

Negative regulation of male development in *Caenorhabditis elegans* by a protein–protein interaction between TRA-2A and FEM-3

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The *tra-2* gene of the nematode *Caenorhabditis elegans* encodes a predicted membrane protein, TRA-2A, that promotes XX hermaphrodite development. Genetic analysis suggests that *tra-2* is a negative regulator of three genes that are required for male development: *fem-1*, *fem-2*, and *fem-3*. We report that the carboxy-terminal region of TRA-2A interacts specifically with FEM-3 in the yeast two-hybrid system and in vitro. Consistent with the idea that FEM-3 is a target of negative regulation, we find that excess FEM-3 can overcome the feminizing effect of *tra-2* and cause widespread masculinization of XX somatic tissues. In turn, we show that the masculinizing effects of excess FEM-3 can be suppressed by overproduction of the carboxy-terminal domain of TRA-2A. A FEM-3 fragment that retains TRA-2A-binding activity can masculinize *fem-3(+)* animals, but not *fem-3* mutants, suggesting that it is possible to release and to activate endogenous FEM-3 by titrating TRA-2A. We propose that TRA-2A prevents male development by interacting directly with FEM-3 and that a balance between the opposing activities of TRA-2A and FEM-3 determines sex-specific cell fates in somatic tissues. When the balance favors FEM-3, it acts through or with the other FEM proteins to promote male cell fates.

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The nematode *Caenorhabditis elegans* develops as a self-fertile hermaphrodite if it has two X chromosomes per diploid cell and a male if it has only one. The hermaphrodite is female except that its germ line transiently expresses a male fate, producing a few hundred sperm before switching to oogenesis. Extensive genetic analysis suggests that the ratio of X chromosomes to sets of autosomes (the X/A ratio) determines sex by regulating a genetic cascade of masculinizing and feminizing activities (Fig. 1A; for review, see Meyer 1997; Kuwabara 1999). This paper deals with one of the interactions in the cascade, involving the *tra-2* gene and the *fem* genes.

The activity of *tra-2* is necessary for female cell fates in XX hermaphrodites (Hodgkin and Brenner 1977), whereas the *fem* genes are required for male cell fates both in XO males and in the germ line of XX hermaphrodites (Doniach and Hodgkin 1984; Kimble et al. 1984; Hodgkin 1986). Genetic epistasis analysis suggests that *tra-2* activity promotes female development by nega-

tively regulating the activity of at least one of the *fem* genes (Doniach and Hodgkin 1984; Hodgkin 1986). The major feminizing activity of *tra-2* resides in a large integral membrane protein known as TRA-2A. Mutations that inactivate TRA-2A masculinize XX hermaphrodites (Kuwabara et al. 1992), and elevated expression of TRA-2A in XO animals is sufficient to transform them into fertile hermaphrodites (Kuwabara and Kimble 1995). The carboxy-terminal domain of TRA-2A, which is predicted to be intracellular, is at least partly responsible for negatively regulating *fem* activity, because expression of this domain alone in XO males results in partial feminization (Kuwabara and Kimble 1995).

The predicted products of the *fem* genes are all intracellular proteins. FEM-1 contains ANK repeats, which in many other proteins mediate specific protein–protein interactions, but its sequence is otherwise novel (Spence et al. 1990). FEM-2 is a protein serine/threonine phosphatase of Type 2C (Pilgrim et al. 1995; Chin-Sang and Spence 1996), and it interacts directly with FEM-3 (Chin-Sang and Spence 1996). The sequence of FEM-3 is unrelated to that of any other known protein (Ahringer et al. 1992).

Both genetic and molecular evidence suggest that the

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fem genes are post-transcriptionally regulated (Doniach and Hodgkin 1984; Hodgkin 1986; Rosenquist and Kimble 1988; Ahringer and Kimble 1991; Ahringer et al. 1992; Gaudet et al. 1996). A simple model (Fig. 1B) is that in XX animals, the carboxy-terminal domain of TRA-2A regulates *fem* activity by interacting with one or more of the FEM proteins (Kuwabara and Kimble 1995). Such an interaction may prevent the FEM proteins from acting on their targets and so allow female development to proceed. A candidate target of FEM activity in somatic tissues is the product of the *tra-1* gene, TRA-1A. It is a sequence-specific DNA-binding protein related to the

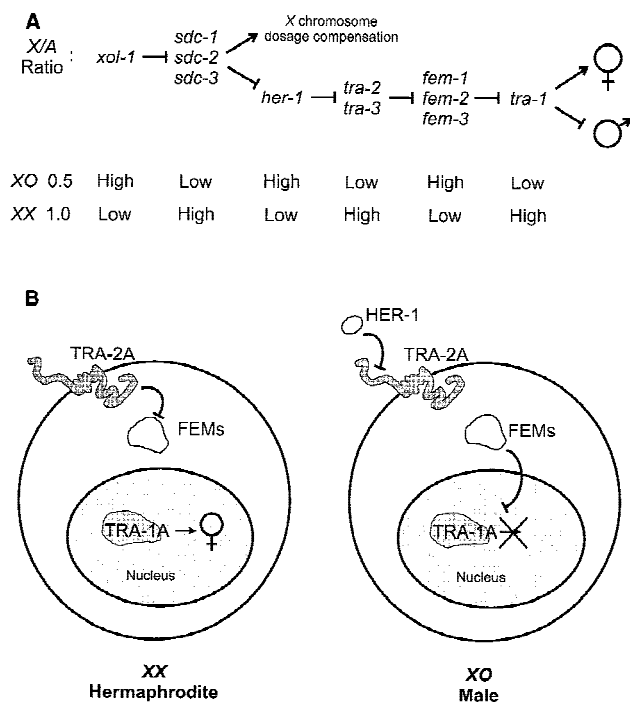


Figure 1. Model of somatic sex determination in *C. elegans*. (A) Genetic hierarchy regulating somatic sex determination. (Barred lines) Negative interactions; (arrows) positive interactions. The X/A ratio controls both X chromosome dosage compensation and sex determination via *xol-1* and the *sdc* genes. The regulatory pathway branches at the level of the *sdc* genes and only the branch that controls somatic sex determination is shown. In XX animals, *tra-2* negatively regulates the *fem* genes, allowing *tra-1* to promote female development. In XO animals, elevated *her-1* activity inhibits *tra-2* and permits the *fem* genes to bring about male development by negatively regulating *tra-1*. The *fem* genes have additional targets in the germ line, because they are required for spermatogenesis irrespective of the state of *tra-1* (Doniach and Hodgkin 1984; Hodgkin 1986). (B) Molecular model of somatic sex determination. (Left) A high X/A ratio prevents HER-1 synthesis, allowing TRA-2A to inhibit the FEM proteins and TRA-1A to direct female somatic development. Whether the three FEM proteins form a complex is not known. (Right) A low X/A ratio results in the synthesis of HER-1, a small, secreted protein that inactivates the membrane protein TRA-2A, thereby releasing the FEM proteins from negative regulation. The FEM proteins then inhibit the activity of the sequence-specific DNA-binding protein, TRA-1A, by an unknown mechanism to cause male development.

