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# Proteasomal Ubiquitin Receptor RPN-10 Controls Sex Determination in *Caenorhabditis elegans*<sup>□</sup>

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The ubiquitin-binding RPN-10 protein serves as a ubiquitin receptor that delivers client proteins to the 26S proteasome. Although ubiquitin recognition is an essential step for proteasomal destruction, deletion of the *rpn-10* gene in yeast does not influence viability, indicating redundancy of the substrate delivery pathway. However, their specificity and biological relevance in higher eukaryotes is still enigmatic. We report herein that knockdown of the *rpn-10* gene, but not any other proteasome subunit genes, sexually transforms hermaphrodites to females by eliminating hermaphrodite spermatogenesis in *Caenorhabditis elegans*. The feminization phenotype induced by deletion of the *rpn-10* gene was rescued by knockdown of *tra-2*, one of sexual fate decision genes promoting female development, and its downstream target *tra-1*, indicating that the TRA-2-mediated sex determination pathway is crucial for the  $\Delta$ *rpn-10*-induced sterile phenotype. Intriguingly, we found that co-knockdown of *rpn-10* and functionally related ubiquitin ligase *ufd-2* overcomes the germline-musculinizing effect of *fem-3(gf)*. Furthermore, TRA-2 proteins accumulated in *rpn-10*-defective worms. Our results show that the RPN-10-mediated ubiquitin pathway is indispensable for control of the TRA-2-mediated sex-determining pathway.

## INTRODUCTION

Ubiquitin is a covalent modifier that produces a polyubiquitin chain functioning as a degradation signal (Hershko and Ciechanover, 1998; Pickart, 2001). Recruitment of polyubiquitinated proteins to the degradation machinery is a key step in the selective degradation of various cellular proteins (Pickart, 1998; Voges *et al.*, 1999; Madura, 2004). Polyubiquitin chains with lengths of at least four ubiquitin moieties can be recognized and degraded by the 26S proteasome, a eukaryotic ATP-dependent protease complex (Voges *et al.*, 1999; Thrower *et al.*, 2000). The 26S proteasome is composed of the catalytic 20S proteasome and the regulatory PA700 complex; the later complex consists of six ATPase subunits (RPT-1-6) and multiple non-ATPase subunits (RPN-1 -3, -5-15), each ranging in size from 11 to 110 kDa (Tanaka, 1998).

Previous studies have shown that the RPN-10 subunit of PA700, originally called S5a, can bind to a polyubiquitin chain linked to proteins *in vitro* and *in vivo*, and it is thought to play a role as a ubiquitin receptor of the 26S proteasome (Deveraux *et al.*, 1994; Ferrell *et al.*, 1996; van Nocker *et al.*, 1996; Kawahara *et al.*, 2000; Wilkinson *et al.*, 2001; Elsasser *et al.*, 2004; Verma *et al.*, 2004). Figure 1 shows an abbreviated model that highlights the RPN-10 ubiquitin-

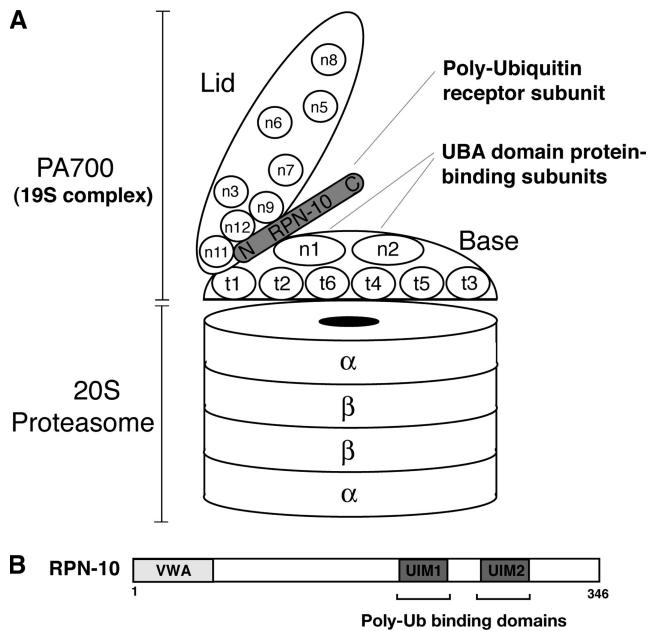
recognition subunits in 26S proteasomes. The RPN-10 has at least two distinct domains: one domain is the 60-residue N-terminal called the VWA domain, which was reported to be involved in the integration of the 26S complex (Glickman *et al.*, 1998; Verma *et al.*, 2004), and the other domain is the C-terminal region containing two independent polyubiquitin-binding sites, named ubiquitin interacting motif (UIM)1 and UIM2 (Young *et al.*, 1998; Hofmann and Falquet, 2001). Although it was thought that ubiquitin recognition is an essential step in the proteasome-mediated degradation process, the role of the RPN-10 subunit as a ubiquitin receptor of the 26S proteasome has become a subject of debate after the observation that its deletion in yeast does not influence the viability of the cells and causes only a mild phenotype with increased steady-state levels of ubiquitin-protein conjugates (van Nocker *et al.*, 1996; Fu *et al.*, 1998; Szlanka *et al.*, 2002). This observation suggested the existence of other different ubiquitin recognition mechanism(s). Indeed, several proteins such as Rad23p and Dsk2p are involved in polyubiquitinated substrate delivery to the proteasome in yeast, because they have a ubiquitin-associated domain (UBA) that can bind to polyubiquitin chains and a ubiquitin-like domain capable of associating with the 26S proteasome subunits (Wilkinson *et al.*, 2001; Saeki *et al.*, 2002; Elsasser *et al.*, 2004). Accordingly, these gene products in the substrate delivery pathway may play a largely redundant role. However, their specific target proteins and biological relevance in higher eukaryotes remain a mystery to be uncovered.

The genetic basis of sex determination has been analyzed extensively in the nematode, which has two naturally occurring sexes, an XX hermaphrodite and an XO male (Kuwabara and Kimble, 1992; Meyer, 2000; Kuwabara

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**Figure 1.** (A) Schematic representation of the 26S proteasome. The 26S proteasome is composed of the catalytic 20S proteasome and a regulatory particle termed PA700, which can be subdivided into the Base and the Lid complex. RPN-10 is an intrinsic ubiquitin receptor of 26S proteasomes and is highlighted in the figure. It was also reported that there are alternative ubiquitin receptors of 26S proteasome, UBL-UBA domain proteins, and that they interact with RPN-1 and possibly RPN-2 subunits of the Base complex of 26S proteasome. (B) Schematic diagram of RPN-10 ubiquitin receptor. Previous studies have shown that RPN-10 can bind to the Base complex via N-terminal VWA domain and bind to polyubiquitinated substrates via its C-terminal domain, including polyubiquitin (poly-Ub)-binding UIMs.

