The *Caenorhabditis elegans* F-box protein SEL-10 promotes female development and may target FEM-1 and FEM-3 for degradation by the proteasome

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The *Caenorhabditis elegans* F-box protein SEL-10 and its human homolog have been proposed to regulate LIN-12 Notch signaling by targeting for ubiquitin-mediated proteasomal degradation LIN-12 Notch proteins and SEL-12 PS1 presenilins, the latter of which have been implicated in Alzheimer's disease. We found that *sel-10* is the same gene as *egl-41*, which previously had been defined by gain-of-function mutations that semidominantly cause masculinization of the hermaphrodite soma. Our results demonstrate that mutations causing loss-of-function of *sel-10* also have masculinizing activity, indicating that *sel-10* functions to promote female development. Genetically, *sel-10* acts upstream of the genes *fem-1*, *fem-2*, and *fem-3* and downstream of *her-1* and probably *tra-2*. When expressed in mammalian cells, SEL-10 protein coimmunoprecipitates with FEM-1, FEM-2, and FEM-3, which are required for masculinization, and FEM-1 and FEM-3 are targeted by SEL-10 for proteasomal degradation. We propose that SEL-10-mediated proteolysis of FEM-1 and FEM-3 is required for normal hermaphrodite development.

Caenorhabditis elegans develops either as a self-fertilizing XX hermaphrodite or as an X0 male (1). The X-to-autosome (X/A) ratio provides the primary sex-determining signal and specifies the activity of *her* (*her*maphrodization)-*1*. Downstream of *her-1*, five genes [*tra* (*tra*nsformer)-*2*, *tra-3*, *fem* (*fem*inization)-*1*, *fem-2*, and *fem-3*] control the activity of *tra-1*, the terminal, global regulator of somatic sexual fate. In XX animals, the *her-1* gene, which encodes a secreted protein, is not expressed (2). The lack of *her-1* expression in XX animals permits the activation of the transmembrane protein TRA-2, which blocks the functions of FEM-1 (a novel protein) (3), FEM-2 (a type 2C protein phosphatase) (4, 5), and FEM-3 (an ankyrin-repeat protein) (6), possibly by interacting directly with FEM-3 (7). This block leads to the activation of the Zn-finger DNA-binding protein TRA-1 (8). Active TRA-1 represses the transcription of genes required for male development, resulting in the formation of an animal with a female soma: a hermaphrodite (9, 10). In X0 animals, the HER-1 protein is present and inhibits TRA-2 (11, 12). The FEM proteins are, thus, relieved from negative regulation by TRA-2, resulting in the FEM-dependent inhibition of TRA-1 and subsequent male development.

The gene *egl* (*egg-l*aying-defective)-*41* was defined by three semidominantly acting mutations, *n1069*, *n1074*, and *n1077*, which were identified in a screen for egg-laying-defective (Egl) hermaphrodites (13). Additional *egl-41* alleles were identified in screens for mutations that suppress a semidominantly acting *tra-2* mutation (*e2055*) (14), which cause the male-specific cephalic companion neurons (CEMs) to survive in hermaphrodites (*n3717*; H.T.S. and H.R.H., unpublished data) or that cause abnormalities in the sex-specific pattern of cell deaths in the ventral cord (*n3854*, *n4041*, and *n4046*; B. Galvin and H.R.H., unpublished results). *egl-41* hermaphrodites are weakly masculinized; for example, in *egl-41* hermaphrodites, the hermaphrodite-specific neurons (HSNs) die (the HSNs normally die by programmed cell death in males and survive in hermaphrodites, in which they are required for egg laying) and the CEM neurons, which normally die in hermaphrodites, survive (13). All characterized *egl-41* alleles cause a semidominant phenotype. Semidominant phenotypes often are consequences of gain-of-function (gf) mutations that cause altered gene function. For this reason, previous studies could not establish whether *egl-41* normally acts in the sex-determination pathway. In this article, we describe the molecular characterization of the *egl-41* gene and the phenotype caused by the loss of *egl-41* function. Our results indicate that *egl-41* is the same gene as the previously characterized gene *sel* (*s*uppressor/*e*nhancer of *l*in-12)-*10* and that *sel-10* normally functions in sex determination.

Materials and Methods

General Methods and Strains. *C. elegans* strains were maintained at 20°C, unless otherwise noted. The strain N2 (Bristol) was the standard wild-type strain. For single-nucleotide polymorphism (SNP) mapping, the wild-type Hawaiian strain CB4856 was also used. The alleles, deficiencies, and duplications that were used in this study are as follows and are described by Riddle *et al.* (15), except where noted otherwise: LGI, *him-1*(*e879*), *nIs133*(*pkd-2*::*gfp*) (ref. 16 and H.T.S. and H.R.H, unpublished data); LGII, *tra-2*(*e1875*, *e2019*, *e2021*, *e2531*, and *n1106*); LGIII, *fem-2*(*b245* and *e2105*) and *lin-12*(*n302*, *n676*, and *n930*); LGIV, *fem-1*(*hc17* and *e1965*), *fem-3*(*e2006* and *e1996*), *him-8*(*e1489*), and *ced-3*(*n717*); LGV, *dpy-11*(*e224*), *her-1*(*e1561*, *n695*, and *hv1 y101*), *unc-42*(*e270*), *lon-3*(*e2175*), *rol-4*(*sc8*), *sel-10*(*ar41*, *n1069*, *n1074*, *n1077*, and *e2055*), *sel-10*(*bc189 n1077*, *bc243*, and *n4273*) (this study), *sel-10*(*n3717*) (H.T.S. and H.R.H., unpublished data), *sel-10*(*n3854*, *n4041*, and *n4046*) (B. Galvin and H.R.H. unpublished data), *him-5*(*e1490*), *unc-76*(*e911*), and *dpy-21*(*e428*); and LGX, *sel-12*(*ar131*) and *sdc-1*(*n485*). *nDf42* is a deficiency spanning the *sel-10* locus (17). *ctDp8*(*V*;*f*) is a free duplication spanning the *sel-10* locus (18).

