Feminizing mutations in *Caenorhabditis briggsae* indicate novel regulation of ovotestis development

by

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Abstract

The evolution of novel traits relies on heritable changes in gene structure or gene expression, but the processes by which these occur is not always clear. Sex determination is a particularly interesting trait with which to model these processes because its regulation seems to be subject to rapid evolution. Androdioecy, or a species' ability to make an ovotestis in an otherwise female animal, has independently evolved three times in the *Caenorhabditis* nematode clade from a dioecious ancestor. We want to understand this regulation at the genomic level in *C. briggsae* and compare it to *C. elegans* to provide insight into the way a common set of genes can be modified to produce a novel trait.

Forward genetic screens allowed genetic identification of the *C. briggsae* orthologs of *C. elegans* genes (*tra, fem*) required for somatic and gonadal sex determination, but unlike in *C. elegans*, none of the feminizing mutants blocked spermatogenesis in the female ovotestis. A second screen, looking for suppressors of masculinizing mutants identified several phenotypes not seen in *C. elegans*. Many of these suppressors permit the development of XX hermaphrodites and XO males in a masculinized *tra-2* background, in contrast to *C. elegans* feminizing mutants where XO animals are feminized. A loss of all *cbr-fem* genes results in the development of XX and XO hermaphrodites, indicating that the regulatory locus controlling ovotestis development is downstream of the *fems. tra-2;fem-X/+* animals are partially feminized males, providing more evidence that the regulation is downstream of the *fems.* Finally, *cbr-ubxn-3* was indicated as a novel member of the *C. briggsae* sex determination regulatory network. Together these findings indicate that the regulatory locus in *C. briggsae* that permits ovotestis development is downstream of the *fem* genes, different than in *C. elegans*.

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List of Abbreviations

°C.....Degrees Celsius
µL.....Microliters
bp.....Base Pairs
Cbr..... *C. briggsae*
cby.....chubby (phenotype)
Cel..... *C. elegans*
Chr.....Chromosome
CUL.....Cullin-Ubiqitin Ligase
DIC.....Differential Interference Microscopy
DNA.....Deoxyribonucleic Acid
dNTP.....deoxyribose nucleoside triphosphate
dpy.....dumpy (phenotype)
DRE.....Direct Repeat Element
dsRNA.....double stranded ribonucleic acid
EtOH.....Ethanol
F1.....Filial 1
F2.....Filial 2
F3.....Filial 3
F4.....Filial 4
FBF.....Fem-3 mRNA Binding Protein
fem.....*fem*inized (Phenotype)
fog.....*f*eminization *o*f *g*ermline
gDNA.....genomic Deoxyribonucleic Acid
gf..... *gain of function*
GLD.....defective in germline development
GRN.....Genetic Regulatory Network
her..... *her*maphrodite (phenotype)
ic.....intracellular
L4.....Larval stage 4
laf.....lethal and feminizing
lf.....*loss of function*
mM.....nanoMolar
mog.....masculinization of germline

mRNA……..messenger Ribonucleic Acid
NOS……..Nanos Related
NP-40……..Tergitol-type nonyl phenoxypolyethoxylethanol
NGM……Nematode Growth Media
PCR……Polymerase Chain Reaction
PME……..Point Mutation Element
PPC2……..Protein Phosphatase type 2C
RNA……..Ribonucleic Acid
RNAi……Ribonucleic Acid interference
RPM……..Rotations per minute
RT……..Room Temperature
SHE……Spermless hermaphrodites in C. briggsae
STAR……Steroidogenic acute regulatory protein
tra……*tra*nsformer (phenotype)
trr……..TRRAP-like (transcription/transformation domain-associated protein)
ts……..temperature sensitive
UTR……...Untranslated Region
WGS……..Whole genome sequencing
WLB……Worm lysis buffer
WT……...Wildtype
xol……XO lethal

1. Introduction

1.1 Evolution of Novel Traits

The evolution of novel traits between species that share a recent common ancestor is a central question of the field of evolutionary development. These novelties often lead to the defining characteristics of a species, but we know very little about how these novelties develop at the molecular level. Although there are many examples of conservation in developmental pathways, we have very little understanding of the molecular mechanisms that allow for a common factor or pathway to be modified to produce novel traits. Conceptually, there are two ways in which novel gene function may arise: first, the gene product has acquired the capacity to perform a novel function, or second, the temporal or spatial expression of a gene has changed while the product's molecular function remains the same. As animal development is controlled though a series of regulatory interactions within a signaling network, changes to interacting factors or in the expression of a factor can lead to effects on the network. Understanding how changes to signaling factors impact genetic regulatory networks (GRNs), is required to understand the evolution of these novel traits at the molecular level.

Davidson and Erwin summarize a number of theoretical models of how developmental genetic regulatory networks can evolve (2006). First, they define 4main components of developmental regulatory networks: 1) the core regulatory pathway that has been conserved over evolutionary time, 2) the easily interchangeable regulatory "plug-ins" that are used to regulate gene expression, 3) the switches that turn the pathway on or off, and 4) the genetic output of the pathway. Many examples of how core regulatory pathway elements are conserved over evolutionary time exist; one of the best examples is the function of *hox* genes

in Metazoan axis specification. *hox* genes are a family of genes whose expression specifies the developmental fate of structures along the anterior/posterior axis (Pearson et al. 2005). The function of *hox* genes is conserved over diverse phyla, regardless of structure produced (*i.e*. pectoral fin in fish *vs*. forelimb in mammals), indicating that there are strong evolutionary pressures upon this core pathway. Instead of developing new pathways, novel traits may arise through the modification of other GRN components acting on the existing, conserved pathway. In many cases, this change may arise through changes to the genetic outputs of the GRN though changes to cis-regulatory elements. This leads to novel genetic factors being turned on or off in response to activation of the network, which then contribute to the development of novel structures. In this work, regulatory "plug-ins" and pathway switches will be considered as a single element as they both modify gene expression. Together, these form the mechanism for the change in gene expression in space or time. Although there is abundant evidence for gene expression changing in space and time, leading to a novel trait, there is very little understanding of how this happens at the molecular level. In other words, how can changes to regulatory elements and other pathway switches lead to novel phenotypes?

Sex determination in *Caenorhabditis* has been used as a model to answer this question because of the multiple independent modifications to a GRN to produce the novelty of hermaphroditism in an otherwise male/female system (Kiontke et al. 2004). Because sex determination genes evolve relatively rapidly compared to genes of other systems, this allows us to isolate and test specific genetic changes in closely related species (Haag 2005).

1.2 Evolution of hermaphroditism in *Caenorhabditis*

Within the *elegans* group of *Caenorhabditis* nematodes, a core sex determination pathway is conserved so that XX animals develop as somatic females,

while XO animals develop as males. The two sexes have several sexually dimorphic features in both the soma and germline. Females develop a double armed somatic gonad, the gonad structures excluding gametes, with oocytes developing in the ovary, then being fertilized while passing through the spermatheca containing sperm, and finally developing as embryos in the uterus (figure 1). In hermaphroditic species, the germline transiently produces sperm that can be used for self-fertilization. Additionally, females have a characteristic tail spike, in contrast to the fan like tail of the male with sensory rays for mating. The male gonad is single armed and produces only sperm (figure 1).

The conserved core sex determination pathway depends upon a series of inhibitory interactions between male-promoting genes, *her-1* and the *fems,* and female-promoting genes, the *tras* (figure 2). The ratio of sex chromosomes to autosomes regulates sex with XX animals developing as females and XO animals developing as males. Hermaphroditism, or the ability of an otherwise female animal to develop an ovotestis, requires a germline-specific modulation of the core sex determination pathway described above. The animal develops as a somatic female, then in late larval stages down-regulates female promoting genes in the germline to allow the production of sperm. Then in early adulthood, the worm needs to down regulate these male genes so that oogenesis can begin. Because this is a case of spermatogenesis in an otherwise female gonad, or androdioecy, these events will be referred to as the "sperm on switch" and "sperm off switch", respectively.

Within the *elegans* group of the *Caenorhabditis* genus, ovotestis development has evolved at least three times: *C. elegans, C. briggsae*, and *C. tropicalis* (Kiontke et al. 2004, figure 3). Because the majority of this group is androdioecious and, more importantly, all dioecious species have androdioecious sister species, it is most likely that these are independent evolutionary events. Although it is very hard to identify morphologically distinct features between discrete species in this group

through observation alone, over time a number of developmental differences have been identified (Felix et al. 2014). Some of these changes are related to changes in the genome structure, such as the rapid loss of non-coding DNA in androdioecious species, while others are morphological changes such as the increased incidence of ray fusion in the *C. briggsae* male tail as compared to *C. elegans* (Baird et al. 2005; Kiontke et al. 2004). However, because of the high degree of similarity in other essential processes, it is easy to make comparisons and identify small changes to regulatory networks that allow for the modification of GRNs and evolution of novel traits.

The evolution of sex determination is a good model to study GRN evolution because sex determination is highly varied across metazoans, and has been shown to be rapidly evolving. There is specific evidence for this rapid divergence at the genetic level in *C. briggsae* as many of the sex determination genes do not share strong sequence similarity with their *C. elegans* homologs (Kuwabara 1996; Zhang et al. 2013). The high relatedness between *C. briggsae* and *C. elegans* as well as the rapid divergence of sex determination genes allows us to model how specific modifications to highly similar GRNs can lead to the evolution of the same novel trait: hermaphroditism.

1.3 A model for *Caenorhabditis* sex determination: *C. elegans*

1.3.1 Core sex determination pathway

In *C. elegans,* sex is determined by the ratio of sex chromosomes to autosomes with 1:1 ratio leading to female development and a 0.5:1 ratio leading to male development (Madl & Herman 1979, figure 4). In this section, only *C. elegans* se determination genes will be discussed, so species identifiers were not used. *xol-1* is responsible for sensing the chromosome ratio and setting off downstream signaling cascades for both sex determination and dosage compensation (Miller et al. 1988).

Mutants in genes of the core *C. elegans* sex determination pathway fit into two classes: masculinizing mutations and feminizing mutations (figure 2). All of these genes were identified through forward genetic screens to identify animals whose sex phenotype was different than expected based on the number of sex chromosomes, or a sex transformation phenotype. *tra-2* and *tra-3* mutants were identified as animals where both XX and XO animals developed as males (Hodgkin & Brenner 1977). However, the XX transformed males were not completely masculinized; although mutants developed a somatically male gonad that produced sperm, the worms also had feminized tail rays. *tra-1* was also identified as an masculinizing allele but the transformation phenotype of XX animals was more complete; many alleles permit the development of male tails and somatic male gonads that produce sperm (Hodgkin 1980; Hodgkin 1987). These phenotypes are referred to as tra (*tra*nsformer).

Alleles of *her-1* were isolated through a genetic screen to isolate loci that prevented male development in *him* (*h*igh *i*ncidence of *m*ales) backgrounds (Hodgkin 1980). XX *her-1* animals develop as normal hermaphrodites while XO animals are transformed into fertile hermaphrodites. Other feminizing alleles were found through suppressor screens of the masculinizing alleles described above. To isolate more feminizing loci, forward genetic screens were preformed to identify alleles that rescued the *tra-3* phenotype, leading to the discovery of the *fem* (*fem*inizing) genes. *Loss-of-function* alleles of all three *fem* genes develop as XX and XO females (Hodgkin 1986). The loss of somatic male features in XO animals and spermatogenesis in the germline of XX and XO animals indicates that the *fem* genes are necessary for both a male somatic and germline fate.

Through epistasis, the *fem* genes have been found to occupy the same position in the genetic pathway, downstream of *tra-2* and *tra-3* and upstream of *tra-1* (figure 2, (Hodgkin 1986; Hodgkin 1987). By using *gain-of-function* (*gf*) alleles for

tra-3, tra-3 has been shown to be upstream of *tra-2* and interact with *tra-2* in a *her-1* independent manner (not shown in figure, Hodgkin 1980). All of the *fem* genes are epistatic to *tra-2* and *tra-3* in the soma and germline while being epistatic to *tra-1* only in the germline (Hodgkin 1986). Together, these genes and the described interactions will be referred to as the core sex determination pathway.

Our understanding of the genetic interactions of the core sex determination pathway is enforced by the molecular interactions of the proteins (Figure 5). This genetic signaling cascade leads to HER-1, an extra-cellular signaling molecule, to be expressed in XO animals but not in XX animals (Hunter & Wood 1992). TRA-2 is a transmembrane protein that contributes to signal transduction, from the extracellular ligand, HER-1 (Kuwabara et al. 1992). It is the binding of HER-1 to the extracellular domain of TRA-2 that is the first sex determination signaling event considered in this work. TRA-3 is a cytosolic calpain protease that cleaves the intracellular domain of TRA-2 (TRA-2ic), to promote female development when HER-1 is present (Barnes & Hodgkin 1996; Sokol & Kuwabara 2000). When TRA-2ic is cleaved, it is believed to translocate to the cytoplasm and associate with the FEM complex, interfering with the binding of the FEM complex to TRA-1 (Zarkower 2006).

The FEM complex consists of FEM-1/2/3 as well CUL-2. FEM-1 is a cytosolic protein with many ankyrin repeat motifs, associated with protein binding and regulation of developmental processes (Spence et al. 1990). FEM-2 is a PPC2C phosphatase and its N-terminal domain is important in facilitating the binding to other members of the FEM complex (Pilgrim et al. 1995; Zhang et al. 2013). FEM-3 has no recognizable domains, and is predicted to act as a scaffold for the entire FEM complex (Rosenquist & Kimble 1988). CUL-2 is a cullin-ubiqitin ligase, that has a role in both sex determination and the meiosis-to-mitosis transition (Feng et al. 1999). Because of this essential role in development, *cul-2* mutants were not isolated in forward genetic screens that assayed for feminizing genes epistatic to *tra-2* or *tra-3*.

In a pull-down assay, CUL-2 was found to associate with the FEM complex, and is able to ubiqitinate TRA-1 which targets it for degradation by the proteasome (Starostina et al. 2007).

tra-1 encodes a zinc finger transcription factor that acts as the terminal regulator of sex-fate in the *C. elegans* soma (Zarkower & Hodgkin 1992). When TRA-1 translocates to the nucleus, it is able to promote the expression of female genes, while indirectly inhibiting expression of male genes through the expression of female genes (Berkseth et al. 2013). The gain-of-function domain of TRA-1, is named for the large number of *gf* lesions isolated in this region. These missense alleles eliminate spermatogenesis in XX animals, and feminize the germline and soma of XO animals (De Bono et al. 1995). This domain is postulated to be important for the binding of TRA-1 to the FEM complex. These mutations disrupt this binding resulting in a constitutively active TRA-1, as the FEM complex cannot sequester it.

1.3.2 Germline pathway regulation – how to make a hermaphrodite

For ovotestis development to occur in an otherwise female animal, germline cells must be able to take on a male fate, producing sperm, then later in development take on female fate, producing oocytes for the remainder of the animal's life (Figure 4). Spermatogenesis is initiated through the expression of *fog-1* and *fog-3*, which are the terminal regulators of sexual fate in the germline (Barton & Kimble 1990; Ellis & Kimble 1995). The expression of *fog-3* is negatively regulated directly by TRA-1. This means that the regulation of ovotestis development depends on modulation of the sex determination pathway upstream of TRA-1 (Chen & Ellis 2000).

The first event required for the ovotestis to initiate spermatogenesis is the down regulation of female-promoting factors; specifically, the down regulation of TRA-2, as the *tra-2* 3' UTR contains a DRE (direct repeat element), which is essential

for regulation of *tra-2* in hermaphrodites. Deletions of all or part of the DRE results in *tra-2(gf)* mutations (Goodwin et al. 1993), as binding of inhibitory complexes to this *tra-2* 3'UTR region is required for the down regulation of TRA-2, and the onset of spermatogenesis in XX worms (Goodwin et al. 1993). A complex formed by the STAR protein GLD-1 and the F-box protein FOG-2 binds directly to the DRE to repress *tra-2* translation (Clifford et al. 2000). In addition, *laf-1* is able to repress translation of *tra-2* downstream of *tra-3* control using the same genetic elements as above, but this mechanism is independent of the GLD-1/FOG-2 repression (Goodwin et al. 1997; Jan et al. 1999).

In addition to the translational repression of *tra-2* regulating the expression of *fog-1* and *fog-3*, TRA-2 interacts with TRA-1 directly to promote spermatogenesis. A second, shorter, *tra-2* transcript exists that encodes for the C-terminal fragment of the TRA-2 ORF only. This shorter transcript is expressed in the germline, particularly in the oocyte, and has an important role in the sperm-off switch. XX animals with mutations in this domain develop as females, but XO animals are unaffected (Kuwabara et al. 1998). This intracellular region directly binds to TRA-1 in addition to the FEM complex, and is critical for promoting spermatogenesis (Wang & Kimble 2001).

A similar translational repression of male-promoting factors is employed to terminate spermatogenesis and initiate oogenesis. Specifically, *fem-3* translation is repressed in the gonad, as *fem-3* transcripts are limited in wild type XX hermaphrodites (Rosenquist & Kimble 1988). A region of the *fem-3* 3' UTR, known as the PME (point mutation element), is associated with gain-of-function mutations and is essential to the down regulation of *fem-3* in hermaphrodites (Ahringer & Kimble 1991). A complex of homologs of *Drosophila* Pumilio and Nanos, FBF-1/2 and NOS-3 respectively, form a complex to bind the PME and repress *fem-3* translation (Zhang et al. 1997; Kraemer et al. 1999). In addition to the repression of *fem-3*, FBF-1/2

binds to the 3'UTR of *fog-1,* although *fog-1* is epistatic to *fbf-1* and *fbf-2* (Thompson et al. 2005). This suggests that this is a second locus, where the FBFs can promote oogenesis in the hermaphroditic germline through repression of the *fog-1* transcript. A family of genes known as the *mogs* (*m*asculinization *o*f *g*ermline) were identified as additional factors that repress *fem-3* translation though the PME independently of FBF-1/2 and NOS-3 (Gallegos et al. 1998). Without FEM-3, the FEM complex falls apart and cannot bind TRA-1. This allows TRA-1 to translocate to the nucleus, repressing *fog-3* and leading to the initiation of oogenesis.