and Perry, 2001; Puoti *et al.*, 2001). Hermaphrodites sequentially produce sperm and oocytes from a single pool of precursors. After progression of L4 to adult molt, spermatogenesis is turned off, and all of the gametes that form subsequently develop into oocytes. Most of the sexual fate decision genes have been identified and have been shown to function in a cascade of negative regulation (Ellis and Schedl, 2006; Zarkower, 2006). Sex determination requires posttranscriptional regulation of two key genes, *tra-2* and *fem-3*. The balance between the activities of TRA-2 and FEM-3 influences the decision to adopt either a female or male fate. Genetic studies have shown that *tra-2* promotes female development because loss-of-function mutations of *tra-2* transform XX animals into pseudomales that do not produce oocytes (Hodgkin and Brenner, 1977), whereas *tra-2 eg* (extra gain-of-function) mutant allele lead to dominant transformation of XO animal from fertile male into fertile female (Hodgkin and Albertson, 1995; Kuwabara, 1996). TRA-2 is a large transmembrane protein that somewhat resembles the Patched receptor (Kuwabara and Kimble, 1992; Kuwabara, 1996), and it was thought that the potency of TRA-2 is regulated by a variety of mechanisms; it is controlled by its ligand HER-1 (Perry *et al.*, 1993; Kuwabara, 1996), by TRA-1-mediated nuclear export of *tra-2* mRNA (Graves *et al.*, 1999; Segal *et al.*, 2001) and by translational regulation through 3'-untranslated region (UTR)-binding proteins (Goodwin *et al.*, 1997; Clifford *et al.*, 2000; Puoti *et al.*, 2001). It has recently been suggested that the C-terminal intracellular domain (ICD) of TRA-2 is likely to be produced

as a soluble fragment in vivo through proteolytic cleavage of TRA-2 (Sokol and Kuwabara, 2000) or through translation of an XX germline-specific alternative transcript (Kuwabara *et al.*, 1998). It has been shown that the heat-shock promoter-induced exogenous intracellular domain of TRA-2 interacts with global sex regulatory transcriptional factor TRA-1 in the nuclei of both XX and XO animals (Lum *et al.*, 2000; Wang and Kimble, 2001) and that this fragment has feminizing activity (Kuwabara and Kimble 1995). The intracellular domain of TRA-2 also interacts with FEM proteins and modulates their activity (Mehra *et al.*, 1999). Both FEM-1 and FEM-3 are targeted for proteasomal degradation by the F-box ubiquitin ligase SEL-10 (Jager *et al.*, 2004). However, there have been only a few studies on the regulation of sex determination by the ubiquitin-mediated proteolytic pathway (Clifford *et al.*, 2000; Jager *et al.*, 2004). To investigate the function of the ubiquitin receptors of the 26S proteasome in higher eukaryotes, we focused on *Caenorhabditis elegans* counterpart of RPN-10. Our results clearly show the biological requirement of the RPN-10-mediated substrate discrimination system that is indispensable for the control of the sex-determining signaling pathway.

## MATERIALS AND METHODS

### Nematode Strains

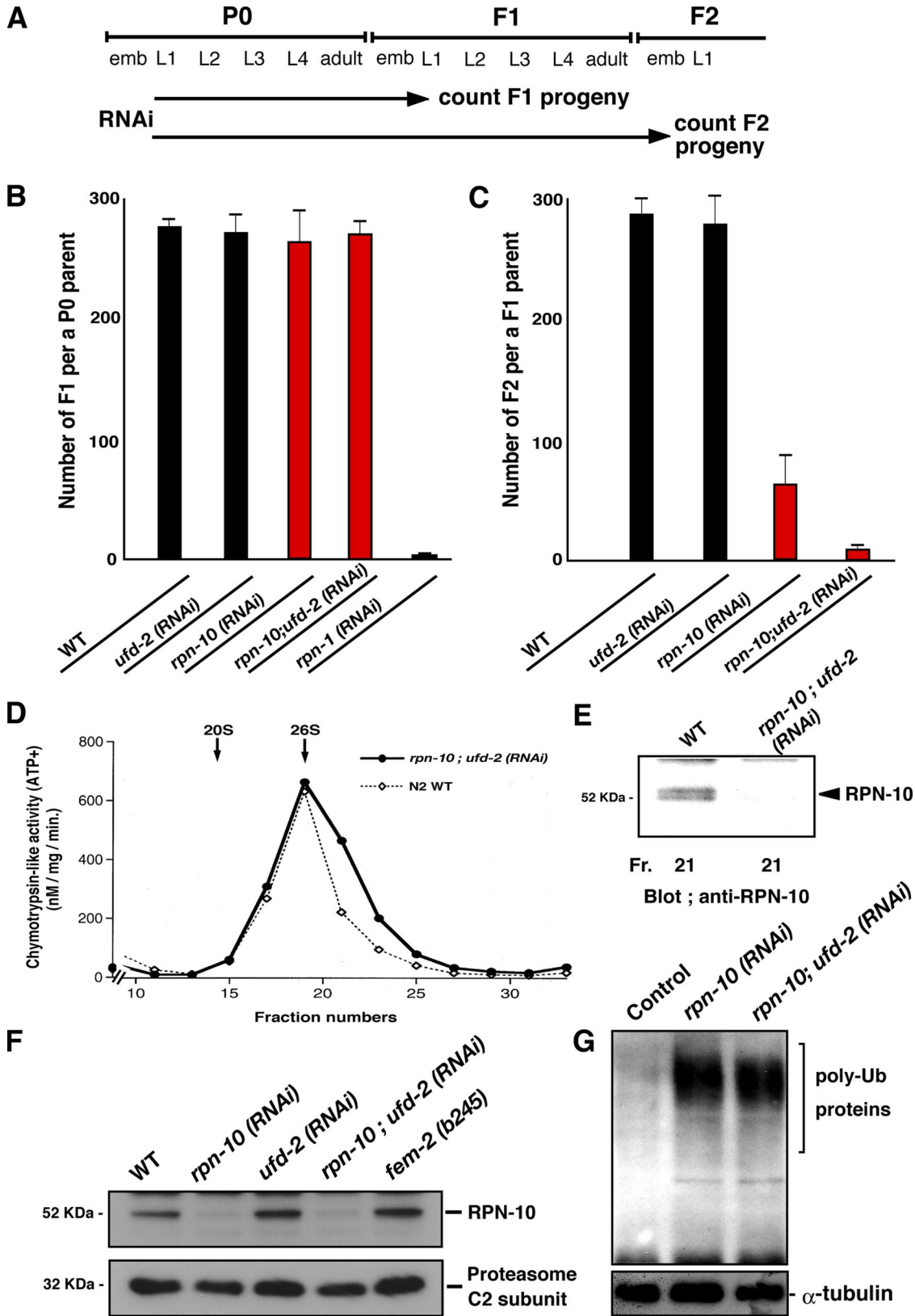
*C. elegans* strains were cultured using standard techniques (Brenner, 1974). *C. elegans* N2 variety Bristol was used for RNA interference (RNAi) analysis and for wild-type observations. The *rpn-10* mutant alleles *tm1180* and *tm1349* was isolated by the National Bioresource Project for the Nematode (NBPN; Department of Physiology, School of Medicine, Tokyo Women's Medical University, Tokyo, Japan). Both *rpn-10* (*tm1180*) and *rpn-10* (*tm1349*) alleles, generous gifts from Dr. S. Mitani (NBPN), were backcrossed at least five times against wild-type males before analysis. Although the original *tm1349* strain from National Bioresource Project shows only modest defects in the production of progeny, we found that repeated rounds of backcross against wild-type males resulted in generation of sterile females, and we used a backcrossed strain as *rpn-10* (*tm1349*) in this study unless otherwise noted. We have confirmed that further backcross procedures did not influence the penetrance of *rpn-10* (*tm1349*) phenotype and that this mutant did not express the corresponding transcript. *fog-2*(*oz40*), *fem-2*(*b245ts*), *tra-2* (*e1095*), *tra-2* (*e2020*), *fem-3* (*q20*), and *fem-3* (*e1996*) mutant worms were kindly provided by Dr. T. Schedl (Washington University, St. Louis, MO) and the *Caenorhabditis elegans* Genetics Center (University of Minnesota, St. Paul, MN), respectively.