Mapping of *egl-41*/*sel-10*. sel-10 gf alleles have been mapped between *sqt-3* and *him-5* on LGV (13). The location of *n3717*gf was refined by using SNP mapping and the following SNPs: *pkP5069*, *pkP5070*, *pkP5086*, *pkP5088*, F55B12 9,811, and R10D12 16,645 (19). To obtain recombinants for LGV between N2 and CB4856, the strains *nIs133*; *rol-4*(*sc8*) *sel-10*(*n3717*) *unc-76*(*e911*) or *rol-4*(*sc8*) *sel-10*(*n1077*gf) *unc-76*(*e911*) were

Abbreviations: Egl, egg-laying-defective; CEM, cephalic companion neuron; HSN, hermaphrodite-specific neuron; gf, gain-of-function; SNP, single-nucleotide polymorphism; lf, loss-of-function; shRNA, short-hairpin RNA; h*sel-10*, human *sel-10*; SCF, Skp1–Cullin–F-box; SEL-10Myc, Myc-tagged SEL-10.

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crossed with CB4856. Recombinants were analyzed for the presence of *n3717*gf or *n1077*gf by scoring for the presence of CEMs and for an Egl phenotype as described below, and SNPs were genotyped by performing PCR and subsequent restriction digests (19).

Isolation of *sel-10* Deletion Mutants. Genomic DNA pools from mutagenized animals were screened for deletions as described (20). Deletion mutant animals were identified by nested PCR, isolated from frozen stocks, and outcrossed at least three times.

Microscopic Analyses of Mutant and Transgenic Animals. The Egl phenotype of *sel-10* gf animals and the presence of HSNs were analyzed as described (21). To score for the presence of CEMs, we anesthetized L4 larvae with 50 mM sodium azide and examined all four CEM positions by using Nomarski microscopy (22). In SNP mapping and epistasis analysis with *sel-10*(*n3717* gf), we scored the presence of CEMs by using the *pkd-2*::*gfp* reporter *nIs133*. Hermaphrodite fertility was tested by picking individual L4 hermaphrodites and, 72 h later, analyzing whether progeny had been generated. Brood sizes were determined by picking individual L4 hermaphrodites, transferring them to fresh plates daily for 4 days, and counting all generated progeny. We scored as males both animals that appeared fully male-like (most of which were presumably pseudomales, defined as XX animals that were essentially completely masculinized) and intersexes with severely masculinized tails, as determined by using a dissecting microscope (23).

Molecular Analysis. pBC262 contains a 6.9-kb *Xba*I fragment of cosmid F55B12 (from base pairs 7,986 to 14,853; all references to F55B12 sequence refer to GenBank accession no. Z79757) ligated into Bluescript KS(+). The sequences of mutant alleles of *sel-10* were determined from PCR-amplified genomic DNA. The plasmids pQNClacZ, pQNCsel-10myc, and pQNCsel-10HA (17, 24) were used for transient transfections. *fem-1*, *fem-2*, and *fem-3* cDNAs were amplified from plasmids AS1000, AS1245, and AS1197 (6) to introduce a Flag-tag or Myc-tag. The *tra-2* fragment encoding TRA-2C (25), was amplified from the plasmid pPK148. The PCR products were cloned into the expression vector pcDNA.3 (Invitrogen). For construction of a plasmid driving the expression of human *sel-10* (h*sel-10*) short-hairpin RNA (shRNA), we used appropriate oligonucleotides that were annealed and ligated into the vector pSHAG-1 (26).

Transgenic Animals. Germline transformation was performed as described (27). Cosmid DNA (5–8.5 μg/ml each) was injected into *sel-10*(*n1077*gf) *unc-76*(*e911*) animals with the *unc-76* rescuing construct p76-16B (50 μg/ml) (28).

Transfections, Immunoprecipitations, and Western Blot Analysis. For coimmunoprecipitation experiments, U2OS cells were grown to 50% confluency in DMEM supplemented with 10% FBS and transfected by using FuGENE 6 (Roche). We added a *LacZ*-containing plasmid (pQNClacz) to keep the total amount of DNA constant. At 24 h after transfection, cells were lysed in Flag lysis buffer (50 mM Tris·HCl, pH 7.8/137 mM NaCl/10 mM NaF/1 mM EDTA/10% glycerin/1% Triton X-100/0.2% sarkosyl) and 1× complete protease inhibitor mixture (Roche). Cell lysates were incubated with anti-Flag M2 affinity gel (Sigma) or anti-Myc agarose (Santa Cruz Biotechnology) for 2 h at 4°C. The beads were washed three times with Flag lysis buffer and boiled in sample buffer. Precipitated proteins were analyzed by using anti-Flag M2 antibodies (Sigma) and polyclonal anti-Myc antibodies (Santa Cruz Biotechnology). For detection of protein steady-state levels, the expression plasmids were transfected into BOSC cells. A plasmid pSHAG-Ff1 expressing firefly luciferase shRNA (26) was used as a negative control (control shRNA). Cell cultures were treated for 8 h with the proteasome inhibitor lactacystin (5 μM; Sigma). The FEM proteins were detected with anti-Flag M2 antibodies.