Gli proteins of vertebrates (Zarkower and Hodgkin 1992, 1993). The activity of *tra-1* is sufficient to direct all somatic tissues in *C. elegans* to adopt female fates (Hodgkin 1987).

In XO animals, the low X/A ratio derepresses transcription of the *her-1* gene (Trent et al. 1991). The product of *her-1* is a secreted protein, HER-1 (Perry et al. 1993), and the model in Figure 1B proposes that it acts as an inhibitory ligand for TRA-2A (Hunter and Wood 1992; Kuwabara et al. 1992; Perry et al. 1993; Kuwabara 1996b). Freed from the influence of TRA-2A, the FEM proteins bring about male development by directly or indirectly negatively regulating TRA-1A in somatic tissues and activating spermatogenesis in the germ line. Male development requires the phosphatase activity of FEM-2 (Chin-Sang and Spence 1996; Hansen and Pilgrim 1998), but otherwise little is known about the mechanism of action of the FEM proteins. Their effect is to transduce a masculinizing signal from the cell surface to the nucleus, the likely site of action of TRA-1A. In this model, interaction between TRA-2A and one or more of the FEM proteins constitutes a key regulatory switch that determines the output of a novel signal transduction pathway.

We tested the hypothesis that the carboxy-terminal domain of TRA-2A regulates sexual fate by interacting directly with one of the FEM proteins. Here we present evidence that the carboxy-terminal domain of TRA-2A can bind to FEM-3 and that this interaction prevents male development in the somatic tissues of XX animals.

Results

FEM-3 interacts with the carboxy-terminal region of TRA-2A in yeast

We used the yeast two-hybrid system (Fields and Song 1989; Durfee et al. 1993) to test for interactions between the carboxy-terminal domain of TRA-2A and the FEM proteins. A hybrid protein consisting of FEM-3 and the Gal4 DNA-binding domain, when expressed together with a second hybrid containing a carboxy-terminal fragment of TRA-2A and the Gal4 activation domain, activated expression of Gal4-regulated *HIS3* and *lacZ* reporter genes (Fig. 2). Expression of the *lacZ* reporter was ~20-fold above background in cells expressing both hybrid proteins. Neither protein stimulated reporter expression when expressed alone or with an unrelated Gal4 hybrid protein. An independent two-hybrid screen of >2,000,000 *C. elegans* cDNAs substantiated the specificity of the interaction reported here (Chin-Sang and Spence 1996). The screen yielded three FEM-3-interacting clones, one of which encoded the carboxy-terminal region of TRA-2A (I. Chin-Sang and A.M. Spence, unpubl.).

The last 45 amino acids of FEM-3 were dispensable for its interaction with TRA-2A, but deletion of 80 amino acids from the carboxyl terminus, or the removal of 70 amino acids from the amino terminus of FEM-3, abolished the interaction (Fig. 2). Full-length and truncated

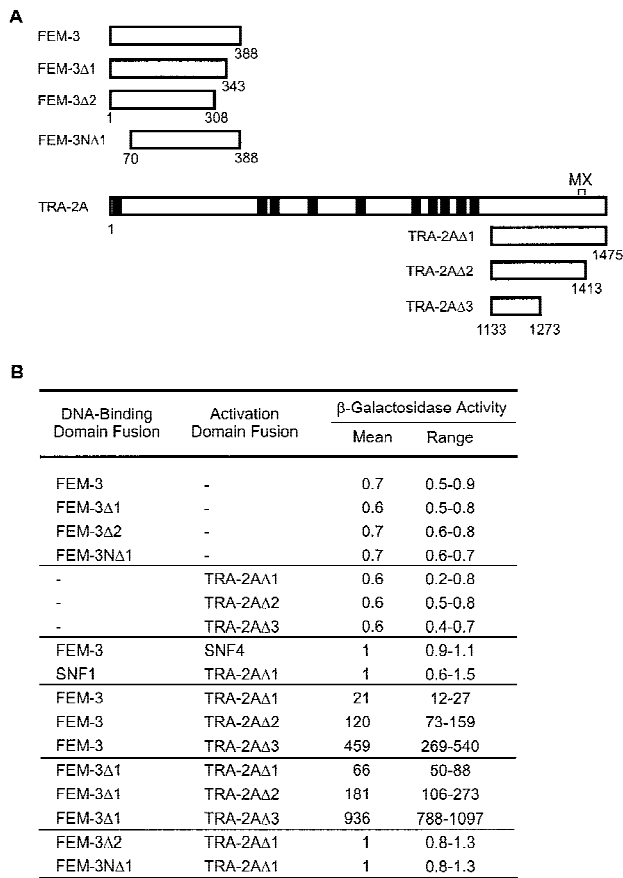


Figure 2. Yeast two-hybrid interaction of FEM-3 and the carboxy-terminal region of TRA-2A. (A) Diagram of fragments of FEM-3 and TRA-2A used in this study. Numbers indicate the amino acids at the termini of each fragment. The predicted signal sequence and transmembrane domains of TRA-2A are indicated as filled rectangles. The MX region is a 22-amino-acid region defined by *tra-2* mutations that transform XX hermaphrodites into females but do not affect XO animals. (Kuwabara et al. 1998). (B) Interaction between FEM-3 and TRA-2A fragments in the yeast two-hybrid system. β-Galactosidase activity was measured in liquid cultures of at least four independent transformants. Activity was normalized to the level in cells coexpressing a FEM-3 DNA-binding domain fusion and a hybrid of the Gal4 activation domain and Snf4. Snf4 and Snf1 are yeast proteins that served as negative controls for interactions involving FEM-3 and TRA-2A.

FEM-3 fusion proteins accumulated to similar levels in yeast (data not shown). Those deletions that disrupted binding to TRA-2A may have affected the proper folding of the binding domain, or they may have eliminated sequences directly involved in the interaction.