### RNA Interference

RNAi experiments were mainly performed by the method of feeding (Timmons *et al.*, 2001), and the results were confirmed by the injection method (Fire *et al.*, 1998; Shimada *et al.*, 2002). Efficacy of gene expression knockdown in each of RNAi experiments were verified by reverse transcription-polymerase chain reaction (RT-PCR) analysis. *rpn-10* (B0205.3), *ufd-2* (T05H10.5), and other cloned genes (*rpn-1*, T22D1.9; *tra-1*, Y47D3A.6; *tra-2*, C15F1.3; *oma-1*, C09G9.6; *oma-2*, ZC513.6; *rad-23*, ZK20.3; and *dsk-2*, F15C11.2) were amplified by PCR from a *C. elegans* mixed stage cDNA library. Primer sequences are available from the author on request. The amplified PCR products were subcloned into a pPD129.36 vector and transfected into *Escherichia coli* strain HT115 (*DE3*). RNA transcription from pPD129.36 vector plasmids was induced by the addition of 0.4 mM isopropyl  $\beta$ -D-thiogalactoside (IPTG) to *E. coli* culture medium (at an optical density of 0.4), followed by incubation for 4 h at 37°C. The harvested *E. coli* was spread on NGM plates (containing 50  $\mu$ g/ml ampicillin, 12.5  $\mu$ g/ml tetracycline, and 0.4 mM IPTG) for the feeding experiments. Feeding was started at the L1 stage, and worms were cultured for the indicated times at 25°C unless otherwise noted. To show self-sterility, worms were picked at the L4/young adult stage to individual plates and assayed for their ability to produce progeny.

### Glycerol Density Gradient Centrifugation

For sedimentation velocity analysis, worms were homogenized with 26S proteasome extraction buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 2 mM ATP, and 10% glycerol) and centrifuged at 10,000  $\times$  g for 20 min to obtain soluble extracts. Extracts of 5 mg of protein were subjected to glycerol density-gradient centrifugation with 10–40% glycerol. After centrifugation at 83,000  $\times$  g for 22 h, the gradient was separated into 33 fractions of 1 ml each. The activity of the 26S proteasome in the fractions was measured by using the synthetic peptide succinyl-Leu-Leu-Val-



**Figure 2.** RPN-10 knockdown results in F1 sterility in *C. elegans*. (A) Schematic diagram of RNAi treatment. (B) Effects of RNAi on the reproduction of P0 parents. The average numbers of F1 progeny per a P0 parent are indicated. *rpn-1* (RNAi) was used as a positive control for this RNAi experiment. (C) Effects of RNAi on the reproduction of F1 parents. The average numbers of F2 progeny per an F1 parent are indicated. Note that double RNAi treatment of *rpn-10* and *ufd-2* results in a nearly complete sterile phenotype. (D) Sedimentation velocity analysis of the 26S proteasome. Worm extracts were fractionated by glycerol density-gradient centrifugation and assayed for 26S proteasomal chymotrypsin-like activity. Elution points of 20S and 26S proteasomes are indicated by arrows. (E) Proteins in the 26S proteasome fraction

Tyr-4-methylcoumarin-7-amide (Suc-LLVY-MCA) as described previously (Kawahara *et al.*, 2000). Purified mouse 20S and 26S proteasomes were used as markers for sedimentation.

### Examination of Spermatheca

To examine the state of the spermatheca, adult hermaphrodites (or females) were cut open to release gonads, and freeze/cracked samples in M9 buffer were fixed by incubation in cold methanol ( $-20^{\circ}\text{C}$ ) followed by cold acetone ( $-20^{\circ}\text{C}$ ). Fixed gonads were stained with Hoechst 33342 at the concentration of 2.5  $\mu\text{g}/\text{ml}$ .

### Mating with Males

Individual F1 sterile worms of *rpn-10;ufd-2* (RNAi) and *rpn-10* (*tm1349*) homozygote at L4 stage were each plated with each of six healthy young-adult males and crossed at  $20^{\circ}\text{C}$ . At 24 h after crossing, parental worms were removed from the plates. After another 24-h incubation, the number of progeny was counted. Oocyte-defective *oma-1;oma-2* (RNAi) worms and feminized *fem-2* (*b245ts*) mutant worms were crossed with males under conditions similar to those described above and used as negative and positive controls, respectively, for this experiment.

### Effect of RNAi on XO Males

XO males (P0) were crossed with XX hermaphrodites (P0) treated with either *rpn-10;ufd-2* (RNAi)- or control vector (RNAi), and resulting F1 larvae were continued to be treated with respective RNAi until the young adult stage at  $20^{\circ}\text{C}$ . After verification of F1 feminization effects in *rpn-10;ufd-2* (RNAi)-treated hermaphrodites, individual F1 males were harvested and subjected to either microscopic observations or RT-PCR analyses.

### Immunological Analysis

The anti-TRA-2 ICD antibody was prepared for this study as follows. Five hundred micrograms of bacterially produced TRA-2 intracellular domain (amino acids 1135–1475 of *C. elegans* TRA-2 protein) was mixed and emulsified with an equal amount of TiterMaxGold (TiterMax USA, Norcross, GA) and then inoculated into a rabbit. The antibody was obtained after six rounds of immunization at 2-wk intervals and used after affinity purification.

Indirect immunofluorescence analyses were performed as reported by Finney and Ruvkun (1990) with modifications as follows. Animals were suspended in 4% paraformaldehyde, 25% methanol, and quick-frozen in liquid nitrogen. After thawing on ice, the animals were incubated on ice for 1 h to complete fixing, washed three times in 1% Triton X-100, 100 mM Tris-HCl, pH 7.5, and incubated in 1% Triton X-100, 100 mM Tris-HCl, pH 7.5, and 1%  $\beta$ -mercaptoethanol for 2 h at  $37^{\circ}\text{C}$  to reduce the cuticle. After three washes in 10 mM Na $\text{BO}_3$ , pH 9.2, the animals were incubated in 10 mM Na $\text{BO}_3$ , pH 9.2, and 0.3% H $_2\text{O}_2$  for 1 h at  $25^{\circ}\text{C}$ . The animals were then washed two times in 10 mM Na $\text{BO}_3$ , pH 9.2, and stored in PTB buffer (1X phosphate-buffered saline [PBS], 0.1% Triton X-100, 0.1% bovine serum albumin, and 0.05% Na $\text{N}_3$ ). After blocking with blocking solution (1X PBS, 0.1% Tween 20, and 5% bovine serum albumin) for 30 min at  $25^{\circ}\text{C}$ , antibody incubations were performed overnight at  $4^{\circ}\text{C}$  in the blocking solution. Affinity-purified anti-TRA-2 ICD antibody was diluted to a concentration of 1  $\mu\text{g}/\text{ml}$  before incubation. After three washes in  $1\times$  PBS and 0.1% Tween 20 (PBS-T), the worms were incubated for 90 min at  $25^{\circ}\text{C}$  with 1:800 Alexa 488-conjugated anti-rabbit IgG (Invitrogen, Carlsbad, CA) in blocking solution. After four washes in PBS-T, the worms were treated with 2.5  $\mu\text{g}/\text{ml}$  Hoechst 33342 for 15 min at  $25^{\circ}\text{C}$  and then washed another three times in PBS-T. Then, small aliquots of worms were mounted on slides with VECTASHIELD (Vector Laboratories, Burlingame, CA) mounting media and examined under an Axioplan II microscope (Carl Zeiss, Jena, Germany) equipped with fluorescence capabilities.

For general immunoblot analyses, extracts from RNAi-treated and mutant worms or cultured cell extracts were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). The membranes were probed with an anti-RPN-10 peptide antibody ( $^{35}\text{S}$ -CHSKTRSNPENNVGLITLAN $^{56}$  as an antigen for *C. elegans* RPN-10), anti-ubiquitin antibody, anti-20S proteasome C2 subunit antibody, anti-tubulin DM1a monoclonal antibody (Sigma-Aldrich, St. Louis, MO), and anti-FLAG M2 antibody (Sigma-Aldrich).