1.4 *C. briggsae* sex determination

1.4.1 Comparing *C. briggsae* and *C. elegans* somatic sex determination

Most of the core *C. elegans* sex determination pathway genes have clear orthologs in *C. briggsae*, although the degree of similarity varies greatly, and are more derived than non-sex related genes (Haag 2005). Despite the divergence in sequence, the roles these genes play in somatic sex determination seem to be conserved. For instance, the sequence of both *cbr-fem-2* and *cbr-fem-3* is highly divergent in *C. elegans* and *C. briggsae,* however the proteins still physically interact (Stothard & Pilgrim 2006). The TRA-2/FEM-3 interaction in *C. elegans* is also conserved in *C. briggsae* (Haag et al. 2002). In both these cases, the interactions are intraspecies specific, *C. briggsae* gene products only weakly bind or are not able to interact with those from *C. elegans* or a dioeciuous member of the group, *C. remanei* (Haag et al. 2002; Stothard & Pilgrim 2006). In addition to changes in protein binding, most *C. briggsae* sex determination genes cannot rescue *C. elegans* mutants when introduced as transgenes. This indicates that these species-specific interactions are critical for sex determination gene roles *in vivo.*

Forward genetic screens in *C. briggsae* produced *loss-of-function (lf)* alleles for the *tra* genes. *Cbr-tra* mutants all develop as masculinized XX animals and

normal XO animals, the same phenotype observed in *C. elegans* (Kelleher et al. 2008; Hill & Haag 2009, figure 6). Both *cbr-tra-2* and *cbr-tra-3* XX animals have a single armed gonad that produces sperm and a blunt tail without rays; indicating that these animals are likely infertile because of incomplete masculinization of their tails (Kelleher et al. 2008). *cbr-tra-1* XX animals develop a single-armed spermatogenetic gonad and a normal male tail, and are capable of mating (Hill & Haag 2009). When *cel-tra-1* mutants are rescued with *cbr-tra-1*, there is a partial somatic feminization of the mutants as well as normal XO animals, however, the somatic germline and gametes are unaffected (De Bono & Hodgkin 1996). This suggests that the germline regulation of *cbr-tra-1* may have diverged enough from *C. elegans* that the *C. elegans* germline regulators have no effect on *cbr-tra-1.* It is interesting that TRA-1 is unable to rescue germline phenotypes in different species, as many of TRA-1 targets are shared between the two (Berkseth et al. 2013). This indicates that although the function of TRA-1 may be conserved in both *C. elegans* and *C. briggsae* pathway, but the modifiers that modulate expression of TRA-1 have changed. This supports the proposed GRN theory that there is a high degree of pressure to retain a core pathway, however the "plug-ins" and modulators of that core pathway can change relatively rapidly.

As in *C. elegans,* a loss of *fem* activity either through RNAi or mutations in *C. briggsae* results in a feminization of XO animals. This feminization only affects the soma as both XX and XO *fem* animals all develop an ovotestis (Stothard et al. 2002; Haag et al. 2002; de Carvalho 2005; Dewar 2011, Figure 6). Only *cbr-fem-2* has been shown to partially rescue the *C. elegans* mutant of its homolog (Hansen & Pilgrim 1998). All other *fem* genes failed to rescue homologous *C. elegans* mutants.

The core sex determination pathway in *C. elegans* is conserved in *C. briggsae* (Figure 2). In addition to the similar phenotypes and conserved physical interactions observed above, the availability of *cbr-lf* mutants has allowed us to test epistatic

relationships within the pathway. A number of *fem* alleles were identified as suppressors of temperature-sensitive *cbr-tra-2* alleles, indicating that the *cbr-fem* genes are epistatic to *cbr-tra-2*, as it is in *C. elegans* (Hill et al. 2006; de Carvalho 2005; Dewar 2011; Reidy 2015). *cbr-tra-1* is also epistatic to the *cbr-fem* genes, with *cbr-fem-2;tra-1* and *cbr-fem-3;tra-1* XX animals developing as Tra animals (Hill & Haag 2009). However, there is a significant increase in endomitotic cells in the *cbr-fem-3;tra-1* gonad, indicating that the *fem-3* mutation has a feminizing effect in the *tra-1* gonad and thus is not completely epistatic to *cbr-tra-1* (Hill & Haag 2009). The genetic interaction between *cbr-fem-1* and *cbr-tra-1* had not been previously tested because a *cbr-fem-1* allele had not yet been identified.

1.4.2 Germline development and evolution of hermaphroditism in *briggsae*

Like in *C. elegans*, to develop an ovotestis there must be a "sperm-on" regulatory switch to initiate spermatogenesis in the otherwise female animal followed by a "sperm-off" switch. In *C. elegans*, the "sperm-on" switch is mediated through a down regulation of *tra-2* by GLD-1 and FOG-2 (Clifford et al. 2000, Figure 7). The DRE elements in the *cel-tra-2* UTR are also found in the *C. briggsae tra-2* UTR and are a good candidate for the down regulation of *cbr-tra-2* (Kuwabara 1996). *fog-2* is not found in *C. briggsae* and likely arose through a *C. elegans* specific gene duplication (Clifford et al. 2000). Close relatives of *cel-fog-2* in *C. briggsae* lack the C-terminal GLD-1 binding domain that is required for the FOG-2/GLD-1 interaction (Nayak et al. 2005). In addition, *gld-1* RNAi of *C. briggsae* animals resulted in spermatogenic germlines, the opposite phenotype as what was observed in *C. elegans* (Nayak et al. 2005). Although the role of *gld-1* has changed between these two species, *cbr-gld-1* is able to completely rescue *cel-gld-1* mutants (Beadell et al. 2011). This indicates that the novel role of *cbr-gld-1* in *C. briggsae* sex

determination is due to other factors in the *C. briggsae* germline, not a change to *cbr-gld-1* sequence as compared to *C. elegans.*

Although *cel-fog-2* homologs do not exist, another F-box protein, SHE-1, has been identified as an upstream regulator of *cbr-tra-2* (Guo et al. 2009). Although this gene is in the same family as *cel-fog-2*, it is not closely related and cannot interact with GLD-1 (Guo et al. 2009). This is an example where genes from the same family can be independently recruited to produce a novelty; as *tra-2* is post-transcriptionally regulated in both *C. elegans* and *C. briggsae*, but this is mediated through different F-box proteins.

Because *cbr-fem* mutants develop as XX and XO hermaphrodites, the *cbr-fem* genes are not required for spermatogenesis and, unlike *C. elegans fem*s, the *C. briggsae fems* cannot be involved in the "sperm-off" switch (Hill et al. 2006; Kelleher et al. 2008; Dewar 2011; Reidy 2015). It is, therefore, unsurprising that homologs of the FBF proteins do not exist in *C. briggsae,* as these down regulate the expression of *Cel-fem-3* (Haag *et al* 2002). However, *cbr-gld-1* works in concert with the FBF homolog, *puf-1.2* to promote oogenesis in *C. briggsae,* possibly through another FBF homolog *puf-8* (Beadell et al. 2011). It is yet to be determined on which component in the sex determination pathway these factors act, as the "sperm off" regulation must be downstream of the *fem* genes.

A possible regulatory point in *C. briggsae* spermatogenesis is *cbr-tra-1,* as it is the final factor downstream of the *cbr-fem* genes in the core regulatory pathway. A previously identified *tra-1 (gf)* mutant develops as an intersex animal, with a male somatic gonad that produces both sperm and oocytes (de Carvalho 2005). This mutation was mapped to the *gain-of-function* domain, a domain that is important for the binding of TRA-1 to the FEM complex in *C. elegans* (Reidy 2015; De Bono & Hodgkin 1996). This indicates that the TRA-1/FEM interaction is still an important regulatory locus in the *C. briggsae* sex determination pathway. *Cbr-trr-1,* a part of

the Tip60 histone acetyl transferase complex, was identified as a male-promoting factor in the *C. briggsae* germline. Both XX and XO *Cbr-trr-1* animals produced only oocytes but had a normal female or male soma (Guo et al. 2013). *trr-1* does not control TRA-1 expression, but likely works with TRA-1 to promote the expression of *fog-3*: a downstream regulator of male fates. *Cel-trr-1* plays a similar role in sex determination, but only a small number of *cel-trr-1* RNAi XX animals still underwent spermatogenesis unless they were sensitized by a weak *fem* allele (Guo et al. 2013). It is possible that *trr-1* plays a bigger role in *C. briggsae* sex determination because *Cbr-tra-1* is the regulatory point in *C. briggsae*, and therefore more sensitive to other regulatory changes.

1.5 Project outline and Goals

The sex determination regulatory network of different *Caenorhabditis* species provides an excellent model of how different genetic modifications to the GRN can lead to the evolution of similar novel traits. This is particularly difficult to study in many other systems because of the high degree of divergence found between species exhibiting the same novel traits open the possibility for larger divergence of their respective GRNs. Because of the high degree of similarity and developmental processes we observe in *C. elegans* and *C. briggsae*, while sex determination systems evolve relatively rapidly, we are able to isolate a single novel trait, hermaphroditism, and understand the ways in which the GRN has been modified to produce this trait.

The goal of my work was to create a more complete understanding of the *C. briggsae* sex determination pathway though the characterization of feminizing alleles and predicted feminizing loci. I was able to achieve this understanding in *C. briggsae* using comparative genomics with *C. elegans* and identified where evolutionary

constraints were placed on the GRN. This will broaden our understanding of GRN evolution as a whole.

The specific goals of this work were to:

(1) Test for redundancy of factors in the FEM complex

(2) Understand the epistatic relationship between *cbr-fem* genes and *cbr-tra-2*

(3) Map and characterize the novel *C. briggsae* feminizing locus, *sup(ed31)*

2.0 Materials and Methods

2.1 Worm Maintenance and Husbandry

Worms were maintained on standard NGM agar plates seeded with OP50 (Brenner 1974). Complete lists of strains used in this work are provided in tables 1 and 2. All worms were maintained at room temperature (RT), with the exception of *tra-2(ed23ts)* strains that were maintained at 16°C, or animals from crosses that were maintained at the temperature indicated in crossing or experimental schemes. All mating crosses were performed at 25°C with a ratio of 20 males: 5 hermaphrodites. After 24 hours, hermaphrodites were transferred to new plates to lay eggs and these progeny were used in subsequent crosses or analysis.

2.2 Microscopy and Image editing

All phenotypes, with the exception of germ line sex phenotypes, were scored on a stereo dissecting scope. Germ line phenotypes were scored and all images were captured on an axioskop 2 microscope with DIC optics (Zeiss). All images were edited using Adobe Photoshop and prepared using Adobe Illustrator.

2.3 Genotyping: PCR and sequencing

2.3.1 Genomic DNA preparation, PCR and sequencing

Genomic DNA was extracted from single worms using 5-15uL of Worm Lysis Buffer (WLB: 50 mM KCL, 10 mM Tris pH 8.3, 2.5 mM MgCl2, 0.45% NP-40, 0.45% Tween-20, 0.01% gelatin) with proteinase K added to a final concentration of 1 mg/mL. This was heated at 65°C for 60 minutes followed by incubation at 95°C for 15 minutes. PCR was performed using 1-2.5 µL of the lysis mixture as template, 0.2 µL of Taq DNA Polymerase (New England Biolabs, Standard Taq and Buffer), 2.5 µL Standard Buffer (New England Biolabs), 1 uL each primer (10 mM) and 0.5 uL dNTP (10 mM) to a final volume of 25µl. PCR conditions were as followed (unless otherwise

noted): 95°C for 2 minutes, [95°C for 15s, annealing temperature for 15s, 72°C for extension time] x35, 72°C for 5 minutes. A full list of primers is given in table 3. PCR products were resolved on a 1% agarose gel. Sanger sequencing reactions were performed by the Molecular Biology Service Unit, following the in-house method (MBSU, University of Alberta)

2.3.2 Whole Genome Sequencing and Analysis

Protocol is based on that described in Reidy, 2015. Worms free of contamination were grown on 3 well-seeded, 60 mm NGM plates until starved for 1 day. Animals were then washed off with M9 and collected into a single 15 ml tube. Worms were pelleted at 2000 RPM for 10 minutes then the buffer was removed and the worms were suspended in sterile water. The worms were rocked for 2 hours in the wash to clear bacteria from gut then washed 2 more times with water. Worms were frozen at -80°C until ready to proceed with DNA preparations.

Each DNA preparation was preformed in duplicate. 100uL of defrosted worm pellet was added to 600 uL of WLB and 20 uL 20 mg/mL proteinase K. DNA was extracted overnight at 65°C in a rotating hybridization oven. Following incubation, RNase A was added to a final concentration of 37.5 ug/mL. Phenol chloroform (PCI) was prepared fresh for each extraction using a 25:24:1 phenol:chloroform:isoamyl alcohol ratio. 400 uL of PCI was added to each tube of worm lysate and mixed by inversion for 5 minutes before centrifuging at 15000 x g for 5 minutes. The aqueous layer was removed to a new tube and 400 uL for 24:1 chloroform:isoamyl alcohol was added. Again the sample was mixed by inversion for 5 minutes before centrifuging at 15000 x g for 5 minutes. The aqueous layer was removed and 40uL of3M 5.2pH Sodium Acetate (about 0.1 volumes) was added. 880 uL 95% EtOH (about 2 volumes) was added to the sample and inverted until DNA began to precipitate. The sample was then incubated at -20°C for 1 hour to overnight. The

sample was centrifuged at 15000 x g for 15 minutes to pellet the DNA. The pellet was washed twice with 70% EtOH with a centrifugation for 5 minutes between each wash. The tubes were left inverted on a paper towel until all EtOH had evaporated. The DNA was then suspended in 20-150 uL of pH 8.0 TE and incubated at RT until dissolved. After a 3 day incubation at -4°C, the concentration of DNA was determined by Nano-drop with the desired concentration of at least 5 ug of DNA at 40 ng/uL. If samples filled this criteria, 5 ug was reserved and the rest was sent for DNA library prep and sequencing. DNA library prep was carried out by Delta Genomics and sequencing by Genome Quebec. Alignments of sequencing data and identification of variants was performed by the Stothard Lab (University of Alberta). Variant list was filtered for mutations that were present in the parent (AF16) strain or the DP237 *tra-2(ed23)* strain.

2.4 Complementation analysis

Complementation analysis was performed using a strategy similar to that previously described (Dewar 2011). Animals homozygous for both *tra-2(ed23ts)* and a suppressor were crossed to spontaneous males from the DP426 *tra-2(ed23ts)* strain (Figure 8). The *tra-2(ed23ts);supA/+* males (indicating a successful cross) were then crossed to *tra-2(ed23ts);supB* hermaphrodites. When possible, the suppressor strain also contained a visible marker (Dpy or Cby) and a loss of this phenotype indicated success of a cross. Successful crosses had phenotypic males (either Tra or WT) in the next generation. Presence of ~50% hermaphrodites in F2 cross generation indicated non-complementation. Reciprocal crosses, *tra-2(ed23ts);supB/+* males to *tra-2(ed23ts);supA* hermaphrodites, were also performed to confirm results.

2.5 Mapping *sup(ed31)* relative to *tra-2(ed23)*

Mapping was used to demonstrate linkage on chrII and map *sup(ed31)* relative to *tra-2(ed23)*. Spontaneous males were picked from a WT AF16 stocks and crossed to *tra-2(ed23ts)sup(ed31).* The success of the cross was assessed by the appearance of ~50% male progeny in the F1 generation. Heterozygous F1 hermaphrodites from successful crosses were picked as L4 animals and allowed to lay eggs at 25°C. Worms were transferred to new plates each day until egg laying stopped. Progeny were raised at 25°C for 2-3 days and adult animals were scored for a hermaphrodite or tra phenotype.

If loci were unlinked, 3/16 of F2 progeny were expected to have a Tra phenotype (figure 9). Chi-squared analysis was used to determine linkage through testing for a significant deviation from this expected ratio. Recombination frequency was calculated using the ratio of recombinant progeny to total progeny. Because only ¼ of possible recombinant classes could be detected using phenotypic analysis, the number of tra animals was multiplied by 4 to estimate the total number of recombinant progeny in the F2 generation.

2.6 Strain construction

2.6.1 *tra-2(ed23ts);fem-1(ed37);fem-2(nm27);fem-3(nm63)*

Spontaneous *tra-2(ed23ts)* XO males were crossed to *cbr-tra-2(ed23ts);cbr-fem-1(ed62)* hermaphrodites to create *cbr-tra-2(ed23ts);cbr-fem-1(ed62)/+* XO males at 16°C. These animals were then crossed to either *tra-2(ed23ts);fem-2(nm27)* or *tra-2(ed23ts);fem-3(nm42)* hermaphrodites (Figure 10). Cross progeny were raised at 16°C and many F_2 hermaphrodites were selfed and F_3 progeny were raised at 25°C. Non-Tra animals are homozygous for the *fem-2* or *fem-3* deletion allele and *tra-2(ed23ts)*. Self-progeny from these non-Tra animals were genotyped for the *fem-1(ed62)* SNP and *fem* deletion allele. Genotyping of deletion alleles was

previously described in Hill *et al* 2006. Genotyping of *fem-1(ed62)* was performed using previously described Sanger sequencing using FEM-1 ID primers (Table 1). *cbr-tra-2(ed23ts);cbr-fem-1(ed62);cbr-fem-2(nm27)* animals could then be crossed to spontaneous *tra-2(ed23ts)* males. *cbr-tra-2(ed23ts);cbr-fem-1(ed62)/+;cbr-fem-2/+* XO males could then be crossed to *Cbr-tra-2(ed23ts);cbr-fem-1(ed62);cbr-fem-3(nm42)* animals. Non-tra F_2 progeny raised at 25°C were homozygous for *tra-2(ed23ts)* and *fem-1(ed62).* Many of these animals were selected and selfed for two generations then genotyped for the *cbr-fem-2(nm27)* and *cbr-fem-3(nm42)* alleles as previously described. *tra-2(ed23ts)* and *fem-1(ed62)* homozygosity was confirmed using previously described Sanger sequencing.