Deletion analysis of TRA-2A identified a region of 141 amino acids that interacted with FEM-3 in the two-hybrid system (TRA-2AΔ3; see Fig. 2). The FEM-3-binding domain lies between the last predicted transmembrane domain of TRA-2A and the MX region, a 22-amino-acid region that is implicated in post-translational regulation of *tra-2* activity in the germ line (Kuwabara et al. 1998). Interaction between TRA-2AΔ3 and

FEM-3 stimulated β-galactosidase expression 450-fold above background (Fig. 2B). This 20-fold increase in reporter activation, compared with the level that resulted from interaction between TRA-2AΔ1 and FEM-3, may reflect an increase in the strength of the interaction, but we cannot exclude other causes (Fields and Sternglanz 1994; Estojak et al. 1995).

Association between FEM-3 and the carboxy-terminal region of TRA-2A in vitro

To verify that the carboxy-terminal domain of TRA-2A can associate with FEM-3, we tested for interaction between the two proteins in vitro. We produced a carboxy-terminal fragment of TRA-2A and a Myc epitope-tagged derivative of FEM-3 in reticulocyte lysates and then immunoprecipitated MycFEM-3 using the Myc monoclonal antibody, 9E10. Figure 3 shows that the carboxy-terminal domain of TRA-2A specifically coimmunoprecipitated with MycFEM-3 from reactions containing both proteins.

A heat shock FEM-3 transgene causes male development in fem-3 XO and in XX animals

The *tra-2* gene behaves as a negative regulator of the *fem* genes. If a direct interaction between TRA-2A and FEM-3 were important for preventing male development in XX animals, then the production of excess FEM-3 might overcome the inhibitory effect of TRA-2A and masculinize XX hermaphrodites. To test the effects of overproducing FEM-3 in transgenic nematodes, we placed a cDNA fragment encoding MycFEM-3 under the

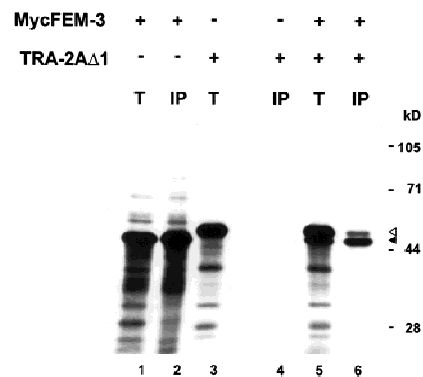


Figure 3. Coimmunoprecipitation of the carboxy-terminal region of TRA-2A with MycFEM-3. [³⁵S]Methionine-labeled MycFEM-3 (lanes 1,2), TRA-2AΔ1 (lanes 3,4) or both MycFEM-3 and TRA-2AΔ1 (lanes 5,6) were produced by in vitro translation in reticulocyte lysates. Equivalent samples were analyzed directly by SDS-PAGE and fluorography [(T) lanes 1,3,5] or subjected to immunoprecipitation with anti-Myc mAb 9E10 prior to electrophoresis [(IP) lanes 2,4,6]. The solid arrowhead marks the position of MycFEM-3; the open arrowhead marks the position of TRA-2AΔ1. Numbers at right indicate the positions and the relative molecular mass in kilodaltons of marker proteins.

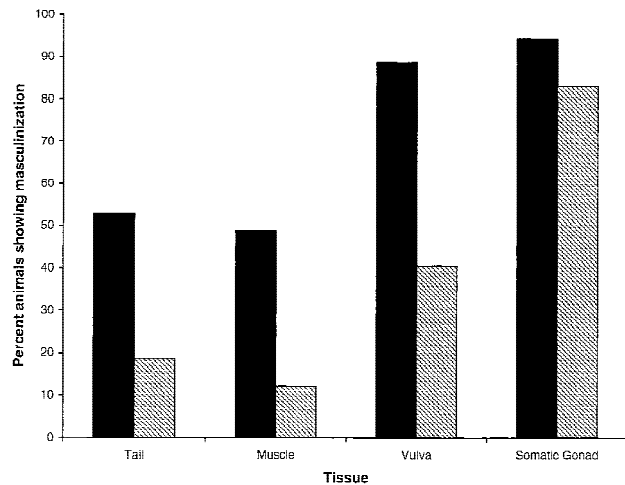


Figure 4. Masculinization of *fem-3 XO* and *XX* mutants by HS-MycFEM-3. Animals of genotypes *unc-24 fem-3; idIs4/O XO* and *unc-24 fem-3; idIs4/+ XX* (solid bars), or *unc-24 fem-3; idIs4/+ XX* only (hatched bars) were subjected to repeated heat shocks during development as described in Materials and Methods. No sign of male development was observed in the absence of heat shock treatment.

control of the nematode *hsp16-2* heat shock promoter, which directs expression in most somatic tissues (Stringham et al. 1992).

We first tested whether the HS-MycFEM-3 transgene could support male development in *XO* animals that lacked endogenous *fem-3* activity. For these experiments, we used a HS-MycFEM-3 transgene array that had been integrated on the *X* chromosome (*idIs4*). The *fem-3* allele that we used, *e1996*, is a putative null allele (Hodgkin 1986; Ahringer et al. 1992). Crossing *fem-3; idIs4* females to heterozygous *fem-3/+* males produced equal numbers of *fem-3 XX* and *XO* progeny that carried the transgene array. In the absence of heat shock, transgenic *XX* and *XO fem-3; idIs4* mutants developed as females. Periodic induction of HS-MycFEM-3 expression throughout development caused partial masculinization in 95% of the animals, suggesting not only that it rescued male development in *fem-3 XO* mutants, but also that it sexually transformed *fem-3 XX* animals (Figs. 4, solid bars, and 5). About half of the cross progeny exhibited extensive male development of the tail, the gonad, and the ventral hypodermis. Most of the remaining animals had strongly masculinized gonads but showed few signs of masculinization of the tail. It is likely that the more strongly masculinized animals were of *XO* karyotype, and the less masculinized animals were *XX* (see below). We did not observe any effects on germ-line development, which is consistent with earlier reports that the heat shock promoter is poorly expressed in the nematode germ line (Stringham et al. 1992).

To specifically examine the effects of HS-MycFEM-3 on *XX fem-3* mutants, we crossed *fem-3/+; idIs4/O XO* males to *fem-3 XX* females (see Materials and Methods for complete genotypes). Because the *idIs4* transgene array was *X*-linked, only the *XX* progeny of this cross in-

herited the array. After periodic induction of HS-MycFEM-3 expression, the transgenic *fem-3 XX* progeny closely resembled the less strongly masculinized class of animals observed in the previous experiment. Transformations of the tail and sex muscles in *fem-3 XX* mutants were infrequent, but the somatic gonad exhibited sexual transformation in 80% of transgenic animals (Figs. 4, hatched bars, and 5). This experiment proves that HS-MycFEM-3 expression is sufficient to cause sexual transformation in *fem-3 XX* animals.

