could lead to a better understanding of the structure and function of these proteins.

In addition to identifying *Cbr-fem* mutants, three suppressors were found which complemented all three *Cbr-fem* genes as well as complementing each other. These three strains have phenotypes never before reported in *C. elegans* or *C. briggsae* and could potentially represent novel genes in the *C. briggsae* pathway or interesting alleles of known genes. Identification and characterization of these mutants is an important step to resolving how *C. briggsae* evolved hermaphroditism. Because these suppressors were made by EMS mutagenesis they are expected to contain mutations in several genes. To identify the causative mutation in these strains I used whole genome sequencing to identify candidates and performed mapping crosses to confirm that these candidates were linked to the lesion responsible for *tra-2(ed23ts)* suppression.

In recent years there has been an increase in the number of nematode species which have been discovered. One such species is *C. nigoni*, the sister species to *C. briggsae. C. nigoni* provides an excellent opportunity for studying the evolution of hermaphroditism as it uses a male/female sex determining system. The presence of hermaphroditism in *C. briggsae* and not *C. nigoni* can be explained in one of two ways. Either their last common ancestor used hermaphroditism and *C. nigoni* lost that trait after they diverged or their last common ancestor was a female/male species and *C. briggsae* evolved hermaphroditism after they diverged. Regardless of which scenario is true,

comparisons between these two sister species will be useful for determining how *C. briggsae* evolved hermaphroditism. I expect *C. nigoni* and *C. briggsae* to be almost genetically identical so any differences in the known SD genes would be indicative of an evolutionary event which may have led to hermaphroditism. *C. nigoni* does not have a fully annotated genome however the 959 nematode genomes database contains contigs of its genome. I constructed the *C. nigoni* SD orthologs by BLASTing *C. briggsae* SD gene sequences and aligning the matching contigs. From these alignments I was able to computationally translate the coding sequence and compare the resulting proteins with the members of the *C. briggsae* SD pathway.

I had three objectives:

I) Complementation analysis to determine the identity of suppressors of the *Cbr-tra-2* null mutants originally isolated from a *C. briggsae tra-2(ed23ts)* suppressor screen.

II) Identify *tra-2(ed23ts)* suppressor alleles which complement *Cbr-fem-1*, *Cbr-fem-2* and *Cbr-fem-3* mutants.

III) Compare the sex determination proteins of *C. briggsae* to its gonochoristic sister species *C. nigoni.*

2.0 Materials and Methods

2.1 Strains

All nematode strains were maintained as described (Brenner 1974) and were kept at room temperature, except temperature-sensitive mutants, which were maintained at 16°C. Worms were maintained on modified NGM agar plates seeded with the OP50 strain of *E. coli*. Modified NGM agar plates contained 5.9 g of worm mix (55 g of Tris-HCl, 24 g Tris base, 310 g tryptone, 200 g NaCl, 800 mg Cholesterol) and 20 g of agar per liter.

In this work two strains of *C. briggsae* were used, AF16 and HK104. *C. briggsae tra-2(ed23ts)* suppressors were created by EMS and ENU mutagenesis screens done in the AF16 background (Carvalho, 2005). Strains used in this work in an AF16 genetic background are provided in table 2, while those in an HK104 background for the purposes of genetic mapping are provided in table 3. Strains where the suppressor lesion has been identified in this work are provided in table 4.

2.2 Crosses and phenotypic analysis

Typically, crosses were set up using 20 males and 6 L4 hermaphrodites. Unless otherwise stated, crosses were performed at room temperature. When the hermaphrodites began laying eggs, they were moved to a new plate. In the absence of an F1 phenotype, successful crosses were identified by the presence of approximately 50% males in the F1 generation.

Phenotypes were scored using a stereo-dissecting microscope. All DIC images were obtained using a Zeiss Axioscope 2 mot plus microscope and a Q imaging retiga EXi camera. Worms were anesthetized with 0.02% sodium azide in

Table 2. List of sex determining strains in an AF16 background used in this work.

Genes	Name	Genotype	Phenotype of homozygote at 16°C	Phenotype of homozygote at 25°C
tra-2	DP297	*tra-2(ed23ts)*	Wildtype	XX: Tra XO: wildtype
tra-2-cby-1-fem-1	DP423	*tra-2(ed23ts); cby-1(s1281); fem-1(ed36)*	XX: Cby, hermaphrodites XO:Cby, hermaphrodites	Same as 16°C
tra-2-cby-15-fem-2	DP369	*tra-2(ed23ts) cby-15(sy5148); fem-2(nm27)*	XX: Cby, hermaphrodites XO:Cby, hermaphrodites	Same as 16°C
tra-2-cby-1-fem-3	DP425	*tra-2(ed23ts); cby-1(s1281); fem-3(nm63)*	XX: Cby, hermaphrodites XO:Cby, hermaphrodites	Same as 16°C
tra-1		*tra-1(nm2)*	Wildtype	XX: Tra XO: wildtype

Table 3. List of sex determining strains in an HK104 background used in this work.

Strain Name	Genotype	Phenotype of homozygote at 16°C	Phenotype of homozygote at 25°C
DP426	*tra-2(ed23ts)*	Wildtype	XX: Tra XO: wildtype

Table 4. Summary of *tra-2; suppressor* strain genotypes and their molecular lesions (if known)

Genotype	Lesion
tra-2(ed23ts); fem-1(ed36)	Splicing defect of intron 7
tra-2(ed23ts); fem-1(ed40)	not yet determined
tra-2(ed23ts); fem-1(ed46)	C to T transition at nucleotide 670 and deletion of nucleotides 841-847 causing a frameshift
tra-2(ed23ts); fem-1(ed62)	Splicing defect of intron 7
tra-2(ed23ts); fem-1 (ed50)	L136S
tra-2(ed23ts); fem-1(cp68)	G to A transition at nucleotide 416 causing a stop codon
tra-2(ed23ts); fem-1(cp71)	G to A transition at nucleotide 416 causing a stop codon
tra-2(ed23ts); fem-1(cp72)	not yet determined
tra-2(ed23ts); fem-1 (ed64)	not yet determined
tra-2(ed23ts); fem-1 (ed65)	not yet determined
tra-2(ed23ts); fem-1 (ed66)	not yet determined
tra-2(ed23ts); fem-2(ed42)	R70C
tra-2(ed23ts); fem-2(ed48)	G to A transition at nucleotide 447, causing a stop codon
tra-2(ed23ts); fem-2(ed49)	not yet determined
tra-2(ed23ts); fem-2(ed51)	G to A transition at nucleotide 447, causing a stop codon insertion
tra-2(ed23ts); fem-2(ed52)	G to A transition at nucleotide 434 causing insertion of a stop codon
tra-2(ed23ts); fem-2(ed63)	Splicing defect of intron 6
tra-2(ed23ts); fem-2 (ed67)	not yet determined
tra-2(ed23ts); fem-2 (ed68)	not yet determined
tra-2(ed23ts); fem-2 (ed69)	not yet determined
tra-2(ed23ts); fem-3(ed43)	C to T transition at nucleotide 202 causing insertion of a stop codon
tra-2(ed23ts); fem-3(ed58)	not yet determined
tra-2(ed23ts); fem-3(ed59)	C to T transition at nucleotide 202 causing insertion of a stop codon
tra-2(ed23ts); fem-3(ed60)	not yet determined
tra-2(ed23ts); fem-3(ed64)	C to T transition at nucleotide 268 causing insertion of a stop codon

M9 buffer (22 mM KH_2PO_4, 42 mM Na_2HPO_4, 85 mM NaCl, 1 mM $MgS0_4$) prior to imaging.

2.3 Complementation

Previous work has shown that the majority of *tra-2(ed23ts)* suppressors contain mutations in one of the three *fem* genes (Hodgkin, 1986; Carvalho, 2005, Dewar, 2011). Null alleles of the three *fem* genes, each in a *tra-2(ed23ts)* background, were made previous to this work (Dewar, 2011). To assess which *Cbr-fem* gene was mutated in a given *tra-2(ed23ts); suppressor* strain, complementation analysis was carried out (figure 14). The three *Cbr-fem* identifier strains each contained a recessive phenotypic marker to easily score outcrossed progeny. Specifically, *tra-2(ed23ts)* males (raised at 16°C) were crossed to *tra-2(ed23ts); cby-1(s1281); fem-1(ed36)* hermaphrodites. The F1 generation was raised at 16 °C, and males from the F1 generation were crossed to *tra-2(ed23ts); suppressor* strains. This cross was performed in triplicate. The F2 generation was raised at 25 °C, and scored. An F2 generation that contained males and Tra worms was evidence of complementation while an F2 generation containing hermaphrodites, males, and Tra worms was evidence of non-complementation. If the suppressor appeared to fail to complement *fem-1(ed36),* 10 F2 hermaphrodites were singled to ensure they were the products of outcrossing and not from self-fertilization of the *tra-2(ed23ts);* suppressor strain. It was expected that approximately half of F2 hermaphrodites would have Cby progeny if they were produced from outcrossing. Similar crosses with *tra-2(ed23ts); dpy-15(sy5148); fem-2(nm27)* and *tra-2(ed23ts); cby-1(s1281); fem-3(nm63)* identification strains were performed for each *tra-2 (ed23ts); suppressor*.

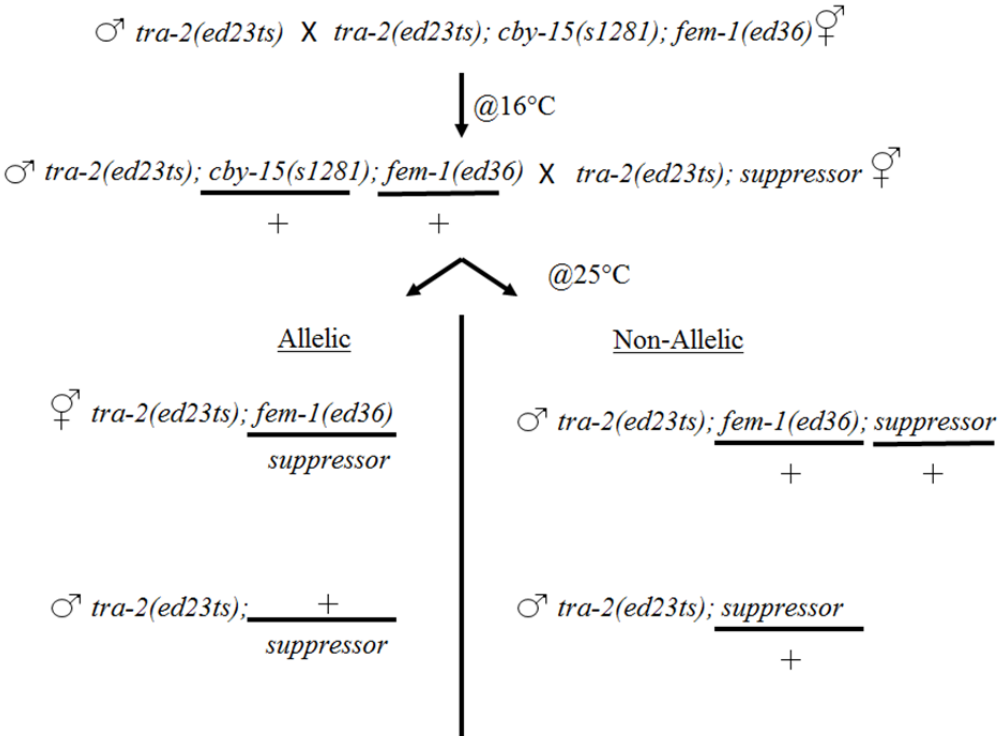

Figure 14. Complementation cross to identify which *fem* gene (if any) a suppressor represented. The above cross was done for each suppressor allele with all three *fem* identifier strains in triplicate. The F1 cross was carried out at the *tra-2(ed23ts)* alleles permissive temperature (16 °C) so that males could be identified and used to cross to the suppressor. The F2 cross was carried out at the *tra-2(ed23ts)* alleles restrictive temperature (25 °C). The suppressor was considered allelic to the *fem* gene in the identifier strain when a ratio of 1:2:1 of hermaphrodites: males: pseudomales was present in the F2. The suppressor was considered non-allelic when a ratio of 1:1 males: pseudomales was present.

2.4 PCR and Sequencing

Genomic DNA was extracted from single worms using 5 µL of Worm Lysis Buffer (50 mM KCL, 10 mM Tris pH 8.3, 2.5 mM $MgCl_2$, 0.45% NP-40, 0.45% Tween-20, 0.01% gelatin) with a final concentration of 1 mg/mL proteinase K. The mixture was heated at 65 °C for 60 minutes, followed by enzyme inactivation at 95 °C for 15 minutes. PCR was performed using 5 µL of the lysis mixture of template, 0.3 µL of Taq DNA Polymerase (New England Biolabs), 2.5 µL of thermobuffer (20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$ 1% Triton X-100, pH 8.8), 1 µL of each primer (10 mM), and 0.5 µL of dNTPs (10 mM) to a final volume of 25 µL. Sequencing was performed by the Molecular Biology Service Unit (MBSU, University of Alberta). Table 5 contains a list of primers used for PCR and sequencing.

2.5 Whole genome sequencing DNA preparation

Clean worms were transferred onto 20 seeded worm plates and allowed to grow until the worms were starved for 1 day. Worms were washed off the plate using M9 buffer and collected in a 15ml tube. Worms were pelleted by centrifugation at 2,000xg for 10 mins before the M9 buffer was aspirated and the worm pellet re-suspended in sterile water. This wash was repeated 2-3 times. The re-suspended worms were rocked at room temperature for 2 hours to allow digestion of any remaining bacteria in their guts. The wash was repeated another 2-3 times before pelleting and freezing at -80°C.

100 µL of the worm pellet was added to 600 µL of worm lysis buffer (50 mM KCL, 10 mM Tris pH 8.3, 2.5 mM $MgCl_2$, 0.45% NP-40, 0.45% Tween-20, 0.01% gelatin) and 20 µL of 20 mg/ml proteinase K. This was incubated in rotating hybridization oven at 60°C overnight.

RNase A was added to a final concentration of 37.5 µg/ml. Phenol chloroform (PCI) was prepared fresh every day in a 25:24:1 phenol: chloroform:

Table 5. List of primers used in PCR and sequencing throughout this work, Forward primers are indicated with an "F". Reverse primers are indicated with an "R".

Name	5'-3' Sequence	Purpose
Fem-1F1	ACA CGA CAG CGT CAT ATT GC	PCR and sequencing
Fem-1R1	TCC AGT TTT GAC GGG AAC TC	PCR and sequencing
Fem-1F2	TTC TCT CGA AAA ATC GCA AA	PCR and sequencing
Fem-1R2	AGT GGT CAA AAG AGC GTT GG	PCR and sequencing
Fem-1F3	AGC CAT CAT TCA AGG ACA CC	PCR and sequencing
Fem-1R3	GAA AGT TAA GCC GCC ATC CT	PCR and sequencing
Fem-1F4	TTC TCC CAC AGC TCC AAA AG	PCR and sequencing
Fem-1R4	GAG CCG AGT TCC TGT AAA CG	PCR and sequencing
Fem-2 seqF1	TGC TAT TCT TCA TTC CAT CCT TG	PCR and sequencing
Fem-2 seqF1B	AGC CTA CGC GTA AAG GTC AA	PCR and sequencing
Fem-2 seqF2	TCC TGT CTG AAA ATT AAA ACT CCA	Sequencing
Fem-2 seqF3B	TGA CCA ATA CGT GAC GGA GA	PCR and sequencing
Fem-2 seqF4	TTT GCT TGG TTG GGA GAT TC	Sequencing
Fem-2 seqF5	TGA CCG AAA AAG TCA AAA AGA G	Sequencing
Fem-2 seqR1	CAA GGA TCC CCG TGG TTA TCT	PCR and sequencing
Fem-2 seqR2	CAT TAC ATC TGC CAC GAA GC	PCR and sequencing
Fem-3F1	TGT TGC ACC GAA AGA CAG AC	PCR and sequencing
Fem-3R1	AGC CAG AGG GAT TGA TGA AA	PCR and sequencing
Fem-3F2	CGA ACG CAT TTC AAT AAC GA	PCR and sequencing
Fem-3R2	GAT GAG GTG GGA TAC GGA GA	PCR and sequencing
Pink-1F1	ATG TCG ATG AAA CGG TTC GG	PCR and sequencing
Pink-1R1	GTA TTT GGC TTG ACT CTC TCG	PCR and sequencing
Pink-1F2	TCC TGA CGC TCT ACA CAC TG	PCR and sequencing
Pink-1R2	GTA AAG ACT GCT CAT GCA CCT	PCR and sequencing
Pink-1F3	CGG ATA TAA CAA TGT GCC TA	PCR and sequencing
Pink-1R3	CCA TAC GTT CTA AGC ATA CGG	PCR and sequencing
Pink-1F4	TGA TGC AGT TAC ATT CTA CG	PCR and sequencing
Pink-1R4	TCC TCC GCA GTT TCT TCG	PCR and sequencing
Tra-1F1	GAA TAC TGA AAG TCG TTC GG	PCR and sequencing
Tra-1R1	ACG TCT TCT GAC TGG TCT	PCR and sequencing
Tra-1F2	CGA AAT GAC TGG TTC CGA TG	PCR and sequencing
Tra-1R2	TCG GGC ATC TGA AAG CAC	PCR and sequencing
Tra-1F3	CTG ATC TTG GAA TAC TTG TGC	PCR and sequencing
Tra-1R3	ACT ATA CTT TGT GTG TGC GT	PCR and sequencing
Tra-1F4	CAG AGT GAC ATT AAG CGT TG	PCR and sequencing
Tra-1R4	ATC CAT CAG AGC AGC GTT G	PCR and sequencing
Tra-1F5	CAC GGA TTA GAA TAT GTG TCG	PCR and sequencing
Tra-1R5	TGC TGT AGA TTC TGC GT	PCR and sequencing
Tra-1F6	GCT AGA CTT GCG ATA GAA CC	PCR and sequencing
Tra-1R6	AAA TGT CAT GGT GAA CAG AC	PCR and sequencing

isoamyl alcohol ratio. 400 µL PCI was added to a 1.5 mL Eppendorf tube. The cell lysate was poured into the 1.5 mL tube containing 400 µL PCI and mixed by inversion for ~5 min before centrifuging at 15 000 x g for 5 min. 400 µL of chloroform: isoamyl alcohol (24:1) was added to another 1.5 mL tube. The aqueous layer (top) was poured from PCI tube to CI tube, and mixed by inversion for 5 min before centrifuging at 15 000 x g for 5 min. 0.1 volumes 3 M pH 5.2 sodium acetate (~40 µL) was added to a new 1.5 mL tube. The aqueous layer (~400 µL) of the sample was poured into the tube containing sodium acetate. 2 volumes of 100% Ethanol (EtOH) (~880 µL) was added and the sample inverted until the solution was fully mixed and DNA was completely precipitated. If very little DNA precipitated the sample was incubated at -20 °C for 1 hr. The sample was centrifuged at 15 000 x g for 15 min before the ethanol was poured off into a waste flask. 1 mL of 70% ethanol was added to wash the pellet which was then centrifuged at 15 000 x g for 5 min. The ethanol was discarded and the wash repeated. The ethanol was poured off and a pipet used to remove excess ethanol from the walls of the tube, without disturbing the pellet. The pellet was dried inverted on a paper towel for no more than 10 min. The DNA was re-suspended in 20-150 µL pH 8.0 TE depending on its size. If needed the pellet was incubate at room temperature until it dissolved (~ 5 min). After a minimum of 3 days, the concentration of DNA was determined using a Nano-drop. The desired amount of DNA was at least 5 µg of DNA at a concentration of at least 40ng/µl. DNA library preparation was carried out by Delta Genomics and sequencing was done by Génome Québec.