For anti-TRA-2 ICD blots, samples were prepared by picking up 50 appropriate animals, boiling in 2X SDS-PAGE sample buffer with 1%  $\beta$ -mercaptoethanol, separating by SDS-PAGE, and transferring onto Immobilon P trans-

fer membranes (Millipore, Billerica, MA). Primary antibody for anti-TRA-2 ICD was used at a concentration of 0.2  $\mu\text{g}/\text{ml}$  in reaction buffer (1X PBS, 5% skim milk, 3% normal goat serum, 3% *E. coli* HT115 extract, and 0.1% Tween 20). Biotinylated goat anti-rabbit IgG and Vectastain ABC detection reagents (Vector Laboratories) were used, followed by detection with ECL Western blotting detection reagents (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom).

## RESULTS

### Knockdown of RPN-10 Results in Induction of F1 Sterility

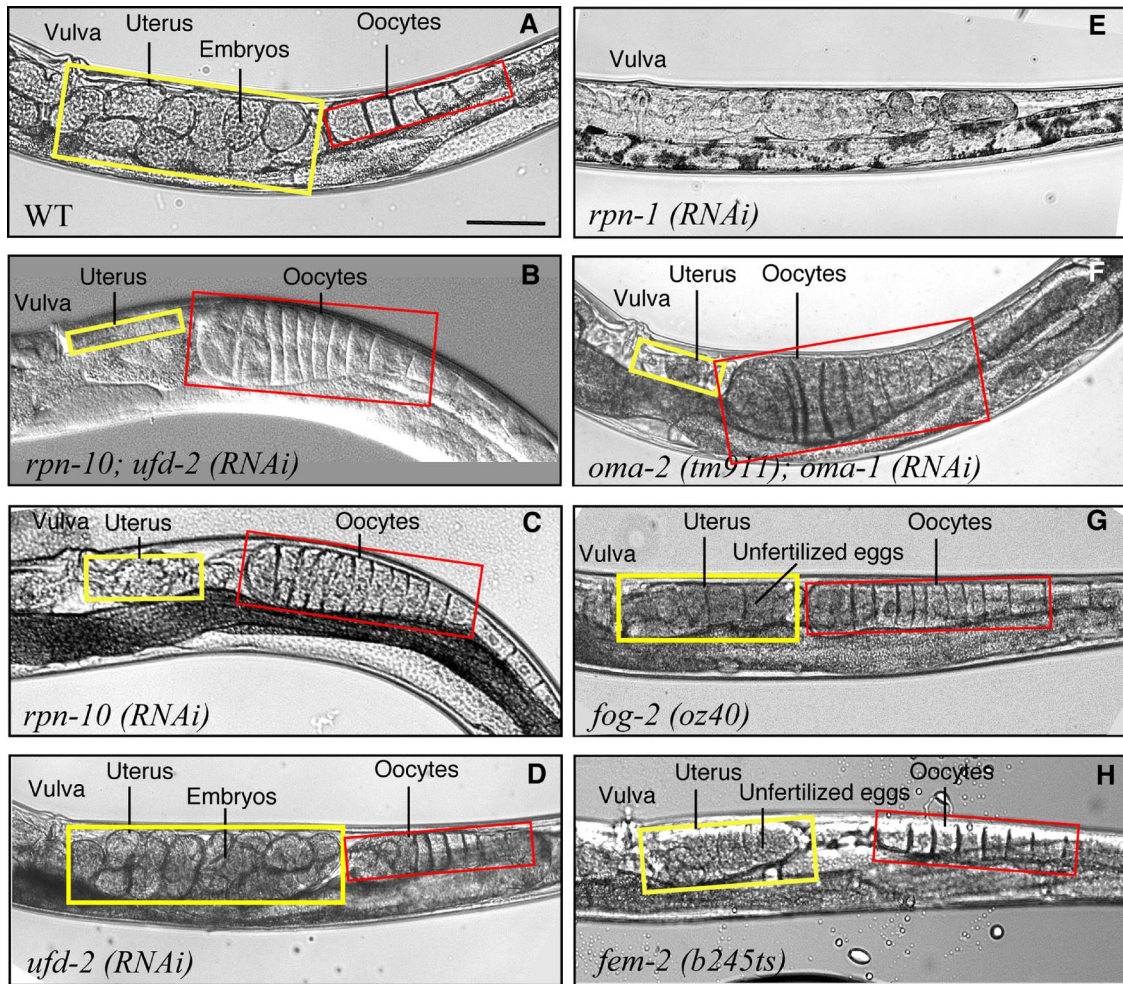
Although reduction of the expression of most proteasomal subunits by RNAi results in immediate growth arrest in *C. elegans*, we found that inhibition of *rpn-10* gene expression did not cause any obvious defects in P0 growth, reproduction, and subsequent development of F1 embryos (Figure 2, A and B). Because mutation in the *ufd-2* gene, which was originally identified by genetic dissection of the UFD pathway (Johnson *et al.*, 1995), with *rpn-10* shows synthetic growth defects in yeast (Koegl *et al.*, 1999), we attempted to inhibit *rpn-10* and *ufd-2* gene expressions simultaneously. Again, we were unable to detect any obvious defects during P0 generation (Figure 2B). Efficacy of gene expression knockdown in each of the RNAi experiments was verified by RT-PCR analysis, confirming that these genes seem to be dispensable for these stages. In *rpn-10;ufd-2* (RNAi) worms, peptidase activity, i.e., chymotryptic Suc-LLVY-MCA-degrading activity, of the 26S proteasome and its sedimentation profile was indistinguishable from that in wild-type worms (Figure 2D), despite the reduction of *rpn-10* gene expression (Figure 2, E and F). These observations indicate that the overall 26S proteasome architecture and functions for cell viability are intact in the *rpn-10*- and *ufd-2*-defective environment. However, we noticed the accumulation of polyubiquitinated proteins in the extract of *rpn-10* (RNAi) as well as *rpn-10;ufd-2* (RNAi) worms (Figure 2G), suggesting certain defects of degradation of polyubiquitinated proteins in these RPN-10-lacking worms without affecting viability.

Although there was no apparent defect at P0 stage in *rpn-10*-knockdown worms, close inspection revealed a prominent reduction in the number of F2 embryos laid from *rpn-10* (RNAi) F1 worms (Figure 2C). Inhibition of *ufd-2* expression alone resulted in an essentially no phenotype in F1 and later stage worms, but double inhibition of *rpn-10* and *ufd-2* expression resulted in a further additional reduction of the number of F2 eggs laid, i.e.,  $>97\%$  reduction in the number of F2 progeny compared with the control (Figure 2C). These observations suggested that the *rpn-10* gene possess important functions for the production of F2 progeny in collaboration with *ufd-2* gene and that their removal results in a sterile phenotype in F1 stage worms.

### Knockdown of RPN-10 Results in a Defect in Sperm Formation

To determine the defect that leads to F1 sterility, we first examined the F1 phenotypes in detail. In wild-type worms, germ cells proliferated by repeated rounds of mitosis on the distal side of the gonad and then progressed into meiosis and moved proximally, producing maturing oocytes on the proximal side. We found that the proximal gonad arm expands greatly in *rpn-10*-inhibited F1 individuals (Figure 3C) compared with that in wild-type gonads (Figure 3A), and germinal vesicle breakdown in proximal oocytes is likely to occur at a very low level. The double RNAi of *rpn-10* and *ufd-2* shows more penetrance but essentially the same phenotype as that of *rpn-10*-compromised individuals (Figure 3B), whereas single RNAi against *ufd-2* induced no abnormality in the gonad (Figure 3D).

**Figure 2 (cont).** (Fr. 21) were precipitated with acetone and subjected to immunoblotting with anti-RPN-10 antibody. (F) Expression of RPN-10 protein is suppressed in *rpn-10* (RNAi) worms. Immunoblotting of the 20S proteasome C2 subunit was used as a control. (G) RPN-10 knockdown results in accumulation of polyubiquitinated (poly-Ub) proteins.  $\alpha$ -tubulin as a loading control.