Results

The *egl-41* Mutation *n1077* Causes Altered *egl-41* Activity That Is Antagonized by Wild-Type *egl-41* Activity. *egl-41*(*n1077*) semidominantly causes a cold-sensitive Egl phenotype (see Table 8, which is published as supporting information on the PNAS web site) (13). *egl-41* is not haploinsufficient for feminization because *nDf42*/+ hermaphrodites (*nDf42* is a deficiency that deletes the *egl-41* locus) (14) were not Egl (see Table 9, which is published as supporting information on the PNAS web site). The semidominant *egl-41* phenotype is not likely to be caused by an increase in wild-type *egl-41* activity. Hermaphrodites carrying the duplication *ctDp8*, which spans the *egl-41* locus (+/+; *ctDp8*) (18), were non-Egl (Table 9), and 54% of *n1077*/+ hermaphrodites but only 24% of *n1077*/+/+ hermaphrodites (*n1077*/+; *ctDp8*) were Egl (Table 9), which also indicates that the semidominant activity of *egl-41*(*n1077*) can be antagonized by wild-type activity. However, *n1077* homozygotes had a more penetrant Egl phenotype than *n1077*/*nDf42* heterozygotes (100% and 26% penetrant for Egl, respectively; Tables 8 and 10, which are published as supporting information on the PNAS web site), which indicates that *n1077* does not simply antagonize wild-type *egl-41* activity and must cause altered gene function. Therefore, we refer to the eight semidominantly acting *egl-41* alleles as gf mutations.

All Eight Independently Isolated *egl-41* (gf) Mutants Carry an Identical Mutation in the *sel-10* ORF. We mapped *egl-41*(*n3717*gf) to a 130-kb interval on linkage group V and found that the Egl phenotype of and masculinization caused by *egl-41*(*n1077*gf) could be suppressed by a 6.9-kb fragment of cosmid F55B12 (base pairs 7,986–14,853) (see Fig. 4*A*, which is published as supporting information on the PNAS web site). This fragment contains the previously characterized gene *sel-10* and the 5′ region of *F55B12.4*, a gene encoding a poly(A) polymerase-like protein (Fig. 4*B*).

sel-10, which encodes a 587-aa F-box protein, was previously defined by the loss-of-function (lf) mutations *ar28* and *ar41* (17, 29). *sel-10* is a negative regulator of *lin* (*lin*eage abnormal)-*12* , which encodes a Notch-like receptor. The SEL-10 protein can interact with the intracellular domain of the LIN-12 protein in mammalian cells (17), and mammalian SEL-10 interacts with the intracellular domain of mammalian Notch, N^{IC}, targeting it for ubiquitin-mediated degradation (24, 30, 31). SEL-10 also appears to be a negative regulator of the presenilin SEL-12, and mammalian SEL-10 targets the presenilin PS1, which has been implicated in Alzheimer's disease, for degradation (32–34). SEL-10 contains eight WD40 repeats, which are located in the C-terminal half of the protein (17, 35). *ar41* and *ar28* are nonsense mutations that truncate SEL-10 in WD40 repeats II and VII, respectively (17). We found that all eight *egl-41*(gf) mutants have an identical mutation leading to a glycine-to-glutamic acid substitution at position 567 in WD repeat VIII (Fig. 4*C*).

***egl-41* and *sel-10* Are the Same Gene.** To identify dominant suppressors of the Egl phenotype of *n1077*gf animals, we mutagenized homozygous *n1077*gf hermaphrodites and screened the F1 self-progeny for rare, non-Egl hermaphrodites. From the 20,000 mutagenized haploid genomes that were screened, we recovered one mutation, *bc189*, that semidominantly suppressed the Egl phenotype and the masculinization caused by *egl-41*(*n1077*gf) (Tables 10–12, which are published as supporting information on the PNAS web site). *bc189* is tightly linked to *egl-41* (data not shown) and is an lf allele of *sel-10*: (*i*) like *sel-10*(*ar41*), *bc189* is a modifier of *lin-12* and a suppressor of *sel-12* (*ar131*) (Tables 13–15, which are published as supporting information on the PNAS web site); (*ii*) *bc189*

Table 1. *sel-10* lf mutations enhance the ability of various *tra* mutations to masculinize hermaphrodites

Genotype	% Tra animals (*n*)			
	+/+	*sel-10*(*ar41*)	*sel-10*(*bc243*)	*sel-10*(*n4273*)
+/+	0 (Many)	0 (Many)	0 (Many)	0 (Many)
sdc-1(*n485*)	10 (223)	52 (105)	76 (82)	73 (70)
her-1(*n695*gf)	28 (113)	89 (155)	ND —	ND —
tra-2(*n1106*)	8 (266)	32 (117)	25 (101)	29 (120)
tra-2(*e1875*)	1 (257)	3 (152)	3 (96)	8 (101)