Expression of the HS-MycFEM-3 transgene in otherwise wild-type *XX* nematodes resulted in strong sexual transformation of the soma: All of the somatic tissues that we examined exhibited some degree of masculinization in 70%–90% of the transgenic animals (Fig. 5; see also Table 1, line 2). Hodgkin (1986) showed that both maternal and zygotic *fem-3* activity are necessary for normal male development. We suggest that maternal *fem-3(+)* in a wild-type genetic background may have en-

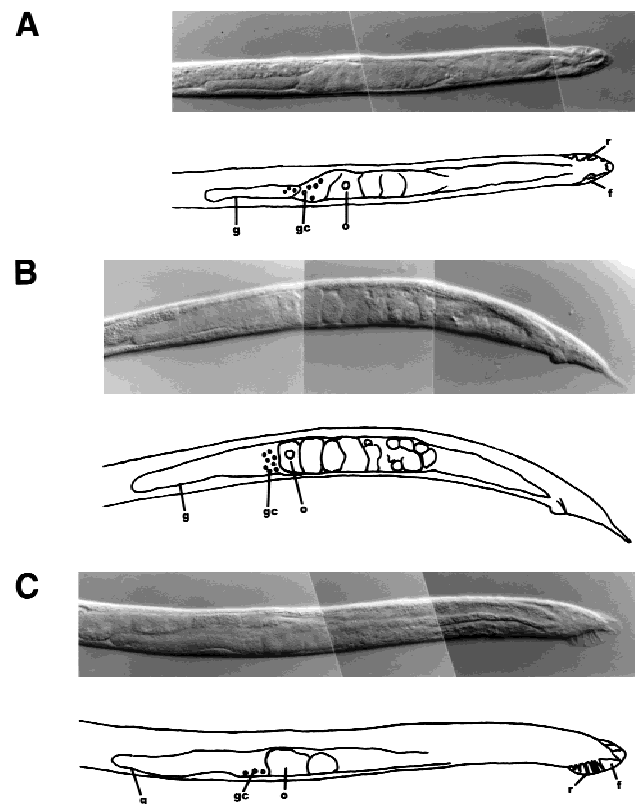


Figure 5. Masculinization of *fem-3* and *fem-3(+)* *XX* animals by HS-MycFEM-3. (A–C) Differential interference contrast photomicrographs and tracings showing gonad and tail morphology (f) fan; (g) gonad; (gc) immature germ cells; (o) oocyte-like cell; (r) ray. (A) *fem-3; idIs4/O* animal, presumed *XO*, showing partial rescue of male development in tail (note incomplete fan with several rays), gonad, ventral hypodermis. (B) *fem-3; idIs4/+ XX* animal, showing male gonad morphology and absence of vulva. The tail shows little masculinization in this animal, and the germ line is unaffected. (C) *fem-3(+); idIs4 XX* animal, showing extensive masculinization of the tail, gonad, and ventral hypodermis.

Table 1. Masculinization of XX animals by a TRA-2A-binding fragment of FEM-3

HS-MycFEM-3 variant ^a	No.	Percent masculinization of		
		gonad	ventral hypodermis	tail
None ^{b,c}	45	0	0	0
FEM-3	50	100	94	88
FEM-3Δ1	167	42	23	7
FEM-3Δ2 ^c	194	0	0	0

^aEach transgenic line carried the indicated heat inducible transgene on an extrachromosomal array. Number of independent lines analyzed: vector, 4 lines; FEM-3, 3 lines; FEM-3Δ1, 10 lines; FEM-3Δ2, 9 lines.

^bThe extrachromosomal array carried the heat shock promoter plasmid pPD49.83 with no insert.

^cOne animal had abnormal gonadal morphology, but it was not recognizably masculinized.

hanced the masculinizing effects of the HS-MycFEM-3 transgene. A similar maternal effect was probably not observed in *fem-3* mutants because the HS-MycFEM-3 transgene was unlikely to have been expressed in the germ line.

Overproduction of FEM-3 was sufficient to direct all somatic tissues in XX animals to adopt male sexual fates. These results suggest that *fem-3* activity must be negatively regulated to prevent inappropriate male development in the wild-type XX soma.

Masculinization by HS-FEM-3 requires the activity of fem-1 and fem-2

Male development in *C. elegans* normally requires the activities of all three *fem* genes. We tested whether excess, unregulated FEM-3 could bypass the requirement for either FEM-1 or FEM-2 by assaying the effects of the HS-MycFEM-3 transgene in *fem-1* and *fem-2* mutants. Table 2 shows that HS-MycFEM-3 does not bypass the requirement for *fem-1*. Transgene expression caused no detectable masculinization in mutants homozygous for the putative null allele *fem-1(e1991)* (Doniach and Hodgkin 1984).

To test whether *fem-2* activity is required for masculinization by HS-MycFEM-3, we assayed the effects of the *idIs4* transgene array in mutants homozygous for a

Table 2. Masculinization by HS-MycFEM-3 requires *fem-1* activity

Cross: *dpy-13/fem-1 unc-24; idIs4 XO* × *fem-1 unc-24; idIs4 XX^a*

Non-Unc F ₁			Unc F ₁		
male	intersex	female	male	intersex	female
214	171	12	0	3	350

^a*unc-24* maps ~1.5 map units from *fem-1*. Hence, ~99% of the Unc F₁ progeny were homozygous for *fem-1*.

putative null allele, *fem-2(e2105)* (Hodgkin 1986; Pilgrim et al. 1995). We examined the self progeny of maternally rescued, heat-shocked *fem-2; him-5; idIs4* homozygotes. The *him-5* mutation causes homozygotes to produce ~30% XO self-progeny (Hodgkin et al. 1979). We observed no sign of masculinization among 30 animals, suggesting that *fem-2* activity is also required for HS-MycFEM-3 to promote somatic male development.

Masculinization of XX hermaphrodites by a TRA-2A-binding fragment of FEM-3

A FEM-3 fragment consisting of the first 343 amino acids of the full-length protein, FEM-3Δ1, failed to rescue male development in *fem-3* mutants when expressed from the heat shock promoter (data not shown). Because FEM-3Δ1 retained the ability to interact with TRA-2A in yeast (Fig. 2), we tested whether its overproduction might promote male development in wild-type XX nematodes. We reasoned that the inactive FEM-3Δ1 might saturate the available TRA-2A and release active, endogenous FEM-3.