2.6 Mapping

Mapping was carried out to test the identification of candidates for the non-*fem* suppressors following whole genome sequencing (figure 15). Hermaphrodites from the suppressor strains *ed30*, *ed31* and *ed34,* which were present in an AF16 background, were crossed to *tra-2(ed23ts)* males, in a HK104 background, at 16

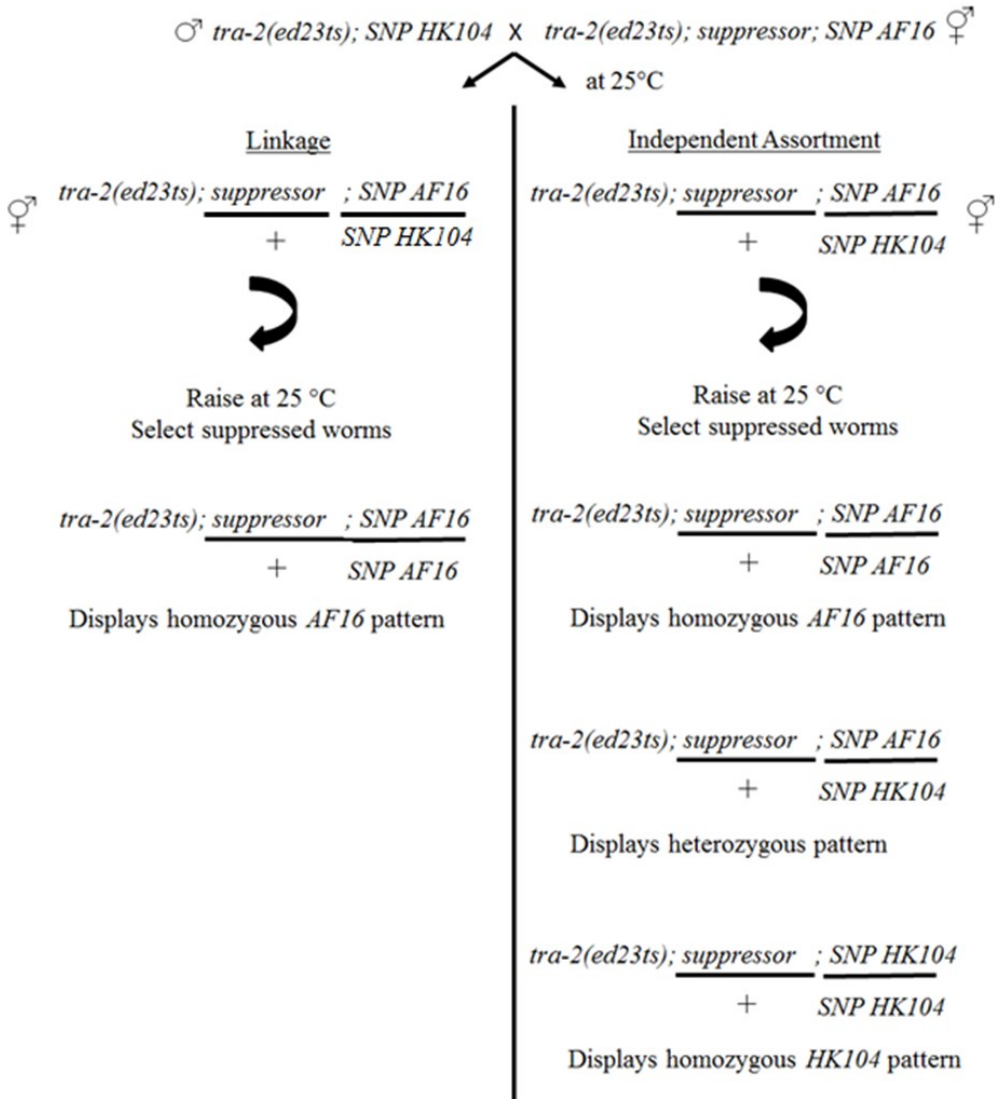

Figure 15: Mapping strategy used to confirm the location of non-fem suppressor candidates. Males from the *tra-2(ed23ts)* strain in the HK104 background were crossed to a hermaphrodite of the *tra-2(ed23ts); sup* strain. The F2 generation was raised at 25 °C, and suppressed worms were individually selected for genotyping. If the suppressed phenotype was difficult to identify, hermaphrodites were singled and allowed to self. A hermaphrodite whose progeny displayed only the suppressed phenotype was used for mapping. An indel ratio of 1 AF16: 2 heterozygous: 1 HK104 indicated that the suppressor was not linked to the indel. Linkage was evident when the Sup animals only displayed homozygous AF16 bands. Modified from (Dewar, 2011)

°C. F1 progeny were then singled and allowed to self-fertilize at 25°C. The F2 progeny were then scored for suppression of the Tra phenotype. In some instances the F2 generation gave a range of phenotypes and it was difficult to determine which individuals were suppressors. Hermaphrodites were allowed to self and those which produced only hermaphrodite offspring were used for mapping. F3 worms were washed into lysis buffer (15 µL) and a DNA extraction was performed. Worm extractions were numbered and split into three. Two samples were examined for indel markers which were located on the same chromosome as the suppressor candidate. The third sample was examined for an indel marker on a different chromosome which was expected to assort independently (table 6) (Koboldt *et al*., 2010). The location of the suppressor was confirmed when all of the isolates displayed the AF16 banding pattern for one indel marker. In contrast, a pattern of 25% homozygous AF16 bands, 25% homozygous HK104 bands, and 50% heterozygous bands for a particular indel marker indicated independent assortment. χ^2 analysis was used to determine whether or not there was a significant deviation from Mendelian ratios to infer linkage.

2.7 Mutant construction and identification

To make *tra-1(ed30)/tra-1(nm2)* mutants *tra-1(nm2)* Tra males were mated to *tra-1(ed30)* hermaphrodites and the progeny were scored. To make *tra-1(nm2); fem-3(ed34)* double mutants *tra-1(nm2)* Tra males were crossed to *fem-3(ed34)* hermaphrodites. Progeny were phenotypically wild-type and heterozygous for both genes of interest. F1 hermaphrodites were singled and allowed to self-fertilize. It was expected that 1/16 of self-progeny would be homozygous for both genes. 30 Worms which displayed the Tra phenotype were selected for genotyping. The same crosses were carried out for constructing *pink-1(ed31); tra-1(nm2)* double mutants. Genotyping was carried out using Simple Allele-discriminating PCR (SAP) (Bui and Liu, 2009). Forward primers were designed that would only amplify the wild-type or mutant allele and paired with a common

Table 6. List of primers used in mapping throughout this work.

Indel	Chromsome	Location (cM)	Forward primer (5'-3')	Reverse primer (5'-3')	AF16 Amplicon	HK104 Amplicon	Restriction Enzyme
cb-m142	1	12.6	AAGGCCTTAAAAATGAAGATAAT	TGAAAATTGAAAAACCTAGAAAA	700	950	N/A
cb-m6	1	43.93	TTAATGCTGGACCAAAAGTC	CCTGCAATTTTTGTGTTTTT	900	1000	N/A
cb-m19	2	11.88-13.10	GAGAGAGAACAGGTGGTTTG	GAGTTGAACGTTTTGATTGAA	1013	750	N/A
cb-m26	2	27.92-33.95	CGTTGAACATTAATTTGAAGAGT	AGACCAGAAGACTGGAATGAG	700	410	N/A
cb-m46	3	6.07	GAATCCATGTGATTTGAGAGAC	TGGCTCAGAGTTGAGAGACT	1020	750	N/A
bdP1	3	31.3	CAGGGAGCGGACAACTTCAAAGG	GGACACAGCCCAGGATTAGCGAC	~1700	~1500	N/A
cb56202	4	37.5236-41.1551	GGTTGACTTCAGTGAAATTGAT	ATGTCTGTGATGCTCCTAATG	60, 190, 489	60, 679	DraI
cb-m177	4	9.1912-9.776	AAAACACTTTCCAAAATTTGATT	GAGAATTTTAAACTTGCATAATGA	800	900	N/A
cb-m103	5	19	AGGTGAGAGTTTTTGACTTTCTT	TTACATTGTTCAGTTGGAAACTT	700	500	N/A
cb-m97	5	46.07	AGATAATGGGCGAAACAGTAG	ATCACTCAAGGTACTGTAGATTTTT	705	490	N/A

reverse primer which worked for both alleles (table 7). DNA from animals was extracted as described above and split into two samples. One sample was genotyped for the wild-type allele, the other for the mutant allele. Amplification in one sample and not the other indicated homozygosity for that allele. Amplification in both samples indicated heterozygosity.

2.8 *C. nigoni* sequence analysis

DNA and protein sequences for *Caenorhabditis* species were obtained from wormbase.org and other organism sequences were obtained using BLAST. All alignments were made using ClustalW. Predictions of functional domains were made using SMART. Sequence analysis of *C. nigoni* was carried out using the sequence data available from the 959 nematode genomes website. *C. briggsae* sequences were BLASTed against a database containing scaffold assemblies of the *C. nigoni* genome. Scaffolds were copied to a text editor and assembled manually to achieve the best fit with known *C. briggsae* sequences (Stothard, 2000).

Table 7. List of primers used in allele Simple Allele-detecting PCR (SAP).

Name	Amplified Allele	5'-3' Sequence
Cbr PINK-1 SAP WT F-1	WT	GCT ACA GCT GTT CCT GGG A
Cbr PINK-1 SAP Mut F-1	*ed31*	GCT ACA GCT GTT CCT GGT T
Cbr PINK-1 SAP R-1	WT & *ed31*	CAG CAA ACG GAG ACT CAC TTC C
Cbr Fem-3 SAP WT F-1	WT	CTA CTT CCA AGA TGA AAC GTG GC
Cbr Fem-3 SAP Mut F-1	*ed34*	CTA CTT CCA AGA TGA AAC GTG GT
Cbr Fem-3 SAP WT R-1	WT & *ed34*	GAG CTG TGT AGT CAA TGT CC
Cbr Tra-1 SAP WT F-1	WT	CTT CCA ATC ATC GCA CCC TAC
Cbr Tra-1 SAP Mut F-1	*ed30*	CTT CCA ATC ATC GCA CCC TAT
Cbr Tra-1 SAP R-1	WT & *ed30*	GGA CGT ACA GCT TGA AAA CTC

3.0 Results

3.1 Complementation and molecular lesion identification

Based on previous identification of *Cbr-tra-2* suppressors, it was expected that the majority of the suppressors would represent *Cbr-fem* mutants (Carvalho, 2005; Dewar, 2011). Of the 32 suppressors that have been identified, there were 3 suppressors that complement all three *Cbr-fem* genes as well as each other. These suppressors were *ed30, ed31* and *ed34*. To continue characterizing the *Cbr-tra-2* suppressors would likely reveal more *Cbr-fem* alleles with the possibility of identifying novel SD pathway members or additional alleles of the suppressor gene in *ed30, ed31* and *ed34*. Sequencing the *Cbr-fem* mutants has shown the location of functionally important domains in these proteins, but the process is time consuming and unlikely to yield much more new information. The *Cbr-tra-2* suppressor were all hermaphrodites, which means that identifying the causative mutations in them are unlikely to tell us how *C. briggsae* makes sperm.

Sequencing was carried out in order to identify the sites of mutations in the *Cbr-fem* alleles isolated in the *Cbr-tra-2* suppressor screen (table 4). It was expected that some of these suppressors contained nonsense mutations that lead to truncations and loss of functional domains. Other suppressors would be the result of point mutations. If these missense mutations occurred in a known functional domain, the substituted residue would likely be a functionally critical one. If the missense mutation occurred outside a known functional domain, it may indicate a previously unrecognized area of importance in the protein. Cbr-FEM-2 is a member of the PP2C phosphatase family and has a well-known protein structure (figure 10). Cbr-FEM-1 cannot be placed into any known protein family, but is known to contain several ankyrin repeat domains (ANK) (figure 9). Similarly, Cbr-FEM-3 cannot be placed in a protein family and does not contain any conserved motifs (figure 11).

3.1.1 C. briggsae fem-1 alleles

Of the mutations isolated from the *tra-2(ed23ts)* genetic screen, 11 novel *Cbr-fem-1* alleles, 9 novel *Cbr-fem-2* alleles and 5 novel *Cbr-fem-3* alleles have been identified (table 4). 2 of the *Cbr-fem-1* alleles are predicted to alter splicing and cause truncation of the protein, while 7 were nonsense mutations and 1 was a missense mutation. One suppressor that failed to complement *Cbr-fem-1* did not contain a mutation in the coding region and so may occur in the regulatory region of that gene. The missense mutation occurred at residue 136 and resulted in the substitution of leucine with serine. This residue was conserved between *C. elegans* and *C. briggsae* and was located within the ankyrin repeat domain of the protein (figure 9).

3.1.2 C. briggsae fem-2 alleles

The mutations in 4 of the 9 novel *Cbr-fem-2* alleles have been identified; 3 of these were nonsense mutations and 1 was a missense mutation (table 4). The nonsense mutations were situated before the phosphatase domain of Cbr-FEM-2 resulting in a truncated protein without this functional region. The missense mutation occurred at residue 70 and was a substitution of arginine with cysteine. Like the *Cbr-fem-1* missense mutation this arginine amino acid was conserved between *C. elegans* and *C. briggsae* (figure 10).

3.1.3 C. briggsae fem-3 alleles

3 of the 4 novel *Cbr-fem-3* alleles have been identified and all are nonsense mutations (table 4). 2 of these alleles were represented by the same molecular lesion, a C to T transition at nucleotide 202, which resulted in a glutamine codon becoming a stop codon. The other identified lesion was an

arginine to stop nonsense mutation. Both lesions occurred near the N-terminus of the protein, at residues 67 and 90 respectively (figure 11).

3.2 Identification of non-*fem* suppressors of *tra-2(ed23ts)*

Three *tra-2(ed23ts)* suppressors, *ed30, ed31* and *ed34*, complemented all three *Cbr-fem* genes and complemented each other (Carvalho, 2005; Dewar, 2011). Previous analysis showed that, like the *Cbr-fem* mutants, these suppressors were autosomal, recessive and not *tra-2(ed23ts)* reversions (Carvalho, 2005). Whole genome sequencing was used to identify which genes differed from the reference genome. It was expected that each of these suppressors would contain indels and point mutations in many different genes as a result of spontaneous mutation and due to the mutagenesis that was used to create them.

To identify the lesions which were the result of spontaneous mutation, the working laboratory strain of AF16 which was originally mutagenized, was sequenced and any identical mutations found between it and the suppressors was subtracted from the pool of suppressor candidates. Each suppressor contained an average of 357 SNPs and 12 indels. Of these 357 SNPs, 10 of them were located in a coding region, on average. Candidates were selected based primarily on whether they were a known SD gene. If there was not a mutation in a SD gene, then genes with a known function and predicted mutant phenotype were selected. The severity of the mutation was also taken into consideration.

3.2.1 tra-2(ed23ts); sup(ed30)

3.2.1.1 tra-2(ed23ts);sup(ed30) phenotype

tra-2(ed23ts);sup(ed30) hermaphrodites were phenotypically normal with a double-armed gonad capable of both sperm and oocyte production and the characteristic long, thin tail of a female soma. Like wild-type hermaphrodites they

were capable of self-fertilization, and self-progeny also appeared to be wild-type (figure 16). Unlike the *tra-2(ed23ts); fem* mutants previously discussed, XO *tra-2(ed23ts);sup(ed30)* worms were capable of developing as males. These males showed incorrect development of the somatic tail tissue and also produced large cells that resembled fertilized eggs after several rounds of division (figure 16). *tra-2(ed23ts);sup(ed30)* males also produced sperm so it is possible that these were fertilized eggs but they did not survive to hatch into larval worms. The males did not display mating behavior and also did not survive long into adulthood (Carvalho, 2005; Dewar, 2011).

3.2.1.2 tra-2(ed23ts);sup(ed30) whole genome sequencing, candidate identification and mapping

Whole genome sequencing identified 8 mutations with predicted effects on genes that could possibly suppress the *tra-2(ed23ts)* phenotype. All of the mutations found were missense mutations in a variety of different genes (table 8). 10 indels were also found but none of these were present in the coding region of a gene. *Cbr-tra-1,* on chromosome III, was selected as the candidate gene in *tra-2(ed23ts);sup(ed30)* which could be responsible for the phenotypes described above. Preliminary mapping showed that *tra-2(ed23ts);sup(ed30)* assorted independently from a region on chromosome I where *Cbr-fog-3* is situated, a region of chromosome II where *Cbr-tra-2* is situated and region of chromosome IV where *Cbr-fem-3* and *Cbr-fem-1* are found (Dewar, 2011). This was consistent with the hypothesis that *tra-2(ed23ts);sup(ed30)* was *Cbr-tra-1*. To test *Cbr-tra-1* as a candidate for *tra-2(ed23ts);sup(ed30)*, the suppressor was mapped to two indel markers, cb-m46 and bdp1, located on opposite ends of chromosome III where *Cbr-tra-1* is located (figure 17).

80 F2 animals which could suppress *tra-2(ed23ts)* were selected and genotyped for cb-m46, bdp1 and cb-m26, an indel marker on chromosome II. It was expected that *tra-2(ed23ts);sup(ed30)* would be linked to one or both of the

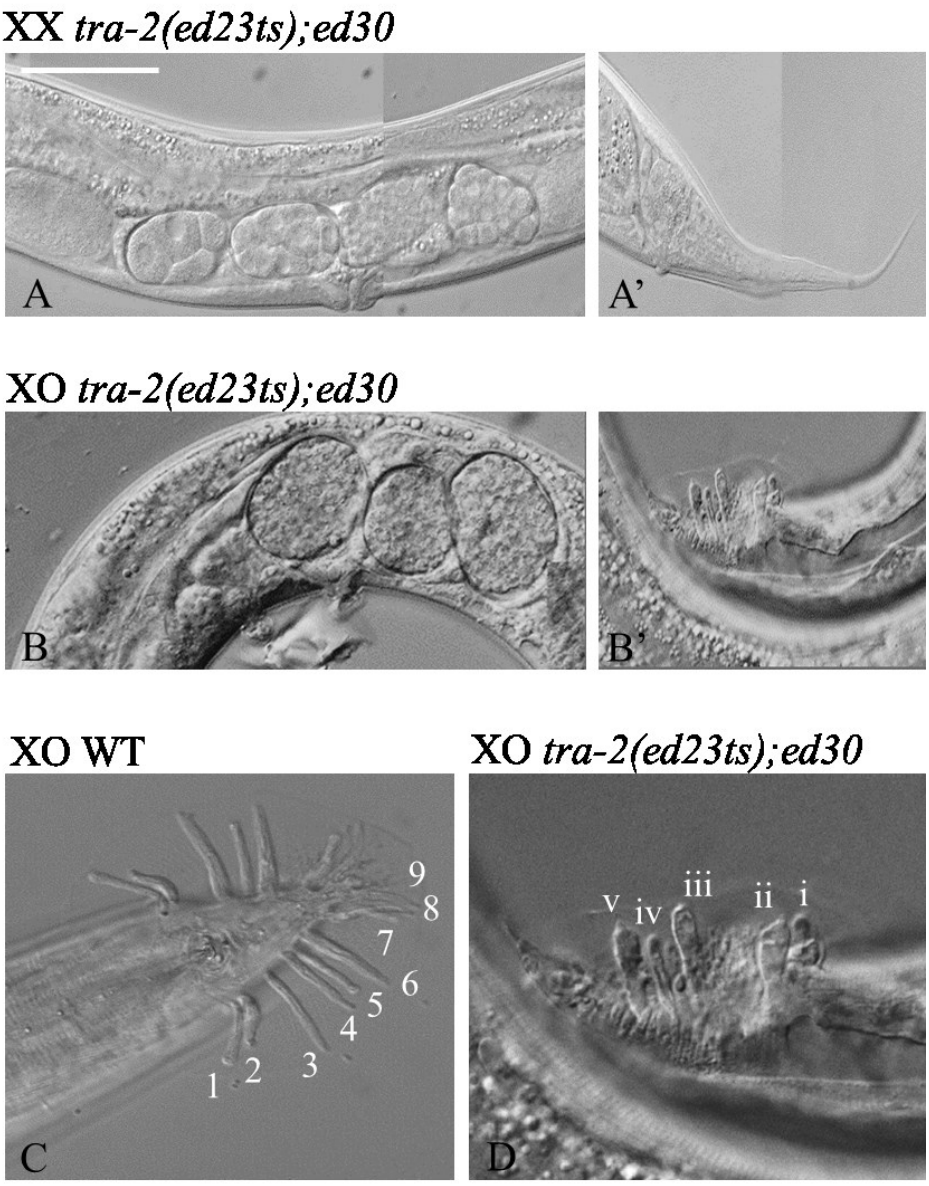

Figure 16. DIC images of suppressor *ed30* phenotypes. XX *ed30* animals develop as hermaphrodites and are phenotypically wild-type (A, A'). Scale bar = 50 μm. They have a double armed gonad which produces sperm and oocytes and are capable of self-fertilization (A). XO *ed30* animals are somatically male but have a female germ line consisting of sperm and oocytes (B). They also show abnormal male tail development as evident by enlarged tail rays (B'). Comparisons between wild-type (C) and XO *ed30* tail rays (D). XO *ed30* animals are missing several tail rays and those that are present are large and misshapen. There is also a loss of bilateral symmetry between the tail rays on either side of XO *ed30* animals (not shown). Wild-type tail rays are labelled 1-9 (C). *ed30* tail rays are labelled i-v (D). Position and numbering of wild-type tail rays does not corresponding to position and numbering of *ed30* tail rays.