The Tra phenotype was scored as described in *Materials and Methods.* The complete genotypes of the analyzed animals were as listed, except that all strains containing *her-1*(*e695*) were homozygous for *dpy-11*(*e224*) and all strains containing *sel-10*(*ar41*) were homozygous for *lon-3*(*e2175*). ND, not determined.

failed to complement *sel-10*(*ar41*) for suppression of *sel-12*(*ar131*) (Table 15); and (*iii*) *bc189* animals have a missense mutation in *sel-10*, leading to an aspartic acid-to-asparagine substitution at position 482 in WD40 repeat VI (Fig. 4*C*). We used a cis–trans test to determine whether *sel-10*(*bc189*) is in the same gene as *egl-41*(*n1077*gf). Specifically, we used *bc189* as a *sel-10* (lf) mutation in cis to *egl-41*(*n1077*gf) (genotype *bc189 n1077*/+) and compared *bc189 n1077*/+ animals with animals carrying the *sel-10* (lf) mutation *ar41* in trans to *egl-41*(*n1077*gf) (genotype *n1077*/*ar41*) (Table 12). *sel-10* (lf) in cis to *n1077*gf, but not in trans to *n1077*gf, suppressed the Egl phenotype of *n1077*, indicating that the mutations affect the same gene. Henceforth, we refer to *egl-41* as *sel-10*.

***sel-10*(*n1077*gf) Shares Selected Characteristics with sel-10 (lf) Mutations.** *sel-10*(*n1077*gf) behaved similarly to the *sel-10* (lf) mutations *ar41* and *bc189 n1077* in elevating *lin-12* function: it suppressed the two-anchor-cell defect caused by the weak *lin-12* lf allele *lin-12*(*n676 n930*) (29) and enhanced the Muv (*mu*lti-*v*ulva) phenotype caused by the weak *lin-12* gf allele *lin-12*(*n302*) (17) (Tables 13 and 14). Unlike *sel-10* (lf), *sel-10*(*n1077*gf) did not suppress the *Sel-12*-Egl phenotype caused by *sel-12*(*ar131*) (29, 32, 36) (Table 15). These findings suggest that the *sel-10* (gf) mutation affects a *sel-10* function that is involved in the regulation of LIN-12 but not of SEL-12.

The *sel-10* Null Phenotype Is a Weak Masculinization of Hermaphrodites. We isolated two deletion mutations in the *sel-10* gene, *bc243* and *n4273*, which delete 851 bp (10,103–10,953 of F55B12) and 956 bp (10,323–11,278 of F55B12) and are predicted to truncate SEL-10 after amino acids 85 and 106, respectively (Fig. 4 *B* and *C*). The resulting proteins should lack the F-box and all eight WD40 repeats. *bc243* and *n4273* most likely are null alleles of *sel-10*. Like *sel-10*(*ar41*) and *sel-10*(*bc189 n1077*) animals, *bc243* and *n4273* hermaphrodites appear grossly wild-type. We found that *bc243* and *n4273* suppressed *lin-12*(*n676 n930*) and *sel-12*(*ar131*) and enhanced *lin-12*(*n302*gf) to a degree similar to that seen with *sel-10*(*ar41*) (Tables 13–15 and data not shown). Thus, as proposed in ref. 17, *ar41* represents a null allele.

Table 2. *sel-10* lf mutations enhance the ability of various *tra* mutations to cause the HSNs to undergo programmed cell death

Genotype	% HSNs missing in hermaphrodites (*n*)			
	+/+	*sel-10*(*ar41*)	*sel-10*(*bc243*)	*sel-10*(*n4273*)
+/+	0 (Many)	2 (60)	7 (60)	9 (60)
sdc-1(*n485*)	34 (110)	76 (50)	78 (60)	77 (60)
her-1(*n695*gf)	90 (50)	92 (50)	ND —	ND —
tra-2(*n1106*)	85 (110)	86 (50)	87 (60)	83 (60)
tra-2(*e1875*)	32 (220)	81 (110)	60 (60)	60 (60)

The presence of HSNs was scored as described in *Materials and Methods.* The genotypes of the animals were as described for Table 1. ND, not determined.

Table 3. *sel-10* lf mutations enhance the ability of various *tra* mutations to cause CEMs survival

Genotype	% CEMs present in hermaphrodites (*n*)			
	+/+	*sel-10*(*ar41*)	*sel-10*(*bc243*)	*sel-10*(*n4273*)
+/+	0 (Many)	2 (168)	4 (160)	7 (152)
sdc-1(*n485*)	21 (376)	46 (80)	35 (80)	39 (80)
her-1(*n695*gf)	80 (80)	85 (80)	ND —	ND —
tra-2(*n1106*)	84 (160)	91 (80)	84 (80)	83 (80)
tra-2(*e1875*)	44 (156)	65 (80)	69 (80)	68 (80)

The presence of CEMs was scored as described in *Materials and Methods.* The genotypes of the animals were as described for Table 1. ND, not determined.

sel-10(*n1077*gf) enhances the Tra phenotype caused by weak lf mutations of *tra-2* (13). Therefore, we tested whether null alleles of *sel-10* could modify the Tra phenotypes caused by a gf mutation of *her-1* or by weak lf mutations of *sdc* (*s*ex *d*etermination and dosage *c*ompensation)-*1* (*sdc-1* negatively regulates *her-1*) or *tra-2.* By several criteria, we found that *sel-10* (lf) enhanced their Tra phenotypes (Tables 1–3). In addition, hermaphrodites homozygous for any of the three *sel-10* null mutations exhibited defects indicative of weak masculinization, including the absence of HSNs and the presence of CEMs (Tables 2 and 3), albeit to a far lesser degree than seen for *sel-10*(*n1077*gf) animals (Table 11). Thus, the *sel-10* null phenotype with respect to sex determination is a weak masculinization of hermaphrodites. We conclude that *sel-10* promotes hermaphrodite development.