**Figure 3.** RPN-10 knockdown induces characteristic reproductive defects. Compared with the WT gonad (A), typical *rpn-10* (RNAi) worms show expanded oocytes (indicated by a red box) and nearly empty uterus (indicated by a yellow box) (C), representing similar morphology to oocytes-defective *oma-2* (*tm911*);*oma-1* (RNAi) worms (F). Double RNAi of *rpn-10* and *ufd-2* results in more penetration but essentially the same phenotype as in C (B). *ufd-2* (RNAi) alone shows no abnormality in gonads (D). Knockdown of the expression of RPN-1, a constitutive subunit of the 26S proteasome, induces immediate growth arrest (E). Feminized *fog-2*(*oz40*) (G) and *fem-2*(*b245ts*) mutant (H), show accumulation of unfertilized oocytes in the uterus due to their stochastic maturation. Images on the left-top side correspond to the ventral uterus. Bar, 50  $\mu$ m.

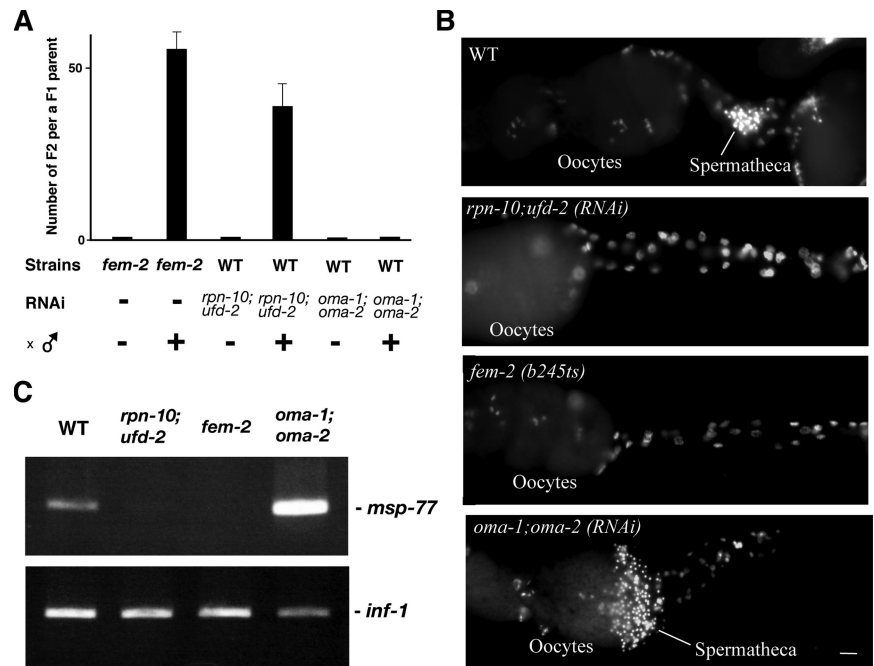
To check whether the sterile but viable phenotype of *rpn-10* (RNAi) is caused by partial retardation of general 26S proteasome ability, we examined the effects of continuous reduction of 26S proteasome function. Efficient knockdown of the expression of RPN-1, a constitutive and essential subunit of the 26S proteasome, resulted in immediate arrest of larval growth and/or extensive death (Figure 3E). Continuous knockdown of *rpn-1* by modest feeding of double-stranded RNA to *C. elegans* hermaphrodites can result in partial depletion of the product of this gene (Kamath *et al.*, 2001 and Supplemental Table S1). Irrespective of any extent of partial inhibition of *rpn-1*, no individuals showed similar reproductive defects as seen in *rpn-10* (RNAi) (Supplemental Figure S1). Furthermore, there was no synthetic effect between *ufd-2* (RNAi) with partial knockdown of *rpn-1* (Supplemental Table SII). Thus, it was concluded that the phenotype of *rpn-10* (RNAi) is not due to general retardation of proteasome activity.

Oocyte maturation defects in *C. elegans* so far reported are feminizing mutations such as *fem-2* (*b245ts*) and *fog-2* (*oz40*) (Figure 3, G and H; Schedl and Kimble, 1988; McCarter *et al.*,

1999; Miller *et al.*, 2001, 2003) as well as defects in oocyte-specific *oma*-family genes (Figure 3F; Detwiler *et al.*, 2001; Shimada *et al.*, 2002, 2006). To determine whether the defects in *rpn-10*-compromised individuals are due to an abnormality in oocyte or sperm, we performed mating experiments with normal males and sterile worms. After being crossed with wild-type (N2) males, *fem-2* (*b245ts*) mutant females were able to produce progeny, whereas oocyte-defective *oma-1*;*oma-2* (RNAi) worms were not rescued by the wild-type male sperm (Figure 4A). It was found that *rpn-10*;*ufd-2* (RNAi) sterile worms can be cross-fertilized by wild-type males to produce normal viable F2 embryos (Figure 4A). These findings support the view that the primary defect induced by *rpn-10*;*ufd-2* (RNAi) is likely to be caused by a defect in the sperm.

To demonstrate directly the presence of defective sperm production, we dissected gonads from worms and stained them with Hoechst. In wild-type (WT) and *oma-1*;*oma-2* (RNAi) hermaphrodites, many sperm were present in the spermatheca, whereas the sperm was completely absent in the spermatheca of *rpn-10*;*ufd-2* (RNAi) worms, as in femi-

**Figure 4.** The primary defect in RPN-10-knockdown worm is sperm formation. (A) Mating experiments with normal males and sterile feminized *fem-2(b245ts)* mutants were able to produce progeny, whereas oocyte-defective *oma-1; oma-2* (RNAi) worms were not. *rpn-10; ufd-2* (RNAi) sterile worms can be cross-fertilized by wild-type (N2) males to produce normal viable F2 embryos. (B) Hoechst staining of dissected gonads from WT, *rpn-10; ufd-2* (RNAi), *fem-2(b245ts)*, and *oma-1; oma-2* (RNAi) worms. A number of sperm signals are evident in WT and *oma-1; oma-2* (RNAi) worms as indicated but are absent in *fem-2(b245ts)* and *rpn-10; ufd-2* (RNAi) worms. Bar, 10  $\mu$ m. (C) RT-PCR analysis of the sperm-specific marker *msp-77*. Transcript of *msp-77* is not expressed in *fem-2(b245ts)* and *rpn-10; ufd-2* (RNAi) worms. *inf-1* transcript (encoding translation initiation factor CelF) amplified under similar conditions was used as a control.



nized *fem-2(b245ts)* worms (Figure 4B). As noted later using *rpn-10* null mutant strain, primary and secondary spermatocytes were absent in these feminized gonads at late L4 or young adult stages (data not shown). Consistent with these morphological observations, RT-PCR quantification of the sperm-specific marker *msp-77* clearly showed that sperm were in fact absent in *rpn-10; ufd-2* (RNAi) worms as in the *fem-2(b245ts)* female (Figure 4C). These results indicate that the primary defect in *rpn-10; ufd-2* (RNAi) worms is in the production of sperm, and sterility induced by reduction of the RPN-10-mediated protein degradation pathway could be explained as a consequence of feminization.