Table 8. List of mutated genes in DP374 (*tra-2(ed23ts)*; *ed30)*

Gene	Chromosome	Description	Phenotypes	Mutation
Cbr-gcy-19	II	predicted transmembrane guanylyl cyclase	No Phenotype	Missense
Cbr-tra-1	III	GLI family transcription factor	feminization of XX and XO animals	Missense
Cbr-elo-1	IV	Component of C-18 polyunsaturated fatty acid (PUFA) elongase	fat content increased	Missense
Cbr-pps-1	IV	orthologous to human PAPSS1	Sterility, shortened lifespan, lethality	Missense
Cbr-egl-4	IV	cyclic GMP-dependent protein kinase	aldicarb resistant, butanone chemotaxis	Missense
Cbr-srb-17	IV	Serpentine Receptor, class B (beta)	No Phenotype	Missense
Cbr-nrs-2	V	Unknown	No Phenotype	Missense
Cbr-ret-1	V	RETiculon protein	No Phenotype	Missense
Cbr-tps-1	X	Trehalose 6-Phosphate Synthase	shortened life span , thermotolerance reduced	Missense
Cbr-spr-3	X	Suppressor of PResenilin defect	maternal sterile, organism development variant, sterile progeny	Missense
Cbr-utx-1	X	putative histone H3 di/trimethyllysine-27	bag of worms, early larval lethal, embryonic lethal	Missense
Cbr-ram-5	X	novel transmembrane protein	male ray morphology variant, ray tips swollen	Splice acceptor

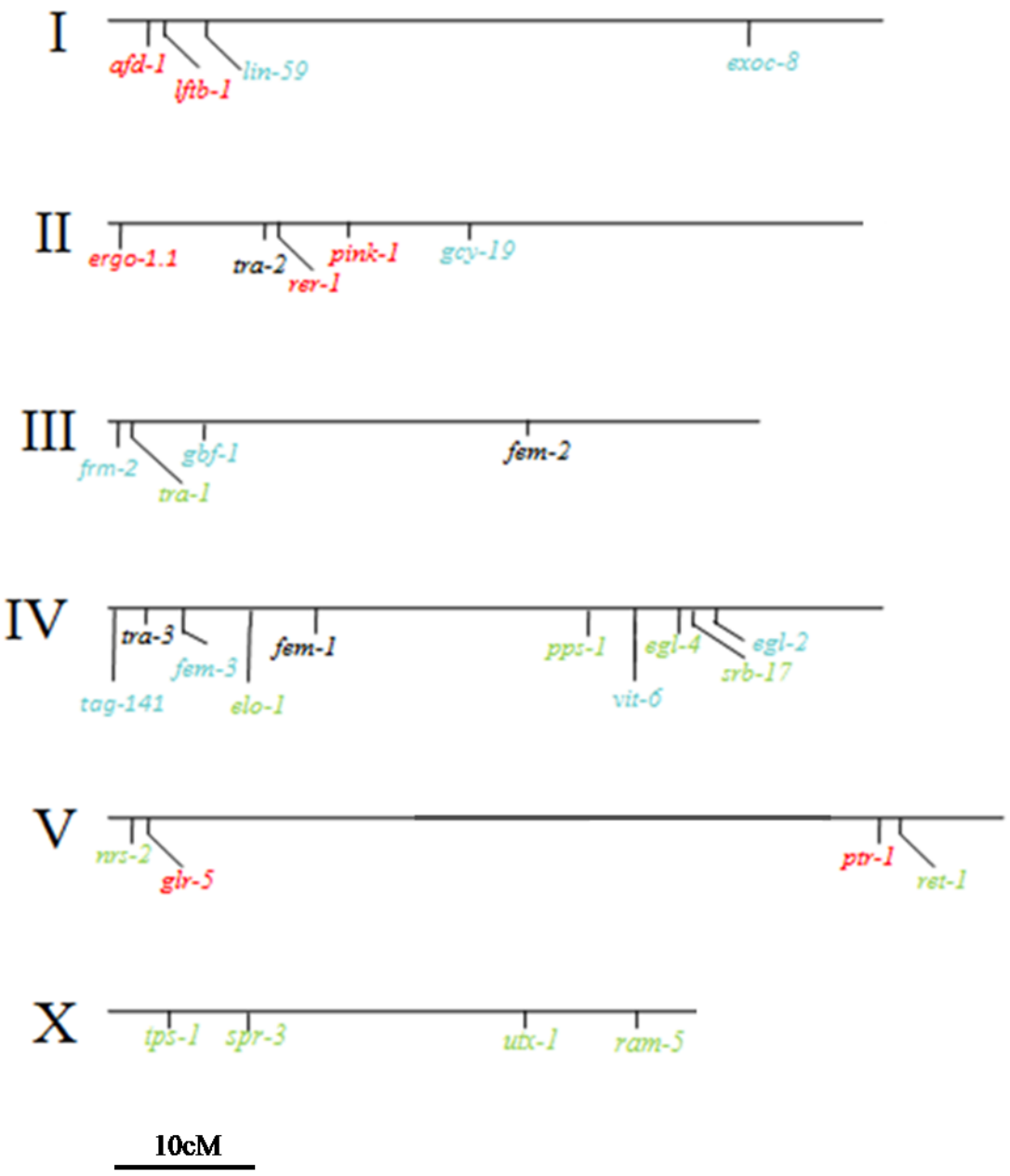

Figure 17. Schematic of the *C. briggsae* genetic linkage map with the candidate genes for suppressors *ed30, ed31* and *ed34*. Genes which were mutated in the suppressor *ed30* are shown in green. Genes which were mutated in the suppressor *ed31* are shown in red. Genes which were mutated in the suppressor *ed34* are shown in blue.

chromosome III markers but would assort independently with cb-m26. 80 animals were genotyped for these three markers. 80 animals were homozygous AF16 cb-m46, which was inconsistent with independent assortment (χ^2 = 160, df=1, p<0.0001). 75 animals were homozygous AF16 bdp1 with 1 animal being a heterozygote, which was also indicative of linkage (χ^2 = 148, df=1, p<0.0001). 15 animals were homozygous AF16 cb-m26, 47 were heterozygotes and 18 animals were homozygous HK104 cb-m26 which showed independent assortment as expected (χ^2 =0.225, df=1, p=0.6353). Conveniently, the only candidate present on chromosome III was *Cbr-tra-1* which is known to play a critical role in *C. briggsae* SD. This allele of *Cbr-tra-1* contained a missense mutation resulting in a proline to leucine substitution at position 90. This substitution was present in a region which corresponds to the gf region of Ce-TRA-1 (de Bono *et al.,* 1994). The phenotypes of XX and XO animals were consistent with *tra-2(ed23ts);sup(ed30) tra-1* being a weak gain of function mutation.

3.2.1.3 ram-5 mutants

To determine the feminizing activity of a single copy of gf *tra-1(ed30),* a *tra-1(ed30)/tra-1(nm2)* mutant was created. Since *tra-1(nm2)* heterozygotes appeared to be completely normal, I expected that the gain of function allele would provide sufficient TRA-1 activity that *tra-1(ed30)/tra-1(nm2)* mutants would not be transformed and XX and XO animals would appear wild-type. In addition, since *tra-1(ed30)* was also a recessive allele I expected *tra-1(ed30)/tra-1(nm2)* XX animals to look normal, as they do in *tra-1(ed30)* animals and that the germline feminization in XO animals would be reduced or absent. The hermaphrodites from this cross appeared wild-type, showing that there was at least enough TRA-1 activity from a single copy of the gf *tra-1(ed30)* allele to facilitate normal development. The males however showed deformed tail rays that appeared more disorganized and swollen than in *tra-1(ed30)* homozygotes (figure 18). The severity of the swelling but not the disorganization of the tail rays resembled Ce-

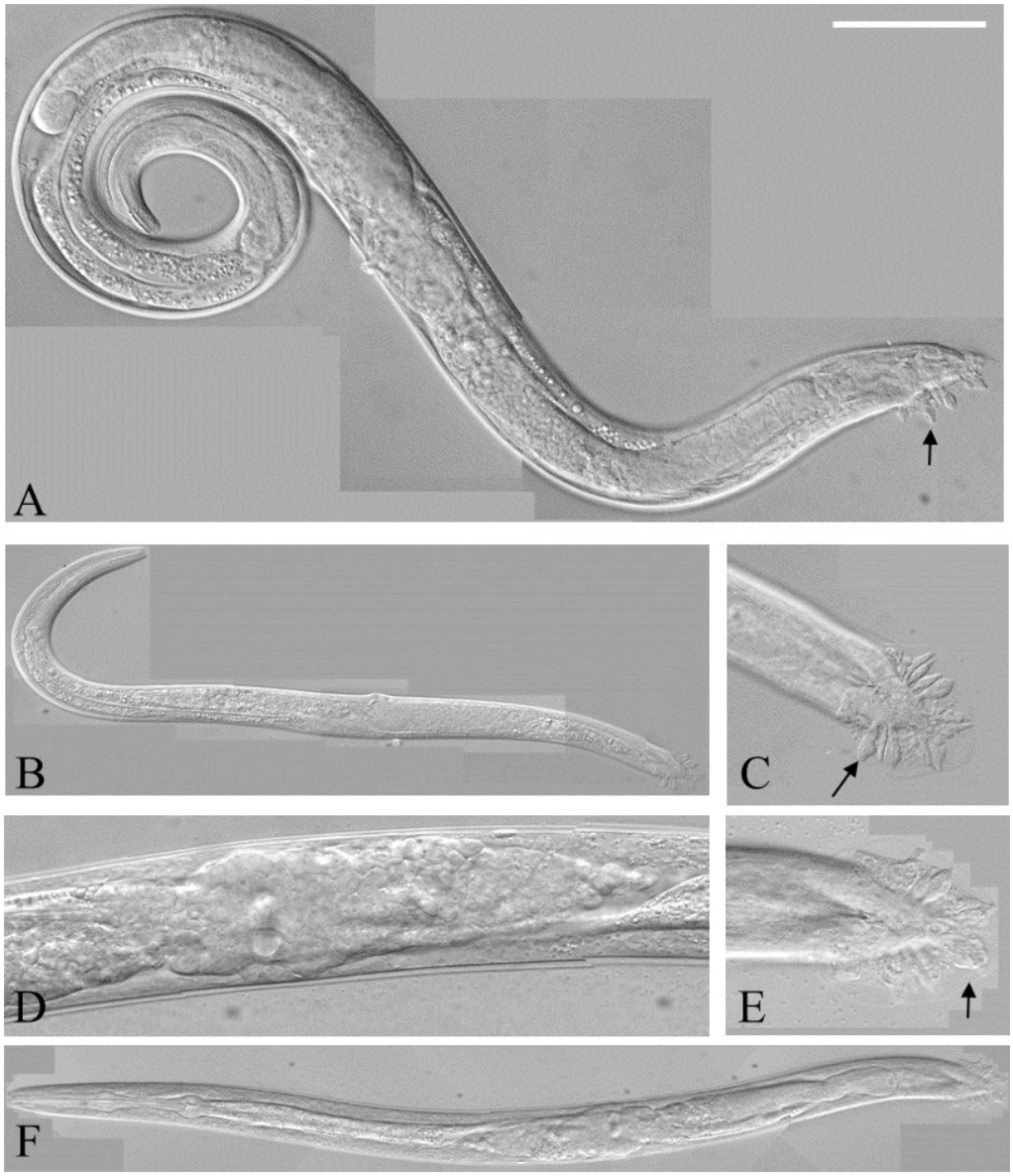

Figure 18. DIC images of *tra-1(ed30)/tra-1(nm2)* males (A-F). Scale bar = 100 µm. XX animals (not shown) were found to be phenotypically normal. These gain of function/ loss of function mutants showed a mildly disorganized gonad but lacked the ooids found in homozygous *tra-1(ed30)* mutants. The tails of the *tra-1(ed30)/tra-1(nm2)* XO animals appear to be missing several tails rays and the ones that are present are enlarged (arrows).

RAM (RAy Morphology) mutant phenotypes in *C. elegans* (Yu *et al.*, 2000). This was consistent with *ed30* having a splice site mutation in the X-linked *Cbr-ram-5* gene. The germlines of the male *tra-1(ed30)/tra-1(nm2)* mutants appeared to be relatively normal in comparison to *tra-1(ed30)* homozygotes. They lacked any kind of ova or embryo like structures and instead more closely resembled the germlines of *tra-1(nm2)* animals. The lumpy tail phenotype is likely caused by a *Cbr-ram-5* mutation but the disorganization is probably a result of the *tra-1(ed30)* allele.

3.2.2 tra-2(ed23ts); sup(ed31)

3.2.2.1 tra-2(ed23ts); sup(ed31) phenotype

tra-2(ed23ts); sup(ed31) XX animals develop as wild-type hermaphrodites, showing complete suppression of the *tra-2(ed23ts)* phenotype (figure 19). XO animals were capable of developing as males; they had a single armed gonad that exclusively produced sperm. Their tails exhibited a small degree of feminization as shown by their incorrect tail ray patterning (figure 19). Despite this they displayed normal mating behavior and are capable of mating. Past work demonstrated that the average brood size for self-fertilizing *tra-2(ed23ts);ed31* hermaphrodites at 16 °C was not significantly different from *tra-2(ed23ts)* or *tra-2(ed23ts); fem-1(ed36)* hermaphrodites at 16 °C (Carvalho, 2005; Dewar, 2011).

It has been previously shown that this strain displayed a range of suppressed phenotypes in the F2 generation during mapping. F2 hermaphrodites were capable of producing either Tra and Sup progeny or Sup progeny alone at 25°C (Dewar, 2011). To overcome this, F2 hermaphrodites were singled and F3 progeny examined. Only hermaphrodites which produced all Sup progeny were used for mapping.

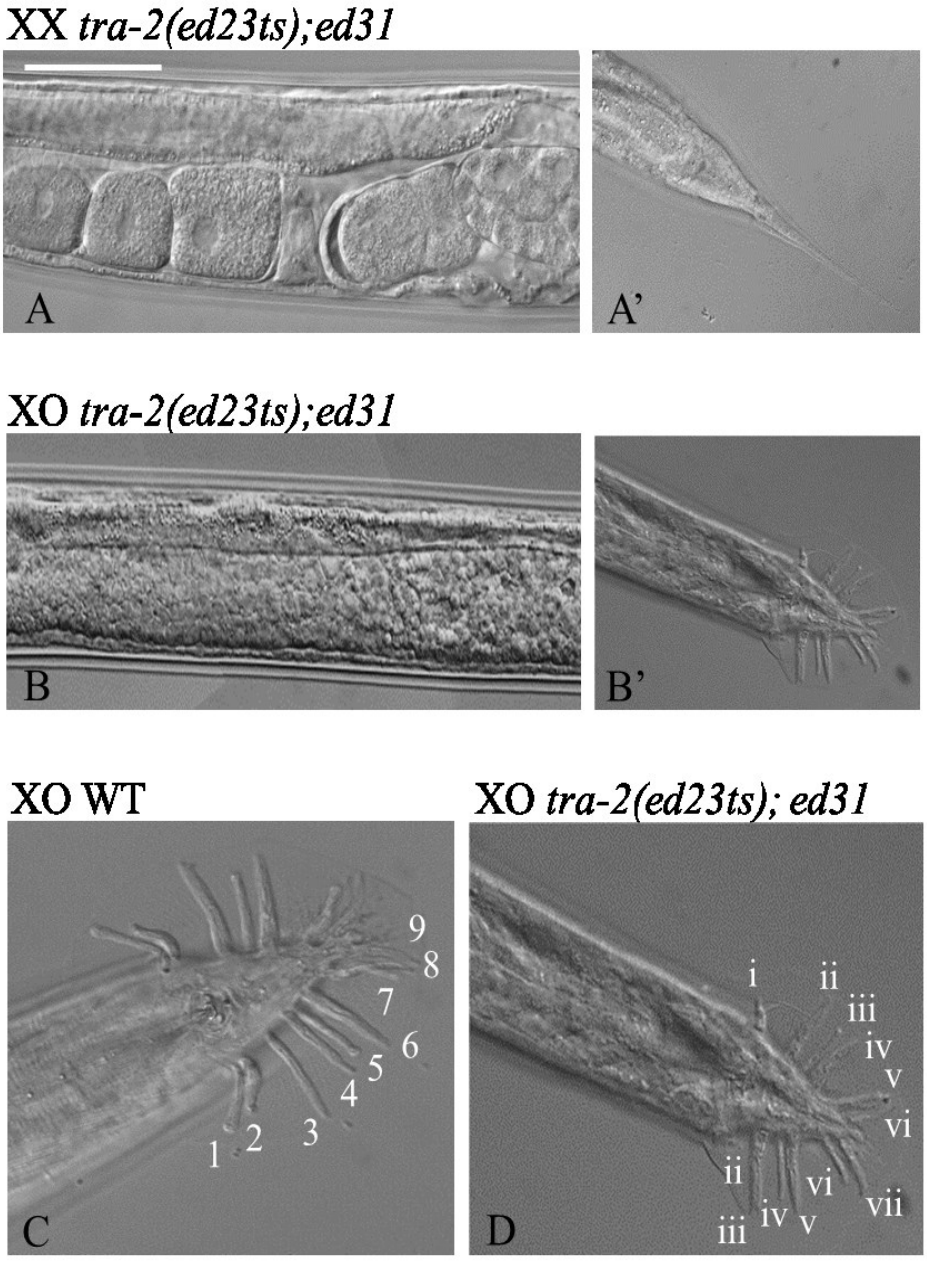

Figure 19. DIC images of suppressor *ed31* phenotypes. Scale bar = 50 μm. XX *ed31* animals develop as hermaphrodites and are phenotypically wild-type (A, A'). They have a double armed gonad which produces sperm and oocytes and are capable of self-fertilization (A). XO *ed31* animals show have a normal male germline (B) but have a slightly feminized soma as indicated by the abnormal arrangement of the tail rays. (B'). Comparisons between wild-type (C) and XO *ed31* tail rays (D). XO *ed31* animals are missing several tail rays and those that are present are large and misshapen. Wild-type tail rays are labelled 1-9 (C). *ed31* tail rays are labelled i-vii (D). Position and numbering of wild-type tail rays does not corresponding to position and numbering of *ed31* tail rays. There is also a loss of bilateral symmetry between the tail rays on either side of XO *ed31* animals.