***sel-10* Acts Upstream of *fem-1*, *fem-2*, and *fem-3* and Downstream of *her-1* and Possibly *tra-2*.** To place *sel-10* function within the sex-determination pathway, we examined the interactions of *sel-10* null mutations with lf mutations in *her-1*, *fem-1*, *fem-2*, and *fem-3*. To ensure detection of the weak masculinizing effects of *sel-10* (lf), we used temperature-sensitive, partial lf mutations of *her-1*, *fem-1*, *fem-2* and *fem-3* under sensitized conditions that cause a partial feminization of X0 animals. *sel-10* (lf) could masculinize X0 animals feminized by *her-1*(*e1561*) but not X0 animals feminized by *fem-1*(*hc17*), *fem-2*(*b245*), or *fem-3*(*e2006*) (Tables 4 and 5). These

Table 4. *sel-10(ar41)* partially suppresses the feminization of X0 animals caused by *her-1(e1561*lf*)*

Genotype	% Males (*n*)	
	15°C	24.5°C
him-8(*e1489*)	30 (209)	34 (273)
him-8(*e1489*); *her-1*(*e1561*)	36 (270)	12 (217)
him-8(*e1489*); *sel-10*(*ar41*)	38 (252)	32 (93)
him-8(*e1489*); *her-1*(*e1561*) *sel-10*(*ar41*)	34 (291)	30 (205)
him-8(*e1489*); *sel-10*(*bc243*)	40 (181)	42 (186)
him-8(*e1489*); *her-1*(*e1561*) *sel-10*(*bc243*)	41 (207)	30 (186)
him-8(*e1489*); *sel-10*(*n4273*)	37 (194)	35 (221)
him-8(*e1489*); *her-1*(*e1561*) *sel-10*(*n4273*)	36 (114)	24 (256)

"Males" were identified based on the criteria described in *Materials and Methods.* The complete genotypes of the animals analyzed were as listed, except for the second through fourth strains, as listed from top to bottom, which were as follows: *him-8*(*e1489*); *her-1*(*e1561*) *unc-76*(*e911*), *him-8*(*e1489*); *lon-3*(*e2175*) *sel-10*(*ar41*), and *him-8*(*e1489*); *her-1*(*e1561*) *lon-3*(*e2175*) *sel-10*(*ar41*) *unc-76*(*e911*).

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Table 5. *sel-10(ar41)* fails to suppress the feminization of X0 animals caused by lf mutations in *fem-1, fem-2,* and *fem-3*

Genotype	% CEMs in X0 (*n*)	
	+/+	*sel-10*(*ar41*)
+/+*	91 (80)	91 (100)
fem-1(*hc17*)*	51 (100)	53 (112)
fem-2(*b245*)*	73 (100)	75 (220)
+/+†	91 (80)	89 (156)
fem-3(*e2006*)†	55 (176)	55 (92)

The presence of CEMs in X0 animals was scored as described in *Materials and Methods.* The complete genotypes of the animals analyzed were as listed save that all strains contain *him-1*(*e879*) and all strains containing *sel-10*(*ar41*) are homozygous for *lon-3*(*e2175*).

*Animals were cultured at 25°C until reaching the second larval stage, and then the temperature was shifted to 16°C.

†Animals were cultured at 20°C.

results suggest that *sel-10* functions downstream of or in parallel to *her-1* and upstream of or in parallel to *fem-1*, *fem-2*, and *fem-3*. Furthermore, *sel-10* (lf) partially suppressed the Fem phenotypes caused by the dominantly acting "enhanced gf" mutation *e2531* (11) and the "mixed character" mutations *e2019* and *e2021* (37) of *tra-2* (Tables 6 and 7). These findings suggest that *sel-10* acts downstream of or in parallel to *tra-2*. Results similar to those obtained with *sel-10* (lf) were obtained for the stronger masculinizing effect of the *sel-10* (gf) mutation: it has been reported that the Egl phenotype of *sel-10*(*e2055*gf) hermaphrodites is suppressed by a null mutation in *fem-1* (14), and we found that CEM survival caused by *sel-10*(*n3717*gf) was suppressed by null mutations in any of the three *fem* genes but was not suppressed by a null mutation in *her-1* (data not shown).

SEL-10 Interacts Physically with the FEM Proteins. F-box proteins, which were first described as exchangeable subunits of the Skp1–Cullin–F-box (SCF) E3 ubiquitin-protein ligase complex, interact with the Skp1 subunit of the complex via their F-box domains (38, 39). Many F-box proteins contain protein–protein interaction domains, such as leucine-rich domains or WD40 repeats that recruit protein substrates for ubiquitination (38, 39).