2.6.2. *tra-2(nm1);supX*

tra-2(nm1);fem-1(ed36), tra-2(nm1);fem-3(ed34) and *tra-2(ed23)sup(ed31)* were all created using the same crossing scheme (figure 11). Spontaneous males in the *tra-2(ed23)* strain were mated to *tra-2(ed23);sup-X* animals to create *tra-2(ed23);sup-X/+* animals, raised at 16°C. These males were mated to *tra-2(nm1)+/+dpy(nm4)* animals. *dpy(nm4)* is a recessive allele tightly linked to the *tra-2* locus (Koboldt et al. 2010). Progeny of this cross were compound heterozygotes for *tra-2* or heterozygous for the *tra-2(ed23)* and *dpy(nm4)* alleles. Because the animals of interest were heterozygous for the suppressor allele, the animals were raised at 16°C. Many animals from the F1 generation were singled and the F_2 generation was raised at 25°C. Animals with non-Dpy, non-Tra progeny in the F_2 had to be homozygous for the suppressor mutation and be homozygous mutant or a compound heterozygote at the *tra-2* locus. These animals were genotyped using Sanger sequencing.

2.6.3. *fem-1(ed36)*

tra-2(ed23);fem-1(ed36) animals were outcrossed to wild-type males and the F1 progeny were singled to produce animals that were *tra-2(+);fem-1(ed36)* in the F2 generation. Animals were identified by Sanger sequencing at both loci.

3. Results

3.1 Analysis of the FEM complex in *C. briggsae* sex determination

3.1.1 *fem-1(ed36)* can suppress multiple alleles of *tra-2* and is a good reference allele

Previous work had identified *fem-1(ed36)* as a suppressor of *tra-2(ed23ts)* and that no *fem-1* transcript was detected in *tra-2(ed23ts);fem-1(ed36)* animals (Dewar 2011). However, because *tra-2(ed23ts)* is a conditional allele of *tra-2,* we wanted to test the ability of *fem-1(ed36)* to suppress a stronger *tra-2* allele, *tra-2(nm1)* as well as the phenotype of *fem-1(ed36)* in a wild-type *tra-2* background (Kelleher et al. 2008). Like *tra-2(ed23);fem-1(ed36)* animals, both *tra-2(nm1);fem-1(ed36)* and *fem-1(ed36)* animals develop as hermaphrodites (table 4). Due to limitations in unambiguously identifying XO *fem* animals, we were unable to definitively conclude that XO animals are indeed hermaphrodites. However, all strains were observed over many generations and phenotypically male animals were never observed. On a wild type plate, a small percentage (about 0.01%) of animals will develop as XO males due to random non-disjunction events.

3.1.2 *Cbr-fem* genes have non-redundant roles in the regulation of somatic and germ line sex

In contrast to *C. elegans,* where strong *lf* mutations in any of the *fem* genes develop as XX and XO females*,* null mutations in any of the *Cbr-fem* genes develop as XX and XO hermaphrodites with a normal ovotestis (Hill et al. 2006; Dewar 2011). This led to the question: in the *C. briggsae* sex determination network, could one or more of the *fem* genes have developed a redundant role in a parallel pathway to regulate ovotestis development? In *C. elegans,* there is a slight additive feminizing effect when multiple *fem* genes are heterozygosed in embryos (Hodgkin 1986).

However, this effect might be due only to stoichiometry and not reflect actual regulatory changes in the pathway. Previous work has identified null alleles of each *fem* gene as well as the phenotype of a double mutant of *cbr-fem-2;fem-3*, all of which developed as XX and XO hermaphrodites (Hill et al. 2006). However, there is some evidence that *fem-3* has an additional role in the *C. briggsae* sex determination pathway as *fem-3(nm63);tra-1(nm2)* animals have an enhanced feminization phenotype while *fem-2(nm27);tra-1(nm2)* animals do not (Hill & Haag 2009).

The first goal of this work was to create two triple mutant strains: *cbr-tra-2;fem-1;fem-2* and a *cbr-tra-2;fem-1;fem-3*. All work was done in a *tra-2* background because all the alleles were known to suppress *tra-2* and this made phenotypic screening simpler. Animals with both these genotypes developed as self-fertile hermaphrodites (figure 12). Additionally, a homozygous *cbr-tra-2;fem-1;fem-2;fem-3* quadruple homozygous strain was constructed. This strain also developed as self-fertile hermaphrodites (figure 12). In all of the created strains, males were never observed on the plates, although these strains were observed over many generations. This indicates that in *C. briggsae,* the *fem* genes act together in the previously identified *fem* complex and there is no parallel pathway involving the FEM complex regulating spermatogenesis in *C. briggsae.*

3.1.3 *fem-1, fem-2* and *fem-3* have a partial dominant feminizing effect in a *tra-2* background

Previous studies have focused on the role of *cbr-fem-2* on the development of XO animals because of the maternal effect of *cel-fem-2* in development (Hodgkin 1986). A knockdown of *cbr-fem-2* through RNAi or through generation of *fem-2/+* and *tra-2;fem-2/+* XO mutant animals resulted in the development of somatically male animals with a gonad that develops both sperm and ooids, indicating that there is a dominant feminizing effect of *cbr-fem-2* on the germline (Hill et al. 2006;

Stothard et al. 2002). However, *cbr-fem-2* m^+z^- XO animals develop as hermaphrodites, indicating that there is no maternal effect of *cbr-fem-2,* unlike in *C. elegans* (Hill et al. 2006). As enhanced feminization was observed in *cbr-tra-1;fem-3* animals compared to that of *cbr-tra-1;fem-2* animals, we wanted to ask if there is also a different feminizing effect of *cbr-fem* genes on *cbr-tra-2.*

By crossing *tra-2(ed23ts)* males to *tra-2(ed23ts);fem-X;cby-X* animals and raising the *tra-2;fem-X/+;cby-X/+* F1 progeny at restrictive temperature (25°C), we were able to observe the effect of a single WT copy of *fem-X* on *tra-2* while the loss of the *cby* phenotype confirmed crossing. For these experiments the previously identified *fem* reference alleles were used: *fem-1(ed36), fem-2(nm27)* and *fem-3(nm63)* (Dewar 2011; Hill et al. 2006). In all cases, *tra-2;fem-X/+* animals had some degree of somatic and germline feminization (figures 12-14). When somatic germline development was assayed, a significant increase in the proportion of worms with abnormal gonads were observed in *tra-2;fem-1/+, tra-2;fem-2/+* and *tra-2;fem-3/+* when compared to *tra-2* homozygous controls F1 progeny raised at 25°C ($p<<0.001$, figure 13E). Many of these gonad abnormalities were defined as "mild abnormalities" such as a swelling of the distal arm of the male gonad, but an otherwise normal gonad (figure 13A,B). "Moderate abnormalities" were defined as gonad phenotypes where a male-like gonad formed but there was a defect such as the distal arm wrapping around the proximal gonad or a particularly thin proximal gonad that did not directly connect to the tail (figure 13C). Finally, "severe abnormalities" were defined as those where a male-like gonad failed to form, often developing as a mass of undefined tissue in the mid-body of the animal or a proximal gonad arm that failed to migrate posteriorly (figure 13D, figure 14). Using the phenotypic categories described, a significantly larger proportion of *tra-2;fem-3/+* worms exhibited severe defects more often than *tra-2* controls ($p=0.002$, figure 13F). Although there was a significant increase in the population of *tra-2;fem-1/+*

and *tra-2;fem-2/+* that had gonad abnormalities compared to *tra-2* controls, the proportions of that population with severe gonad defects was not significantly different than *tra-2* controls (figure 13F). In part, this increase in number of *tra-2;fem-3/+* animals with severe gonad abnormalities may be explained by the increased frequency of gonad cells attaching to the body wall of the animal, resulting in a protruding-vulva (Pvul) phenotype (figure 13). This phenotype is also rarely observed in *tra-2;fem-2/+* animals (figure 13D).

In addition to the somatic feminization, a proportion of all *tra-2;fem-X/+* populations developed an ovotestis while ovotestis development was never observed in *tra-2* control animals (figure 15). Animals were observed at 2 days old, young adults, or three or more days old, old adults for the presence of large oocyte-like structures in the germline with the presence of these structures indicating ovotestis development (figure 15). In both *tra-2;fem-1/+* and *tra-2;fem-3* animals, ooids were only observed in old adults (figure 15). In WT *C. briggsae,* the switch from spermatogenesis to oogenesis occurs in young adult worms, so this is a delayed oogenesis phenotype in the animals. Although some *tra-2;fem-2/+* animals had initiated oogenesis as young adults, a significantly larger proportion of the population had initiated oogenesis as old adults ($p<0.01$, fig 15). This is also a significantly larger proportion of the population than observed for either *tra-2;fem-1/+* or *tra-2;fem-3/+* animals.

3.1.4 *fem-3(ed34)* and *fem-3(ed44)* fail to complement other *fem-3* alleles

Both *fem-3(ed34)* and *fem-3(ed44)* had been reported to be *tra-2(ed23ts)* suppressors that complemented all of the *fems* and homozygotes were capable of producing both XX hermaphrodites and XO fertile males in a *tra-2(ed23ts)* background (Dewar 2011). However, the complementation analysis of these alleles was complicated by the presence of males in these strains (de Carvalho 2005; Dewar

2011). Because Tra animals can look very similar to normal males using the stereoscope, it could be difficult to distinguish between normal males arising in a population, a rare event, and tra males due to complementation.

Previous WGS had identified *fem-3* as the cognate gene for the *ed34* suppressor as *fem-3* was the only known sex determination gene mutant in this strain (Reidy 2015). I prepared genomic DNA from homozygous *tra-2(ed23ts);fem-3(ed44)* animals for Whole genome sequencing and found 255 SNP mutations and 2 indel mutations predicted to affect gene expression or function. *fem-3* was also identified as the cognate gene in this case because it was the only known sex determination gene mutant in this strain and because of complementation results (see below). The *fem-3* gene in the *tra-2(ed23ts);fem-3(ed44)* strain contains two nonsense mutations, Q68* and W245* (figure 16). Interestingly, the Q68* mutation was also identified in two other *fem-3* mutants, *ed43* and *ed59* (Reidy 2015, figure 15).

I also performed a complementation test between both the *ed34* and *ed44* alleles and the canonical *fem-3* allele, *fem-3(nm63)*, in the *tra-2* background (see complementation strategy). In both cases, *tra-2(ed23ts);fem-3(ed34)/fem-3(nm63)* and *tra-2(ed23ts);fem-3(ed44)/fem-3(nm63)* animals developed as hermaphrodite (10/10 crosses). These animals appeared to be fertile, as a large number of eggs and young animals were present on plates 3 days after F2 generation was laid. This confirmed that both *ed34* and *ed44* are alleles of *cbr-fem-3* and previous complementation results were false positives.

3.1.5 *tra-2(nm1);fem-3(ed34)* animals may develop as XX hermaphrodites

tra-2(ed23);fem-3(ed34) animals are still able to interrupt the chromosome ratio to determine sex, leading me to ask if this was because of the weaker *tra-2(ed23)* allele (Dewar, 2011). *tra-2(nm1)* is a stronger allele that lacks the

intracellular domain of TRA-2 and can not bind the FEM complex (Kelleher *et al* 2008). *tra-2(nm1);fem-3(ed36)* XX animals develop as hermaphrodites (table 4, created with the help of undergraduate student S. Fox). Following multiple heat shocks, XO phenotypic males were never observed, leading to the prediction that XO animals develop as hermaphrodites in this background. Therefore, *tra-2(nm1);fem-3(ed34)* animals no longer correctly interrupt the chromosome ratio.

3.2 *sup(ed31)* is a candidate for a novel regulatory locus in *C. briggsae* sex determination

3.2.1 *sup(ed31)* is located on ChrII

sup(ed31) is a unique *tra-2* suppressor allele because XX animals develop as hermaphrodites and XO animals develop as males, unlike *tra-2;fem-X* strains where XO animals develop as hermaphrodites (Reidy, 2015, figure 17). In addition, *sup(ed31)* complements all of the *fem* genes (Dewar 2011). Previous work in the lab had linked the *sup(ed31)* allele to chrII, however the molecular analysis in this work was complicated by the presence of *tra-2* in the chrII linkage group (Reidy 2015). The previous approach had used a strain of worms where *tra-2(ed23ts)* had been regressed into the HK104 genetic background, as opposed to the AF16 background, and crossed to *tra-2(ed23ts)sup(ed31)* (AF16 background) and selected recombinant animals for molecular mapping using previously described polymorphic markers (Koboldt et al. 2010). Although *tra-2(ed23)* had been regressed into the HK104 background in the stain used, there was still some degree of linkage to AF16 polymorphisms located near *tra-2.* Therefore, it was not possible to unambiguously map *sup(ed31)* with this method.

This work took a different approach to mapping *sup(ed31)* relative to *tra-2* by recombination. This approach provided two benefits over the previously used molecular approach. First, I was able to eliminate bias resulting from AF16

polymorphisms being linked to *tra-2(ed23ts).* And second, it allowed us to draw conclusions about the relative location of *sup(ed31)* to *tra-2* on ChrII. From the previously described mapping cross, 17 F_1 heterozygotes were selfed and their progeny were scored for a hermaphrodite or Tra phenotype. In the F_2 generation, 2094 animals developed as hermaphrodites while 150 developed as Tra animals. This was inconsistent with a pattern of independent assortment ($X=82.24$, $df=16$, $p<0.005$). Because the tra phenotype represented ¼ of the possible recombinant phenotypes, the number of tra progeny was multiplied by 4 to estimate the total number of recombinant progeny present for all calculations. Using this approach, we calculated the recombination frequency between *tra-2(ed23ts)* and *sup(ed31)* to be 0.27.

3.2.2 Candidate genes for *sup(ed31)*

sup(ed35) represents a second member of the *sup(ed31)* complementation group (Dewar 2011). I collected WGS data for the *tra-2(ed23)sup(ed35)* strain with the hypothesis that the cognate gene for this group would be mutant in both strains. The identified polymorphisms in *sup(ed35)* were compared to the list of 403 variants previously identified in *tra-2(ed23)sup(ed31)* (Reidy 2015). Of these, 49 genes were found to be variant in both strains and 7 of those genes with common variants were found on chrII (table). None of these match *pink-1,* the previously identified candidate for *sup(ed31)*(Reidy 2015). The previous analysis excluded all genes without identified *C. elegans* homologs and thus, excluded all candidates shared with the *tra-2(ed23ts)sup(ed35)* strain.

Of these 7 identified genes on chrII: CBG00947, CBG00860, CBG20451, CBG23567, CBG23572, CBG07032, CBG20935 (Table 5). Two stood out as particularly strong candidates: CBG00947 and CBG07032 because of the nature of the identified lesions. First CBG00947 was chosen as a candidate because it is the

only identified variant that would have a clear effect on the coding region of the protein. There are two lesions in CBG00947, A103T and A107T that are present in both *ed31* and *ed35*. However, this mutation was not homozygous in both strains and likely not the suppressor. When *tra-2(ed23)sup(ed31)* or *tra-2(ed23)sup(ed35)* animals are crossed to *tra-2(ed23)* males, the resulting *tra-2(ed23)sup(ed31)/+* or *tra-2(ed23)sup(ed35)/+* animals all develop as Tra animals, thus *sup(ed31)* and *sup(ed35)* are completely recessive (N= 44/44 and 48/48 crosses, respectively).

The second candidate was CBG07032, a gene that was found to contain homozygous lesions in both suppressor strains. This was the only identified lesion to fill this criterion. It is not clear how the deletion of 4bp 4692bp upstream of the start codon found in *sup(ed31)* and the A→G transition in the middle of the first exon would have on the CBG07032 transcript. CBG07032 is the *C. briggsae* homolog of *C. elegans slc-36.2,* a solute carrier enriched in the intestine, germ line and some neurons (Shaye & Greenwald 2011).

However, upon further investigation, an INDEL in the *sup(ed31)* strain was located within the coding sequence of the *ubxn-3* gene. The *C. briggsae* genome annotation used in the *sup(ed31)* analysis was older and did not identify the *ubxn-3* gene within the sequence when analysis was preformed. This deletion would result in a frame shift and a nonsense mutation after 26AA into the protein (figure 18). Additionally, *sup(ed35)* has a splice donor site mutation in the fourth intron that is homozygous in the suppressor strain (figure 18). Because this is the only gene with homozygous lesions in both *sup(ed35)* and *sup(ed31)* on ChrII, I identified it as the most likely cognate gene.

3.2.3 ubxn-3 in Caenorhabditis

A BLAST search of *Caenorhabditis* genomes returned orthologs in *C. elegans, C. tropicalis* and *C. japonica* (Table 6). There is about 73% identity at the nucleotide

level of both the *C. elegans* and *C. briggsae* ortholog, similar to other genes in the sex determination pathway (Haag 2005). The presence of *ubxn-3* orthologs and similar degree of conservation in the *C. japonica* outgroup indicates that *ubxn-3* is orthologs all arose from a single common ancestor. In addition, the nucleotide alignment of *C. briggsae, C. tropicalis* and *C. elegans* orthologs show a high degree of identity in the 5' end of the gene, however, the ORF of *cbr-ubxn-3* is expanded in the 3' direction compared to the other genes (figure 19). This region contains the UBA-like domain, indicated to enhance binding to ubiquitin. *Cel-ubxn-3* has a role in sex determination as RNAi knockdown of *cel-ubxn-1/2/3* results in a feminization of the germline (Sasagawa et al. 2010). This is a possible novel regulatory locus in *C. briggsae* sex determination as *cbr-ubxn-3* is not redundant with other *ubxn* genes in *C. briggsae,* unlike *C. elegans*.

4. Discussion

4.1 *C. briggsae fem* genes occupy the same step in the core sex determination pathway as in *C. elegans*

Strong *loss-of-function* alleles exist for each of the *cbr-fem* genes and these animals develop as XX and XO hermaphrodites (Hill et al. 2006; Dewar 2011). This does not exclude the alternative hypothesis that one or more of the *C. briggsae fem* genes are also involved in a parallel pathway in the germline such that knocking out any single *fem* gene is not sufficient to prevent ovotestis development. However, because animals that lack functional copies of all three *fem* genes develop as hermaphrodites, there is no redundancy or parallel pathway for the *cbr-fem* genes in the germline. The fourth member of the *C. elegans* FEM complex, CUL-2, has not yet been investigated in *C. briggsae* sex determination and it is possible that CUL-2 is necessary for *C. briggsae* ovotestis development. When coupled with the previous evidence that the *cbr-tra-1* and *cbr-tra-2* are structurally conserved and have the same *loss-of-function* phenotype in *C. elegans,* this supports the theory that core pathway genes and their interactions are conserved within the *Caenorhabditis* genus and it is the regulators of these genes that are modified to produce novel traits.