3.2.2.2 tra-2(ed23ts); sup(ed31) whole genome sequencing candidate identification and mapping

Sequencing of the *tra-2(ed23ts); sup(ed31)* genome showed 7 mutations with predicted effects on genes that could be responsible for suppressing the *tra-2(ed23ts)* phenotype and causing the unique phenotypes in *tra-2(ed23ts); sup(ed31)* males (table 9). 10 indels were also found but none of these were present in the coding region of a gene. None of these genes had been previously reported to function in a SD capacity. *Cbr-pink-1* was the only gene in *tra-2(ed23ts); sup(ed31)* which contained a nonsense mutation. Previous mapping showed that this suppressor was unlinked to *Cbr-fem-1, Cbr-fem-3*, *Cbr- fog-3* or *Cbr-tra-2* (Dewar, 2011). To test if *Cbr-pink-1* is genetically linked to *tra-2(ed23ts); sup(ed31)*, the suppressor was mapped with two indel markers, cb-m19 and cb-m26 on opposite ends of chromosome II, where *Cbr-pink-1* is located (figure 17).

In total, 144 F2 animals which could suppress *tra-2(ed23ts)* were selected and genotyped for cb-m19, cb-m26 and cb-m46, an indel marker on chromosome III. It was expected that the suppressor in *tra-2(ed23ts); sup(ed31)* would be linked to one or both of the chromosome II markers but would assort independently with cb-m46. 144 animals were genotyped for these three markers. 136 animals were homozygous AF16 cb-m19, which was inconsistent with independent assortment (χ^2 = 257.778, df=1, p<0.0001). 37 animals were homozygous AF16 cb-m26 with 95 animals being heterozygotes. No HK104 homozygotes were found. These results were also indicative of linkage (χ^2 = 20.742, df=1, p<.0001). 26 animals were homozygous AF16 cb-m46, 66 were heterozygotes and 34 animals were homozygous HK104 cb-m46 which showed independent assortment, as expected (χ^2 =1.016, df=1, p=0.3135).

Chromosome II contained three candidate genes for *tra-2(ed23ts); sup(ed31)*; *Cbr-ergo-1.1*, *Cbr-rer-1* and *Cbr-pink-1*. In *C. elegans Ce-ergo-1.1* encodes an argonaute protein that stabilizes endogenous siRNAs (Yigit *et al.,*

Table 9. List of mutated genes in DP373 (*tra-2(ed23ts); ed31)*

Gene	Chromosome	Description	Phenotypes	Mutation
Cbr-pink-1	II	PTEN-Induced ser/thr Kinase	mitochondrial DNA repair variant, protein aggregation variant, transgene, abnormal brood size	Nonsense
Cbr-afd-1	I	AFaDin. Actin filament binding protein homolog	antibody staining increased, meiosis variant, chromosome instability, fewer germ cells, organism development variant	Missense
Cbr-iftb-1	I	Translation initiation factor 2 beta ortholog	apoptosis fails to occur, apoptosis reduced, cell membrane organization	Missense
Cbr-ergo-1.1	II	Endogenous-RNAi deficient arGOnaute	Unknown	Missense
Cbr-rer-1	II	Retention in Endoplasmic Reticulum homolog	Unknown	Missense
Cbr-glr-5	V	Kainate (non-NMDA)-type ionotropic glutamate receptor subunit	Unknown	Missense
Cbr-ptr-1	V	Sterol sensing domain (SSD) protein	body vacuoles, breaks in alae, lethal, locomotion defects, molt defects	Missense

2006; Guang *et al.,* 2008; Genet *et al.,* 2010). In *tra-2(ed23ts); sup(ed31), Cbr-ergo-1.1* contains a missense mutation that causes a leucine to phenylalanine substitution, both of which have similar properties. *Cbr-rer-1* is predicted to be involved in protein transport in the endoplasmic reticulum, although there are no phenotypes associated with its mutation in *C. elegans*, as this function is inferred from homology (*C. elegans* sequencing consortium, 1999). In *tra-2(ed23ts); sup(ed31)* it contained an isoleucine to asparagine substitution, that could have potentially been deleterious. *Cbr-pink-1* codes for a serine/threonine kinase that has orthologs in *Drosophila* and Humans. Human Hs-PINK-1 has been implicated in familial forms of Parkinson's disease (reviewed in Nuytemans *et al*., 2010). In *C. elegans* Ce-PINK-1 is required for mitochondrial homeostasis, oxidative stress response and correct neurite outgrowth (Park *et al*., 2006; Clark *et al*., 2006; Samann *et al.*, 2009). Ce-PINK-1 is broadly expressed throughout different tissue types, including neurons, muscles and vulval tissue and has been shown to localize to both the cytoplasm and mitochondria. The *tra-2(ed23ts); sup(ed31) Cbr-pink-1* mutation resulted in a lysine codon being replaced by a stop codon resulting in a truncated Cbr-PINK-1 which was missing the C-terminus of its kinase domain. Due to the severity of the mutation and the broad roles it has, *Cbr-pink-1* may be a novel member of the *C. briggsae* SD pathway.

3.2.2.3 tra-2(ed23ts); sup(ed31) pathway position

From its ability to suppress the *tra-2(ed23ts)* allele, *ed31* acted downstream of Cbr-TRA-2 and from the feminized phenotypes of the *tra-2(ed23ts); sup(ed31)* males I hypothesized that it acted at the same point of the pathway as the *Cbr-fem* genes. Alternatively, it may have acted downstream of Cbr-TRA-1. To test this a *tra-1(nm2)* mutant was crossed into the *ed31* strain. If *ed31* is upstream of *Cbr-tra-1* than some animals that display a Tra phenotype would be homozygous for *ed31*. 32 Tra animals were genotyped and 2 were found to be homozygous for *ed31*. These Tra animals appear identical to *tra-1(nm2)* homozygotes. If *Cbr-pink-*

1 is part of the *C. briggsae* SD pathway, these results put it upstream of *Cbr-tra-1* but downstream of *Cbr-tra-2*.

3.2.3 tra-2(ed23ts); sup(ed34)

3.2.3.1 tra-2(ed23ts); sup(ed34) phenotype

tra-2(ed23ts); sup(ed34) hermaphrodites had a normal female soma and a double-armed gonad capable of both sperm and oocyte production, indicative of *Cbr-tra-2* suppression. They were capable of self-fertilization and self-progeny appeared to be wild-type hermaphrodites (figure 20). XO *tra-2(ed23ts); sup(ed34)* worms were also capable of developing as males. The males showed mild feminization of the soma as indicated by incorrect development of the tail rays. There was also disorganization of the single-armed gonad but these males were still capable of sperm production and were able to successfully mate (figure 20) (Carvalho, 2005; Dewar 2011).

Like *tra-2(ed23ts)*; *sup(ed31),* it has been previously shown that the *tra-2(ed23ts); sup(ed34)* mutant displayed a range of suppressed phenotypes in the F2 generation during mapping. F2 hermaphrodites were capable of producing either Tra and Sup progeny or Sup progeny alone at 25°C (Dewar, 2011). To overcome this, F2 hermaphrodites were singled and F3 progeny examined. Only hermaphrodites that produced all Sup progeny were used for mapping.

3.2.3.2 tra-2(ed23ts); sup(ed34) whole genome sequencing, candidate identification and mapping

Whole genome sequencing revealed a total of 9 mutations with predicted effects on genes which could suppress *tra-2(ed23ts)*. It also contained 17 indels, 3 of which were present in hypothetical proteins and the remainder were in non-coding regions of the genome (table 10). *Cbr-fem-3* was selected as the candidate gene in

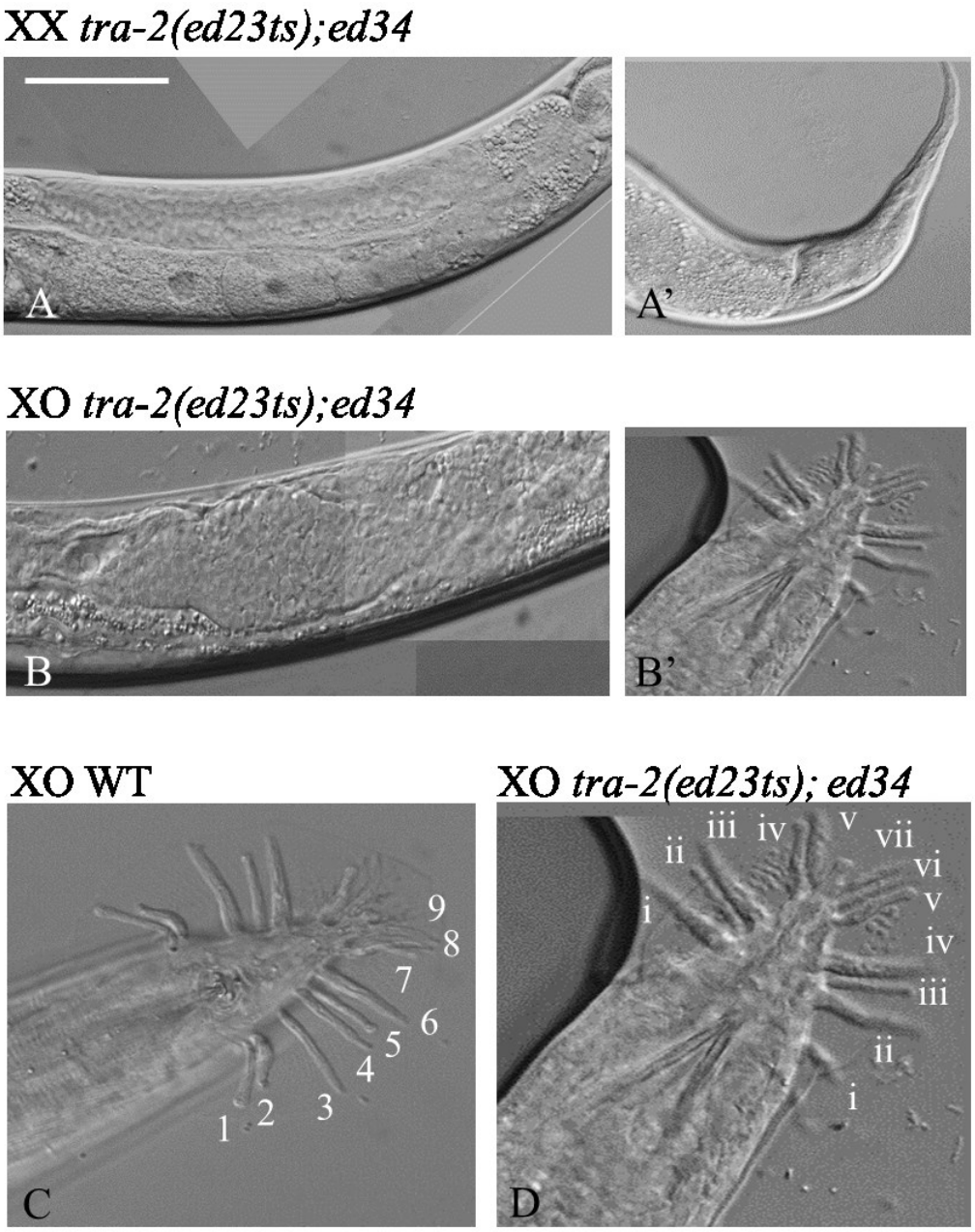

Figure 20. DIC images of suppressor *ed34* phenotypes. Scale bar = 50 μm. XX *ed34* animals develop as hermaphrodites and are phenotypically wild-type (A, A'). They have a double armed gonad which produces sperm and oocytes and are capable of self-fertilization (A). XO *ed34* animals show mild feminization of the germline (B) and soma (B') as indicated by the disorganized gonad and missing tail rays. Comparisons between wild-type (C) and XO *ed34* tail rays (D). XO *ed34* animals are missing several tail rays and those that are present are large and misshapen. There is also a loss of bilateral symmetry between the tail rays on either side of XO *ed34* animals. Wild-type tail rays are labelled 1-9 (C). *ed34* tail rays are labelled i-vii (D). Position and numbering of wild-type tail rays does not corresponding to position and numbering of *ed34* tail rays.

Table 10. List of mutated genes in DP377 (*tra-2(ed23ts); ed34)*

Gene	Chromosome	Description	Phenotypes	Mutation
Cbr-fem-3	IV	FEMinization of XX and XO animals	Hermaphroditization of XX and XO animals	Nonsense
Cbr-lin-59	I	SET domain-containing protein	Cord commissures fail to reach target, early larval lethal, larval lethal, lethal, loss of left right asymmetry, mRNA levels reduced, receptor mediated endocytosis defective, shortened life span, slow growth, sterile, sterile progeny	Missense
Cbr-exoc-8	I	EXOCyst component	*Bacillus thuringiensis* toxin hypersensitive, dumpy, embryonic lethal, larval arrest, lethal, maternal sterile, nuclei enlarged, pore forming toxin hypersensitive, reduced brood size, slow growth, small, sterile progeny	Missense
Cbr-gcy-19	II	Predicted transmembrane guanylyl cyclase	No phenotypes	Missense
Cbr-frm-2	III	FERM domain (protein4.1-ezrin-radixin-moesin) family	No phenotypes	Missense
Cbr-gbf-1	III	GBF1 (Golgi-specific Brefeldin-A-resistant Factor 1) homolog	Adult lethal, apoptosis reduced, early embryonic lethal, embryonic lethal, larval arrest, larval lethal, lethal, maternal sterile, nuclei enlarged, receptor mediated endocytosis defective, sick, sterile,	Missense
Cbr-tag-141	IV	Temporarily Assigned Gene name, zinc transporter	Unknown	Missense
Cbr-vit-6	IV	vitellogenin precursor protein	Nicotine hypersensitive	Missense
Cbr-egl-21	IV	EGg Laying defective, carboxypeptidase	Aldicarb resistant, coiler,egg laying defective, egg laying imiprine response, egg laying serotonin resistant, egg retention, transient bloating	Missense
CBG04283	III	Hypothetical protein	Unknown	Frameshift
CBG04283	II	Hypothetical protein	Unknown	Frameshift
CBG22064	I	Hypothetical protein	Unknown	Inframe deletion

tra-2(ed23ts); sup(ed34) which could be responsible for the phenotypes described above. Preliminary mapping showed that the *tra-2(ed23ts); sup(ed34)* suppressor was not present on chromosome V and was unlikely to represent an allele of *Cbr-fog-3*, *Cbr-fem-1* or *Cbr-fem-3 (*Dewar, 2011). To test *Cbr-fem-3* as a potential candidate, the suppressor was mapped to two indel markers, cb-m177 and cb56202 on opposite ends of chromosome IV (figure 17). 110 F2 animals which could suppress *tra-2(ed23ts)* were selected and genotyped for cb-m177, cb56202 and cb-m26, an indel marker on chromosome III. It was expected that *ed34* would be linked to one or both of the chromosome IV markers but would assort independently with cb-m46.

110 animals were genotyped for these three markers. 110 animals were homozygous AF16 cb-m177, which was inconsistent with independent assortment (χ^2 = 220, df=1, p<.0001). 33 animals were homozygous AF16 cb56202 with 53 animals being a heterozygote and 19 animals being homozygous for HK104 cb56202. This was consistent with independent assortment (χ^2 = 3.733, df=1, p=0.053). 13 animals were homozygous AF16 cb-m46, 67 were heterozygotes and 23 animals were homozygous HK104 cb-m46 which showed independent assortment as expected (χ^2 =1.942, df=1, p=0.1635).

The suppressor was tightly linked to the cb-m177 indel, a region of chromosome IV which contains *Cbr-fem-3* as well as *Cbr-tag-141* (*t*emporarily *a*ssigned *g*ene name). There is no information on the function of *Cbr-tag-141* but based on its sequence it was predicted to function as a zinc transporter (wormbase). It contained two missense mutations, a L12V substitution and an I13V substitution. With these substitutions and the proposed function of *Cbr-tag-141* it was unlikely that *Cbr-tag-141* is suppressing *tra-2(ed23ts)* and feminizing the germline and soma of *tra-2(ed23ts); sup(ed34)* males. *Cbr-fem-3* is already known to play a role in the *C. briggsae* SD pathway and contained a nonsense mutation, changing an arginine residue at position 204 into a stop codon. This made *Cbr-fem-3* the most likely candidate for *tra-2(ed23ts); sup(ed34)*.

3.3 Comparisons with *C. nigoni*

I expected that since *C. briggsae* and *C. nigoni* are sister species that diverged recently their proteins will be mostly identical. Those proteins that showed a high degree of divergence are likely under positive selection. Even though the expectation was that these two species' proteins would be >90% identical there must be some difference between them to allow for spermatogenesis to occur in *C. briggsae* hermaphrodites. This difference could either have been a structural change in an already existing protein such as gain or loss of a functional domain, modification of an existing functional domain or introduction of a novel protein into the *C. briggsae* SD pathway. Alternatively, spermatogenesis in *C. briggsae* hermaphrodites could have evolved due to novel regulatory mechanisms of existing SD genes.

If *C. briggsae* spermatogenesis was the result of structural changes to the pathway members then I would have expected to see large changes, such as novel functional domains, to be present in an ortholog of one species and not the other. I might have also expected that introduction of a novel protein to the SD pathway would come with structural changes but that may not necessarily have been true as novel regulation could be responsible for integrating it into the pathway. Amino acid changes in functional domains could indicate positive selection of existing SD pathway members. This was expected as previous evidence has shown that several members of the SD pathway rapidly co-evolve together such as TRA-2 and FEM-3. Some residue mismatches would have been the result of neutral evolution, random mutations in the genome which led to a change in an amino acid. These are expected to occur in both functional and non-functional domains.

Trying to determine if there was a change in the regulation of existing SD pathway genes was difficult to do from a comparison of gene and protein sequences. However, since some of the *C. briggsae* germline specific factors were known, comparisons between these could have indicated whether or not they were present in *C. nigoni* and if so, were they different enough that they could account

for spermatogenesis in *C. briggsae* hermaphrodites? It was expected that the germline specific genes, *she-1* and *gld-1*, would exist in *C. nigoni* but that orthologs would be less similar than orthologs of core pathway genes as germline specific genes are more rapidly evolving (Stothard and Pilgrim, 2003). Absence of *she-1* in *C. nigoni* would have indicated that it evolved in *C. briggsae* after *C. nigoni* and *C. briggsae* diverged. Absence of *gld-1* in *C. nigoni* would have indicated that it lost *gld-1* after *C. nigoni* and *C. briggsae* diverged since *gld-1* is present in *C. elegans*. If both of these genes were present and unchanged in *C. nigoni* then they alone could not have been responsible for the evolution of hermaphroditism in *C. briggsae.*

All of the *Cni-fem* genes, *Cni-cul-2, Cni-tra-3, Cni-pink-1* and *Cni-gld-1* were present in *C. nigoni* and full length or close to full length sequences were made for all of them from the available scaffold data. *Cni-tra-2* and *Cni-she-1* were also present but absence of some scaffold data led to sequences which contained large gaps. *Cni-tra-1* appeared to be present in *C. nigoni* but a sequence was not assembled due to the absence of a large amount of scaffold data. A *Cni-unc-45* sequence was also assembled to show the degree of conservation of a non-sex determining gene. All *C. nigoni* sequences were compared to *C. briggsae* and *C. elegans*. Where sequences were incomplete or contained gaps due to missing scaffold, only those parts of the sequence that were present were used determine percentage amino acid identity (table 11).