Our epistasis studies suggest that the *fem* genes are negatively regulated by *sel-10*. Therefore, we tested whether the FEM proteins interact with SEL-10 by performing coimmunoprecipitation experiments using U2OS human osteosarcoma cells transiently transfected to express Flag-tagged FEM-1, FEM-2, or FEM-3; Myc-tagged SEL-10 (SEL-10Myc); or both a Flag-tagged FEM protein and SEL-10Myc (Fig. 1). We immunoprecipitated the Flag-tagged proteins and detected SEL-10Myc only in the precipitates from lysates expressing both SEL-10Myc and any Flag-tagged FEM protein. Similarly, the immunoprecipitation of SEL-10Myc resulted in the detection of Flag-tagged FEM proteins only in the precipitates of cell lysates expressing both SEL-10Myc and any Flag-tagged FEM protein (Fig. 1). Flag-tagged TRA-2C did not precipitate SEL-10Myc (Fig. 1). Thus, when expressed in mammalian cells, SEL-10 can physically interact with each of the three *C. elegans* FEM proteins either directly or through other proteins.

Table 6. *sel-10(ar41)* partially suppresses the feminization of X0 animals caused by *tra-2(e2531*eg)/+

Genotype	% CEMs present in X0 (*n*)
sel-10(*ar41*)	91 (100)
tra-2(*e2531*eg)/+	15 (120)
tra-2(*e2531*eg)/+; *sel-10*(*ar41*)	40 (172)

The presence of CEMs in X0 animals was scored as described in *Materials and Methods.* The complete genotypes of the analyzed animals were, from top to bottom, as follows: *him-1*(*e879*); *lon-3*(*e2175*) *sel-10*(*ar41*), *tra-2*(*e2531*)/+, *tra-2*(*e2531*)/+; *lon-3*(*e2175*) *sel-10*(*ar41*). eg, Enhanced gf.

Table 7. *sel-10(ar41)* partially suppresses the germline feminization in XX animals caused by *tra-2* (mx) mutations

Genotype	% Fertile animals (*n*)	Average no. of progeny	Range	*n*
sel-10(*ar41*)	100 (58)	277	243–328	6
tra-2(*e2019*mx)	10 (102)	70	18–104	6
tra-2(*e2019*mx); *sel-10*(*ar41*)	22 (102)	127	20–180	6
tra-2(*e2021*mx)	13 (101)	60	7–108	7
tra-2(*e2021*mx); *sel-10*(*ar41*)	46 (101)	129	34–210	6

The number of fertile animals and the number of progeny were analyzed as described in *Materials and Methods.* The complete genotypes of the animals analyzed were as listed except that all strains containing *sel-10*(*ar41*) were homozygous for *lon-3*(*e2175*). mx, Mixed character.

The Levels of FEM-1 and FEM-3 Are Regulated by SEL-10 and the Proteasome. The ability of SEL-10 to interact with the FEM proteins suggested that SEL-10 might target the FEM proteins for proteasomal destruction. The coexpression of SEL-10Myc and Flag-tagged FEM-1 in BOSC human embryonic kidney cells did not result in decreased FEM-1 protein levels (data not shown). However, FEM-1 protein levels were increased in the presence of

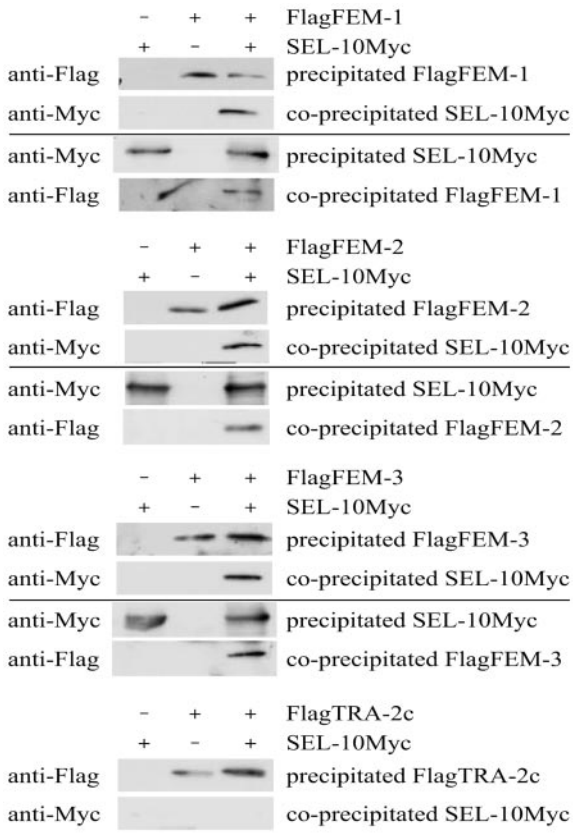

Fig. 1. The FEM proteins interact with SEL-10 in mammalian cells. Extracts from mammalian U2OS cells expressing SEL-10Myc; Flag-tagged FEM-1, -2, or -3; Flag-tagged TRA-2C; or both SEL-10Myc and the indicated Flag-tagged protein were immunoprecipitated by anti-Flag M2 or anti-Myc antibodies. The precipitated proteins were analyzed for the presence of SEL-10Myc with anti-Myc antibodies and the Flag-tagged proteins with anti-Flag M2 antibodies.