#### *rpn-10* Mutant Worms Show Feminization Phenotype

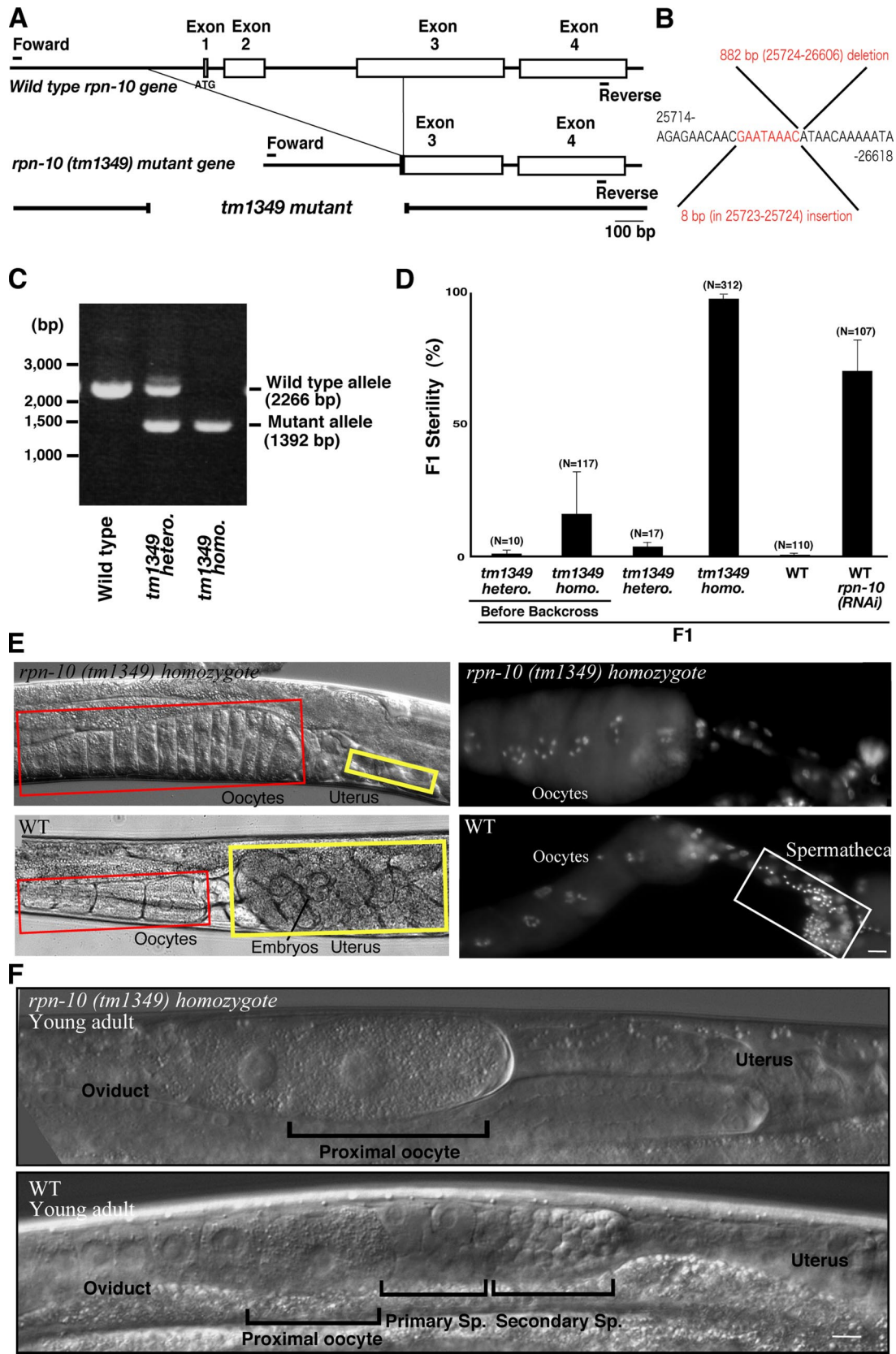
*tm1349* is a null *rpn-10* allele that includes a 882-base pair deletion and an 8-base pair insertion of the *rpn-10* gene locus (Figure 5, A–C), resulting in loss of the first three exons of the *rpn-10* gene (Figure 5A). As shown in Figure 5D, adequately backcrossed *rpn-10* (*tm1349*) homozygotes show nearly complete F1 sterility. The morphological phenotype of *rpn-10* (*tm1349*) homozygote is identical to that of *rpn-10; ufd-2* (RNAi) worms: essentially no sperm in the spermatheca, no embryos in the uterus, and characteristic expansion of the proximal oocytes at a relatively young adult stage (54 h from L1 at 25°C) (Figure 5E; our unpublished observations). Furthermore, we found that primary and secondary spermatocytes were absent in *rpn-10* (*tm1349*) homozygote gonads at young adult stages having only one or at most two oocytes (Figure 5F). As in *rpn-10; ufd-2* (RNAi) worms, exogenously supplied sperm restored fertility; an average of 155 progeny were produced from an *rpn-10* (*tm1349*) homozygote female that was crossed with wild-type males. These data further support the idea that the *rpn-10* (*tm1349*) homozygote has defects in sperm production.

RPN-10 protein is comprised of the N-terminal VWA domain and the C-terminal domain containing UIMs (Figure 1). In yeast, the modest stress-sensitive phenotype of an RPN10 deletion mutant is completely rescued by the N-terminal fragment of Rpn10p, indicating that the C-terminal

domain is dispensable (Fu *et al.*, 1998). To determine whether the C-terminal domain of *C. elegans* RPN-10 is essential for the feminization phenotype, we used another mutant strain *rpn-10* (*tm1180*) (kindly provided by Dr. S. Mitani). In this mutant, exon 3 of *rpn-10* gene was disrupted with a 356-base pair deletion and the gene product was expressed as a C-terminal truncated form (Supplemental Figure S2B, left, and F). As a result, polyubiquitinated proteins accumulated in *rpn-10* (*tm1180*) worms (Supplemental Figure S2B, right) as seen in *rpn-10* (RNAi) worms (Figure 2E). The *rpn-10* (*tm1180*) is a hypomorphic allele that shows modest impairment of progeny production (Supplemental Figure S2C), but it also represents a synthetic effect with *ufd-2* (RNAi) (Supplemental Figure S2C), characteristic expansion of oocytes (Supplemental Figure S2D), and reduction of sperm in the gonads of young adult hermaphrodites (Supplemental Figure S2E), supporting our idea that the C terminus of RPN-10 can cooperate with the UFD-2 pathway to metabolize the protein(s) required for production of male zygotes. These findings indicate that the C-terminal domain of RPN-10, which contains two sets of UIM, is essential for regulation of sex determination under the condition of *ufd-2* reduction. In addition, we found that the ability of reproduction in *rpn-10* (*tm1180*)/*rpn-10* (*tm1349*) worms is identical to that of *rpn-10* (*tm1180*) homozygote hermaphrodites.

#### RPN-10 Regulates the TRA-2-mediated Sex Determination Pathway

Next, we focused on the role of RPN-10 in specification of sexual fate. Most of the sexual fate decision genes have been identified and shown to function in a cascade of negative regulation (Ellis and Schedl, 2006; Zarkower, 2006). Figure 6A shows a somatic sex determination pathway that highlights functional relationships among genes at the end of the pathway. In hermaphrodite somatic cells, three SDC proteins inactivate the extracellular ligand HER-1, which blocks its transmembrane receptor protein TRA-2 (Meyer, 2000) (Figure 6A), and the pathway ultimately controls activity of the nuclear transcriptional factor TRA-1 (Zarkower, 2006).