About 40% of the transgenic animals expressing HS-FEM-3Δ1 exhibited significant masculinization of the somatic gonad (Table 1, line 3). In contrast, a smaller FEM-3 fragment, FEM-3Δ2, that failed to interact with TRA-2A in yeast, failed to cause sexual transformation when expressed from the heat shock promoter in transgenic nematodes (Table 1, line 4). Figure 6 shows that both FEM-3Δ1 and FEM-3Δ2 accumulated to similar levels following heat shock.

The carboxy-terminal domain of TRA-2A suppresses the masculinizing effects of HS-FEM-3

Kuwabara and Kimble (1995) showed that overexpression of the carboxy-terminal domain of TRA-2A partly

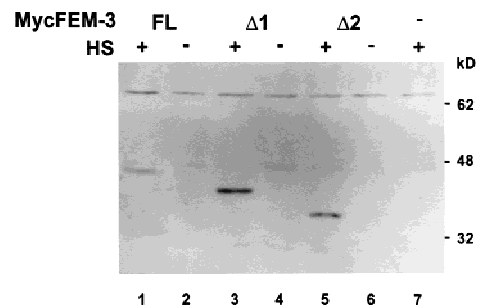


Figure 6. Expression of MycFEM-3 and truncated derivatives in transgenic nematodes. Lysates were prepared from animals maintained continuously at 20°C (no heat shock; HS -, lanes 2,4,6) and from animals subjected to heat shock at 33°C followed by a 2-hr recovery period at 20°C (HS +, lanes 1,3,5,7). The lysates were analyzed by SDS-PAGE and immunoblotting with antibody 9E10. Transgenic nematodes carried plasmids encoding MycFEM-3 (FL, lanes 1,2), MycFEM-3Δ1 (Δ1, lanes 3,4), or MycFEM-3Δ2 (Δ2, lanes 5,6), or empty heat shock vector (lane 7). Numbers at right indicate the relative molecular masses in kilodaltons and the positions of migration of molecular mass markers.

suppresses male development in wild-type *XO* animals. Having found that a carboxy-terminal fragment of TRA-2A binds to FEM-3, we tested whether its overproduction might also suppress the masculinizing effects of HS-MycFEM-3 in *XX* animals. We compared the phenotype of *XX* animals coexpressing HS-MycFEM-3 and a second transgene, HS-TRA-2A Δ 1, that encodes the carboxy-terminal domain of TRA-2A, to that of animals coexpressing the HS-MycFEM-3 transgene with an inactive *tra-2* transgene, HS-TRA-2Afs, that carried a frameshift mutation to prevent production of TRA-2A Δ 1 (see Materials and Methods).

Periodic heat shock caused significant somatic masculinization of *XX* nematodes carrying HS-MycFEM-3 and HS-TRA-2Afs. As before, the gonad and ventral hypodermis were most strongly affected. The somatic gonad exhibited male morphology in 80%–90% of the transgenic animals in each of three independent strains (Fig. 7A). In contrast, only 3% of the animals from five independent lines (range 0%–9% for individual lines) expressing both HS-FEM-3 and HS-TRA-2A Δ 1 had strongly masculin-

ized somatic gonads. A further 23% exhibited incomplete somatic gonad masculinization, but in 74% of the animals, the gonad showed no detectable masculinization (Fig. 7A). The level of expression of MycFEM-3 varied between strains, but strains expressing only MycFEM-3 did not consistently express higher levels than those expressing both MycFEM-3 and TRA-2A Δ 1 (Fig. 7B). We conclude that TRA-2A Δ 1 inhibited the activity of MycFEM-3. This result suggests that the sexual fate of the somatic tissues in *C. elegans* depends on a balance between the opposing activities of TRA-2A and FEM-3.

Discussion

Because male development in *C. elegans* requires the activity of all three *fem* genes (Doniach and Hodgkin 1984; Kimble et al. 1984; Hodgkin 1986), negative regulation of one is sufficient to prevent the others from acting. Previous genetic analysis has shown that *tra-2* is the principal negative regulator of *fem* activity (Doniach and Hodgkin 1984; Hodgkin 1986; Kuwabara and Kimble 1995). Our results suggest that this regulation is achieved at the level of protein–protein interactions: TRA-2A negatively regulates FEM activity by binding directly to FEM-3.

Limiting FEM-3 activity prevents male development in the *XX* soma

The ability of the HS-FEM-3 transgene to cause sexual transformation of somatic tissues in *XX* hermaphrodites establishes that *fem-3* acts as a developmental switch in the soma. In the absence of *fem-3* activity, male development cannot occur, whereas elevated *fem-3* activity is sufficient to cause male somatic development in an *XX* animal that would otherwise develop as a hermaphrodite. Overexpression of either *fem-2* or *fem-1*, in contrast, fails to masculinize the *XX* soma (Chin-Sang and Spence 1996; J. Gaudet and A.M. Spence, unpubl.). These observations imply that the activity of *fem-3* must ordinarily be limited to prevent *XX* somatic tissues from adopting male fates. Since both *fem-1* and *fem-2* are not only available, but essential for HS-FEM-3 to masculinize the *XX* soma, it appears that limiting *fem-3* activity is normally the key step in preventing *XX* somatic tissues from adopting male fates. It is unlikely that negative regulation of FEM-3 activity is achieved via regulation of either of the other FEM proteins.

Interaction with TRA-2A limits FEM-3 activity

The observation that the carboxy-terminal region of TRA-2A interacts specifically with FEM-3 in the yeast two-hybrid system and in vitro supports the conclusion that FEM-3 is the direct regulatory target of TRA-2A. Interaction between the two proteins apparently does not require other nematode proteins. We were not able to detect interactions between the carboxy-terminal portion of TRA-2A and either of the other FEM proteins in