Additionally, the identification of the *cbr-tra-1(ed30gf)* allele supports the hypothesis that the FEM complex/TRA-1 molecular interaction is conserved in *C. briggsae*. The analogous lesion of this allele in *C. elegans* TRA-1 interferes with the binding of TRA-1 to the FEM complex and I predict that *cbr-tra-1(ed30gf)* behaves in the same way (De Bono et al. 1995). *cbr-fem-2(lf);tra-1(lf)* and *cbr-fem-3(lf);tra-1(lf)* animals develop a male soma and an ovotestis (Hill & Haag 2009). Because *cbr-tra-1(ed30gf)* animals also develop an ovotestis, this suggests that the TRA-1/FEM molecular interaction is lost in *cbr-fem-X(lf);tra-1(lf)* double mutants. However, this molecular interaction needs to be confirmed with biochemical experiments and the

determination of the phenotype of a *cbr-fem-X(lf);tra-1(ed30gf)* double mutant. If the *cbr-tra-1(ed30gf)* mutation behaves as we predict, the double mutants should phenocopy the *cbr-tra-1(ed30gf)* mutation alone and not become more feminized as the lesion is predicted to interfere with the TRA-1/FEM interaction

This is an example of the core GRN pathway being conserved, while the regulators of this pathway are modified to produce a novelty. Because the *cbr-fem* genes remain in the same epistatic location in the sex determination pathway but are not required for ovotestis development, there must be a different "sperm off" regulatory point in *C. briggsae* than in *C. elegans.* This novel regulatory point cannot involve the *fem* genes and must be downstream of this point.

4.2 A semi-dominant suppressor effect of *fem* alleles on *tra-2(ed23)* indicates the sperm off switch is downstream of the *fem* alleles

Although the phenotype of *C. elegans tra-2;fem-X/+* animals has not been reported, an increased number of females in animals heterozygous for two or three *fem* genes has been reported (Hodgkin 1986). This enhanced feminization phenotype is also seen in *C. briggsae* animals, however, the phenotype must be observed in a masculinized *cbr-tra-2* background because *C. briggsae fem* mutants develop as hermaphrodites. Because all of these *cbr-tra-2;fem-X/+* animals develop an ovotestis, the "sperm off" switch must be downstream of the *fems* as restoring partial function of the FEM complex is also sufficient to restore the onset of oogenesis in the gonad.

There are a number of explanations for the different effects of the *fem* mutations, although they all form a single complex and are all required for the complex to function properly. First, it could be an artifact of the alleles. *cbr-fem-2(nm27)* and *cbr-fem-3(nm63)* are both large deletions, 1.6 kbp and 1.1 kbp respectively, that are predicted to produce a non-functional protein, if the protein is

produced at all (Hill et al. 2006). *cbr-fem-1(ed36)* is a splice acceptor mutation that leads to an early truncation of the protein and has also been shown to be degraded through nonsense-mediated decay (Dewar 2011). Despite the molecular nature of these mutations, it is possible that in the case of *cbr-fem-2* or *cbr-fem-3* alleles, some protein functionality is retained in the mutant and this leads to the stronger dominant effect of one particular allele. However, this is unlikely as there is a stronger and earlier effect of *cbr-fem-2* on germline feminization while the feminizing effect of *cbr-fem-3* is stronger in the soma. If this was an artifact of some residual FEM complex activity the effect would likely be true for both the soma and germline as FEM activity is required in both systems.

The second explanation, and more likely one, is a different role of each of the FEM proteins on somatic and sex determination. In this model, a particular *fem* gene is somewhat dispensable for either somatic or germline sex determination. The three FEM proteins are distinct proteins, each with a specific role in the FEM complex (Zarkower 2006). For example, in *C. elegans,* FEM-1 is required for ubiqitination of TRA-1 because it is required to bind CUL-2, however, this is enhanced when all three FEM proteins are expressed as they are required for enhanced binding of TRA-1 (Starostina et al. 2007). Because there is increased feminization of the germline of *cbr-tra-2;fem-2/+* animals and increased feminization of the soma of *cbr-tra-2;fem-3/+* animals, there is evidence for different roles of these genes in the soma and germline. It is possible that different factors act on these genes or the protein products to regulate their activity.

4.3 *C. briggsae fem-3* alleles that permit the development of XO animals are unique

cbr-fem-3(ed34) is the only identified *fem* allele that allows the development of XO males and XX hermaphrodites (Dewar 2011). I predict that because *cbr-fem-3(ed34)* truncates the protein at AA203, some FEM-3 function is still retained. No

cbr-fem-3 mutations with more C-terminal lesions have been identified from previous screens for *cbr-tra-2* suppressors (Dewar 2011; Reidy 2015; de Carvalho 2005). It is possible that this is because more C-terminal mutations have no effect on FEM-3 function, although this still needs to be tested. FEM-3 is a protein with no identified domains that is thought to act as a scaffold for the FEM complex (Zarkower 2006). Our prediction is that the *cbr-fem-3(ed34)* protein product is sufficient for the FEM complex to form and regulate TRA-1 activity. However, this leaves the question of how *cbr-tra-2(ed23);fem-3(ed34)* animals are still able to properly interrupt the chromosomal signal. Because *cbr-tra-2(ed23ts)* is a weaker conditional allele and males have not been observed in the *cbr-tra-2(nm1);fem-3(ed34)* background, it is possible that some *tra-2* signal is still present in the *cbr-tra-2(ed23ts)* background and this is sufficient for the animal to interrupt the chromosomal signal.

4.4 Identified non-fem/non-tra suppressor alleles are conserved regulatory loci between *C. elegans* and *C. briggsae* sex determination pathways

In previous work, a single distinct complementation group of *cbr-tra-2(ed23ts)* suppressors complemented all of the *fem* genes and WGS revealed no mutations in known sex determination genes. Through WGS and mapping, these alleles have been identified as lesions in *cbr-ubxn-3,* a ubiquitin ligase adaptor protein.

C. elegans ubxn-3 has been implicated in germline sex determination as *ubxn-1/2/3* RNAi results in a feminization of the germline (Sasagawa et al. 2010). In this case, three *ubxn* parologs must be knocked down to produce the Fog phenotype because of redundancy. This is not the case in *C. briggsae* as the identified *ubxn-3* animals do not have mutations in other *ubxn* parologs. In *C. briggsae ubxn-3* therefore has an essential role in sex determination as compared to *C. elegans.* This phenomenon has been observed before when comparing *C. briggsae* and *C. elegans*

sex determination genes. The *C. briggsae* germline regulator *trr-1* feminizes *fem-3* mutants but the feminization of *C. elegans fem-3* mutants is temperature dependent (Guo et al. 2013). Because of the reduced role of the *C. briggsae fem* genes in germline sex determination, it has been hypothesized that other downstream factors, such as *cbr-trr-1,* have become more important in determining *C. briggsae* sex as compared to *C. elegans* (Guo et al, 2013).

4.5 *C. briggsae* ovotestis development has a different regulatory "sperm off" switch that may be germline specific

In *C. elegans,* the *fem* genes act as a master regulator switch for determining the sexual fate of both the soma and the germline. Forward genetic screens for *C. briggsae* feminizing alleles have identified alleles that feminize the XO soma, but the germline remained an ovotestis or feminized the germline of both XX and XO animals but the soma was unaffected (de Carvalho, 2005; Guo et al, 2013). This indicates that there was no "master regulatory" switch that controls both somatic and germline sex determination in *C. briggsae* as there is in *C. elegans.* Instead, these data suggest that the germline and somatic regulation of sex determination are independent in *C. briggsae* but not in *C. elegans*. Specifically, the *C. briggsae* regulatory switch is downstream of the *fem* genes, likely acting on *tra-1* its self or the targets of *tra-1.*

One previously identified gene, *cbr-trr-1,* is an excellent candidate for this regulatory locus (Guo *et al,* 2013). *cbr-trr-1* animals develop as XX somatic females and XO somatic males but both XX and XO animals develop a feminized germline that produces only oocytes. This phenotype would be expected for a gene required for ovotestis development, but not male somatic fates.

Taken together this fits a model of GRN evolution where the core network pathway is conserved while modifiers of the pathway are changed to produce a

novelty, as it is a novel regulation of the sex determination pathway that leads to ovotestis development in both species. In addition, the differences in regulating ovotestis development in *C. briggsae* and *C. elegans* indicate that there is not a single way to modify a GRN to produce a novelty, as the "sperm off" switch in *C. elegans* mediated by the *fem* genes is not conserved in *C. briggsae*. The high degree of constraint placed on the core pathway does not constrain the loci at which the pathway can be modified to produce a novelty.

The regulation of ovotestis development has not been investigated in the third dioecious species, *C. tropicalis.* Ovotestis development has independently evolved in this species, as it has in *C. briggsae* and *C. elegans* (Kiontke et al. 2004). By identifying the regulatory loci in this species and comparing that to *C. elegans* and *C. briggsae* regulation would provide more evidence for the constraints places on the modification of the sex determination GRN in *Caenorhabditis.*

In addition, the genome of *C. briggsae*'s androdioecious sister species, *C. nigoni* has recently been published (Yin et al. 2018). This provides the opportunity to preform direct comparisons between *C. briggsae* and *C. nigoni* sex determination genes and identify changes that permit ovotestis development. Using genome editing, these specific changes can be tested for the ability to confer ovotestis development to animals. These other species are an excellent tool with which to test these hypotheses related to GRN evolution.

In conclusion, this thesis demonstrates that the regulation of the *C. briggsae* sex determination pathway that permits ovotestis development is different than the *C. elegans* regulation. This supports a theory of GRN evolution where core network genes, in this case the core sex determination pathway, and their interactions are conserved but the "plug-ins" or regulators of these pathways, in this case the "sperm on" and "sperm off" switches, change to produce a novel trait. In addition, these

modifiers do not have to change in the same way or modify a common locus in the core pathway to produce a novel trait.

Tables

Table 1. List of strains used in this work from other sources

Name	Genotype	Phenotype of Homozygote at 16°C	Phenotype of Homozygote at 25°C
AF16	Wildtype	XX: Hermaphrodite XO: Male	XX: Hermaphrodite XO: Male
DP426	*tra-2(ed23)**	XX: Hermaphrodite XO: Male	XX: tra XO: Male
	tra-2(nm1)+/+dpy(nm4)	XX: tra XO: Male	XX: tra XO: Male
DP374	*tra-2(ed23);tra-1(ed30)*	XX: Hermaphrodite XO: Intersex	XX: Intersex XO: Intersex
DP423	*tra-2(ed23);fem-1(ed36);cby-1(s1281)*	XX: Hermaphrodite XO: Hermaphrodite	XX: Hermaphrodite XO: Hermaphrodite
	fem-2(nm27)	XX: Hermaphrodite XO: Hermaphrodite	XX: Hermaphrodite XO: Hermaphrodite
DP369	*tra-2(ed23);fem-2(nm27);cby-15(sy5148)*	XX: Hermaphrodite XO: Hermaphrodite	XX: Hermaphrodite XO: Hermaphrodite
CP87	*fem-3(nm63)*	XX: Hermaphrodite XO: Hermaphrodite	XX: Hermaphrodite XO: Hermaphrodite
DP425	*tra-2(ed23);fem-3(nm63);cby-1(s1281)*	XX: Hermaphrodite XO: Hermaphrodite	XX: Hermaphrodite XO: Hermaphrodite
DP377	*tra-2(ed23);fem-3(ed34)*	XX: Hermaphrodite XO: Male	XX: Hermaphrodite XO: Male
DP387	*tra-2(ed23);fem-3(ed44)*	XX: Hermaphrodite XO: Hermaphrodite	XX: Hermaphrodite XO: Hermaphrodite
DP389	*tra-2(ed23);sup(ed31)*	XX: Hermaphrodite XO: Male	XX: Hermaphrodite XO: Male
DP390	*tra-2(ed23);sup(ed35)*	XX: Hermaphrodite XO: Male	XX: Hermaphrodite XO: Male

* This strain is regressed into an HK104 WT background. For details see: Dewar 2011.

Table 2. List of strains created during this work.

Name	Genotype	Phenotype of Homozygote at 23°C
DP381	*tra-2(ed23);fem-1(ed36)fem-3(nm62)*	XX/XO hermaphrodite
DP382	*tra-2(ed23);fem-1(ed36);fem-2(nm27)*	XX/XO hermaphrodite
DP383	*tra-2(ed23);fem-1(ed36)fem-3(nm62);fem-2(nm27)*	XX/XO hermaphrodite
DP388	*fem-1(ed36)*	XX/XO hermaphrodite
DP386	*tra-2(nm1);fem-3(ed34)**	XX/XO hermaphrodite

*This strain was made with help from BIOL399 student Sabrina Fox

Table 3. PCR primers used for genotyping

Name	Forward Primer	Reverse Primer	Purpose/Notes
Fem-2OUT	agtttccagGATCTCCACTTGG	cgtatcgagaagagatctcg	[1] ID of *fem-2(nm27)* deletion
Fem-2IN	TCATGACGTTTTCGGAGATGC	tcctaagcctgtacttaagcc	[1]Nested primers for ID of *fem-2(nm27)* deletion
Fem-2WTO	TGCTCCCAATACGCTGCTGGGC	CGAGATCATCGGTCGGCCAGGG	[1]ID of WT *fem-2* allele
Fem-3ID	tgttgcaccgaaagacagac	AGCCAGAGGGATTGATGAAA	[2] ID of *fem-3(nm63)* deletion
Fem-3WTO		CATCGTGATACAGTAGTCGACACG	[1] ID of WT *fem-3* allele
Fem-1PCR	GTGTTGGAGACATGCGATG	tgagattcatgtgaacgatgc	ID of *fem-1(ed62)* lesion
Fem-1seq	GAGCCGAAAGAAGTCTATG	N/A	Sequencing of *fem-1(ed62)* lesion
Ed23PCR	CGAGTCACCTTCATTTGCTATC	GATCCATGATGAAGGACACC	ID of *tra-2(ed23)* lesion
Ed23seq	CATTAGGATCGGgtagtcac	CCTTTTGCAGTCGTTGAATCG	Sequencing of *tra-2(ed23)* lesion
Nm1PCR	TTCGTCACTGCTACCATG	gtccagtgtatctttccgac	ID of *tra-2(nm1)* lesion
Nm1seq	GTTGCCTCGAGAAGAAGATC	ggcgtagaaaactcaacttgc	Sequencing of *tra-2(nm1)* lesion

[1]Primers from Hill *et al* 2006
[2]Primers from Dewar 2011

Table 4. Phenotypes of novel *cbr-fem* alleles in *tra-2* backgrounds. XO phenotypes are predicted based on the progeny seen following multiple heat shock treatments, without the production of male worms.

Genotype	**XX Phenotype**	**XO phenotype**
tra-2(ed23);fem-1(ed36)	Hermaphrodite*	Hermaphrodite*
tra-2(nm1);fem-1(ed36)	Hermaphrodite	Hermaphrodite
tra-2(ed23);fem-3(ed34)	Hermaphrodite*	Male*
tra-2(nm1);fem-1(ed34)[1]	Hermaphrodite	Hermaphrodite

*From Dewar, 2011

[1]Created with the help of undergrauate student S. Fox

Table 5. List of candidate genes for *sup(ed31)* filtered for lesions shared between *sup(ed31)* and *sup(ed35)* on ChrII. Genes are listed in their order on the chromosome (left to right) according to the Jan. 2007 genome draft provided on the UCSC genome browser. *tra-2* is located between CBG00860 and CBG20451. Cells indicated in grey are homozygous alternate lesions in the whole genome sequencing data. Gene descriptions are from worm book unless otherwise noted.

Gene	***ed31* lesion**	***ed35* lesion**	**Description**
CBG00947	C107T & A103T Missense (both)	C107T & A103T Missense (both)	No known orthologs: protein crosslinking domain
CBG00860	Intron SNP	Upstream (1733bp from start) SNP	*Cel* F33H1.6 ortholog: enriched in neurons (Spencer *et al* 2011)
CBG20451	Downstream (250bp from stop) SNP	3 Upstream (1827bp, 1819bp & 1574bp from start) SNPs	*Cel-ant-1.2* ortholog: mitochondrial membrane transporter (Farina *et al* 2008)
CBG23567	Upstream (764bp from start) SNP	Intron SNP	*Cel* Y39F10B.1 ortholog: positive regulator of growth, may interact with RAB11 (Maeda *et al* 2001)
CBG23572	2 Upstream (3646bp &4138bp from start) SNPs	Upstream (4138bp from start) SNP	*Cel-fbxc* family ortholog
CBG07032	Upstream (3629bp from start) INDEL	Intron SNP	*Cel-slc-36.2*ortholog: a solute carrier
CBG20935	Downstream (250bp from stop) SNP	3 Intron SNPs	*Cel-marc-5* ortholog: known to have zinc binding activity

Table 6. Nucleotide identity of *C. briggsae ubxn-3* with orthologs in other *Caenorhabditis* species. *C. elegans* and *C. tropicalis* are other androdioecious species and *C. japonica* is an out group to the *elegans* clade. A txBLASTtx search was performed using the *Caenorhabditis* genomes database.