3.3.1 C. nigoni male promoting pathway members

All proteins, or parts of proteins, examined were almost identical at the structural level between *C. nigoni* and *C. briggsae* (table 11). Comparisons between *C. elegans* and *C. briggsae* showed that *fem-3* was the most rapidly evolving SD gene however there was little difference in the *C. nigoni* and *C. briggsae* orthologs. FEM-1 is 98.64% identical between *C. briggsae* and *C. nigoni* with the majority of differences represented by similar amino acids. Two thirds of the

Table 11. Percentage amino acid identity of SD pathway members between *C. nigoni* and *C. briggsae, C. nigoni* and *C. elegans* and, *C. briggsae* and *C. elegans.*

	% amino acid identity		
Protein	*C. nigoni* & *C. briggsae*	*C. nigoni* & *C. elegans*	*C. elegans* & *C. briggsae*
UNC-45	98	89	90
TRA-2 pt I	92	50	51
TRA-2 pt II	92	37	37
TRA-3	99	90	90
FEM-1	98	71	71
FEM-2	94	62	63
FEM-3	95	38	39
CUL-2	98	83	80
GLD-1	98	84	85
PINK-1	97	85	85
SHE-1	83	N/A	N/A

differences between *C. nigoni* and *C. briggsae* occurred in the N-terminal ankryin repeat domains with the other third present at the C-terminus of the protein in a region with no conserved functional domain (figure 21). Cni-FEM-2 was marginally the least conserved of the Cni-FEM proteins. All of the differences occurred in functional domains of Cni-FEM-2, the majority of them were found in the N-terminal α-helices which are important for establishing the Cni-FEM complex. The other difference was in Cni-FEM-2's phosphatase domain (figure 22). Cni-FEM-3 was highly conserved, the most prominent difference was a 4 residue deletion in the Cni-FEM-3 N-terminus (figure 23). Since Cbr-CUL-2 is embryonically lethal when mutated it is expected that such an important protein would be highly conserved, which it was. Most of the differences in Cni-CUL-2 appear in the C-terminal Cullin-Nedd8 domain. Interestingly this region, which is different in *C. nigoni*, was found to be highly conserved between *C. elegans* and *C. briggsae* (figure 24). Differences exist in the functional domains of male promoting *C. nigoni* SD proteins but they share a high percentage amino acid identity with *C. briggsae* orthologs.

3.3.2 C. nigoni female promoting pathway members

The Cni-TRA-2 sequence was analyzed in two parts due to a gap in scaffold sequence available from the 959 nematode genome database. The first available part contained the first of nine trans-membrane loop (TML) domains. The second part contained TML domains 6-9 as well as the TRA-2ic domain. Most of the differences in TRA-2 between *C. nigoni* and *C. briggsae* occurred in non-functional parts of the protein, areas in between the TMLs (figure 25). Interestingly there appeared to be two small areas (<20 amino acids) of the Cni-TRA-2 intracellular domain which have diverged more than other parts of the protein. One of these areas was located in the Cni-FEM-3 binding domain the other in the Cni-TRA-1 binding domain. Ce-TRA-3 is 90% identical to Cbr-TRA-3, so it was not surprising to find that Cni-TRA-3 and Cbr-TRA-3 are 99%

Figure 21. Alignment between *C. nigoni* and *C. briggsae* (CBG15267) FEM-1 orthologs. Ankryin repeat domains are indicated in blue. * indicates identical amino acids, : indicates highly conserved amino acids, . indicates mildly conserved amino acids. Alignment was made using ClustalW.