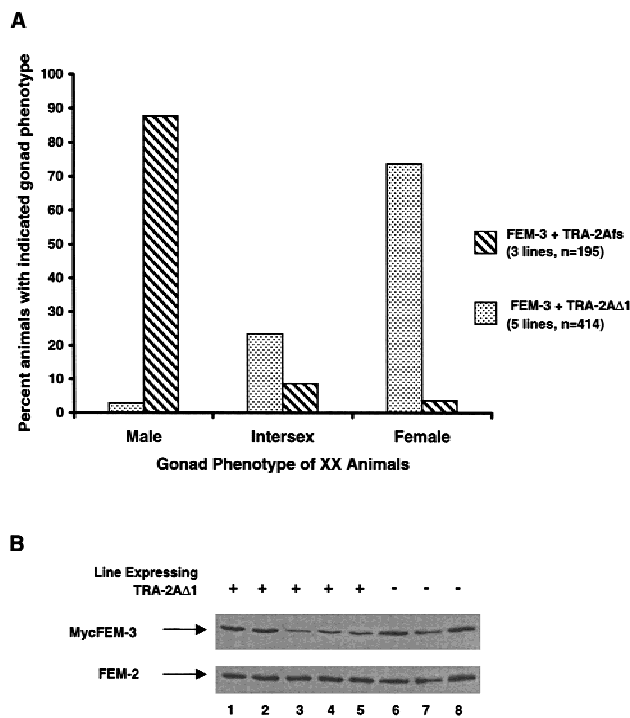


Figure 7. Suppression of the masculinizing effect of HS-MycFEM-3 by the carboxy-terminal domain of TRA-2A. (A) Masculinization of the gonad in *XX* animals expressing HS-MycFEM-3 with an inactive TRA-2A construct, HS-TRA-2Afs (hatched bars; three transgenic lines, $n = 195$) or with HS-TRA-2A Δ 1 (stippled bars; five transgenic lines, $n = 414$). (B) Expression of MycFEM-3 in the transgenic lines used in A. Lysates from lines coexpressing MycFEM-3 and TRA-2A Δ 1 (lanes 1–5) or from lines expressing MycFEM-3 and TRA-2Afs (lanes 6–8) were analyzed by SDS-PAGE and immunoblotting with the anti-Myc mAb 9E10 to test whether they contained similar levels of MycFEM-3. An equivalent blot was probed with a FEM-2 antiserum to verify that similar quantities of protein were present in each sample.

the yeast two-hybrid system (A. Mehra, I. Chin-Sang, D. Lum, U. Vivegananthan, and A.M. Spence, unpubl.).

Two other observations favor the conclusion that TRA-2A regulates *fem* activity by binding to FEM-3. First, the FEM-3 fragment, FEM-3 Δ 1, masculinized the XX soma in a manner dependent on endogenous *fem-3*(+). This effect is most simply explained by proposing that FEM-3 Δ 1 titrated a negative regulator of endogenous FEM-3. Because FEM-3 Δ 1 retained TRA-2A-binding activity, and since a shorter FEM-3 fragment, FEM-3 Δ 2, that lacked TRA-2A-binding activity in yeast failed to masculinize transgenic nematodes, we suggest that the negative regulator in question was TRA-2A. The dependence of FEM-3 Δ 1 on endogenous FEM-3 for its masculinizing activity probably explains why its effects were significantly weaker than those resulting from overexpression of full-length FEM-3: The somatic tissues of XX hermaphrodites accumulate only low levels of *fem-3* transcripts (Rosenquist and Kimble 1988), suggesting that the abundance of FEM-3 is likely to be low. Full-length FEM-3 also interacts with FEM-2 (Chin-Sang and Spence 1996), but FEM-3 Δ 1 failed to interact with FEM-2 in the yeast two-hybrid system (I. Chin-Sang and A.M. Spence, unpubl.), supporting our contention that its masculinizing activity arose entirely from its ability to titrate TRA-2A and liberate endogenous FEM-3.

Second, overexpression of the carboxy-terminal fragment of TRA-2A suppressed the masculinizing effects of excess FEM-3, suggesting that sexual fate in somatic tissues depends on a balance between the activities of FEM-3 and the carboxy-terminal region of TRA-2A. We suggest that the direct interaction of TRA-2A with FEM-3 provides a mechanism for measuring the relative levels of the two opposing activities.

Regulation of TRA-2A activity in XO males

How is TRA-2A activity regulated in XO animals to allow male development? The model of sex determination presented in Figure 1A suggests that in XO animals the secreted protein HER-1 inhibits TRA-2A and allows the FEM proteins to promote male development. One possibility is that HER-1 binding induces a conformational change in the carboxy-terminal domain of TRA-2A that disrupts the interaction between TRA-2A and FEM-3. Alternatively, HER-1 might instead destabilize TRA-2A, reducing its steady-state level and shifting the balance in favor of the masculinizing activity of FEM-3. It will be of great interest to determine whether HER-1 directly affects the affinity of the interaction between TRA-2A and FEM-3.

Significance of TRA-2/FEM-3 interaction for germ-line sex determination

Although our experiments focus on the role of the TRA-2A/FEM-3 interaction in somatic tissues, the interaction is likely also to be important in the germ line. First, the loss of *tra-2* activity causes *fem*-dependent masculinization of both the soma and the germ line of XX animals

(Hodgkin and Brenner 1977). Second, the germ line expresses both the mRNA encoding TRA-2A and a germ-line-specific *tra-2* transcript encoding a protein known as TRA-2B (Okkema and Kimble 1991; Kuwabara et al. 1998). TRA-2B is identical to the cytoplasmic domain of TRA-2A (Kuwabara et al. 1998) and therefore may be expected to interact with FEM-3. Third, the sexual fate of the germ line is governed by a balance between the activities of *tra-2* and *fem-3* (Barton et al. 1987; Schedl and Kimble 1988). Both *tra-2* and *fem-3* are subject to post-transcriptional repression, mediated by their 3' untranslated regions (3' UTR), in the germ line (Doniach 1986; Barton et al. 1987; Ahringer and Kimble 1991; Goodwin et al. 1993). We suggest that the protein-protein interaction between TRA-2A (or TRA-2B) and FEM-3 that we have described provides a second level of control over *fem-3* activity in the XX germ line. Whereas 3' UTR-mediated repression presumably controls the levels of TRA-2A, TRA-2B, and FEM-3 in the germ line, the TRA-2(A or B)/FEM-3 interaction may serve to measure the relative levels of the proteins. Their relative levels in turn determine the sexual fate of the germ line, excess FEM-3 triggering spermatogenesis, and an excess of TRA-2 proteins resulting in oogenesis (see Gallegos et al. 1998 for further discussion).

Mechanism of inhibition of FEM-3 by TRA-2A

How does the binding of TRA-2A to FEM-3 prevent adoption of male fates in XX animals? A simple hypothesis is that the interaction sequesters FEM-3 and prevents it from gaining access to its targets or cofactors. Because TRA-2A is predicted to be a transmembrane protein (Kuwabara et al. 1992), one might expect the interaction between TRA-2A and FEM-3 to occur in vivo at the inner surface of the cell membrane. However, membrane localization of the FEM-3-binding domain of TRA-2A is probably not strictly essential for FEM-3 regulation. In our experiments, a carboxy-terminal fragment of TRA-2A that lacks apparent transmembrane sequences was nevertheless capable of suppressing the effects of HS-FEM-3. Similarly, Kuwabara and Kimble (1995) showed that overexpression of the carboxy-terminal domain of TRA-2A feminizes *tra-2* XX pseudomales and *tra-2*(+) XO males.