**Figure 5.** *tm1349*, a null allele of *rpn-10*, shows a feminized phenotype. (A) Schematic diagram of wild-type and *tm1349* mutant allele of the *rpn-10* gene. *C. elegans* *rpn-10* gene is composed of four exons, and the *tm1349* mutant completely lacked the first and second exons with partial deletion of the third exon. Boxes, exons; lines, introns. Extent of the 882-base pair *rpn-10* (*tm1349*) deletion is indicated by a gap. (B) Nucleotide sequence of the mutated region of the *rpn-10* gene in *tm1349* allele. *rpn-10* cDNA (genome) obtained from a *tm1349* homozygote

Figure 6B shows a simplified version of the germline sex determination (Goodwin and Ellis, 2002; Bachorik and Kimble 2005; Ellis and Schedl, 2006). In this pathway, the onset of hermaphrodite spermatogenesis depends on the control of TRA-2 germ-line activity. *tra-1* is not a terminal sex regulator in germline, but the activity of *tra-1* and *tra-2* inhibits sperm production. Thus, both TRA-1 and TRA-2 promote feminization in the sex-determining pathways. We addressed the question of whether the defects in *rpn-10* (*tm1349*) homozygote or *rpn-10; ufd-2* (RNAi) worms would result in excess activation of the above proteins, which may lead to induction of inappropriate feminization (Figure 6, A and B). The results showed that *tra-1* (RNAi) (which inhibits *tra-1* expression to almost 55%) reversed the sterile phenotype of *rpn-10* (*tm1349*) homozygote, supporting the view that TRA-1 and/or its upstream cascade pathway are responsible for the sterility of *rpn-10* (*tm1349*) (Figure 6C). Knockdown of *tra-2* by RNAi to >90% resulted in masculinization as reported previously in *tra-2* mutants (Hodgkin and Brenner, 1977), but we found that partial knockdown of the *tra-2* gene product (57% reduction of its mRNA) effectively rescued the sterile phenotype of *rpn-10* (*tm1349*) homozygote (Figure 6C). The results of morphological observations supported the above results; knockdown of *tra-2* with *rpn-10* (*tm1349*) restored sperm formation and resulting viable embryos (Figure 6D). We obtained similar results using co-knockdown of *rpn-10; ufd-2* (RNAi) (as knockdown at P0 generation) with either *tra-1* or *tra-2* (as a knockdown at F1 generation without affecting the efficacies of *rpn-10; ufd-2* knockdown) (Figure 6E). The results of RT-PCR analysis indicated that co-knockdown of *tra-2* with *rpn-10; ufd-2* restored the expression of *msh-77*, a sperm marker (Figure 6F). Considered together, the above-mentioned findings support the view that RPN-10 acts in the sex determination pathway at a point of *tra-2* to regulate their activity negatively, either directly or indirectly, and thus promotes sperm production in the *C. elegans* hermaphrodite.

#### RPN-10 Functions Cell Autonomously in the Germline

To verify whether the product of the *rpn-10* gene acts cell-autonomously in the germline, we performed a feminization assay under an *rrf-1* (*pk1417*) genetic background. In this mutant, RNA interference for genes expressed in somatic tissue was lost, whereas interference was retained for genes expressed in the germline (Sijen *et al.*, 2001). We observed a typical sign of feminization of the germline in *rrf-1* (*pk1417*) homozygotes that were treated with *rpn-10; ufd-2* (RNAi) (Figure 7A), whereas control *rrf-1* (*pk1417*) worms were vi-

able, self-fertile, and none showed any obvious morphological or growth defects. These results support the notion that RPN-10 function in the germline is needed for the production of sperm. To show that the sex determination pathway is a target of RPN-10 in the germline, we established a double homozygote mutant of *rrf-1* (*pk1417*); *rpn-10* (*tm1349*) (Figure 7B). Although this double mutant worm shows less sterility to *rpn-10* (*tm1349*) single homozygotes for an unknown reason, knockdown of either *tra-1* or *tra-2* in double mutants greatly enhanced self-fertility (Figure 7C). These results collectively suggest that the product of the *rpn-10* gene functions cell autonomously in the germline tissues to promote male differentiation.

#### Knockdown of *rpn-10* Overcomes the Germline-musculinizing Effect of Gain-of-Function Mutant of *fem-3*

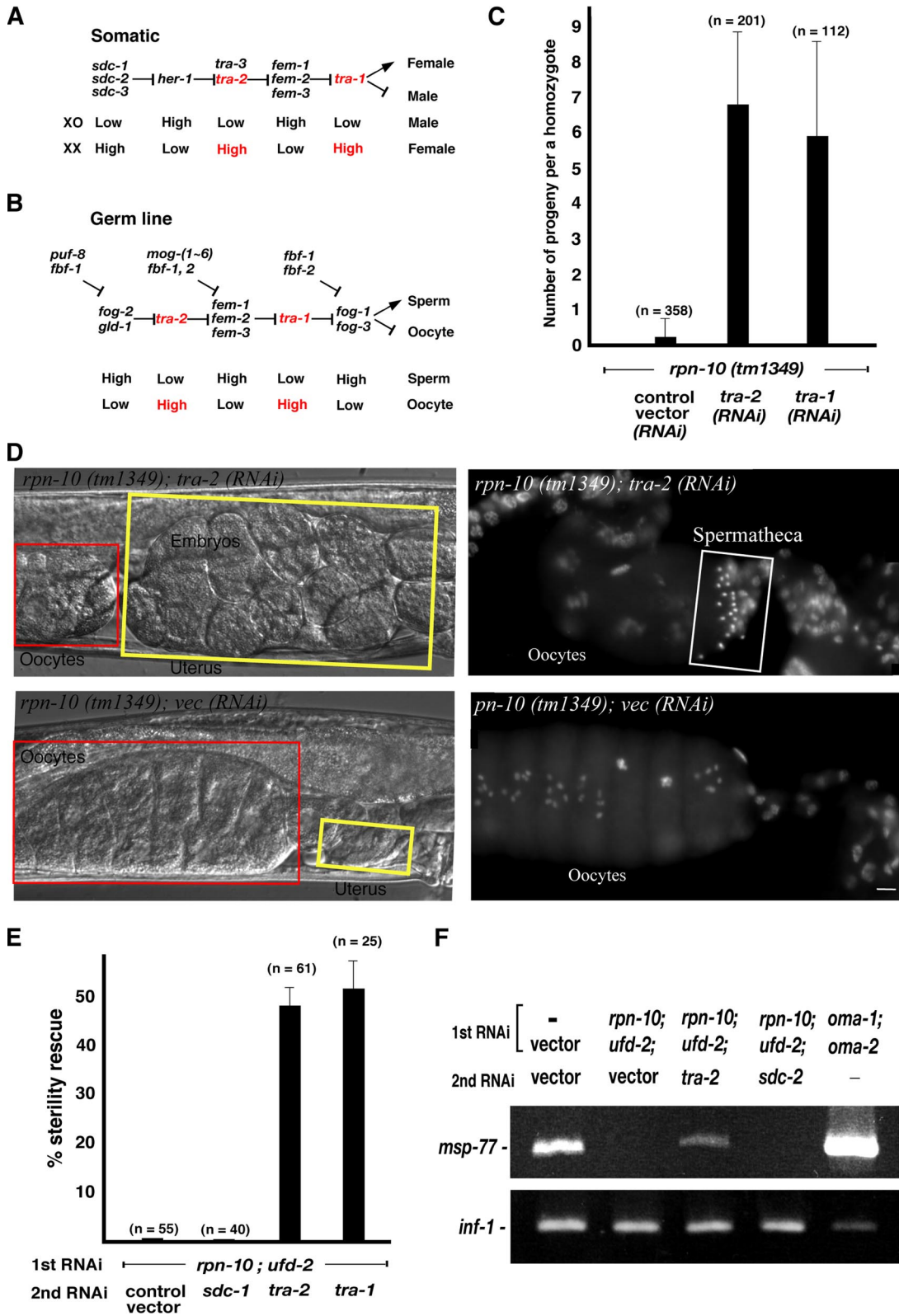
The *fem* genes are required for male development in the XO soma and for spermatogenesis in both XO and XX germlines. It has been reported that *fem-3* (*gf*) alleles masculinize only hermaphrodite germlines (Barton *et al.*, 1987). Thus, the XX *fem-3* (*gf*) mutant at a restricted temperature (25°C) has a normal hermaphrodite soma, but the germline produces a vast excess of sperm and no oocytes. Interestingly, it has been reported that an intermediate allele of *fem-3* (*gf*, *q20*), when homozygous in combination with *tra-2* (*gf*) or *fog-2* (*lf*), gave a fertile hermaphrodite at 25°C (Barton *et al.*, 1987), suggesting that overproduction of *tra-2* product during adulthood will counteract the excess FEM-3 produced by *fem-3* (*gf*). We therefore investigated whether *rpn-10; ufd-2* (RNAi) overcomes the masculinizing effect of *fem-3* (*gf*) *q20*. We found that oocyte production was restored in >70% of *fem-3* (*gf*) mutant worms by treatment with *rpn-10; ufd-2* (RNAi) (Figure 8). The oocytes it makes can be fertilized and can give rise to progeny (Figure 8A). These results clearly indicate that, as has been reported for *tra-2* (*gf*), co-knockdown of *rpn-10* and *ufd-2* pathway can suppress the masculinizing effect of *fem-3* (*gf*).