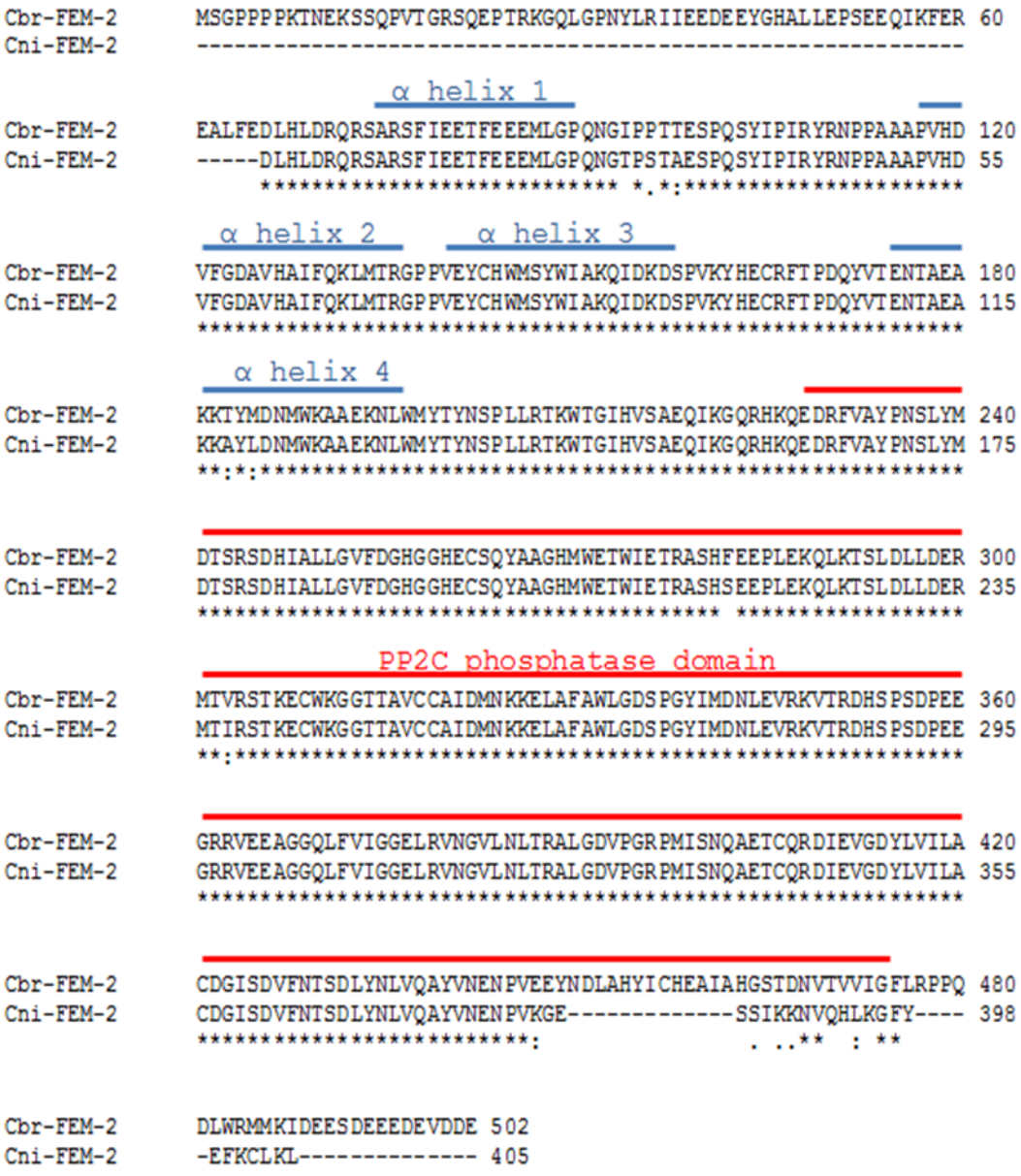

Figure 22. Alignment between *C. nigoni* and *C. briggsae* (CBG15267) FEM-2 orthologs. α helices implicated inFEM-2s role as a scaffolding protein are indicated in blue. The PP2C phosphatase domain is indicated in red. * indicates identical amino acids, : indicates highly conserved amino acids, . indicates mildly conserved amino acids. The first 65 amino acids are missing from the *C. nigoni* due to unavailable sequencing data. Alignment was made using ClustalW.

```
Cbr-FEM-3       MVPDDVEPMEVDDDGALIVDLNETVEEDEETKKEKKRRKRFREKLKRFDHYSQFSGISIA 60
Cni-FEM-3       MVPDDVEPMEVDDDGALIVDLNETVEEDEEPE----RRKRFREKLKRFDHYSQFSGICIE 56
                ******************************.:    ********************.*

Cbr-FEM-3       QIDWPLIQGRSLQRSPLTGQSFNADENIFRIDEWPRETFLQITSTLTFCAGAALLSNEKI 120
Cni-FEM-3       QIDWPLIQGRSLQRSPLTGQSFNADENTFRIDEWPRETFLQITSTLTFCAGAALLSNEKI 116
                ************************** ********************************

Cbr-FEM-3       TLFVFQRTMKTLVAYCNFMYHRAITHNRRQINRIDVHELISRNPLRFHMFLQKFLPHPDI 180
Cni-FEM-3       TLFVFQRTMKTLVAYCNFMYHRARTHNRRQINRIDVHELISRNPLRFHMFLQKFLPQPDI 176
                ********************** ********************************:***

Cbr-FEM-3       NRTHFNNEFLYYFHNLYFQDETCRLLYHDVARYSPIINQQGTRMSLQHQIYYPDVMRNPA 240
Cni-FEM-3       NRTHFNNEFLYYFHNLYFQDETCRLLYHDVARYSPIINTQGTRMSLQHQLYYPDVMRNPA 236
                ************************************ **********:**********

Cbr-FEM-3       FDALWFTSFINPSGYSFSRFHAYRFHEALGMPPLESELIIVLDWLAKLIICDIGYKVLAW 300
Cni-FEM-3       FDALWFTSFINPSGYSFSRFHAYRFHEALGCPPLESELIIVLDWLAKLIICDIGYKVLAW 296
                ****************************** ****************************

Cbr-FEM-3       RDARGFQGLPDLLSFQMAMLEEGDPLFDLDIDYTAPPTRLFSEPTRFQTYPKFQPRRRID 360
Cni-FEM-3       RDARGFQGVPDLLSFQMAMLEEGDPLFDLDIDYTAPPTRLFSQPTRFQTYPKFQPRRRID 356
                ********:**********************************:*****************

Cbr-FEM-3       FPSRFDGFYKKRRLERGLEEIQESFIMNHFPTKPLRTVYVYTHPEERRR 409
Cni-FEM-3       FPSRFDAFYKKRRLQRGLEEIQESFIRNHFPTGPLRTVYIYTHPEERRR 405
                ******.*******:*********** ***** ******:*********
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Figure 23. Alignment between *C. nigoni* and *C. briggsae* (CBG21774) FEM-3 orthologs. * indicates identical amino acids, : indicates highly conserved amino acids, . indicates mildly conserved amino acids. Alignment was made using ClustalW.

Figure 24. Alignment between *C. nigoni* and *C. briggsae* (CBG18282) CUL-2 orthologs. The cullin domain is indicated in blue. The neddylation domain is indicated in red. * indicates identical amino acids, : indicates highly conserved amino acids, . indicates mildly conserved amino acids. Alignment was made using ClustalW.


TML 1
Cbr-TRA-2 MKLAFNKLLVASVVFTVLSFGLLLASLFTTTATTPSEWTILLPEFRFPVNKKQTTEQFLV 60
Cni-TRA-2 MKLAFNKLLVASVVFTVLSFGLLLASLFTNTATTPSEWTSLLPEFRFPITRKQSTEEFLV 60
Cbr-TRA-2 EKIVHEHEEGEDVRSALYLTHHGYFMNAIANMKVTYRQKSYTVNDVCFKPHSAIFENVPA 120
Cni-TRA-2 EKIVHEHDEGEDVRSALYLTHHGYFMNAIANMKVTYRQKSYTVNDVCFKPHSAIFEKVLS 120
Cbr-TRA-2 PENIDKLPAYFQRLLLEMQRLSPCLIVTPLNCFYDSYHIHSEISNWNASTDYLNRRLRNS 180
Cni-TRA-2 PENIDKLPAYFQRLLLEMQRLSPCLIVTPLNCFYDSYRIHSEMSNWNASTDYLNRRLRNA 180
Cbr-TRA-2 YLEAIEEKDSRPYVKSTYGPELIKEWARHMFAIPSKPLSNFSKSDLYSRVKTWLSSIAAR 240
Cni-TRA-2 YLEAIEEKDSRPYVKSTCGPELIKEWARHMFAIPSKPLSNFSKSDLYSRVETWLSSITPR 240
Cbr-TRA-2 KKICADPMRSCDETLDAENYFNVCTVMQQINDYDERRKQRLKFQLEYGDEEFTTRLDCVE 300
Cni-TRA-2 KKICADPMRSCDETLDAENYFNVCTVMQQINDYDERRKQRLKFQLEYGDEEFTTRLDCVE 300
Cbr-TRA-2 DREKFIEWMQERNLRDMLKLFASSVEIPDHKEIPNQVCDGIYHDLDTSSGLELFRGARSF 360
Cni-TRA-2 DREKFIEWMQERNLRDMLKLFASNVEIPDHRDIPSQVCDGIYHDLDTITGLELFRGARSF 360
Cbr-TRA-2 SNNTSAYDTINVELGFMTPENLLTTMRHSDFVNGFESIWTIERARELLNEFRLALKVEVT 420
Cni-TRA-2 SNNTSAYDTINVEFGFMTPENLLATMRHSDYVNGFESIWTIERARELLNEFRLALRAEVT 420
Cbr-TRA-2 KFSESRSSRRVKVTTRIVNQIEEEGSDEEMEYHMIYFILGACALMVALFAAFAFSEAFLT 480
Cni-TRA-2 KFSEGRSSRRVKVTTRIVNQIEEEGSEEEIEVYMIYFILGACGLMFALFAAFAFSEAFLT 480
TML 2
Cbr-TRA-2 SLSMFLLRGFITGLLFIFLCKSGGLILIDSNFLCYITMHLAFNLVMTARVTFICYRIGGC 540
Cni-TRA-2 SLSMFLLRAGITGLLFILLCKSGGLIMIDSNFL---------- 513
TML 3
Cbr-TRA-2 VQSEKDFVKSNFSSLGSVPVDSLKEDSCKRHVQYVLAKYTKFQVAQDAYSEEPFEKLPKY 600
Cni-TRA-2 ----------
TML 4
Cbr-TRA-2 WFLIAIVLVPVIGVYWFFIDSDVQKICIVLLPAFLIAAFEEMRVKNQLLRERRIKKAIQR 660
Cni-TRA-2 ----------
Coiled coil
Cbr-TRA-2 LQKEENTRIMSRGEIDNLLSGNAELSGEKSHYESKQGVLHHGSAGGLFELSRSTYDVSLI 720
Cni-TRA-2 ----------
TML 5
Cbr-TRA-2 MAYPNQMIRNLRLCALGAYFRLFKMKYCAVVVSSVAALLILLSIGLLFIPVQRSSVPKEL 780
Cni-TRA-2 ----------

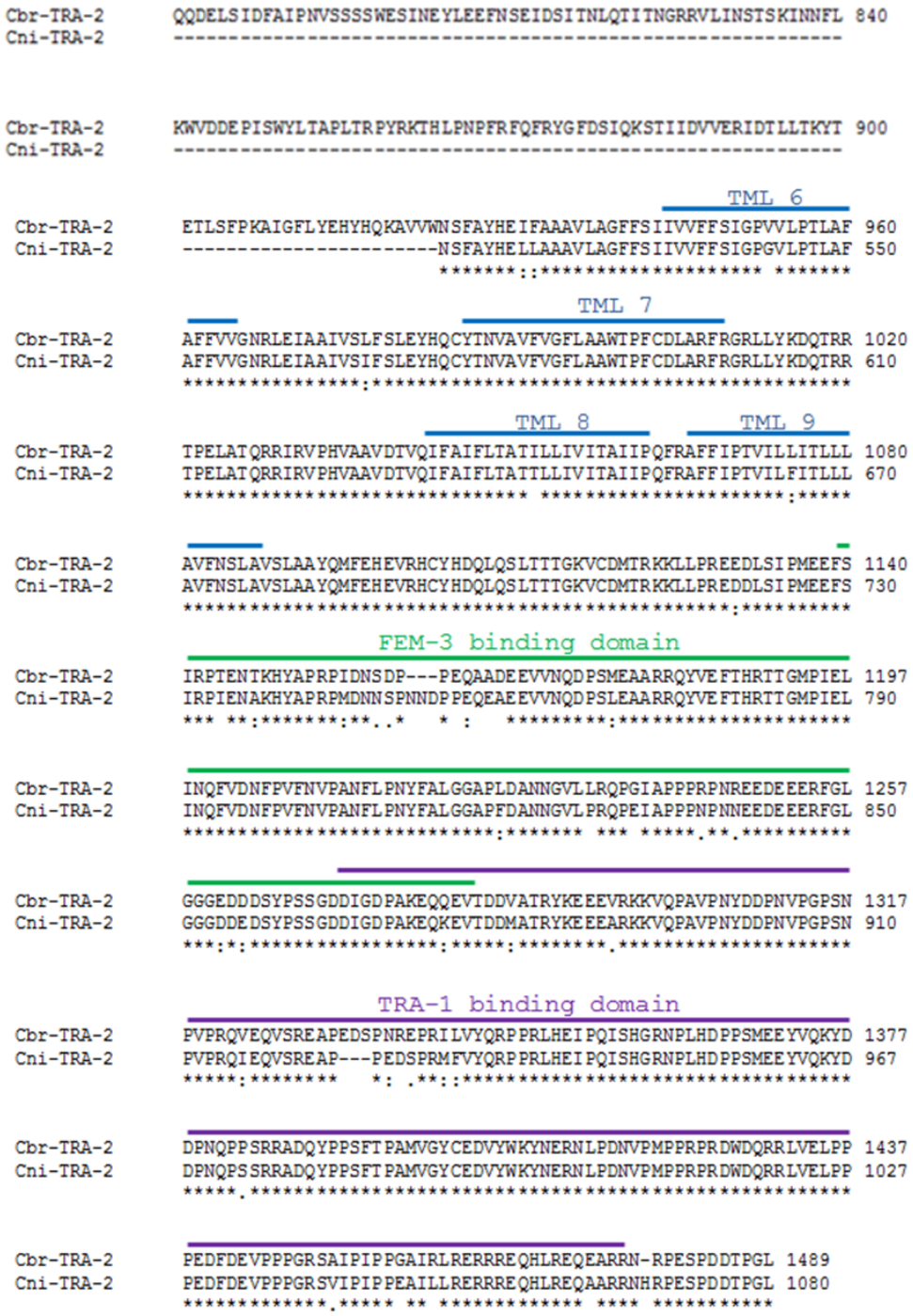

Figure 25. Alignment between *C. nigoni* and *C. briggsae* (CBG11193a) TRA-2 orthologs. Trans membrane loop (TML) domains are indicated in blue. The coiled-coil domain is indicated in red. The FEM-3 binding domain is indicated in green. The TRA-1 binding domain is indicated in purple. * indicates identical amino acids, : indicates highly conserved amino acids, . indicates mildly conserved amino acids. There are several large gaps in the *C. nigoni* sequence due to unavailable sequencing data. Alignment was made using ClustalW.

identical (table 11). The few differences that were present were changes to residues with some degree of similarity and all occurred in the protease domain (figure 26). Differences exist in the functional domains of female promoting *C. nigoni* SD proteins but they share a high percentage amino acid identity with *C. briggsae* orthologs.

3.3.3 C. nigoni pink-1 and germline members

Cni-pink-1 scaffolds were available in full and the protein was very highly conserved with its *C. briggsae* ortholog (97.8%). The differences between the two were mostly present in the kinase domain (8 out of 11) but they were mostly changes to residues of similar properties (figure 27). Like Cni-PINK-1, Cni-GLD-1 was present in its entirety and was highly conserved (figure 28). It was unclear whether or not *she-1* would be present in *C. nigoni* as it has previously only been described in *C. briggsae.* The scaffold data that was available for it was incomplete but what was present was 83.3% identical at the amino acid level. This was much lower than any other SD protein examined in this study however it was still a very high percentage of identity. As with the other members of the SD pathway the majority of the differences between Cni-SHE-1 and Cbr-SHE-1 were in residues with similar properties (figure 29). SD pathway members that were thought to be unique to *C. briggsae* show large percentage amino acid identity to *C. nigoni* orthologs.

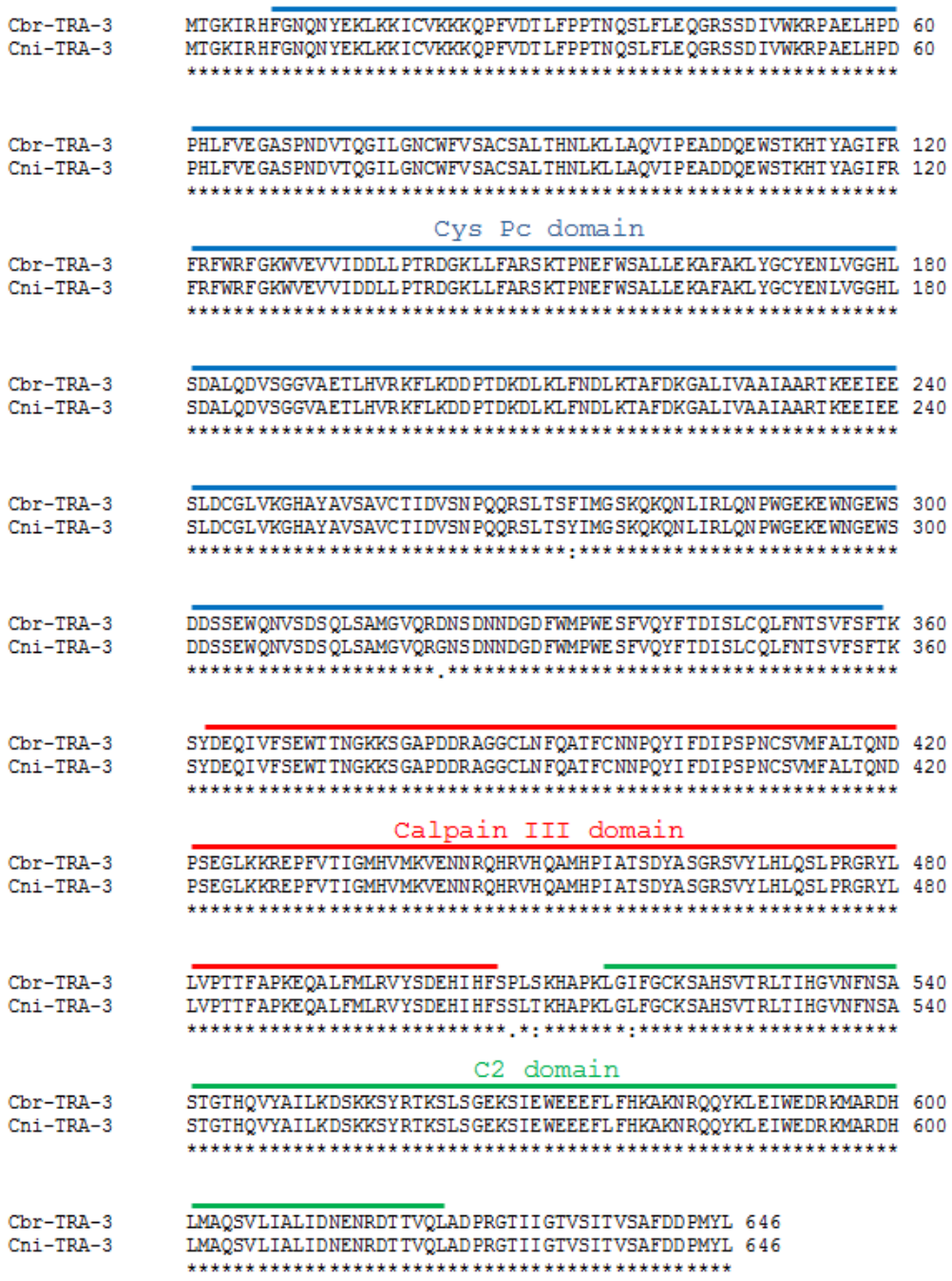

Figure 26. Alignment between *C. nigoni* and *C. briggsae* (CBG21580) TRA-3 orthologs. The protease domain is indicated in blue. The calpain III domain is indicated in red. The calcium binding C2 domain is indicated in green. * indicates identical amino acids, : indicates highly conserved amino acids, . indicates mildly conserved amino acids. Alignment was made using ClustalW.

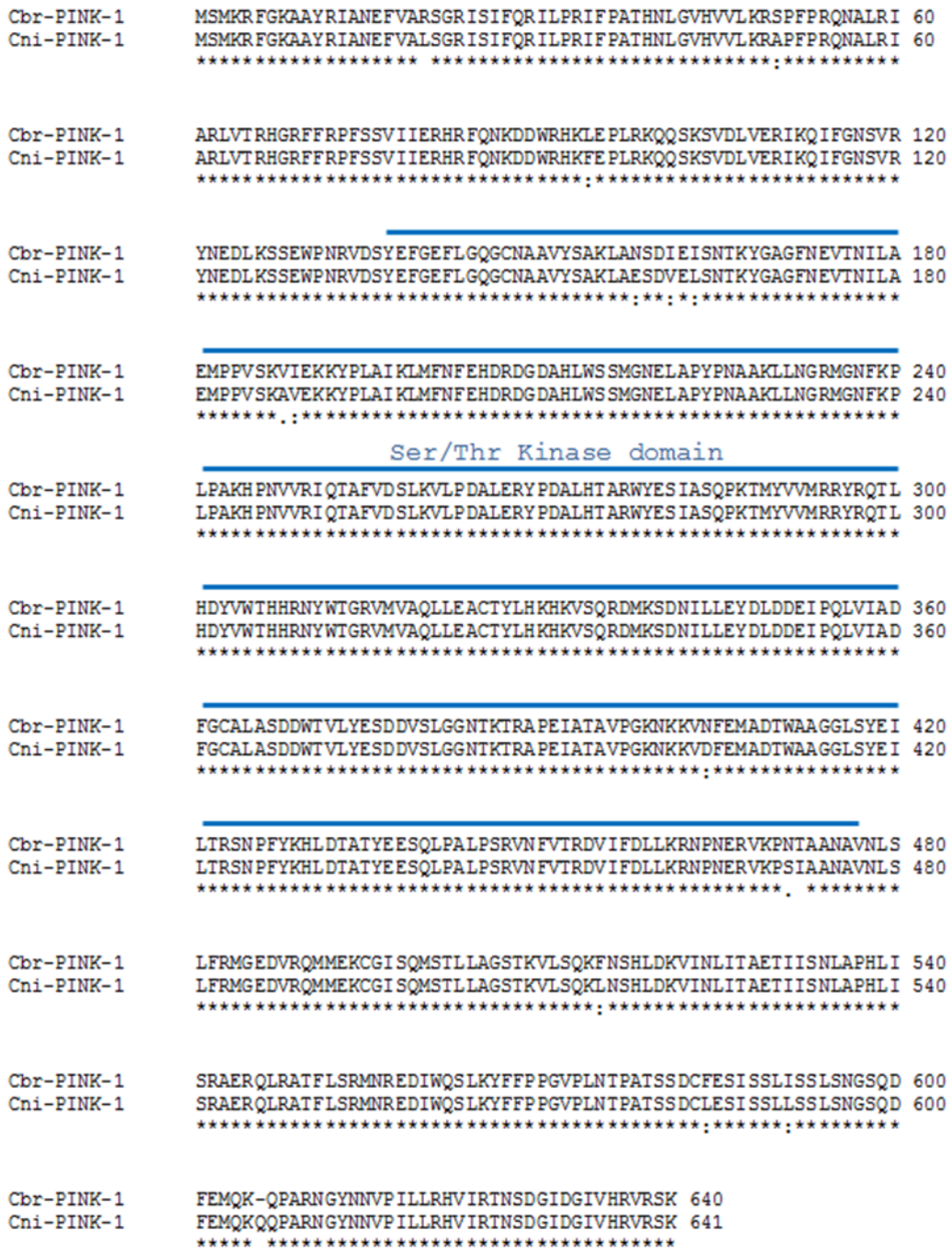

Figure 27. Alignment between *C. nigoni* and *C. briggsae* (CBG02385) PINK-1 orthologs. The serine/threonine kinase domain is indicated in blue. * indicates identical amino acids, : indicates highly conserved amino acids, . indicates mildly conserved amino acids. Alignment was made using ClustalW

Figure 28. Alignment between *C. nigoni* and *C. briggsae* (CBG00303) GLD-1 orthologs. The RNA binding domain is indicated in blue. * indicates identical amino acids, : indicates highly conserved amino acids, . indicates mildly conserved amino acids. Alignment was made using ClustalW

Figure 29. Alignment between *C. nigoni* and *C. briggsae* (CBG11663) SHE-1 orthologs. F-box domains are indicated in blue. * indicates identical amino acids, : indicates highly conserved amino acids, . indicates mildly conserved amino acids. There are several large gaps in the *C. nigoni* sequence due to unavailable sequencing data. Alignment was made using ClustalW

4.0 Discussion

4.1 Complementation and Sequencing

Identifying mutations in the *Cbr-fem* genes has the capacity to reveal critically important residues in the protein as well as allow us to glean further insight to the different functional domains of this protein and how they contribute to its overall function. The majority of *Cbr-fem* mutants found in the *tra-2(ed23ts)* genetic screen are nonsense mutations, which can be informative in showing important C-terminal regions. For example, If a *tra-2(ed23ts); sup* mutant encodes a truncated form of Cbr-FEM-1 and the worm develops as a hermaphrodite (i.e. the Tra phenotype is suppressed) it can be inferred that the missing part of the protein is necessary for Cbr-FEM-1 function. The same inferences can be applied to Cbr-FEM-2 and Cbr-FEM-3.

4.1.1 Analysis of Cbr-fem-1 alleles

Several of the nonsense alleles truncated Cbr-FEM-1 very early on in its ankyrin repeat domain so it is not surprising that these proteins are nonfunctional (figure 9). Animals with *Cbr-fem-1* nonsense mutations which occur after the large ankyrin repeat domain were phenotypically identical to animals with early nonsense mutations showing that the remaining 340 amino acids are necessary for Cbr-FEM-1 function. A mutagenic screen investigating the function of Ce-FEM-1 in *C. elegans* found that out of twenty missense mutations, five of them occurred in the CTD outside of any known functional domains. It was hypothesized that these missense mutations negatively affected the folding of the protein which could inhibit its interactions with the rest of the Ce-FEM complex (Vivegananthan, 2004). In support of this *C. elegans* and *C. briggsae* FEM-1 has been shown to contain a VHL-box motif in its CTD, which is necessary for interacting with CUL-2 (Starostina *et al.,* 2007).

A single missense mutation was also found in Cbr-FEM-1 at position 136, a region in the N-terminal ankyrin repeat domain. This resulted in a leucine-to-serine substitution, which resulted in mutant animals that were phenotypically identical to those with N-terminal *Cbr-fem-1* nonsense mutations. Leucine is a hydrophobic amino acid so replacing it with a hydrophilic serine could potentially disrupt the coil structure of the ankryin repeat domain, rendering Cbr-FEM-1 unable to bind Cbr-TRA-1 and fulfill its male-promoting role. Several missense mutations have been found in the *C. elegans* Ce-FEM-1 NTD. One of these missense mutations resulted in a glycine to arginine substitution at position 134. Like the Cbr-L136S substitution the change from glycine to arginine was a change from a hydrophobic to a hydrophilic amino acid. Several other missense mutations were found in *Ce-fem-1*, none of which localized to ANK 2 or 4 indicating these may be less important than the other ANK repeats (Viveganantha, 2004). The majority of substitutions were found in ANK 5 indicating the importance of this domain in *C. elegans* however the same conclusion cannot be made for *C. briggsae,* as no mutations were found in ANK 2, 4 or 5. The mutations found in this work and comparisons with *Ce-fem-1* mutants reiterates the importance of the ankryin repeat domains in Cbr-FEM-1 function.

4.1.2 Analysis of Cbr-fem-2 alleles

Cbr-FEM-2 is a member of the PP2C phosphatase family. Cbr-FEM-2 differs from related PP2C phosphatases in that it has a large N-terminal domain (NTD) which is suspected to be specific to its role in sex determination (Hansen and Pilgrim, 1998). It also has a conserved C-terminal domain which is responsible for its phosphatase activity and necessary for its function. Cbr-FEM-2 shows dephosphorylating activity *in vitro* (Pilgrim *et al*., 1995; Chin-Sang and Spence, 1996) however its substrates have thus far remain unidentified in *C. elegans* or *C. briggsae.* Amino truncated Ce-FEM-2 does not show a difference in *in vitro*

phosphorylation activity compared to normal Ce-FEM-2. Despite not being necessary for phosphorylation the N-terminus is required for Ce-FEM-2 function as N-terminal truncated Ce-FEM-2 cannot rescue *Ce-fem-2* null mutants (Hansen and Pilgrim, 1998). The NTD of Cbr-FEM-2 consists of four α helices which are predicted to facilitate assembly of the Cbr-FEM complex. Both Cbr-FEM-1 and Cbr-FEM-3 are able to interact with the NTD of Cbr-FEM-2 simultaneously in the presence and absence of the Cbr-FEM-2 CTD (Zhang *et al*., 2013).

Several nonsense mutations have been identified in the N-terminal coding region of *Cbr-fem-2* that occurred in codons for non-conserved tryptophan residues (figure 10). A singe missense mutation has also been identified in the N-terminal coding region. This mutation causes a conserved arginine residue to be substituted with a cysteine at position 70 (R70C). R70C is located at the beginning of the first α helix of Cbr-FEM-2. Deletion of the first α helix in Ce-FEM-2 has been shown to severely impair the binding of Ce-FEM-1 and Ce-FEM-3 to Ce-FEM-2 (Zhang *et al*., 2013). It is not clear how the R70C substitution could knock out the function of Cbr-FEM-2. However, changing a hydrophilic residue to a hydrophobic one in this critical α helix is likely to have a negative impact on protein-protein interactions. The introduction of a cysteine could also lead to disulphide bonds being formed within Cbr-FEM-2 thereby disrupting its correct folding. A splice donor site mutation was also found between the fifth and sixth exon. This leads to translation of the fifth intron which contains an in frame stop codon, leading to truncation of Cbr-FEM-2 near the end of its phosphatase domain. Even though a large portion of the protein is made, it is likely that lack of the C-terminal part of the phosphatase domain removes or reduces the phosphatase activity of this Cbr-FEM-2 allele to the extent that it can suppress the *tra-2(ed23ts)* mutation. These mutants highlight the necessity of both the N-terminal and C-terminal for Cbr-FEM-2 function in *C. briggsae*.

4.1.3 Analysis of Cbr-fem-3 alleles

fem-3 is the most rapidly evolving member of the *Caenorhabditis* SD pathway and is likely one of the most rapidly evolving loci in the genome (Kent and Zahler, 2000). Comparisons of the FEM-3 proteins from *C. elegans*, *C. briggsae* and *C. remanei* show that it is a highly divergent protein and ranges from 31.2%-37.5% pair-wise amino acid identity (Haag *et al*., 2002). It lacks any known functional domains but it contains localized regions of conservation, defined as four or more consecutive amino acids (Haag *et al.*, 2002). Null and temperature-sensitive missense mutations have been found in *fem-3* of all three species. In several cases the affected residue is conserved between the three (Ahringer *et al.,* 1992). However, none of these conserved critical residues occur in any region of conservation (Haag *et al*., 2002).

Two nonsense mutations resulted in a Q67stop and R90stop (figure 11). Neither of these amino acids are conserved between *C. elegans*, *C. briggsae* or *C. remanei* but Q67 is the only residue in between two consecutive regions of conservation. As these mutations result in a severely shortened protein it is not surprising that they would result in a non-functional Cbr-FEM-3. These mutants show that the first 90 amino acids alone are not able to provide Cbr-FEM-3 activity to suppress Cbr-TRA-1.

4.2 Non-fem suppressors

4.2.1 A model for tra-1(ed30)

tra-1 codes for a transcription factor similar to the *Drosophila* pattern forming genes *cubitus interruptus dominant* and *odd-paired* and the GLI family of human oncogenes and developmental regulators such as THP. The most striking feature of TRA-1, which is highly conserved in the above mentioned factors, is a series of five zinc finger domains that are involved in DNA binding. In *C. elegans* two splice forms of Ce-TRA-1 exist; a full length Ce-TRA-1A, which contains all of