Species	Subject ID	% Identity	Alignment length	Mismatches	Gap length	E value
C. elegans	F48A11.5a	73.318	862	180	5	4.4e-152
C. tropicalis	G19276.t1	73.171	861	184	4	5.5e-151
C. japonica	CJA07122	67.981	862	216	10	2.8e-15

Figures

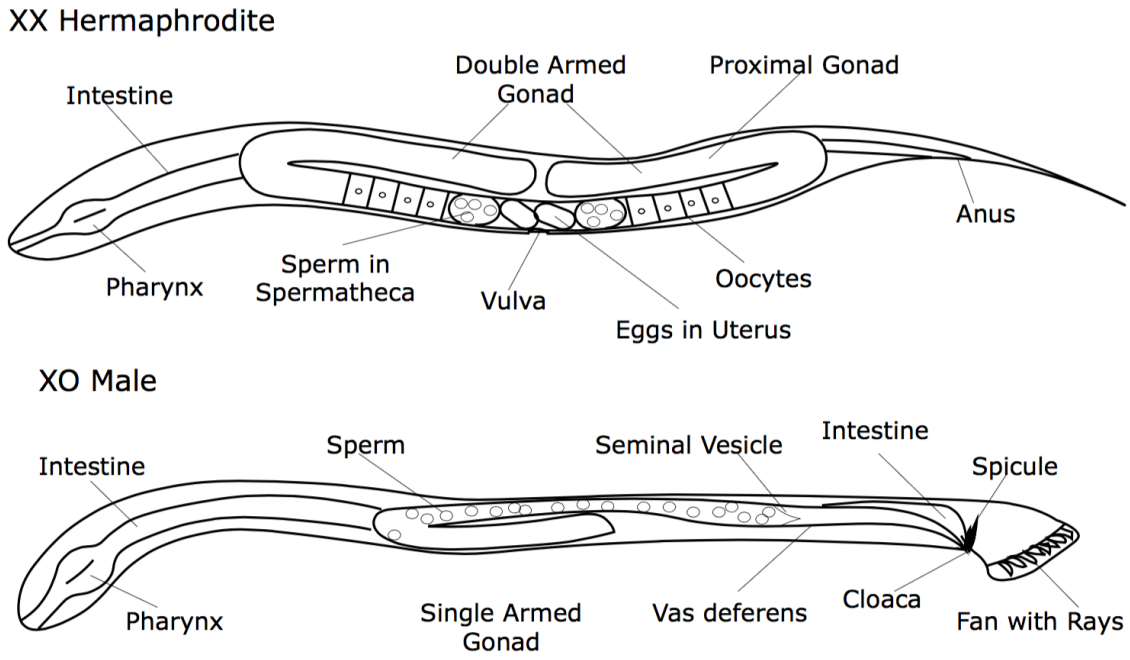
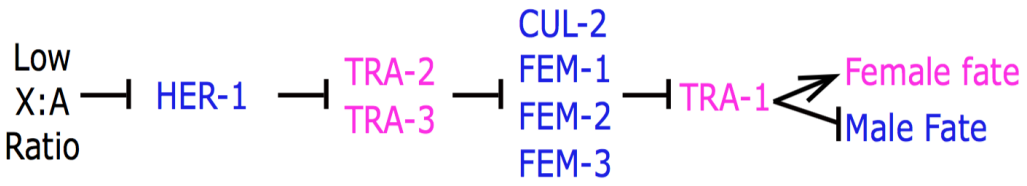


Figure 1. Anatomy of XX hermaphrodite and XO male *Caenorhabditis* worms. Hermaphrodites are larger and have a characteristic whip like tail and double-armed somatic gonad. Gametes begin to divide in a syncytium in the proximal gonad and then become cellularized as they move toward the distal gonad. Oocytes pass through the spermatheca, a specialized organ that stores both self and outcross sperm, where fertilization occurs. Embryos begin to develop in the uterus before being laid through the vulva. Males have a blunt tail with sensory rays used for mating and a single armed somatic gonad. The spicule is a male specific organ used for copulation.



C. elegans Spermatogenesis	OFF	ON	OFF	Sperm
C. elegans Oogenesis	ON	OFF	ON	Oocytes

Figure 2. Simplified core sex determination pathway in *Caenorhabditis* species. This genetic pathway for both somatic and germline sex determination exists in all assayed species. Sex is determined by the ratio of sex chromosomes to autosomes (X:A) with XX animals developing as females and XO animals developing as males. In hermaphroditic species, the activity of genes in this pathway must be differentially regulated in the gonad to allow for spermatogenesis and oogenesis to occur. The differential regulation in the C. elegans germline is indicated here as an example. Reviewed in: Ellis & Schedl, 2007.

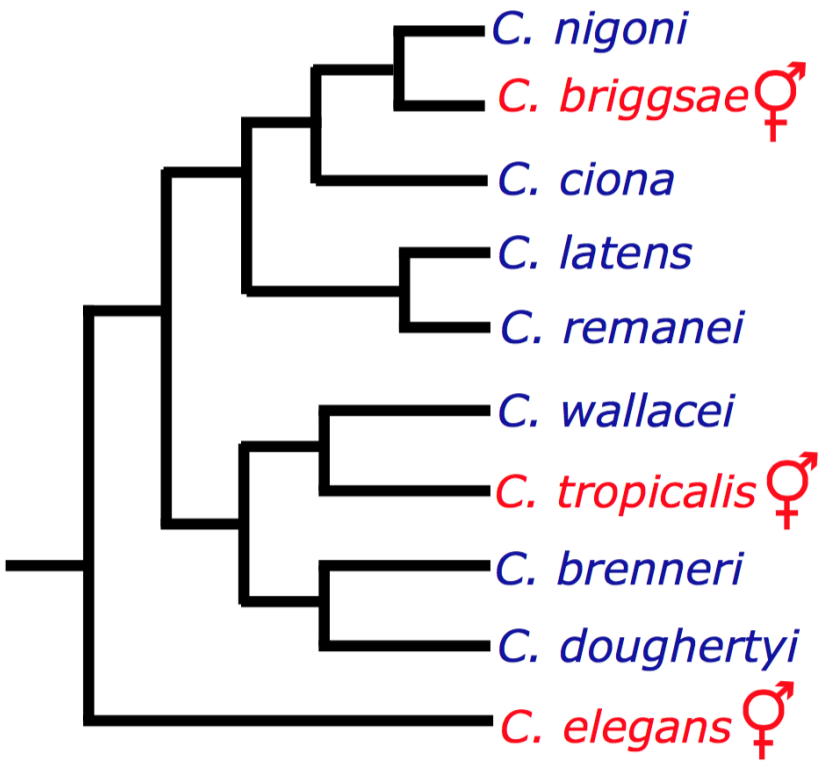


Figure 3. Hermaphroditism has independently evolved three times in the *elegans* group of *Caenorhabditis.* Phylogeny of *Caenorhabditis* nematodes as adapted from Kiontke et al 2014. Blue species have a dioecious (male/female) sex determination system while red species have an androdioecious (male/hermaphrodite) system. Hermaphroditism, or ovotestis development, has evolved independently in each case.

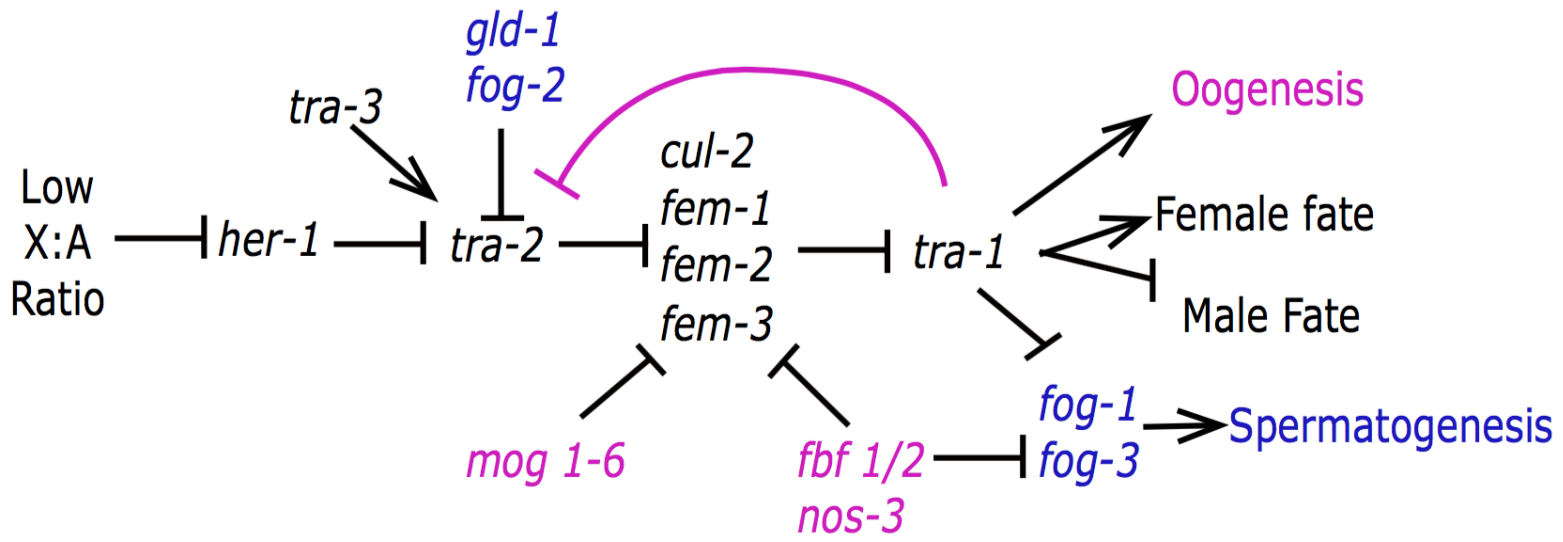


Figure 4. Key regulatory events in *C. elegans* somatic and germline sex determination. The common core pathway is indicated in black. Some regulatory loci upstream of *her-1* have been omitted for simplicity. Germline specific regulatory elements are indicated in pink and blue; pink indicates genes promoting oogenesis and blue indicates genes promoting spermatogenesis. The effect of *gld-1* and *fog-2* on *tra-2* is the "sperm on" switch, while the effect of *fbf-1/2* with *nos-3* and *mog-1-6* is the "sperm off" switch. This "sperm off" switch regulates *fem-3* mRNA. Other germline regulatory loci exist and are indicated. Reviewed in: Ellis & Schedl, 2007.

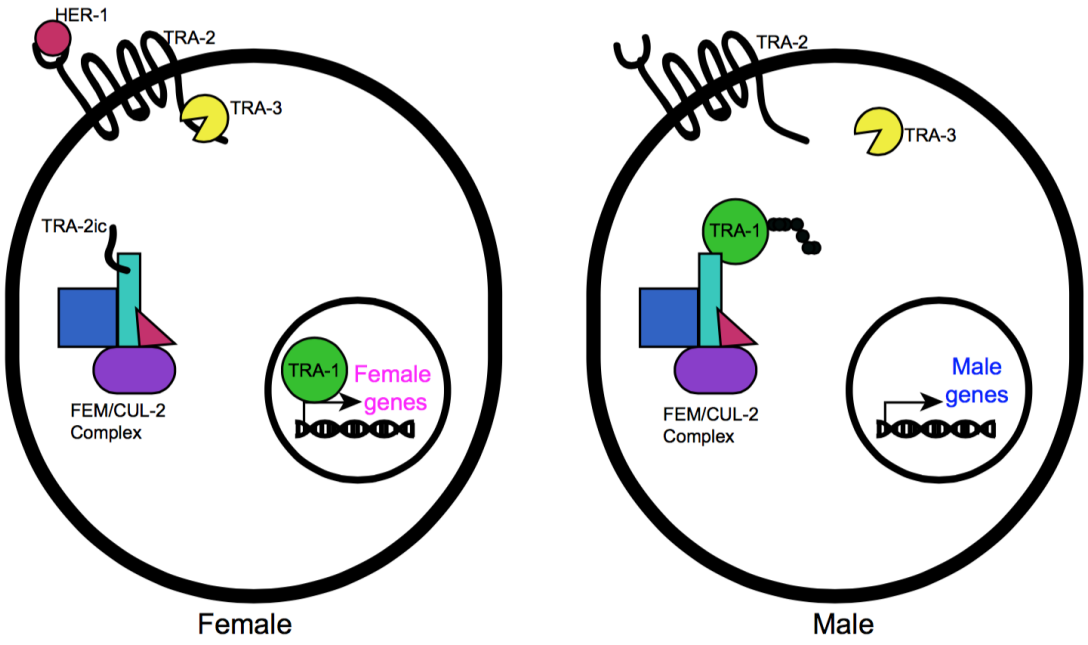


Figure 5. Cellular interactions in *Caenorhabditis* sex determination. In XX somatic (female) development, HER-1 promotes a conformational change in TRA-2 so that it can be cleaved by TRA-3. This intracellular portion of TRA-2 can then bind the FEM/CUL-2 complex and TRA-1 can promote the expression of feminizing genes. In the XO (male) soma, HER-1 is not expressed so TRA-1 is bound to the FEM/CUL-2 complex and polyubiqitinated. These interactions are based on our understanding of the *C. elegans* sex determination. Adapted from: Zarkower, 2006.

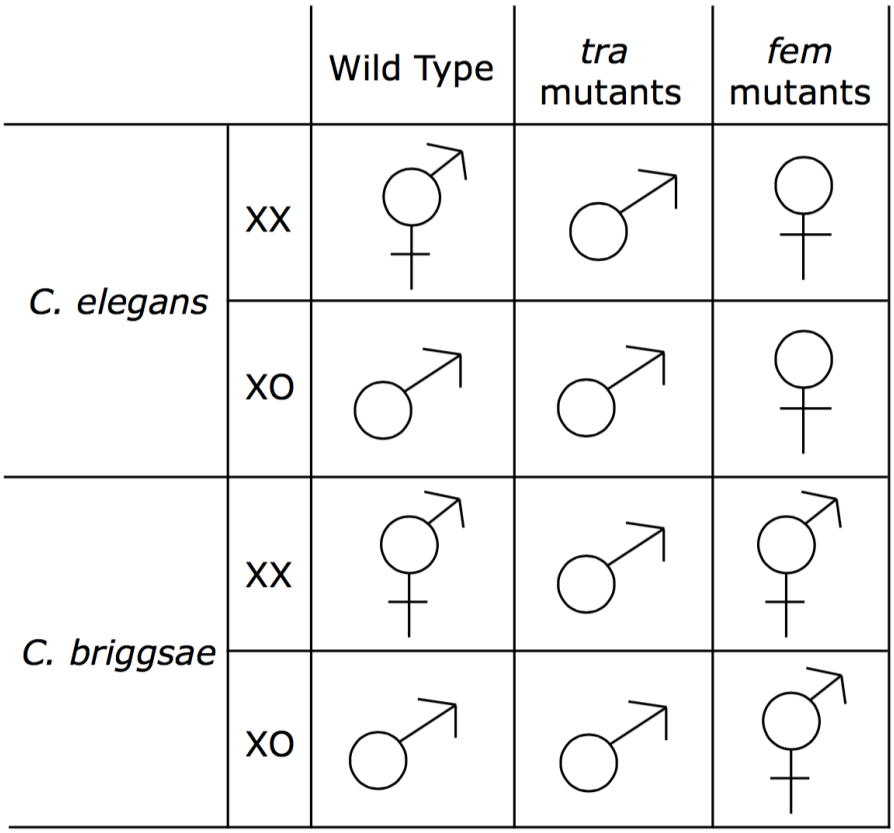


Figure 6. Summary of core sex determination in *C. elegans* and *C. briggsae*. In both species, wildtype XX animals develop as hermaphrodites and XO animals develop as males. *tra* mutants develop as XX and XO males, although XX *tra* animals are often partially feminized. In *C. elegans*, *fem* animals develop as XX and XO females. In contrast, *Cbr-fem* animals develop as XX and XO hermaphrodites.

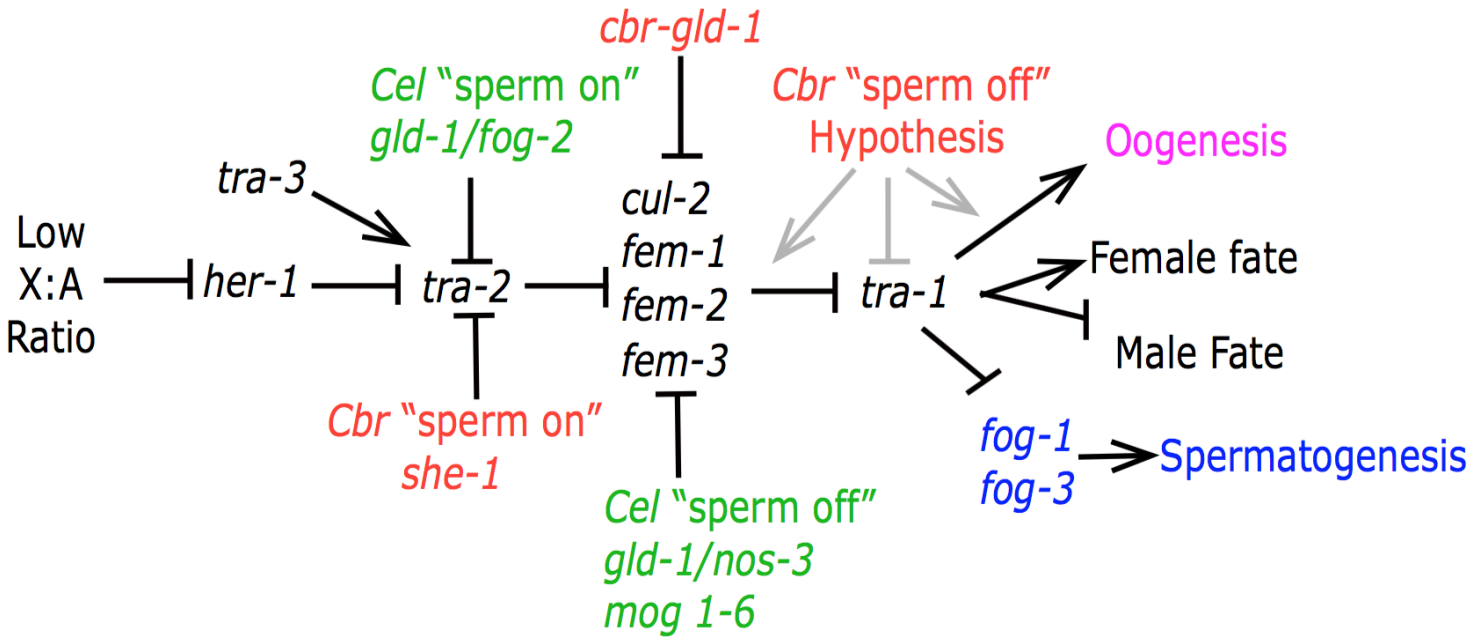


Figure 7. Comparison of the germline regulation in *C. briggsae* and *C. elegans* sex determination. The key "sperm on" and "sperm off" regulatory events are indicated for *C. elegans* sex determination (green). In *C. briggsae*, the "sperm on" switch is indicated (red). However the "sperm off" switch is unknown, with three possible regulatory loci indicated with grey arrows. In addition, *cbr-gld-1* has a novel role in promoting spermatogenesis and is an upstream regulator of the FEM complex.