FEM-3 interacts with FEM-2 (Chin-Sang and Spence 1996), and their interaction is likely to be required for male development. TRA-2A might regulate FEM-3 by preventing its interaction with FEM-2, either by direct competition or through allosteric effects. Alternatively, TRA-2A might bind to the FEM-3-FEM-2 complex and prevent its activation or its correct targeting.

Sequence requirements for the TRA-2A/FEM-3 interaction

Although the carboxy-terminal 45 amino acids of FEM-3 are dispensable for its interaction with TRA-2A, further deletion analysis failed to define a TRA-2A-binding domain in FEM-3 (Fig. 2; data not shown). Mutations in *fem-3* that selectively interfere with the TRA-2A/FEM-3

interaction would be expected to cause constitutive activation of FEM-3 and inappropriate masculinization of somatic tissues in XX animals. No *fem-3* gain-of-function mutations with these properties have been isolated. Such mutations might be expected to cause dominant sterility in XX animals; if so, their phenotype would have precluded their recovery in the screens performed to date. It is also possible that *fem-3* mutations that disrupt TRA-2A binding inevitably compromise other aspects of FEM-3 function, such as binding to FEM-2, and result in a loss of function.

A 141-residue fragment of TRA-2A is sufficient for interaction with FEM-3 in the yeast two-hybrid system. Mutations that affect the minimal FEM-3-binding region have not yet been described. If such mutations interfered with FEM-3-binding, we would expect them to reduce *tra-2* activity. Mutations have been identified that truncate TRA-2A carboxy-terminal to the FEM-3-binding region, and they reduce *tra-2* activity in somatic tissues (Kuwabara et al. 1992). Their effects might be at least partly attributable to reduced stability of the mRNAs, or of the truncated proteins themselves. As well, sequences carboxy-terminal to the minimal FEM-3-binding region may affect the affinity of the interaction in vivo.

Evolutionary divergence of the FEM-3-binding region in TRA-2A

The sequence of TRA-2A from *C. elegans* has diverged extensively from its counterpart in the closely related nematode *Caenorhabditis briggsae* (Cb-TRA-2A). The predicted TRA-2A proteins from the two species exhibit only 43% amino acid identity, although the level of conservation is significantly higher in the region surrounding a potential site of HER-1 interaction known as the enhanced gain-of-function (EG) site (Kuwabara 1996a). In marked contrast to the region around the EG site, the minimal FEM-3-binding region identified in this study is particularly divergent, with only 20% identity between the two proteins and a 16-amino-acid insertion in Cb-TRA-2A. *C. briggsae* develops as either a male or a self-fertile hermaphrodite, as does *C. elegans*, and RNA interference experiments suggest that Cb-TRA-2A is required for hermaphrodite development in *C. briggsae*, as is Ce-TRA-2A in *C. elegans* (Kuwabara 1996a). The rapid sequence divergence of the carboxy-terminal region of TRA-2A during evolution may conceal a higher level of structural conservation; alternatively, it may suggest a similarly rapid divergence of the target or mechanism of action of TRA-2A. No FEM-3 homolog from *C. briggsae* has yet been described, but it will be interesting to investigate whether a TRA-2A/FEM-3 interaction regulates sexual fate in *C. briggsae*, as it does in *C. elegans*.

Materials and methods

C. elegans strains and culture methods

Standard methods for culture and manipulation of nematodes were used (Brenner 1974; Sulston and Hodgkin 1988). MYOB

medium (Church et al. 1995) was used in place of NGM (nematode growth medium) in some experiments. The incubation temperature was 20°C unless otherwise noted. We used the standard laboratory wild-type strain of *C. elegans* var. Bristol, N2. All of the other strains used in this work are derivatives of N2. Following are the mutant alleles used in this study; the affected genes are described in Hodgkin (1997): LG III *fem-2(e2105)*; LG IV *dpy-13(e184)*, *fem-1(e1991)*, *mor-2(e1125)*, *unc-24(e138)*, *fem-3(e1996)*, *dpy-20(e1282ts)*; and LG V *him-5(e1490)*.

Plasmids

Standard molecular biological methods were used (Sambrook et al. 1989). Plasmids and details of their construction are available on request. J. Kimble (University of Wisconsin, Madison, WI) kindly provided a *fem-3* cDNA in plasmid pJK453. Ligation of appropriate restriction fragments of pJK453 into pAS1 (Durfee et al. 1993) produced plasmids AS#1197, AS#1278, AS#1279, and AS#1283, which respectively encode FEM-3, FEM-3Δ2, FEM-3Δ1, and FEM-3NΔ1 as Gal4 DNA-binding domain fusion proteins. Full-length FEM-3 is 388 amino acids long: FEM-3Δ1 includes amino acids 1–343, FEM-3Δ2 includes amino acids 1–308, and FEM-3NΔ1 includes amino acids 70–388.

Plasmid AS#1184 is *fem-3* cDNA in T7plinkTag (Bardwell and Treisman 1994), and it encodes FEM-3 with an amino-terminal Myc epitope tag (MycFEM-3).

The HS–MycFEM-3 plasmid AS#1196 was produced by subcloning the insert of plasmid AS#1184 into the *C. elegans* heat shock vector pPD49.83 (Mello and Fire 1995). Plasmids AS#1230 and AS#1231 are derivatives of AS#1196 that direct the expression of MycFEM-3Δ2 and MycFEM-3Δ1, respectively.

Plasmid pPK126 consists of an *EcoRI* fragment of *tra-2* cDNA, encoding amino acids 1133–1475 of TRA-2A (referred to here as TRA-2AΔ1), in the vector T7plink (Bardwell and Treisman 1994).

Insertion of the *NcoI*–*XhoI* fragment from pPK126 into pAC-TII (Durfee et al. 1993, S. Elledge, pers. comm.) yielded plasmid AS#1191, encoding TRA-2AΔ1 fused to the activation domain of Gal4. Plasmids pPK165 and pPK166, respectively, encode TRA-2AΔ2 and TRA-2AΔ3 as Gal4 activation domain fusion proteins. TRA-2AΔ2 includes amino acids 1133–1413 of TRA-2A, and TRA-2AΔ3 includes amino acids 1133–1273.

The HS–TRA-2AΔ1 plasmid AS#1241 carries the *NcoI*–*XhoI* fragment from pPK126 in vector pPD49.83. Plasmid AS#1242, referred to here as HS–TRA-2Afs, differs only in that it carries a frameshift mutation in the polylinker immediately preceding the *tra-2* cDNA insert, to prevent expression of TRA-2AΔ1.