the regions necessary for correct transcriptional control, and an alternatively spliced Ce-TRA-1B, which results in a protein that contains the nuclear localization signal but only two of the five zinc finger domains (Zarkower & Hodgkin, 1993).TRA-1A shows 44% identity between *C. elegans* and *C. briggsae.* A *Cbr-tra-1A* transgene is capable of rescuing many tissues of *Ce-tra-1* null mutants but not in the somatic gonad or in the germline (de Bono and Hodgkin, 1996).

The *C. briggsae* transgene also results in non-gonadal feminization of XO animals which is indicative of incorrect sexual regulation of the transgene (de Bono & Hodgkin, 1996). *Cbr-tra-1* can also produce a second Cbr-TRA-1B isoform that lacks N-terminal features of the full length Cbr-TRA-1. Both *C. elegans* and *C. briggsae* TRA-1A is present through all four stages of larval development and in the adult worm respectively however Ce-TRA-1B is only present during the first three larval stages of development and Cbr-TRA-1B is present during L3 and L4 only. One feature of these two alternative isoforms is that the splice donor and acceptor sites that produce Ce-TRA-1B are conserved but not used in *C. briggsae* (de Bono & Hodgkin, 1996).

The 44% identity between *C. elegans* and *C. briggsae* TRA-1A can be clustered into 5 highly conserved regions which are likely to have functional significance (figure 30). One of these regions is known as the gain-of-function (gf) region and is the area to which Ce-FEM-1 binds and inhibits Ce-TRA-1A. Mutations in this region strongly feminize XO animals and abolish spermatogenesis in *C. elegans.* Several *C. elegans* Ce-*tra-1*(gf) alleles have been identified and characterized. All of the XX animals examined displayed an intersex or male tail and a feminized, frequently disorganized germline when heterozygous for the *Ce-tra*-1(gf) allele (de Bono *et al.*, 1994).

The *C. briggsae ed30* strain contains a mutation in the conserved Cbr-TRA-1 gf region but the gf phenotype is much weaker in *ed30* than in any *C. elegans Ce-tra-1*(gf) mutant. In addition, *ed30* contains the *tra-2(ed23ts)* mutation

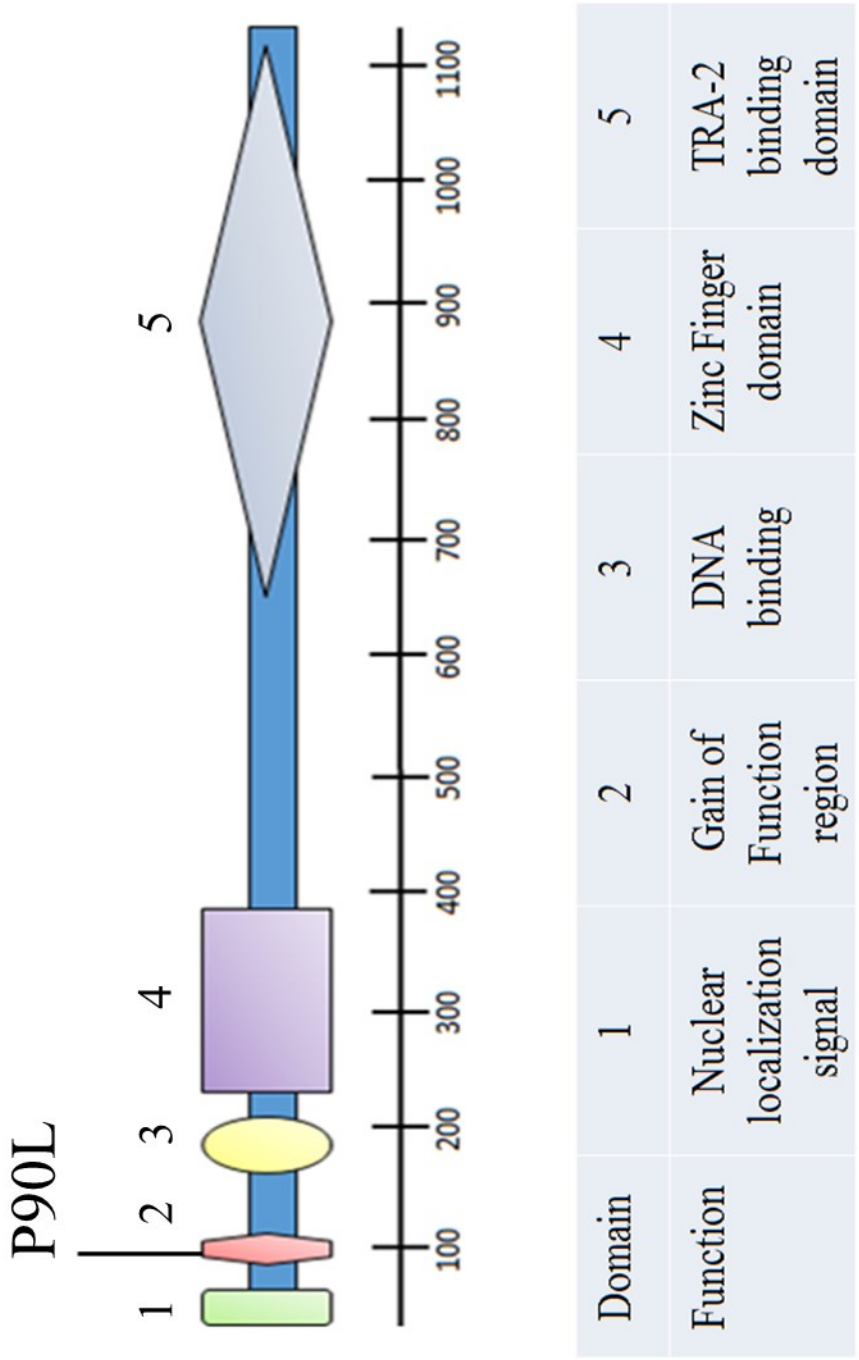

Figure 30. Diagram of TRA-1 which shows the five functionally conserved regions between *C. elegans* and *C. briggsae (*modified from Zarkower *et al.,* 1996). The location of the mutated residue in *tra-1(ed30)* is shown. Functions were assigned to regions 1-5 based on previous work (Zarkower and Hodgkin, 1992; Zarkower and Hodgkin, 1993; de Bono *et al.*, 1995).

that is sufficient to knock out Cbr-TRA-2 repression of the Cbr-FEM complex. As this is the only known *tra-1*(gf) mutation found in *C. briggsae* it is currently impossible to tell whether this is due to a difference in Cbr-TRA-1 function and/or regulation between *C. elegans* and *C. briggsae* or if the *tra-1(ed30)* allele is strong enough to overcome Cbr-FEM inhibition but weak enough that it requires two copies of the mutant gene to do so with little effect on XX animals and the XO soma. I propose a model to explain the phenotypes seen in *ed30* animals (figure 31). The *tra-1(ed30)* allele is a weak gain-of-function mutation that overcomes Cbr-FEM inhibition by reducing the binding of Cbr-FEM-1 to Cbr-TRA-1A. This allows sufficient Cbr-TRA-1A activity to promote female development in the XX soma and germline. XO soma are unaffected as the slight increase in Cbr-TRA-1A activity is not enough to cause feminization. However, in the male germline a small amount of Cbr-TRA-1 is required for spermatogenesis and a slight increase in Cbr-TRA-1 activity, facilitated by the *tra-1*(gf) mutation, is enough to push Cbr-TRA-1 activity over a threshold and switch spermatogenesis to oogenesis.

4.2.2 Cbr-PINK-1 as a potential novel member of the C. briggsae sex determining pathway

Hs-PINK-1 has been shown to bind to the E3 ligase Hs-PARKIN, which catalyzes ubiquitin transfer to mitochondrial substrates. E3 activity of Hs-PARKIN is usually repressed via auto-inhibition. In the absence of Hs-PINK-1, Hs-PARKIN adopts a conformation where its catalytic RING domains are inactive. Hs-PINK-1 relieves this inhibition in a two-step fashion. Hs-PINK-1 is capable of phosphorylating serine 65 (S65) in the ubiquitin-like domain (UBL) of Hs-PARKIN while also phosphorylating the same residue on ubiquitin itself. This results in Hs-PARKIN's phosphorylated UBL domain to bind to its RING 0 domain, which in turn frees up the RING 2 domain. This other domain contains a functionally important cysteine residue, to recruit ubiquitin and ligate it to Hs-

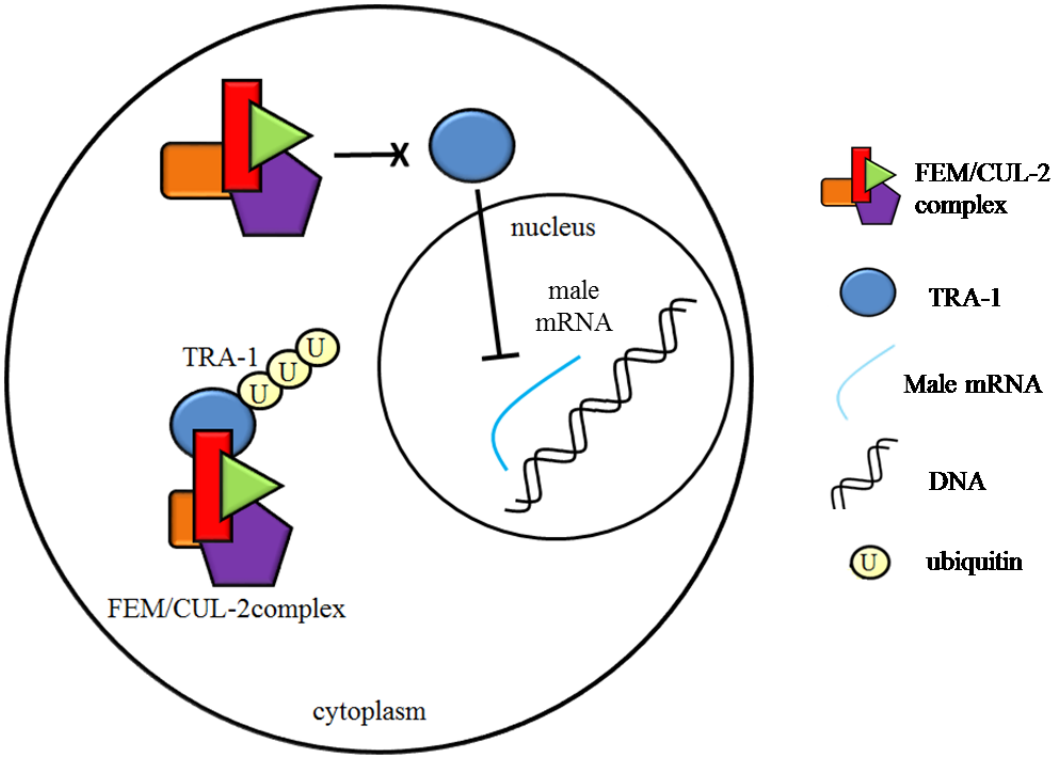

Figure 31. Proposed model for the molecular action of *tra-1(ed30)*. There are multiple TRA-1 proteins and multiple FEM/CUL-2 complexes in a cell at any given time. In some instances the FEM-complex will be able to bind TRA-1*(ed30)* and ubquitinate it as normal but in others TRA-1*(ed30)* is able to move to the nucleus to repress male promoting genes. In XX animals this is the normal state of the cell so no phenotype is observed. In XO somatic cells the marginal increase in TRA-1 activity is not enough to feminize the soma. However in XO germ cells a small amount of TRA-1 activity is required for normal spermatogenesis. In *tra-1(ed30)* mutants the extra TRA-1 present in germ cells is enough that overall TRA-1 activity is pushed over a threshold transforming sperm into the ooids seen in XO *tra-1(ed30)* gonads.

PARKINs substrate. Alternatively, the phosphorylated ubiquitin can bind to Hs-PARKINs RING 0 domain and relieve the inhibition on the RING 2 domain, allowing Hs-PARKIN to ubiquinate its target. Hs-PINK-1 has been shown to phosphorylate free ubiquitin in the vicinity of the outer mitochondrial membrane and may be involved in recruiting Hs-PARKIN to the mitochondria. In addition, Hs-PINK-1 is capable of functioning in the absence of Hs-PARKIN, phosphorylating free ubiquitin *in vitro* (Kane *et al.,* 2014)

Due to its location in the pathway (downstream of *Cbr-tra-2* and upstream of *Cbr-tra-1*) and its male promoting role, it appears that Cbr-PINK-1 is contributing to the negative regulation of Cbr-TRA-1. How it is doing this is not clear. However there are several pieces of evidence which lead me to hypothesize that it is acting to promote Cbr-CUL-2 mediated ubiquination of Cbr-TRA-1. CUL-2 belongs to the cullin family of proteins, whose members function as central components in multi-subunit cullin-RING E3 ubiquitin ligases. CUL-2 participates in a variety of cellular processes such as establishing anterior-posterior polarity, progression through cell division and *Caenorhabditis* sex determination. CUL-2 acts as a rigid scaffold to which RING finger subunits (in the case of the *Caenorhabditis* SD pathway this is Rbx1/ Roc1) bind, forming the catalytic core of the E3 ligase (Petroski & Deshaies, 2005). Elongin C binds the N-terminus of CUL-2 which binds to the ubiquitin-like protein Elongin B as well as binding a variable substrate recognition subunit (SRS). In the case of sex determination this SRS has been shown to be FEM-1 with FEM-2 and FEM-3 as cofactors (Starostina *et al*., 2007). CUL-2, along with the FEMS, is responsible for negatively regulating TRA-1 by tagging it for degradation by the proteasome. Hs-PINK-has been experimentally located to peroxisomes and lysosomes and recruits and activates Hs-PARKIN (Lazarou *et al.*, 2012). In the same manner Cbr-PINK-1 could localize to peroxisomes and lysosomes and activate Cbr-CUL-2 (figure 32). Polyubiquitination chain linkage occurs at the K63 residue, which is proximal to the S65 phosphorylation site providing the possibility that phosphorylation could have an effect on polyubiquitination (Kane *et al.*, 2014). In summary, it appears possible that Cbr-PINK-1 negatively regulates Cbr-TRA-1

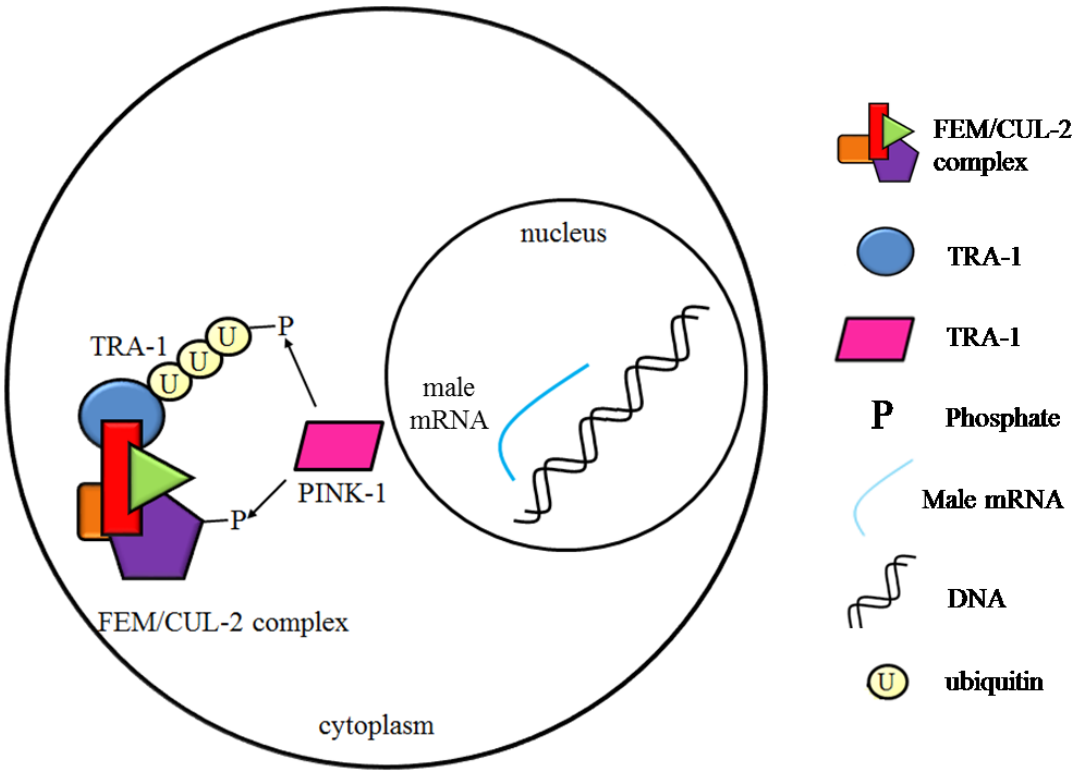

Figure 32. Proposed model for the molecular action of *pink-1* in the *C. briggsae* sex determining pathway. PINK-1 is capable of phosphorylating ubiquitin and ubiquitin like domains of other proteins. It is possible that PINK-1 could be facilitating TRA-1 inhibition by phosphorylating CUL-2 and/or ubiquitin.

through direct or indirect (or both) promotion of Cbr-CUL-2 mediated ubiquitination.

The *C. elegans* genome contains a number of duplications in a small number of kinase classes which has led to a dramatic increase in the number of kinase genes (438) compared to *Drosophila* (241) (Manning, 2005). Nematodes appear to have lost 6 subfamilies of kinases, based on their presence in *Drosophila*, Humans and more basal animals, but also share eight subfamilies with Humans that are absent from *Drosophila*. There are also 8 unique Nematode kinase subfamilies. The expansions that caused this appear to be relatively recent and some have occurred after the *C. elegans* and *C. briggsae* split. The members of these kinase expansions are closely related by sequence and chromosomal location, with many of them having reduced or no function. A similar expansion is also evident in the *C. briggsae* genome but not to as large a degree in *C. elegans*, resulting in 341 kinase genes (Manning, 2005). There is evidence of at least two kinases produced from these expansions that contribute to reproductive function; Ce-SPE-6 and Ce-SPE-8 are both kinases expressed during spermatogenesis (Reinke *et al.*, 2000). The majority of *C. briggsae* kinases appear orthologous to *C. elegans* kinases but there are 21 unique *C. briggsae* kinases, 19 of which are the result of genomic expansions (Manning, 2005).

The idea that a gene that has previously been undescribed in a sex determining role could evolve to take on new functions is not a novel one. An excellent example of this is *fem-2*. Ce-FEM-2 is a PP2C phosphatase, one of the two types of phosphatase that act on serine/threonine residues. There are three other PP2C phosphatases in *C. elegans,* allowing for the possibility of Ce-FEM-2 evolving into a SD role while other members of the family take over its previous roles (Stothard *et al.*, 2002). In the same way, perhaps another serine/threonine kinase replaced Cbr-PINK-1 in its oxidative stress protection and neurite growth roles or perhaps Cbr-PINK-1 simply evolved a new role in the *C. briggsae* sex determining pathway. Cbr-PINK-1 is also the first candidate as Cbr-FEM-2's

antagonistic kinase but rather than fulfilling opposite roles it appears that they both promote a male fate in *C. briggsae.*

4.2.3 A model for fem-3(ed34)

The *fem-3(ed34)* allele is unique in that it is the only *Cbr-fem* mutant known which allows XO animals to develop into males rather than hermaphrodites. *fem-3* is the most divergent of the *Caenorhabditis* SD genes and contains no known structural motifs. Even though its structure is not well conserved much is known about it. It is required for FEM mediated inhibition of TRA-1 and is post-transcriptionally down-regulated in *C. elegans* hermaphrodites to switch from spermatogenesis to oogenesis (Ahringer *et al.,* 1992). It is also the part of the FEM complex which directly interacts with TRA-2. Even though *fem-3* is a rapidly evolving gene so too is *tra-2* and their interaction has been maintained in *C. briggsae* (Haag *et al.*, 2002). The nonsense mutation in *fem-3*(*ed34)* is expected to leads to a truncated 204 amino acid long protein. It appears that FEM-3*(ed34)* is partially functional due to the presence of males that are capable of sperm production. This partial function also explains why fem-3(*ed34)* complements the *fem-3(nm63)* deletion allele. Despite having enough of a function to complement a null *Cbr-fem-3* allele, *fem-3(ed34)* shows hypomorphic activity that can suppress the *tra-2(ed23ts)* mutation.

Cbr-fem-3 null mutants resemble *Cbr-fem-1* and *Cbr-fem-2* mutants, implying that they are all responsible for maintaining the integrity of the Cbr-FEM/CUL-2 complex. *fem-3(ed34)* animals must have enough Cbr-FEM/CUL-2 activity to repress Cbr-TRA-1 in order to produce males. I hypothesize that *fem-3(ed34)* produces a protein that is able to form a complex with Cbr-FEM-1, Cbr-FEM-2 and Cbr-CUL-2 but that this complex is unstable. This complex is able to inhibit Cbr-TRA-1 as when it is assembled but it cannot maintain this state and comes apart over time. At any given time in a cell there would be a number of assembled and unassembled Cbr-FEM/CUL-2 complexes, the latter of which

would result in a net increase in the levels of Cbr-TRA-1. This increase in Cbr-TRA-1 activity would not completely repress male development but could cause feminization of the soma and germline in these males. This overall loss in Cbr-FEM/CUL-2 activity could also be how *fem-3(ed34)* suppresses *tra-2(ed23ts)* (figure 33).

4.2.3.1 fem-3(ed34) may reveal a functionally important region in FEM-3

FEM-3 integrates into the FEM complex by binding to FEM-2 *in vitro,* in a species-specific manner, (Stothard and Pilgrim, 2006; Zhang *et al*., 2013) while the intracellular cleavage product, TRA-2ic, directly interacts to FEM-3 to negatively regulate the FEM/CUL-2 complex (Figure 4.) (Mehra *et al.*, 1999). The site of FEM-2 to which FEM-3 binds is known and the region of TRA-2ic that interacts with FEM-3 is known, however, it is still unclear which parts of FEM-3 are involved in these interactions.

The *fem-3(nm63)* deletion allele is missing part of exon 2, all of exons 3 and 4 and part of exon 5, from residue 73-227, but all other exons are translated in the peptide (Hill *et al*., 2006). This allele is phenotypically identical to nonsense alleles that truncate Cbr-FEM-3 after only 67 residues and is likely a null allele. *fem-3(ed34)* produces a peptide containing the first 204 amino acids of Cbr-FEM-3. Since *fem-3(ed34)* retains some male promoting activity, this suggests that the area between residues 73 and 204 is potentially important for some Cbr-FEM-3 function. Based on the male-promoting behavior, this truncated Cbr-FEM-3 protein likely retains the Cbr-FEM-3/2 interaction which is essential for this function rather than Cbr-FEM-3/TRA-2ic interaction. In addition, *fem-3(ed34)* complements *fem-3(nm63)* hinting that there are two separate functional domains in Cbr-FEM-3.

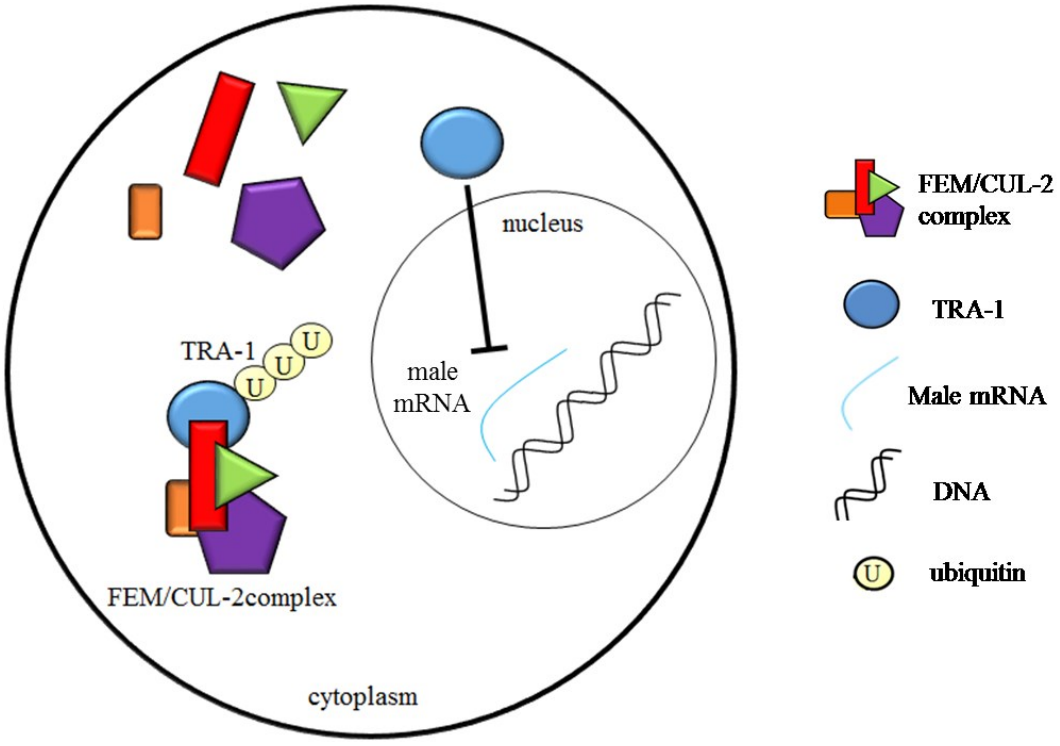

Figure 33. Proposed model for the molecular action of *fem-3(ed34)*. There are multiple TRA-1 proteins and multiple FEM/CUL-2 complexes in a cell at any given time. In some instances the FEM-complex will be able to bind TRA-1 and ubquitinate it as normal but in others FEM-3*(ed34)* is unable to integrate into the FEM complex correctly. This results in an unstable FEM complex which is unable to inhibit TRA-1.The overall affect is that there is an increase in active TRA-1 in the nucleus. In XX animals this is the normal state of the cell so no phenotype is observed. In XO cells an increase in TRA-1 activity causes mild feminization of the soma and germline.

4.3 RAM phenotype in *tra-1(ed30)/tra-1(nm2)* mutants

C. briggsae tra-1(ed30)/tra-1(nm2) mutants produce malformed tail rays (figure 18) similar to those seen in *Ce-ram* mutants. The tail rays appear larger than wild-type male tails and some appear to not develop. This malformation is likely caused by a mutation in the *ram-5* gene present in the *tra-1(ed30)/tra-1(nm2)* mutants. Ce-RAM-5 is a transmembrane protein that is essential for correct tail ray morphogenesis (Sulston and Horvitz, 1977; Yu *et al.*, 2000) and exclusively expressed in structural and hypodermal cells of L4 male larvae. After the tail ray has formed it is rapidly turned over and is not required for the maintenance of the structure. *Ce-ram-5* is not expressed in the neuronal cells of the tail and null *C. elegans* mutants show normal, successful mating behavior (Yu *et al*., 2000).s