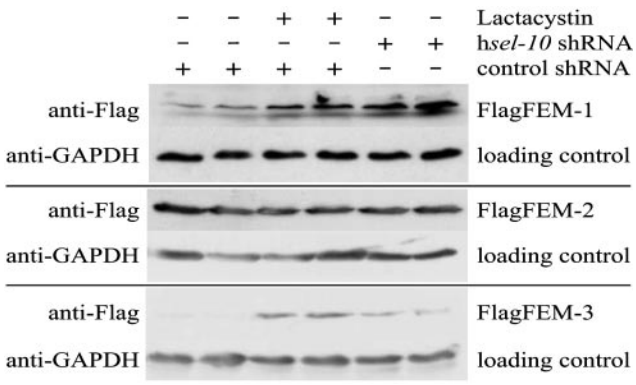

Fig. 2. FEM-1 and FEM-3 may be targeted by hSEL-10 for degradation by the proteasome. To analyze protein steady-state levels, we treated BOSC cells expressing Flag-tagged FEM-1, -2 or -3, respectively, with lactacystin to inhibit the proteasome or with h*sel-10* shRNA to partially inactivate h*sel-10*. The untreated and lactacystin-treated cells were cotransfected with a plasmid expressing control shRNA (firefly luciferase). Whole-cell lysates were analyzed by using anti-Flag M2 antibodies. Representative data from three independent experiments are shown.

lactacystin, a proteasome inhibitor (Fig. 2). We postulated that transfected FEM-1 might be targeted by hSEL-10 (FBW7), which is 46% identical to *C. elegans* SEL-10. To reduce the amount of endogenous hSEL-10, we generated specific shRNA (26) against the h*sel-10* gene. We transiently transfected BOSC cells to express Flag-tagged FEM-1 and either control firefly luciferase shRNA or h*sel-10* shRNA. When compared with control cells, the steady-state level of FEM-1 was increased in the h*sel-10* shRNA cells to a level similar to the level of FEM-1 found in cells treated with lactacystin (Fig. 2). In analogous experiments, lactacystin and h*sel-10* shRNA increased the protein level of FEM-3 but did not affect the protein level of FEM-2 (Fig. 2). Together, these results indicate that the steady-state levels of transfected FEM-1 and FEM-3 in BOSC cells depend on the presence of hSEL-10 and a functional proteasome.

Discussion

Our genetic analysis indicates that *egl-41* mutations cause masculinization as a result of altered function of *sel-10* and further demonstrates that *sel-10* wild-type function is required for normal hermaphrodite development. That null mutations of *sel-10* cause a weak phenotype might be explained by the fact that the genome of *C. elegans* is predicted to encode at least 326 F-box proteins (40). Hence, *sel-10* might be functionally redundant with other, similar proteins. Alternately, the sex determination processes in which *sel-10* is involved, for example the degradation of FEM-1 and FEM-3, might be redundant (i.e., pathways other than a proteasome-dependent pathway might negatively regulate the activities of the *fem* genes).

The *sel-10* (gf) mutation results in the alteration of a conserved residue in WD40 repeat VIII. We propose that rather than decreasing binding to substrate or the SCF complex, this mutation might result in the formation of stable but nonfunctional $SCF^{SEL\text{-}10(gf)}$ complexes. By causing the formation of such complexes, SEL-10 (gf) protein could prevent wild-type SEL-10 protein as well as additional functionally redundant F-box proteins from entering SCF complexes and from mediating the ubiquitination and degradation of their substrates. This model could explain why different processes are affected to differing degrees by the *sel-10* (gf) mutation and the *sel-10* (lf) mutations. SEL-10 might be the sole or principal F-box protein responsible for regulating *lin-12* activity, which is affected similarly by *sel-10* gf and lf mutations. By contrast, in sex determination, F-box