Plasmid pSE1111, encoding a hybrid of the Gal4 activation domain and the yeast protein Snf4, and pSE1112, which encodes a Gal4 DNA-binding domain–Snf1 hybrid (Durfee et al. 1993), respectively served as negative controls in yeast two-hybrid tests with FEM-3 and TRA-2A derivatives.

Yeast two-hybrid tests

Yeast was cultured on standard complete and synthetic medium at 30°C (Sherman 1991). Transformation was performed by the methods of Schiestl and Gietz (1989). Two-hybrid tests were carried out in strain Y153 as described (Durfee et al. 1993). Standard methods were used to assay B-galactosidase activity (Ausubel et al. 1989).

Immunoprecipitations

[³⁵S]-Methionine-labeled MycFEM-3 and TRA-2AΔ1 were produced by coupled in vitro transcription and translation of

AS#1184 and pPK126 in a TNT lysate (Promega). Immunoprecipitations with monoclonal antibody 9E10 (Evan et al. 1985) were carried out as described previously (Chin-Sang and Spence 1996). In some experiments, protein A-Sepharose beads (Pharmacia) were incubated with rabbit anti-mouse IgG antiserum (Jackson Laboratories) prior to use. Immunoprecipitates were analyzed by SDS-PAGE and fluorography.

Western blotting

Gal4 fusion proteins encoded by plasmids derived from pAS1 and pACTII carry the HA epitope tag recognized by mAb 12CA5 (Wilson et al. 1984). Their expression was verified by Western analysis of yeast lysates, prepared as in Ausubel et al. (1989). Western analysis was performed as described (Gaudet et al. 1996) with 12CA5 and an alkaline phosphatase-conjugated goat anti-mouse secondary antibody (GIBCO).

To assay the expression of MycFEM-3 and its truncated derivatives in nematodes after heat shock, a sample of young larvae of each transgenic line to be tested was distributed to two plates. After 3 days of incubation at 20°C, one plate in each pair was subjected to two heat shocks of 1 hr each at 33°C, separated by a rest interval of 20 min at 20°C. After the second heat shock, animals were allowed to recover at 20°C for 3 hr before harvesting. The second plate in each pair remained at 20°C until the time of harvesting. Harvesting, lysis, and Western analysis with mAb 9E10 were carried out as described (Chin-Sang and Spence 1996).

Expression of MycFEM-3 in transgenic strains coexpressing HS-MycFEM-3 and either HS-TRA-2AΔ1 or HS-TRA-2Afs was similarly assayed by Western blotting with mAb 9E10. Duplicate blots were assayed with a polyclonal anti-FEM-2 antiserum (J. Gaudet, unpubl.) to verify equal loading of the lysates.

Nematode transformation

Nematode transformation was carried out as described by Mello et al. (1991). The plasmid pRF4, carrying the *rol-6(su1006)* allele, served as a transformation marker: It confers a dominant Roller (Rol) phenotype. The mixture of DNA injected included pRF4 (50 µg/ml), the plasmid(s) to be tested (10 µg/ml, or in some experiments, 50 µg/ml), and pBluescript (Stratagene) as needed to adjust the total DNA concentration to 100 µg/ml. Lines carrying heritable extrachromosomal arrays were established from among the Rol F₂ progeny of injected animals. At least two independent extrachromosomal arrays were tested for each *fem-3* construct at a given concentration.

To produce the integrated HS-MycFEM-3 array, *idIs4*, animals carrying plasmid AS#1196 on an extrachromosomal array were γ-irradiated with 3600 rads from a ¹³⁷Cs source. F₂ Rol animals were screened for individuals that transmitted the array to all of their progeny (Mello and Fire 1995). Crosses between *idIs4* Rol males and non-Rol hermaphrodites produced broods consisting entirely of non-Rol males and Rol hermaphrodites, suggesting that *idIs4* was X-linked.

Heat shock experiments

Adult transgenic hermaphrodites or mated females were allowed to lay eggs for 2–16 hr and were then removed. Their progeny were subjected to periodic heat shock throughout development, beginning in late embryogenesis or early larval development. In most experiments, heat shocks were administered at 18-hr intervals, and each consisted of two 1-hr periods at 33°C separated by a 20-min period at 20°C. In the experiment shown in Figure 7, animals were heat-shocked at 33°C for 2 hr

at 12-hr intervals. We examined Rol animals for signs of masculinization when they reached adulthood. In most experiments, masculinization of various tissues was assessed by differential interference contrast microscopy at a magnification of 400×. Sex muscles were scored by polarized light microscopy. The following features were taken as indicative of masculinization: an asymmetric, unilobed gonad, absence of a vulva or presence of a nonfunctional pseudovulva, presence of a tail fan, rays, or spicules, presence of sex muscles in the tail region. In some experiments, as noted, animals were scored as either female or masculinized intersexes following examination in a dissecting microscope at a magnification of 50X.

HS-MycFEM-3 in *fem-3* XX animals

Roller males of genotype *unc-24 fem-3/dpy-20; idIs4* were crossed to *unc-24 fem-3 dpy-20* females. The Unc Rol progeny (XX animals of genotype *unc-24 fem-3 dpy-20/unc-24 fem-3; idIs4/+*) were scored for masculinization. In this cross and the following one, Unc recombinants heterozygous for *fem-3* could be excluded because they were also Dpy.

HS-MycFEM-3 in *fem-3* XX and XO animals

Males of genotype *unc-24 fem-3/dpy-20* were crossed to *unc-24 fem-3 dpy-20; idIs4* females. Unc Rol progeny (*unc-24 fem-3 dpy-20/unc-24 fem-3; idIs4/+* XX and *unc-24 fem-3 dpy-20/unc-24 fem-3; idIs4/O* XO) were scored for masculinization.

HS-MycFEM-3 in *fem-1* XX and XO animals

Males of genotype *dpy-13/fem-1 unc-24; idIs4* were crossed to *fem-1 unc-24; idIs4* females. All progeny were scored for somatic sexual phenotype with the dissecting microscope.

HS-MycFEM-3 in *fem-2* XX and XO animals

Males of genotype *him-5/+; idIs4* were crossed to *fem-2; him-5* females. F₁ Rol animals were picked to separate plates, and *him-5* homozygotes were identified as those that segregated male F₂ self-progeny. Several *him-5* Rol hermaphrodite F₂ animals were picked as L4 larvae to separate plates and were transferred to new plates daily. The first plate was incubated at 15°C, and subsequent plates were incubated at 25°C and subjected to heat shock treatment as described above. Among the F₂ progeny that were homozygous for *idIs4*, those also homozygous for *fem-2* were identified as those that produced intersexual XO F₃ progeny at 15°C. Their heat shock-treated progeny were scored for masculinization.

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