Unlike the *tra-1(ed30)/tra-1(nm2)* mutants, *Ce-ram-5* mutants have correct positioning of their lumpy tail rays, however the *tra-1(ed30)/tra-1(nm2)* positioning could be explained by abnormal levels of *Cbr-tra-1* expression. The reason why this phenotype is more pronounced in the *tra-1(ed30)/tra-1(nm2)* mutants could be related to the levels of *Cbr-tra-1* expression. It is possible that Cbr-RAM-5 may be under Cbr-TRA-1 regulatory control so that in the *tra-1(ed30)* homozygous mutant the phenotype is repressed but in the *tra-1(ed30)/tra-1(nm2)* mutants the mutant allele of *Cbr-ram-5* is expressed leading to the lumpy phenotype. *C. elegans* and *C. briggsae* RAM-5 appear to be structurally very similar and are likely to play similar roles in male ray development. As in *C. elegans* I expect *Cbr-ram-5* mutants to be fully fertile and capable of outcrossing with hermaphrodites. This makes *Cbr-ram-5* a potentially attractive X-chromosome phenotypic marker for *C. briggsae*.

4.4 *C. nigoni* SD protein structure comparisons

While the degree of identity can be useful for determining functional conservation between orthologs it is by no means a certain indicator of such a relationship. An

extreme example of this is the *gld-1* gene. GLD-1 is 85% identical between *C. elegans* and *C. briggsae,* which would normally be considered similar enough that both proteins should have the same role in these two species. However GLD-1 plays opposite roles in these closely related species by promoting spermatogenesis in *C. elegans* while promoting oogenesis in *C. briggsae* (Jan *et al.*, 1999; Beadel *et al.*, 2011). Even in less extreme cases, when trying to determine functional conservation it can be more informative to examine where within a protein change has occurred, rather than how much of the protein has changed. Specifically, small changes in functional domains, like protein binding sites or cellular localization signals could lead to large changes in a protein's role. In addition, changes to regulatory sequences of a gene could lead to a change in function of that gene product but that is more difficult to measure if those regulatory sequences are not known. The comparisons between *C. nigoni* and *C. briggsae* SD pathway members did not reveal any obvious clues as to how *C. briggsae* facilitates hermaphroditism but it did show that there is a large degree of structural conservation in the SD genes in both species and that the germline specific factor *she-1* which was thought to be unique to *C. briggsa*e is also present in *C. nigoni* (table 11).

One of the foundations of molecular genetics assents that gene function is determined by gene structure, and as such, any region that is absolutely required for said function should not be changeable. An interesting observation made is that some of the SD proteins exhibit divergence in functional domains, compared with other parts of the protein. Cni-TRA-2, Cni-FEM-1, Cni-FEM-2, Cni-CUL-2 and Cni-PINK-1 all follow this trend. TRA-3 is almost completely identical between these two species. The differences which do exist are changes to amino acids with similar properties and there appears to be an equal number of substitutions in functional and non-functional domains which is indicative of neutral evolution (figure 26). While there are several changes in Cni-TRA-2's TMLs, most of the differences occur in its intracellular domain which is involved in independent interactions with Cni-FEM-3 and Cni-TRA-1 (figure 25). Presumably these interactions are maintained in *C. nigoni* and these proteins

continue to co-evolve. The localized sequence divergence seen in the Cni-FEM-3 and Cni-TRA-1 binding domains is not terribly surprising given that these interactions have been shown to occur in a species specific manner and one reason why they are relatively similar is due to *C. nigoni* and *C. briggsae* having undergone speciation so recently.

Indeed FEM-3 is so well conserved that it may be possible that Cni-FEM-3 can function in *C. briggsae* and vice versa. FEM-3's lack of known functional domains makes it impossible to come to any conclusions about what the differences between *C. nigoni* and *C. briggsae* may mean, if anything (figure 23). Cni-FEM-1's differences appear mostly in its ankryin repeat domains (figure 21) which are important for protein-protein interactions. FEM-1 directly interacts with both FEM-2 and TRA-1 so changes in its ANK domains may necessitate changes to these two other proteins in order to maintain these interactions. Alternatively they may simply be due to random mutations which lead to changes in the amino acid sequence. The same conclusions can be made for Cni-FEM-2. There are several differences in the FEM-1 and FEM-3 binding α-helices, which could be to accommodate minor changes in these associated proteins or be the result of neutral selection (figure 22). Cni-FEM-2 also contains a change from a serine to a non-conserved phenylalanine in its kinase domain. This serine is also not conserved between *C. briggsae* and *C. elegans* and unlikely to be a functionally critical residue.

While the number of differences between Cni-CUL-2 and Cbr-CUL-2 are small they are clustered in the cullin neddylation domain (figure 24). Neddylation is the process where the ubiquitin like protein Nedd8 binds to CUL-2 and is necessary for facilitating the ubiquitin ligase activity of the CUL-2 complex. Interestingly this region of CUL-2 is conserved between *C. elegans* and *C. briggsae* so these changes would have occurred after *C. nigoni* and *C. briggsae* diverged. If Cbr-CUL-2 is contributing to hermaphroditism in *C. briggsae* then this would hint that the last common ancestor of *C. nigoni* and *C. briggsae* was a hermaphrodite and *C. nigoni* lost hermaphroditism after they separated. PINK-1 is

almost identical between *C. nigoni* and *C. briggsae* (figure 27) and as divergent from *C. elegans* as *C. briggsae* is, allowing for the possibility that PINK-1 is not unique to *C. briggsae* SD and is used by its sister species as well. Of course it is possible that it is the regulation of Cbr-PINK-1 which has allowed it to integrate into the *C. briggsae* pathway rather than a change in structure.

Cni-GLD-1 is almost identical to its *C. briggsae* ortholog and has a slightly higher percentage amino acid identity to the *C. elegans* ortholog than Cbr-GLD-1 (figure 28). As such, it is likely that Cni-GLD-1 also functions in a female-promoting capacity in *C. briggsae*. The germline specific factor *Cni-she-1* appears to be present in *C. nigoni,* indicating that the duplication event that led to its existence occurred before *C. nigoni* and *C. briggsae* diverged. From the limited amount of sequence that was obtained it appears that Cni-SHE-1 is the least well conserved SD protein (figure 29). The presence of *Cni-she-1* in *C. nigoni* shows that it alone is not responsible for the evolution of hermaphroditism in *C. briggsae*. It appears that the germline specific pathway is much more rapidly evolving than the global pathway as evident by loss and gain of entire genes (e.g. *Ce-fog-2* and *Cbr-she-1* respectively) and the reassignment of roles as in *gld-1* rather than small scale differences found between somatic pathway members.

4.5 A model for pathway evolution

Although *C. elegans* and *C. briggsae* both evolved hermaphroditism independently, the genetic pathways that underpin this trait have diverged substantially since they shared a common ancestor, a phenomenon known as Developmental Systems Drift (Verster *et al*., 2014). Developmental Systems Drift suggests that even if a trait, such as hermaphroditism, is under strong stabilizing selection, the genes that control it can evolve new roles. A prime example of this are the *fem* genes, which in *C. elegans* regulate spermatogenesis but in *C. briggsae* do not. A systemic study comparing knock-downs in 1,300 *C. elegans* and *C. briggsae* orthologs showed 91 cases of likely functional divergence

(Verster *et al*., 2014). This set of genes showed a large number of transcription factors and recently evolved genes with unknown function. Relatively few genes involved in ancient cellular processes such as protein synthesis were found. They proposed that the differences in molecular function of these genes could be due to (1) changes in expression pattern caused by promoter evolution, (2) changes in the coding region of the genes which leads to altered protein structure and (3) changes in regulatory genes. They concluded that all the above points can contribute to Developmental Systems Drift.

From comparisons made between *C. elegans* and *C. briggsae* SD mutants and sequence comparisons between *C. briggsae* and *C. nigoni* SD proteins it appears that members of the core pathway are under stabilizing selection to maintain their interactions. The large difference in amino acid identity seen between *C. elegans* and *C. briggsae* orthologs is likely due to neutral drift, substitutions accumulating in non-functional parts of the protein (Stothard and Pilgrim, 2003). Sterile hybrids formed between *C. briggsae* and *C. nigoni* is unlikely to be explained by protein sequence divergence and so may be the result of differences in spatial-temporal expression patterns. Some of the most striking differences between the pathways of these two species are found in the germline regulators. Species specific duplications have given rise to these genes (e.g. *Ce-fog-2* and *Cbr-she-1*) which have then been co-opted into their respective pathways. Together these observations lead to a model which suggests that the *Caenorhabditis* sex determining pathway is evolving via Developmental System Drift.

4.6 Future directions

4.6.1 Identifying functional domains in the Cbr-FEM proteins

There are 45 remaining *tra-2(ed23ts)* suppressors to be sorted into complementation groups and sequenced with the goal of identifying missense

mutations. Missense mutations have so far been valuable in identifying regions of functional significance in the Cbr-FEM proteins. The Cbr-FEM proteins are involved in many protein-protein interactions, they form a complex with each other and Cbr-CUL-2 and directly interact with Cbr-TRA-2 and with Cbr-TRA-1.

The first missense mutation in *Cbr-fem-1* was identified in this work. As the mutation occurs in an ankryin repeat domain it is likely that it negatively affects Cbr-FEM-1's interaction with another protein. However it is not clear whether it is FEM complex formation or Cbr-TRA-1 binding that fails to occur. This can be determined using yeast two-hybrid assays or complex immunoprecipitation. A lot is still unclear about the structure and function of Cbr-Cbr-FEM-3 and so far no missense mutations have been found for *Cbr-fem-3*. Cbr-FEM-3 has been shown to directly interact with Cbr-FEM-2 and Cbr-TRA-2 although which part of Cbr-FEM-3 is involved in these evolutionarily conserved interactions is still a mystery. Comparisons between *fem-3(nm63)* and *fem-3(ed34)* deletion alleles hint that an important region may be between residues 70-200. Further complementation and sequencing of *tra-2(ed23ts)* suppressors could identify missense mutations in *Cbr-fem-3* and using yeast 2-hybrid assays with different truncated *Cbr*-FEM-3 could identify evolutionarily conserved functional regions. It has been shown that the phosphatase activity for Ce-FEM-2 is required for SD in *C. elegans* (Chin-Sang and Spence, 1996). A lot of work has been done to show that Cbr-FEM-2, has scaffolding functions and is critical in assembling the Cbr-FEM complex however it has not been shown that Cbr-FEM-2s phosphatase activity is required for SD. Finding missense mutations located in the phosphatase domain of Cbr-FEM-2 that are capable of suppressing *Cbr-tra-2* mutations, would confirm that phosphorylation is necessary for SD in *C. briggsae*.

Knowing the functional domains of these proteins is important for determining the rate of evolution of individual members of the SD pathway. This is particularly useful for comparisons with *C. nigoni*. A higher number of amino acid changes in functional domains is indicative of positive selection while a more

even spread of changes in functional and non-functional domains relative to their size is more indicative of neutral selection. Further comparisons with *C. nigoni* will also prove useful as more of the *C. briggsae* pathway becomes known. It would also be interesting to see if *C. nigoni* SD orthologs are similar enough to their *C. briggsae* counterparts that inter species complementation would be possible.

4.6.2 Confirm that Cbr-tra-1 and Cbr-fem-3 are the causative mutated genes in ed30 and ed34

Although *Cbr-tra-1* and *Cbr-fem-3* appear to be likely candidates for *ed30* and *ed34* respectively, it will need to be confirmed that they are indeed responsible for the phenotypes shown. There are several other lesions in each of these strains so it will be important to isolate them from other background mutations including *tra-2(ed23ts)*. It will be interesting to see what phenotypes (if any) these alleles will display when present in an otherwise wild-type background. I expect that their phenotypes will be similar to those seen in *tra-2(ed23ts)*; *sup*(*ed30)* and *tra-2(ed23ts)*; *sup(ed34)* provided that *tra-1(ed30)* and *fem-3(ed34)* are the causative mutations. In addition to isolating *fem-3(ed34)* in a normal background, microinjection of *tra-2(ed23ts); (ed34)* with wild-type *Cbr-fem-3* mRNA would confirm that *fem-3(ed34)* is indeed the causative mutation if the Tra phenotype is observed. Similarly RNAi could be used on *tra-2(ed23ts); sup(ed30)* animals to knock down the amount of *tra-1*(gf) present in the worm. Again, observation of the Tra phenotype would point to *tra-1(ed30)* as being responsible for the *ed30* phenotype. In the past the ability of RNAi to phenocopy mutants in *C. briggsae* was thought to be unreliable. However that idea may be incorrect and it will be worthwhile to retry RNAi to phenocopy *C. briggsae* SD mutants.

4.6.3 Confirm that Cbr-pink-1 is a novel member of the C. briggsae sex determining pathway

Since *Cbr-pink-1* may represent a novel SD gene this will also need to be confirmed by microinjecting wild-type *Cbr-pink-1* mRNA into *tra-2(ed23ts); sup(ed34)* adult gonads. If *Cbr-pink-1* is a *tra-2(ed23ts)* suppressor, I expect this to rescue the *ed31* phenotype and animals to appear Tra. Like *ed30* and *ed34* it would also be valuable to examine *ed31* in the absence of *tra-2(ed23ts)*. If *Cbr-pink-1* is indeed a novel member of the *C. briggsae* pathway, what exactly is its purpose in the pathway and is it still fulfilling the role of oxidative stress management that it does in *C. elegans*? Making mutations in different parts of the protein, as well as conducting yeast 2-hybrid experiments, could reveal its functional role in SD and show what other members of the pathway it's acting with. It would also be interesting to see how *ed31* worms respond to increases in oxidative stress to determine if *pink-1* has a conserved function between *C. elegans* and *C. briggsae* or if a different kinase has taken over this particular role.

4.6.4 Comparisons with a third androdiocious species, C. tropicalis

C. tropicalis is the third androdiocious species in the *Caenorhabditis* genus and provides another reference point for the evolution of hermaphroditism. It either has the same sex-determining system as *C. elegans* or the same as *C. briggsae* or it evolved an entirely different way of making a hermaphrodite. To identify the members of *C. tropicalis'* SD pathway mutagenic screens could be used in the same manner as described previously. However since the emergence of next generation sequencing and new technologies to cause precise targeted mutations such as CRISPRs it may be easier to create specific null mutants in *C. tropicalis* SD gene orthologs and compare phenotypes with *C. elegans* and *C. briggsae.* This could potentially show one more way that hermaphroditism could evolve and further demonstrate how biological pathways evolve.

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