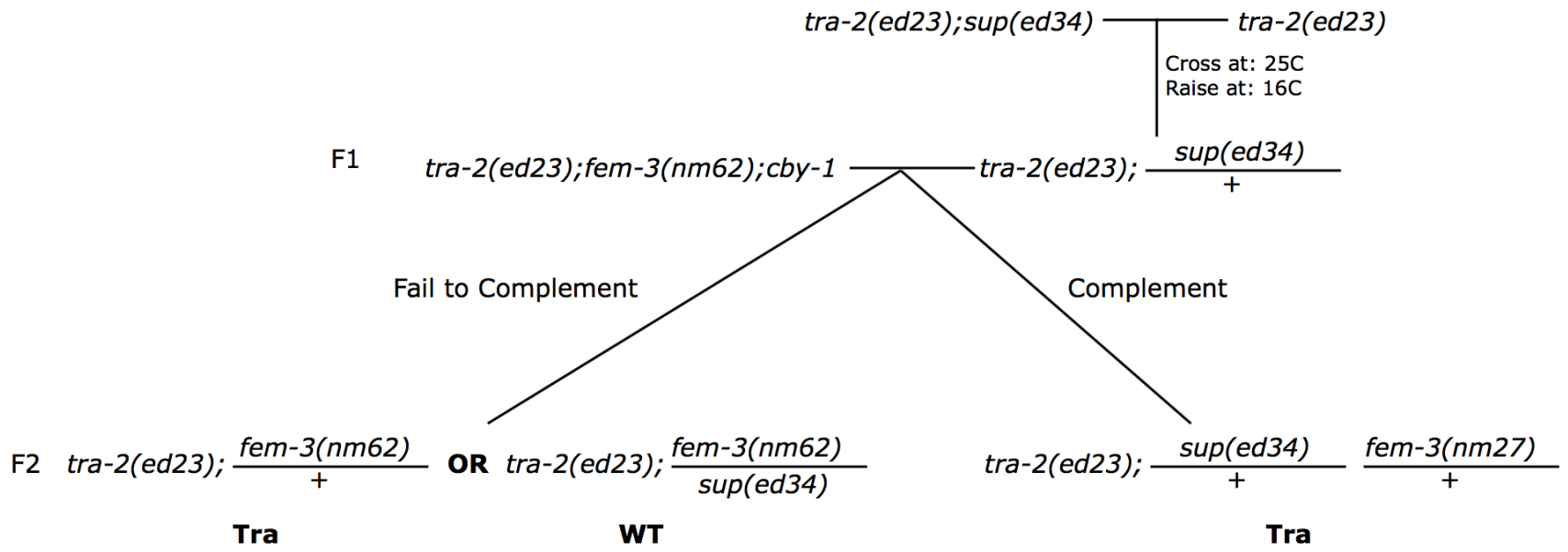


Figure 8. Complementation strategy for *fem-3* alleles. The *tra-2;fem-3(nm62)* strain used a cby phenotypic marker to confirm crossing, however this was not included in progeny genotype. Male genotypes are on the right in all crosses. Non-complementation was confirmed when F2 animals developed as fertile hermaphrodites, as indicated by the presence of eggs on the plate 3 days post hatching.

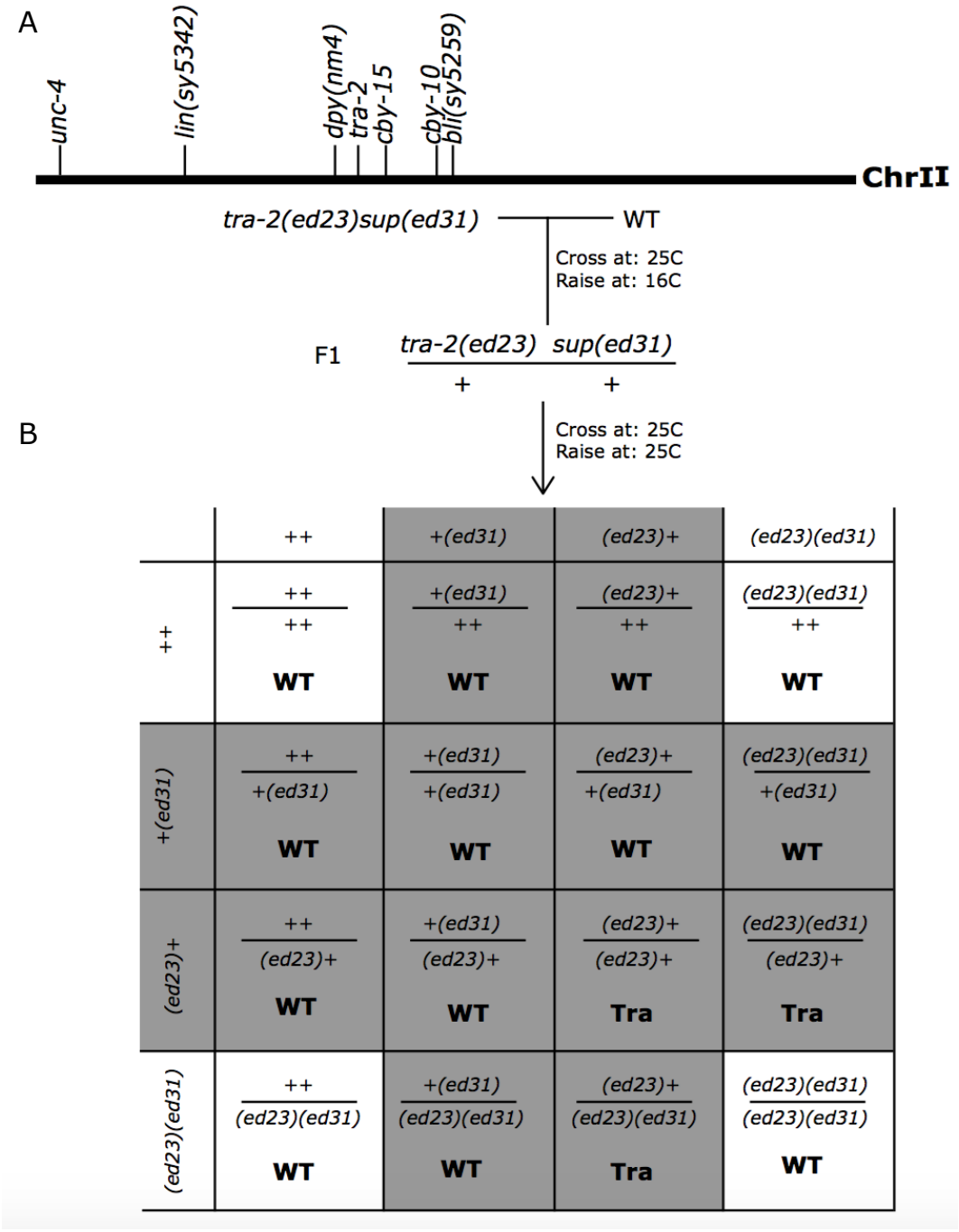


Figure 9. Mapping *sup(ed31)* relative to *tra-2* on ChrII. (A) Physical map of known *C. briggsae* genes on ChrII. (B) Crossing scheme and resulting F2 generation used for mapping. Phenotypes are indicated in bold in the Punnett square and recombinant gametes, and resulting progeny are indicated in grey.

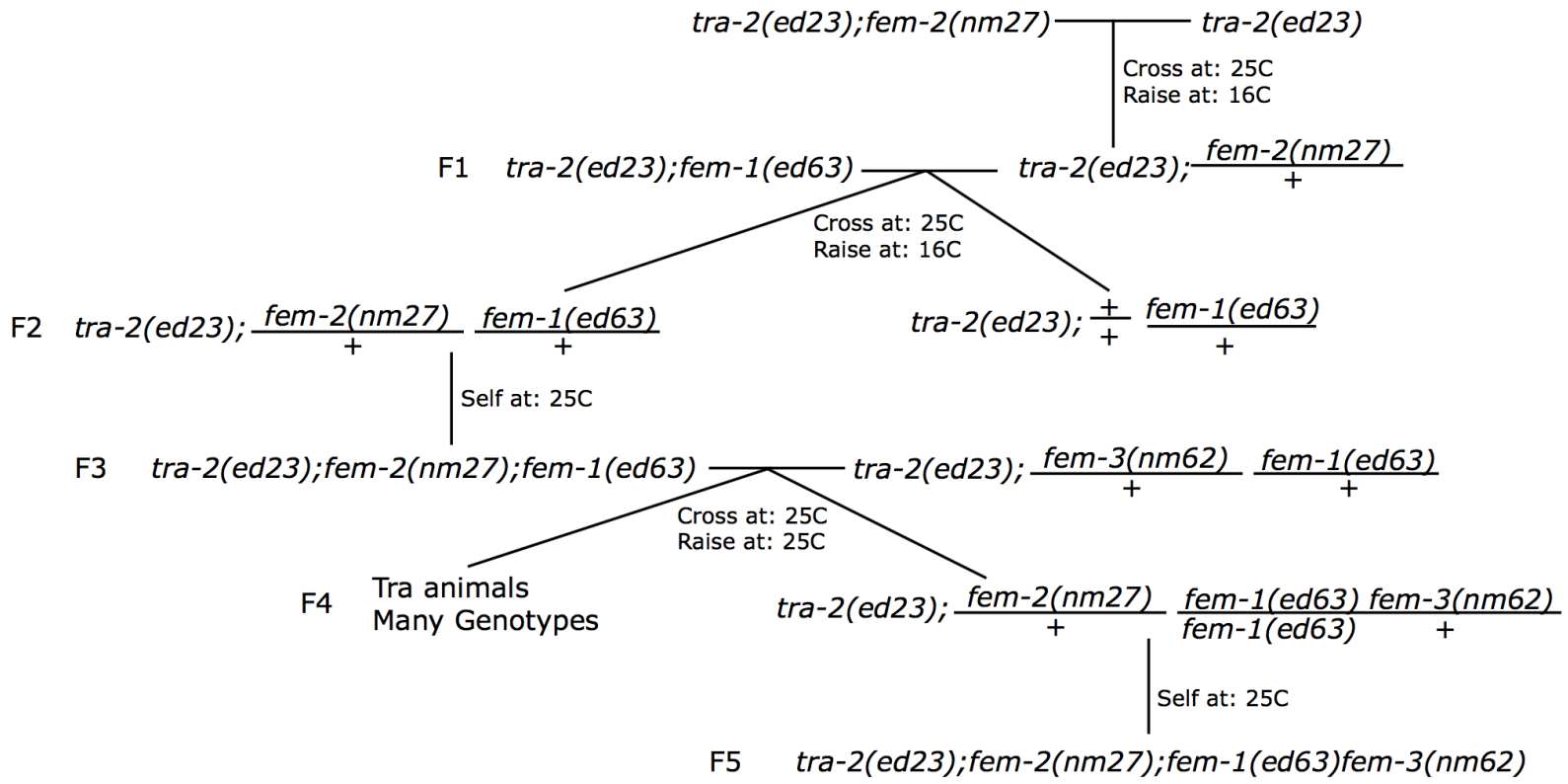

tra-2(ed23);fem-2(nm27)
tra-2(ed23)
Cross at: 25C
Raise at: 16C
F1
tra-2(ed23);fem-1(ed63)
tra-2(ed23); fem-2(nm27) / +
Cross at: 25C
Raise at: 16C
F2
tra-2(ed23); fem-2(nm27) / + fem-1(ed63) / +
tra-2(ed23); + / + fem-1(ed63) / +
Self at: 25C
F3
tra-2(ed23);fem-2(nm27);fem-1(ed63)
tra-2(ed23); fem-3(nm62) / + fem-1(ed63) / +
Cross at: 25C
Raise at: 25C
F4
Tra animals
Many Genotypes
tra-2(ed23); fem-2(nm27) / + fem-1(ed63) / fem-1(ed63) fem-3(nm62) / +
Self at: 25C
F5
tra-2(ed23);fem-2(nm27);fem-1(ed63)fem-3(nm62)

Figure 10. Cross diagram for making *tra-2(ed23);fem-1(ed62)fem-3(nm63);fem-2(nm27).* All fem strains used a cby/dpy phenotypic marker to confirm crossing, however this was not included as dpy/cby animals were selected against in all crosses. Male genotypes are on the right in all crosses. In the F2 generation, animals carrying the *fem-2(nm27)* allele were selected for using PCR. *tra-2;fem-1fem-3/*++ males in the generation were created by crossing the tra-2;fem-1fem-3 homozygote (created using the same crossing scheme as depicted for fem-2) to a tra-2 male. As the indicated genotype was the only possible genotype to produce hermaphrodites in the F4, all other genotypes (tra animals) were ignored. Quadruple homozygous animals were confirmed using PCR and sequencing.

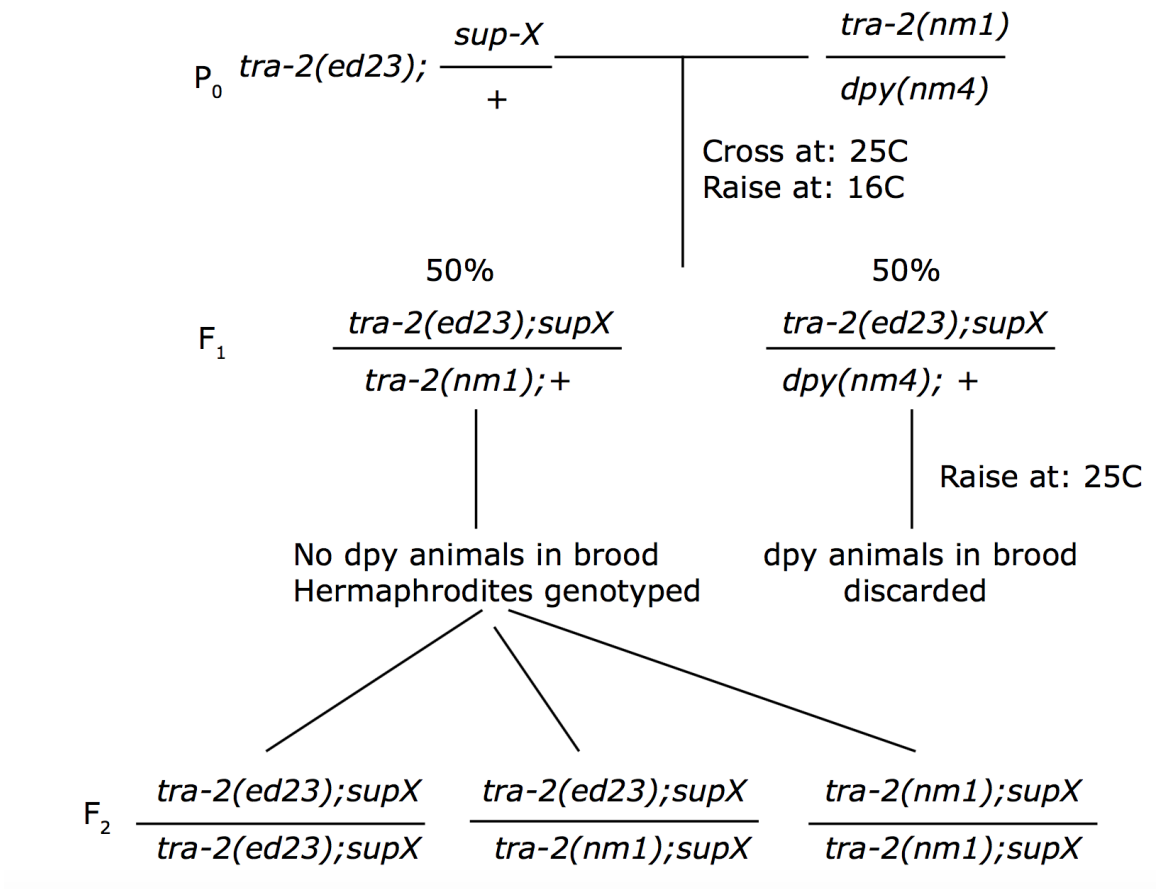


Figure 11. Cross diagram for making *tra-2(nm1);sup-X*. Male genotypes are on the right in all crosses. Generation of *tra-2(ed23);sup-X/+* animals is not indicated as these animals were created in the same way as the previous cross. Animals from non-dpy F2 broods were genotyped at the *ed23, nm1* and suppressor locus by DNA sequencing

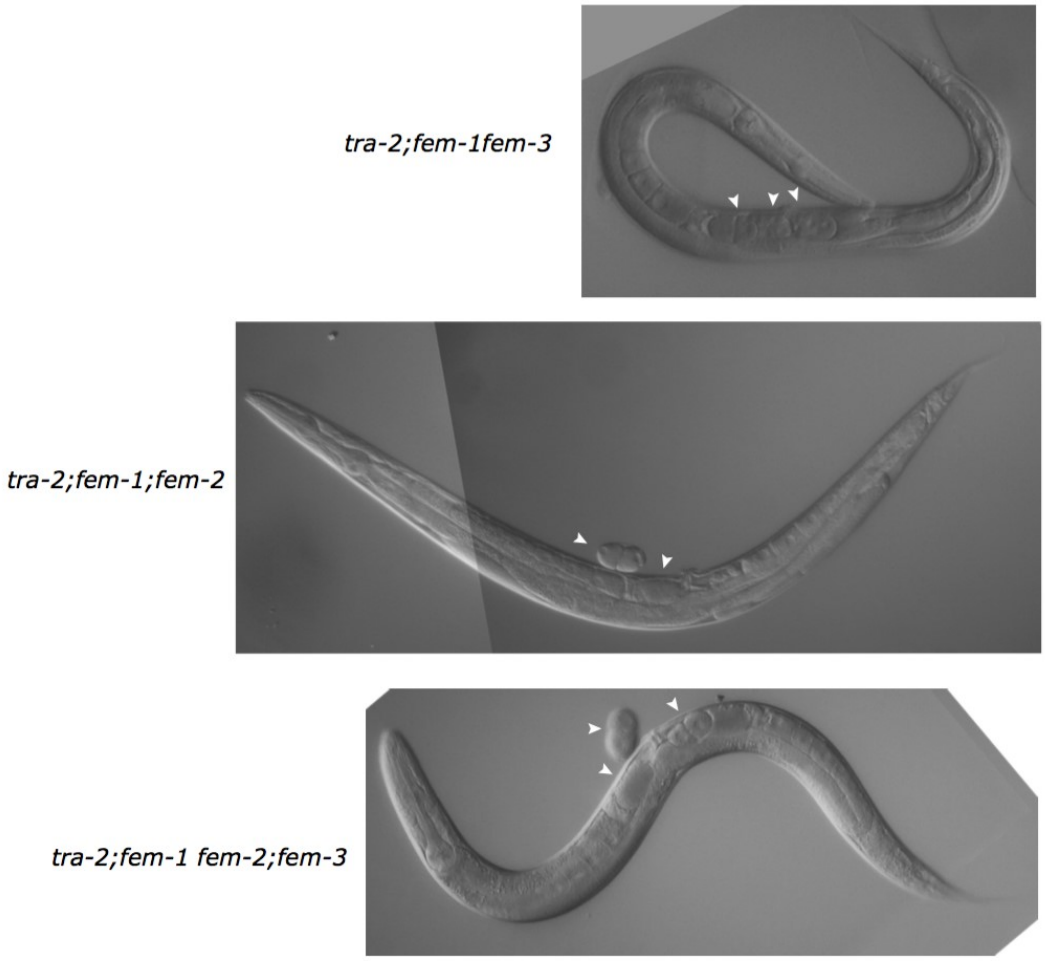


Figure 12. *C. briggsae fem* genes have non-redundant roles in regulating ovotestis spermatogenesis. *tra-2(ed23ts);fem-1(ed62);fem-3(nm63), tra-2(ed23ts);fem-1(ed62)fem-2(nm27)* and *tra-2(ed23ts);fem-1(ed62)fem-2(nm27);fem-3(nm63)* animals all develop as hermaphrodites. Arrowheads indicate developing embryos. All worms are virgin worms so the production of viable embryos is inferred to mean the gonad is a functional ovotestis, producing both viable oocytes and sperm.