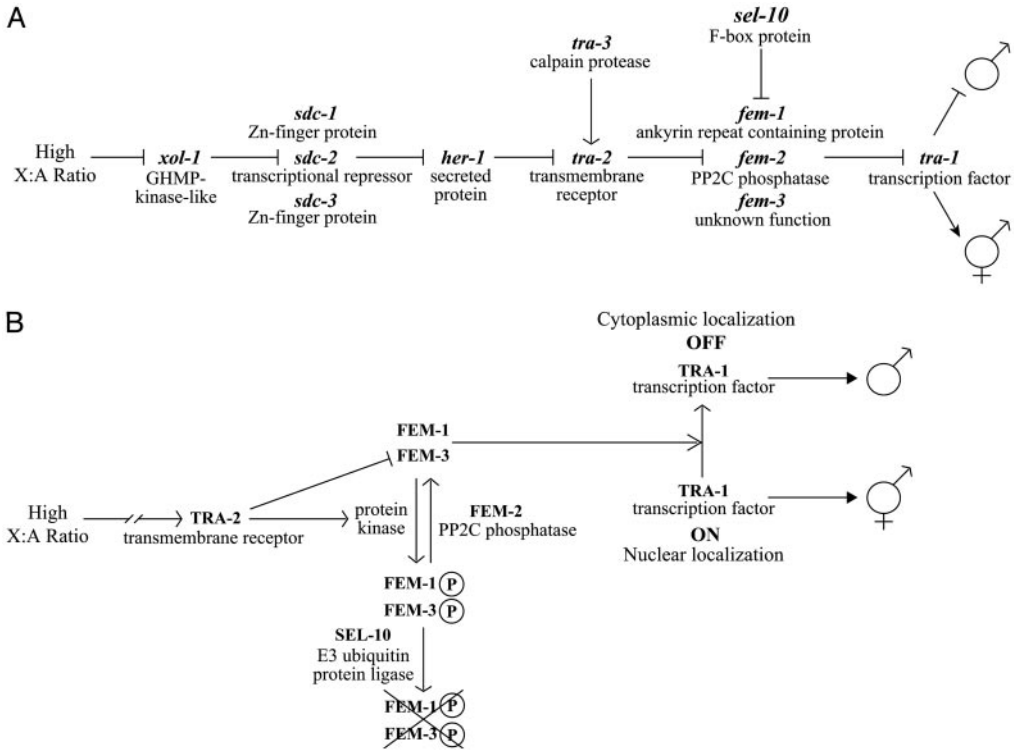

Fig. 3. Genetic and molecular pathways of somatic sex determination in *C. elegans.* (*A*) A simplified genetic pathway for sex determination in the *C. elegans* soma is shown. *sel-10* is a new gene in this pathway and acts as a negative regulator of the *fem* genes. (*B*) A model for the molecular interactions among SEL-10, the FEM proteins, TRA-1, and TRA-2. SEL-10 negatively regulates FEM-1 and FEM-3 by promoting the degradation of their phosphorylated forms. A negative arrow from TRA-2 to FEM-3 reflects the possibility that TRA-2 directly binds and inhibits FEM-3 (7). See text for details.

proteins in addition to SEL-10 might mediate the degradation of FEM-1 and FEM-3. In *sel-10* (lf) animals, these redundant F-box proteins could largely substitute for SEL-10 function in FEM-1 and FEM-3 degradation, resulting in a weak defect in sex determination; in *sel-10* (gf) animals, nonfunctional $SCF^{SEL\text{-}10(gf)}$ complexes would prevent redundant F-box proteins from substituting for SEL-10 function, leading to a stronger defect.

The finding that *sel-12* (lf) is not suppressed by the *sel-10* (gf) mutation indicates that $SCF^{SEL\text{-}10(gf)}$ complexes might still be functional with respect to *sel-12* function. The interaction between SEL-10 and SEL-12, therefore, might differ from other SEL-10-substrate interactions, a difference that may be evolutionarily conserved in the interaction of the homologous proteins hSEL-10 and PS1 in Alzheimer's disease (32–34).

Genetically, *sel-10* wild-type function is likely to act downstream of or in parallel to *tra-2* as a negative regulator of *fem-1*, *fem-2*, and *fem-3* (Fig. 3*A*). When expressed in mammalian cells, SEL-10 interacted with FEM-1, FEM-2, and FEM-3, and hSEL-10 mediated the degradation of FEM-1 and FEM-3 by the proteasome. We propose that *sel-10* promotes female development by down-regulating *fem-1* and *fem-3* activities, which are required for male development. It has been proposed that *fem-1* and *fem-3* are regulated posttranscriptionally (3, 41–43). In the germline of XX animals *fem-3* activity is down-regulated at the level of translation (44). Mutations that disrupt this regulation masculinize the XX germline but do not detectably affect the sexual fate of the XX soma (45). Thus, a different or an additional mechanism must be invoked in the soma. Our data suggest that, in the soma, *fem-1* and *fem-3* activities are regulated at least in part at the level of protein stability by means of a SEL-10-mediated process.

The direct or indirect target of the FEM proteins is the transcription factor TRA-1. One mechanism that controls TRA-1 activity seems to be the regulation of TRA-1 localization. TRA-1 is preferentially exported from the nucleus in males or masculinized XX animals, a process that requires a functional *fem-1* gene (46). Therefore, it has been proposed that the FEM proteins might act to promote the export of TRA-1 from the nucleus (47). Mammalian SEL-10 has been shown to localize to and function in the nucleus (30, 31). It is possible that in XX animals SEL-10 binds to nuclearly localized FEM-1 and FEM-3 proteins and mediates their degradation, thereby preventing FEM protein-mediated export of TRA-1 and allowing TRA-1 to remain inside the nucleus and promote female development. A model in which SEL-10 mediates the degradation specifically of nuclearly localized FEM-1 and FEM-3 could also explain the finding that the overall level of FEM-1 protein appears to be similar in XX and X0 animals (41). In X0 animals, by contrast, SEL-10 would be prevented from binding FEM-1 and FEM-3 protein, resulting in the FEM-dependent export of TRA-1 out of the nucleus and subsequent male development (Fig. 3*B*).

A prerequisite for substrate recognition by the SCF complex seems to be substrate phosphorylation (39). $SCF^{SEL\text{-}10}$-mediated degradation of FEM-1 and FEM-3 might, therefore, depend on their phosphorylation. The type 2C protein phosphatase FEM-2 acts at the same step of the sex-determination pathway, and its phosphatase activity is required for male development (4, 5). FEM-2 can interact with FEM-3 (5) and also with FEM-1 (48). Therefore, we suggest that in XX animals, FEM-1 and FEM-3 are phosphorylated by an unidentified protein kinase and that this phosphorylation is promoted by TRA-2 in XX animals and antagonized by FEM-2 in X0 animals (Fig. 3*B*).

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