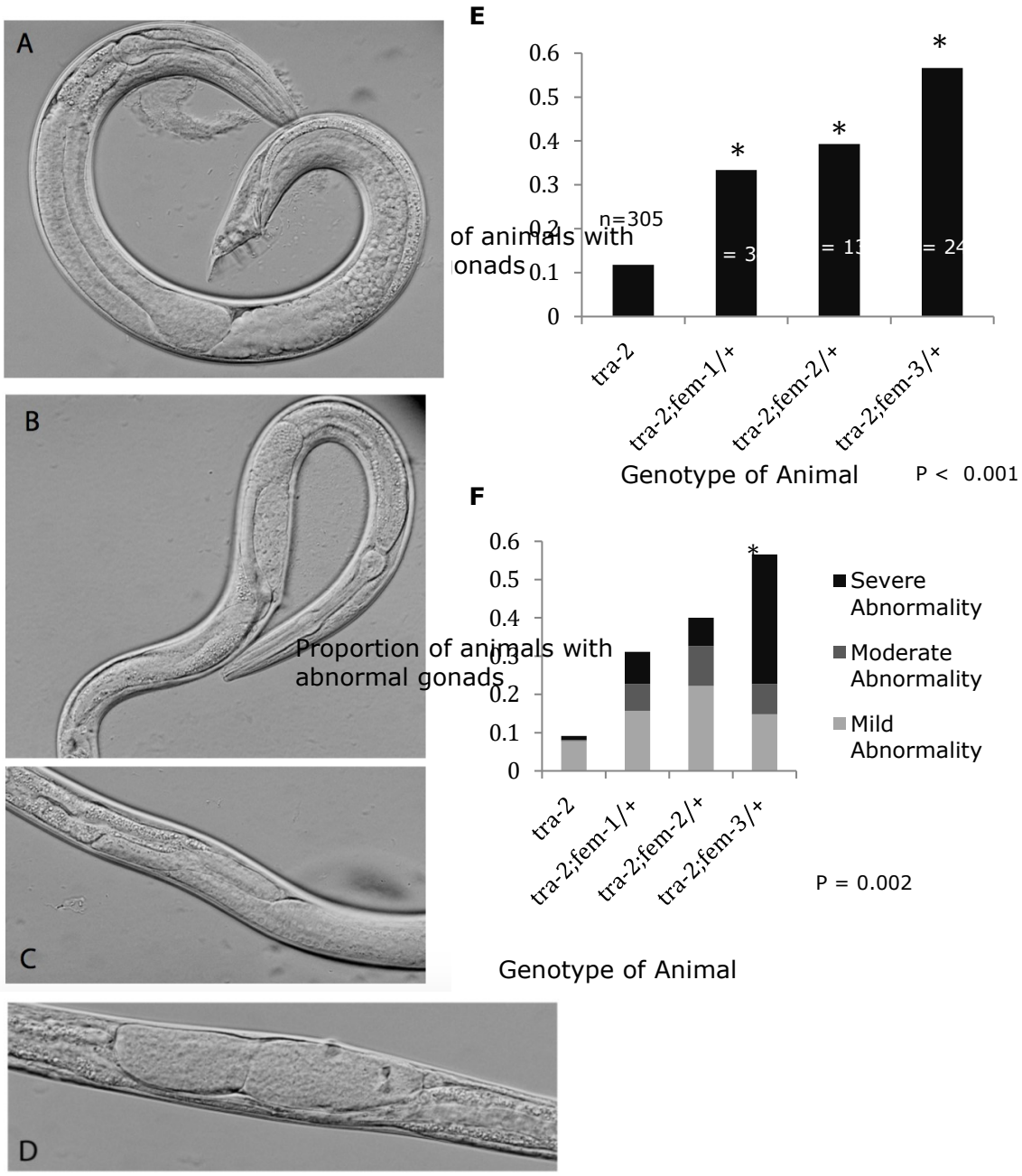

A
B
C
D
E
0.6
0.5
0.4
0.3
0.2
0.1
0
n=305
= 3
= 11
= 24
*
*
*
tra-2
tra-2;fem-1/+
tra-2;fem-2/+
tra-2;fem-3/+
of animals with
onads
Genotype of Animal
P < 0.001
F
Proportion of animals with
abnormal gonads
Severe
Abnormality
Moderate
Abnormality
Mild
Abnormality
P = 0.002
Genotype of Animal

Figure 13. *tra-2(ed23);fem-X/+* animals show a dominant feminizing effect in a masculinized background. Representative animals chosen from genotypes. (A) Example of mild gonad defect, a swelling of the distal arm. (B) Example of moderate defect, where the proximal arm is thin. (C) Example of moderate defect, where the distal arm of gonad is wrapped around the proximal gonad. (D) Example of severe defect, a mass of gonad tissue forms that does not resemble a male or female gonad. (E) Proportion of animals with gonad defects. Asterisk indicates significant differences from *tra-2* control using a binomial distribution test. (F) Proportion of abnormal populations with mild, moderate and severe defects. Asterisk indicates a significant difference in proportions with severe abnormalities compared to *tra-2* controls. N numbers are the same as in (E).

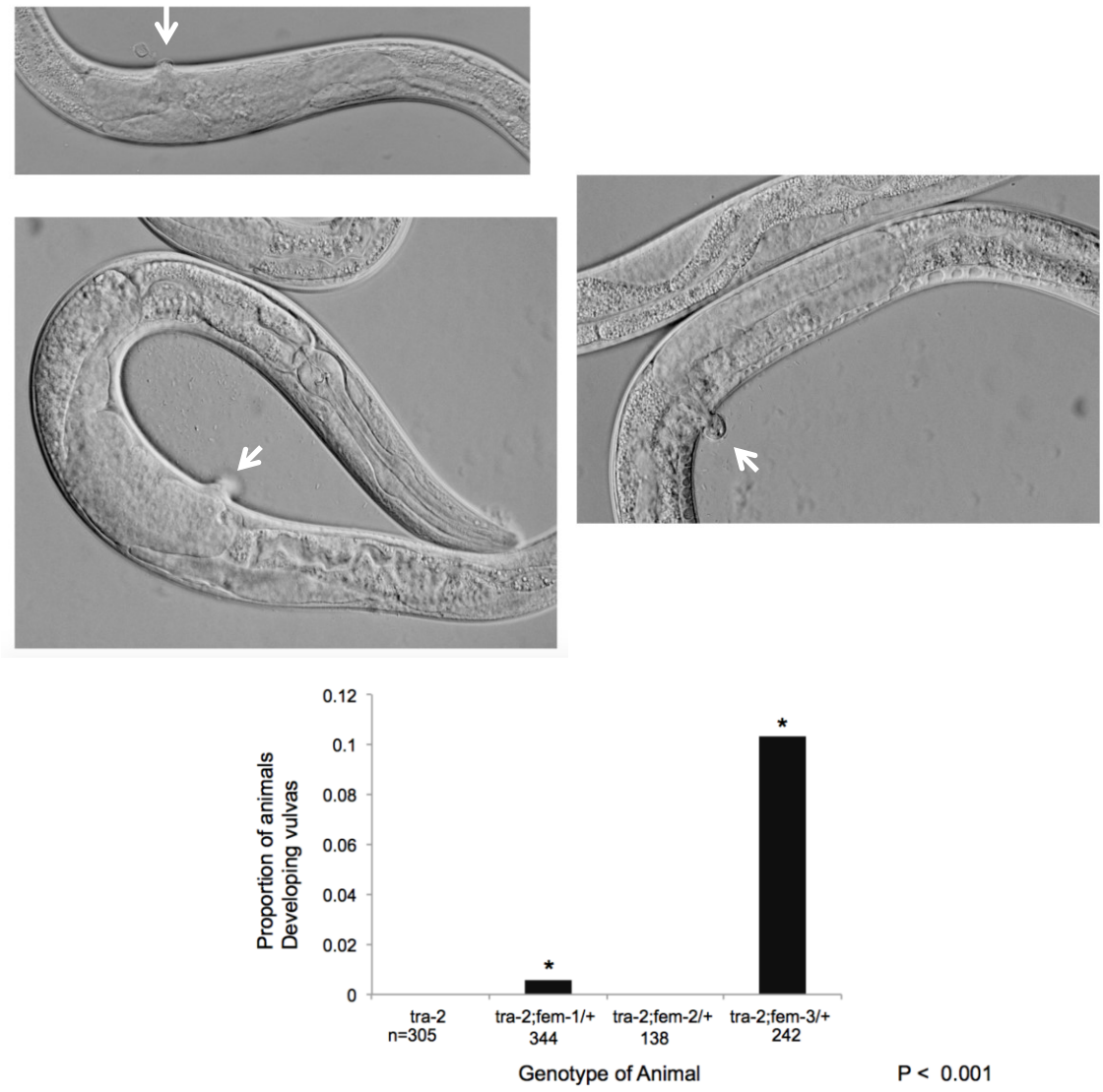


Figure 14. *tra-2;fem-2/+ and tra-2;fem-3/+* animals have an abnormal attachment of the distal gonad, resulting in a protruding vulva (P-vul) like phenotype. (A-C) representative animals, all are *tra-2;fem-3/+* animals. In these animals, the proximal gonad arm fails to migrate to the tail of the animal and anchors to the body wall, indicated by an arrow. This is approximately the correct location for the female gonad to anchor and develop the vulva. (D) Observed frequency of P-vul like phenotype. This phenotype is never seen in *tra-2;fem-1/+* or *tra-2* animals. Asterisk indicates a significant difference in proportions with severe abnormalities compared to *tra-2/tra-2* controls.

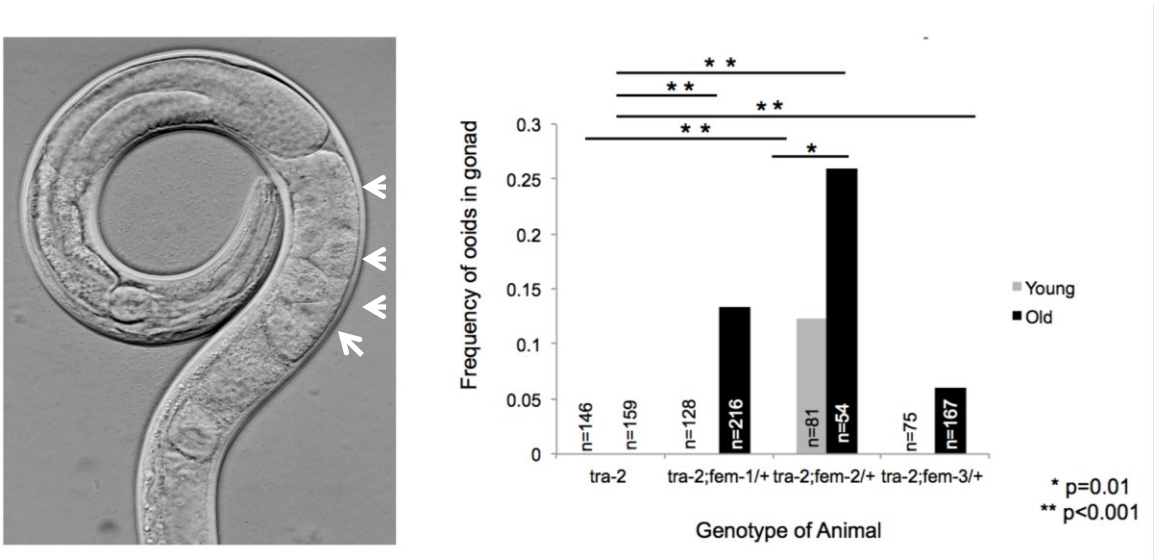


Figure 15. *tra-2;fem-3/+* and *tra-2;fem-2/+* animals raised at 25°C develop an ovotestis. Large ooids can be seen in the gonad of some animals, indicating that the germline is an ovotestis. Ooids are indicated with arrow heads. Developing embryos were never observed in these animals, however, development was never followed past four days. Young animals are 2 days post hatching while old adults are 3 or 4 days post hatching. Asterisk indicates significant differences using a binomial distribution test.

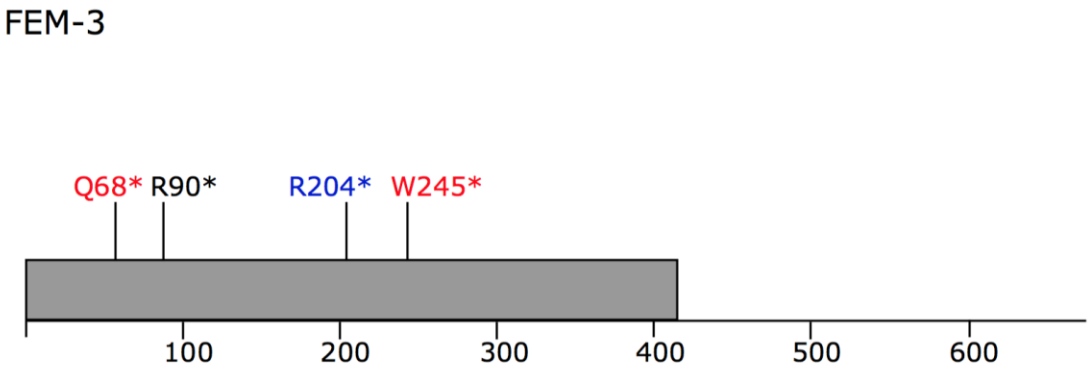


Figure 16. Identified *tra-2* suppressor mutations in *C. briggsae* FEM-3. All identified lesions are nonsense mutations. Identification and analysis of other lesions can be found in Reidy 2015 and Dewar 2011. *ed34* lesion is indicated in red and *ed44* lesions are identified in blue. Q68* lesion was identified in three independent strains: *ed43, ed44* and *ed59*. Grey box indicated FEM-3 protein, scale bar indicates amino acid.

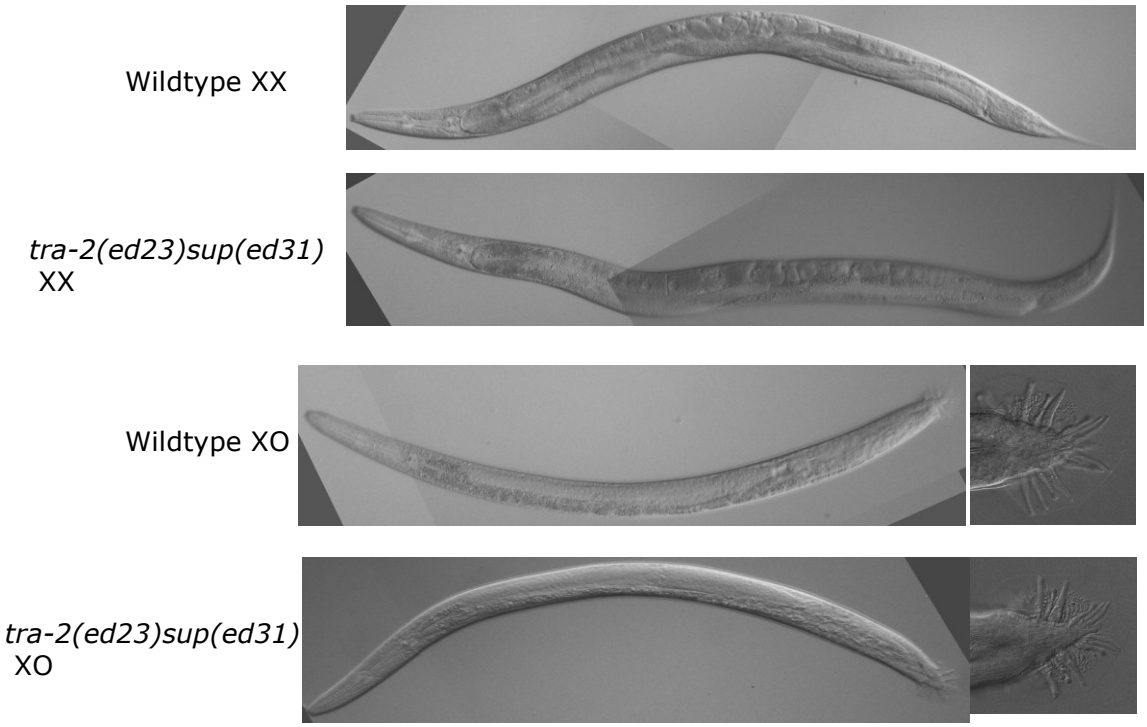


Figure 17. *tra-2(ed23)sup(ed31)* XX animals develop as normal hermaphrodites and XO animals develop as normal males. DIC images of hermaphrodites and males created with a heat shock. Male tail images taken at high magnification to compare tail ray morphology

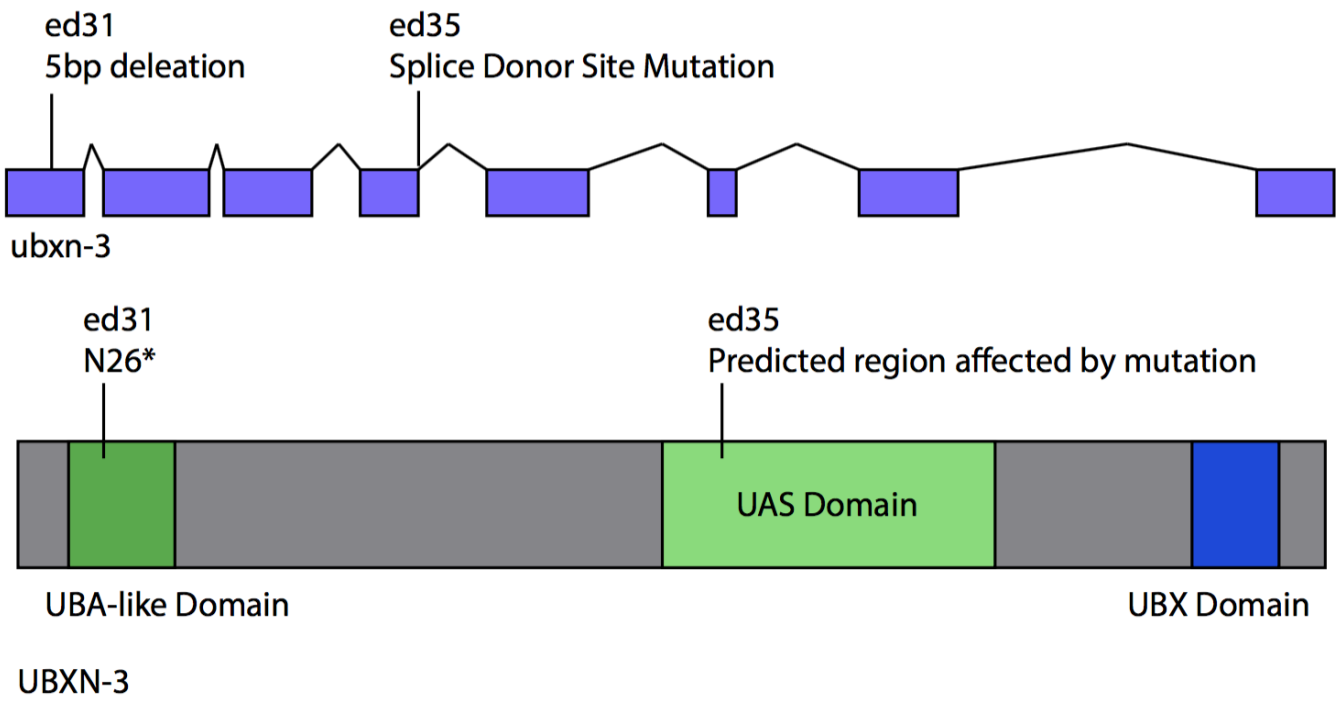


Figure 18. Suppressor lesions in *ubxn-3* and predicted effects on protein. *sup(ed31)* is predicted to have a 5bp deletion in the first exon that results in a nonsense mutation 26AA into the protein. *sup(ed35)* is predicted to have a mutation in the exon 4 splice donor site. Although the exact effect of this mutation is difficult to predict, it is likely to create a nonsense mutation shortly after the incorrect splice site. UBXN-3 contains a UBA-like domain that resembles ubiqitinA, a UAS domain and the UBX domain, predicted to be necessary for the binding of UBXN-3 to other adaptor proteins.

```
Cbr      ATGAGAAGAGGACGAGACATAACACGCAATAGAAGAGACGCTAGACAAGACCAGACAAAA
Cel      ------------------------------------------------------------
Ctr      ------------------------------------------------------------

Cbr      AAGTGTCCGGTTTTCAATAGAGCGTGTGGAGAGGCTCTTCTAGATGCGCAGACAGTGACA
Cel      ------------------------------------------------------------
Ctr      ------------------------------------------------------------

Cbr      CGGACACTGCTGGCTGTCAAGGATTACCACTCGCTATCACATAACTATACGAAAATGGAT
Cel      ------------------------------------------------------------
Ctr      ----------------------------------------------------ATGGAT

Cbr      CATCTAGATTTAGAAGACGATCAGAGGGAGAAGCTTCGACAGTTTACGGATATCACTCAA
Cel      ------------------------------------------------------------
Ctr      CATCTTGATTTGGAGGAAGATCAAAGAGAAAAACTTCGACAGTATACGGAATTCACTCAT

Cbr      CTGCACGACTATGACGTCGCGGTCGGGACGCTCGCATCGTTGAATTGGAATCTGGAGCAG
Cel      ------------------------------------------------------------
Ctr      CAACACGACTACGATGTCGCCGTCGGTACTCTGGCCTCACTGAATTGGAATCTTGAGCAA

Cbr      GCGATCGAGGCGCATCTGATGCAAGAGGACATCAACGACGACGATGAGGATCCAGAAATT
Cel      ------------------------------------------------------------
Ctr      GCTATCGAAGCGCATTTGATGCAAGAAGACATGAATGACGACGATGATCCAGAGATACTG

Cbr      CTGGAGACGATACCACCGAGGATTCCGGCC---GC----------GAGTGCTCCAG----
Cel      ------------------------------------------------------------
Ctr      G---AAGCAGTTCCACCACGATCATCTTCCAACCAGAACAAAAGTGGCAGCTCGACATCG

Cbr      -------------CTTTGCAAGAAGAGGTCATCGAAGTAGAACCTAAC---AGTATGCCA
Cel      ----------------------------------------------------ATGCCA
Ctr      TCAAATGGAAGGAGGGTCGAACCGGAAGTCATTGACATTGAAGCCGAAGAAATGCCAGCA
                                                                  **

Cbr      GCTGCACGTGGACGTCGCCGCGGACGCCCAGCCAACAACGGGGCTGCTTCAGATGAGAGG
Cel      GCTACACGTGGACGCCGTCGCGGACGTGCC------------GTGACTCCAGACGAGACG
Ctr      GCTCGAGGAGGACGTCGTCGTGGACGAACT------------GCTACTTCAGACGAGGCG
         ***  * * ***** ** ** *****  *             *   ** **** ***  *

Cbr      GAATCGGTCGATAACCAGATTGATGATCGTGTGGAAACGAGACAGACC---------A--
Cel      ACGACGGTCGATAACCAGGTGAAAAGACTGCGGATTGACGATGGAAGCAGCAGCAGCAGC
Ctr      ACGACGGTCGATAACCAGATCGATGATGATCCAGAGGATAGGAGCAAAA---CGAGCCG-
                       ************** *  *                       *

Cbr      -----AGAA--------------GGGACAGCCG---ATTGCAAGAAGAGGACGTCAGCCC
Cel      AACGGAGCCGCTACACATCATCGTGGAGCAGCGATACCGAGACAGAAACGTGGTCAAGCC
Ctr      -----ACGAGG------------TGACCCGATTCCACGACAAGGAAAACGTGGTCAAGCC
              *                 *                 *   * * *  ****  **

Cbr      GCTGAAGCAACACCATCTTCATCCTCATCATCTGCTTCATCATCCGCTGGACCAGCAACG
Cel      ACAGAGCCAACTCCGTCCTCATCAGGCTCCTCATCGGCTTCTTTCTCGTCACGCCGTGGG
Ctr      GCTGAGCAAGGAACTTCGTCAGCTTCGTCTTCTACATCTTCTTCGGCGGCATCTTCTTCA
                    * **   *    * ** *** *    ** **  *  * ** *   *   *

Cbr      CGTCGTTCTACTCGTGCCAACCCGA---------------CACCATCAGAGCCATCTCAA
Cel      ACACGTGCCAACCCGGTCCCACCAC---------------CGAATCAAGAGCCCGCTCAT
Ctr      ACTCATTCGAGACGTCACGCACGAGGGCATCCAGTGCCATCGAACCAAGAACCTGCTGAA
             * * * *  *    *   *                  *      *** **  ** *
```

```
Cbr      CCGGAACCGGCTCGTCAGAACGGAGTGCTTGC----------------------------
Cel      CCAGAATCGGCTCGTCAGAATGGCGGAATTCTTGCCTCTCGTCACAACAATCACAATAAC
Ctr      CCAGAAGCAGCCAGGGAAAATGGAACGTCTTTGC-GACGAGTTCCGATGGTTCC------
                                        ** *** * **  *  * ** **      *

Cbr      ------------------------------------------------------CGCATCG
Cel      CAGCAGAATAATCATCATCATCATCACCAGCGTATTCCGATTAATCCACGTCGCGTAGAC
Ctr      --------------AAGGCATGCACGACAAGA---------------GGTGGCGGCAGCT
                                                                * *

Cbr      CGACA---GCAACAACCACCAGTGGCGC------AGGCGCCAATGCAGGACTCGGACGAC
Cel      GTTTTCAACGTTGATTCTGACGAGGATGATGACTCGATGGCTATCGCATACGAGGATGAT
Ctr      CCTCAAATAGAAGATTCCGATGACGATGAAGATGCTATGAACATTGCCTACGACGACGAT
                      *  *    *  *             *   **     **   ** **

Cbr      GATGATGATGATGAGATGATTTACGAAGACGATCATAATGAGCCAGTAGCGCGTCAAGCT
Cel      GATGACGGC---------GTTCATGAGGTTCACCATAGTGAGGTCGTCGCTCGTGGCTCG
Ctr      GATGATATGGATCAGAATCATCAAAACGGAGAATATAATGAGACGGGTCCACGTGGTGGT
               *****               * *  * *   *  *** ****   *   * ***

Cbr      GCTGTTCAAAATGGTCGTGTGCCGATGATTCCTGATGGATACACTTCGGTTCCAGATGCG
Cel      GGTCCGCCGAACGGTCGTATTCCGATGATTCCGGATGGATTTTCGTCCGTCTCGGATGCT
Ctr      GCTCTCACCAATGGGCGAGTTCCGATGATCCCAGACGGTTATACGTCCGTTCCTGATGCT
          * *      ** ** **  * ******** ** ** ** *   * ** **  * *****

Cbr      CTTCGTAACTTTGTCACAGTGTTCTCTGATCGTTTCTGT----------------------
Cel      CTTCGCAACTTTGTCGCGATTTTCTCGGATCGTTTCTGCTCGACTCCGCAGACTCAGGCC
Ctr      CTCAGAAACTTTGTTTCCGTTTTCTCTAACCGTTTCTGCTCGACACCTGCCACACAGGCA
                           **  * ********  *  * *****  * ********

Cbr      ------------------------------------------------------TCACAGTCG
Cel      TTTATGCCACCATTCTACACGGAGCCACTTCCGGCAGCTGTTAAGGAAGCGTTTGATCAT
Ctr      TTCATGCCACCATTCTACACGGATACTCTTCCAAACGCTCTTAAGGAAGCGTTCGAAAAC
                                                                *   *

Cbr      CTTCTCAGTGAGCTTCGTCGCCCGCTCGTCTTCTACATTAATCACGATCGTTCCATCGCT
Cel      CCGAACAGTGAGCATCGTCGTCCACTGCTCTTCTACATCAACCATGATCGATCGATCGCG
Ctr      CCAAACATGGAGCTTCGTCGACCGTTAGTCTTCTATATCAATCATGACCGTTCTATCGCT
          *    **  **** ****** **  *  ******* ** ** ** ** ** ** *****

Cbr      GCCAACATCTTTGCGTCGCAAGTGCTGTGCTCAGAAGCCGTGTCGTCGCTGATCCGCCAT
Cel      GCGAACATCTTTGCGTCGCAAGTCTTGTGCTCGGAAACTGTTTCCACGCTTATTCGTCAT
Ctr      TCCAATATTTTCGCGTCTCAAGTAATGTGCTCCGAAGATGTATCATCACTCATCAGACAT
          * ** ** ** ***** *****  ******* ***   ** **  * ** **  * ***

Cbr      CAATACGTGCTCTTCCCATGGGATATTTCAAGTGACTCGAATCTAATGCATTTTATGGAG
Cel      CAATACGTCCTGTTTCCATGGGATATCACTAGCGACTCGAACTTGATGCTCTTCCTGGAA
Ctr      CAATACGTTCTCTTCCCGTGGGATATTTCGAGCGATTCGAATTTAATGCACTTTTTGGAC
          ******** ** ** ** ********  * ** ** *****  * ****  **  ****

Cbr      TTCCTCCAAGCCTCCAACATGGCTGACGTCCGAAACATGGTCCAACGTCTCGCCATGCAC
Cel      TACCTGCAAGCTGCCAATATGGGTGATGTGCGTACAATAATTCAACGTTTGGCGATGAGC
Ctr      TTTCTTCAAGCATGCAACATGGGAGATGTGCGCAACATGATTCAACGTTTGGCAGTCAAC
         *  ** *****   *** ****  ** ** ** *  **  * ****** * **  *   *
```


Cbr AAGGTTGAGAATTTCCCAATGATGATGGTCGTGACA----------
Cel AAGATCGAATCGTTCCCGTTGATGGCTATCGTTGTCAAGGAGCGAAACTCGTATCGTCTC
Ctr AAAATCGAGACATTCCCAATGATGGCTATCGTCATTCGCGAGAGAAACTCGTATCGTCTC
Cbr ----------CTTGCTGATCAGGTGTTGGAGAAGCTTTTGGCTGGC
Cel GTGGATTACTGTAGAGGAACCGATACCAGTGACCAAGTGATGGAGAAGCTTTTGAGCGGT
Ctr GTTGACTACTGCAGAGGAACTGATACAGCGGATCAAGTGATGGGAAAGCTGCTGGCCGGT
Cbr GTGGAACAGTACTCGAGCATCCGTATGAATGAGGCAGCCGAGAGACGTGAACGCGAAGAA
Cel GTCTCGGAGTACTCGGATATTCGAATGAATGAACAATCTGAGAGACGAGAGCGTGAAGAA
Ctr GTTGAAGAATACTCGAACGTTCGTCTAAACGAAGCCTCGGAACGTCGCGAACGAGAAGAG
Cbr CGCGAGGCTATCAGAAATCAACAAGAAGCCGAATATAAGGCATCTCTGGCTGCTGACAAG
Cel CGAGAGGCTATTCGGAATCAGCAGGAGGCTGAGTATAAGGCATCTCTGGCTGCTGACAAG
Ctr CGCGAGGCTATAAGAAACCAGCAAGAAGCTGAATACAAGGCTTCACTTGAAGCCGACAAG
Cbr GCTCGTATGGAAGCTAAGCAGAAGGAAATCGAGGAGCAACGTCTTGAGGAGGAGCGAAAG
Cel GCACGTATGGAGGCTAAACAGCAAGAAATTGAGGAGCAGCGTCTTGAGGAGGAACGCAAA
Ctr GCGCGAATGGAGGCAAAGCAACGTGAAATCGAAGAGCAAAGACTGGAAGAGGAACGAAAG
Cbr TTGAAAGAAGAAGAGGATGAGGCGCTTCGTCGTCAACTCGTAGCATCACAGCTTCCCGAT
Cel CTTCGTGAGGAGGAGGAAGAATGTGTCCGTCGTCAAACTGTCGCCTCTACAGTTCCAGAA
Ctr CAGAAAGAGATTGAAGATGAAGCTATTCGTCGTCAAACAGTTGCATCTACTCTTCCAGAA
Cbr GAGCCACCAGCCAGTGCCCCAGTCGCAGAGATCATCAATGTCAAATTCAGACTACCAGAA
Cel GAGCCACCAGCGAGTGCTCCACTTGCTGAGATTATCAATGTCAAGTTTAGATTGCCAGAA
Ctr GAGCCGCCAGCTAACGCGCCAGCTGCCGAAATCATTAATGTTAAGTTCAGACTTCCAGAA
Cbr GGTGGACAGGACATGCGCCGCTTCCGCCGTGTCGAAAGCATCCAGACTCTGATCGATTAT
Cel GGTGGACAGGATATGCGTCGTTTCCGTCGCCTCGAATCGATCCAGACACTGATCAACTAT
Ctr GGAGGACAGGATATGCGCCGTTTCCGACGCAGCGAGTCGATTCAAACTCTCATCGATTAC
Cbr TTGTCGTCGAAAGGGTTCTCGCCGGACAAGTATAAATACTTCAACTCGGACTTCCCGAAA
Cel TTGTCGTCAAAGGGATACTCGCCGGACAAGTTTAAATATTTCAACTCGGATTTCCCGAAA
Ctr TTGTCATCGAAGGGCTTCGCGCCTGACAAATACAAATATTTCAACTCGGATTTCCCGAAG
Cbr AAGGAGATCACTCGTCACTTTGATCTTTCGACAAACTTCACCGACTCAAAATGGCCAGCT
Cel AAGGAGATTACTCGCCACTTTGATTTGTCTCACAACTTTGCCGACACCAAATGGCCTGCC
Ctr AAGGAGATTACTCGTCATTTCGATCTGTCGAATAACTTCACCACTTCAAAATGGCCTGCC
Cbr AGGGAGCAAATTTTCGTCGAGGAAATTTGA
Cel AGGGAGCAGATTTTTGTCGAGGAAATCTAA
Ctr AGGGAGCAAATCTTTGTCGAAGAAATTTGA

Figure 19. Nucleotide alignment of *ubxn-3* in androdioecious *Caenorhabditis* species. Alignment preformed using sequence identified from the *Caenorhabditis* genomes database (see table 7) and alignment preformed using Clustal Omega. *C. briggsae* UBA-like domain indicated with purple bar. *C. briggsae* UBX domain indicated with green bar.

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