#### Knockdown of *rpn-10* Had No Obvious Effect on Morphology of XO Males but Caused Weak XO Intestinal Feminization

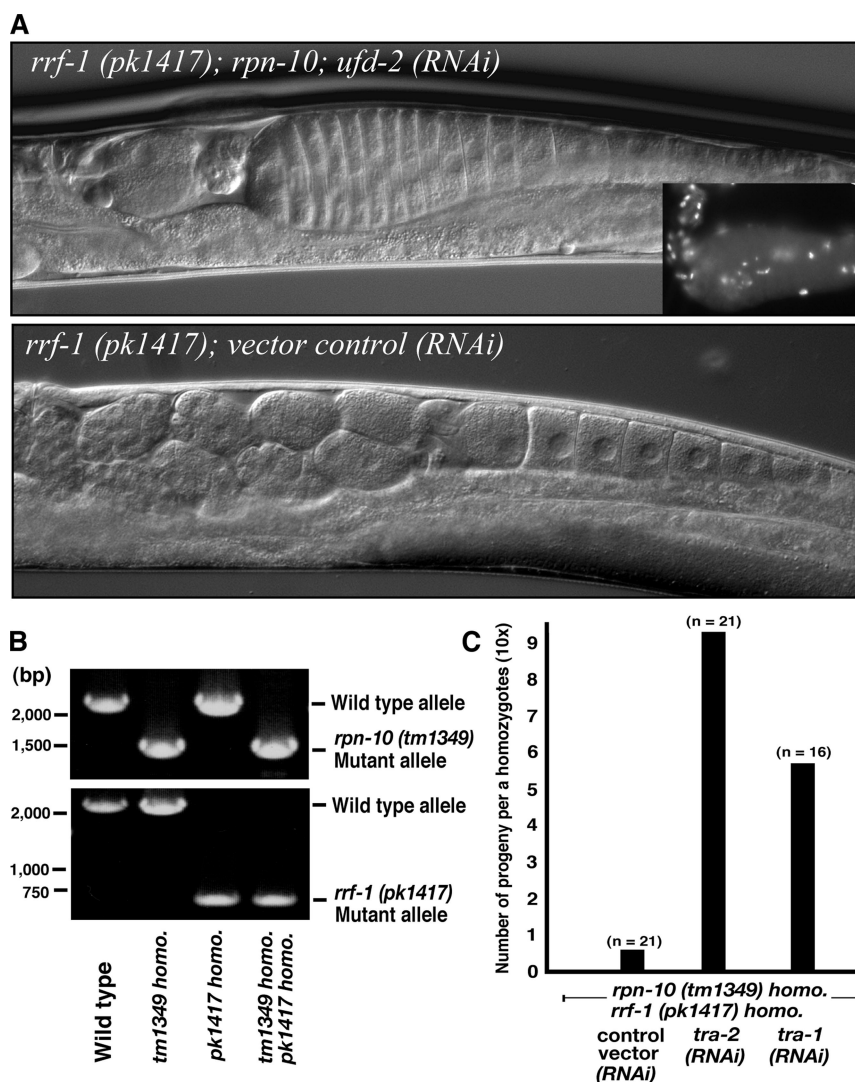
In XO males, secreted HER-1 is postulated to bind and to inactivate TRA-2A (Perry *et al.*, 1993; Kuwabara, 1996). It has been reported that most gain-of-function alleles of *tra-2* feminize the germline of XX animals but do not affect the germline or soma of XO males (Doniach, 1986; Schedl and Kimble, 1988). In overexpression of TRA-2 protein driven from a strong promoter, it has been reported that the germline of XO animals was transformed into hermaphrodites and partially feminized the intestine of XO animals (Kuwabara and Kimble, 1995). We therefore became interested in whether knockdown of *rpn-10* could potentially feminize not only XX hermaphrodites but also XO males.

We found that simultaneous knockdown of *rpn-10* and *ufd-2* did not affect apparent morphology of XO males (Figure 9A) under the condition in which XX hermaphrodites were completely feminized. As shown in Figure 9A, tails of XO males treated with *rpn-10; ufd-2* (RNAi) are indistinguishable from those of control males, and there is no sign of oocyte formation in the gonads. To detect the potential feminization in XO males more precisely, we performed semiquantitative RT-PCR analysis of somatic and germline sex determination markers. The *C. elegans* vitellogenin gene *vit-2* is subjected to sex- and tissue-specific expression (Kimble and Sharrock, 1983). It is expressed solely in the adult hermaphrodite intestine, a female somatic tissue, and it has been reported that no *vit-2* mRNA is present in the

**Figure 5 (cont).** was sequenced, and a 882-base pair deletion and 8-base pair insertion were identified in the region indicated. No sequence difference was identified around this region before and after backcrossing. The numbers of the sequence denote the wild-type genome nucleotide number of chromosome I. (C) Genomic PCR analysis confirming deletion of the *rpn-10* gene in *rpn-10* (*tm1349*) heterozygote and homozygote worms. (D) The average F1 sterilities at 25°C are indicated. Note that F1 of the *rpn-10* (*tm1349*) homozygote shows nearly complete sterility. (E) *rpn-10* (*tm1349*) mutant adult worms show expanded oocytes (indicated by a red box) and a nearly empty uterus (indicated by a yellow box). Hoechst staining of dissected gonads shows essentially no sperm in *rpn-10* (*tm1349*). Spermatheca is indicated by a white box. Photographs were taken at the adult stage (54 h from L1 at 25°C). (F) Nomarski observations of gametogenesis at the young adult stage of an *rpn-10* (*tm1349*) homozygote worm. Proximal oocytes and primary and secondary spermatocytes (labeled "Primary Sp." and "Secondary Sp.," respectively) are indicated. Bar, 10 μm.



**Figure 6.** Sterility induced by deletion of the *rpn-10* gene was rescued by knockdown of *tra-2*. Schematic diagram of the somatic (A) and germline (B) sex determination pathway in *C. elegans*. Accumulation of TRA-2 and TRA-1 proteins, indicated by red, induces the feminization phenotype. (C) Results of a “rescue” experiment. RNAi of either *tra-1* or *tra-2* rescued the sterile phenotype of *rpn-10* (*tm1349*) to produce normal viable F2 embryos. Control vector RNAi did not influence the sterile phenotype. (D) Oocyte maturation defects of *rpn-10* (*tm1349*) can be restored by knockdown of the expression of *tra-2* and *tra-1*. Note that the expanded oocytes and empty uterus observed in *rpn-10* (*tm1349*) worms were restored in *tra-2* (RNAi) individuals. Hoechst staining of dissected gonads from *rpn-10* (*tm1349*);vector control (RNAi) and *rpn-10* (*tm1349*);*tra-2* (RNAi) worms. Restored spermatheca is indicated by a white box. Bar, 10  $\mu$ m. (E) At P0 stage, all worms were treated with first



**Figure 7.** RPN-10 acts cell-autonomously in the germline. (A) RNAi analysis under an *rrf-1 (pk1417)* genetic background, which is fully proficient for RNAi responses in the germline but not the soma. Nomarski observations indicate a typical sign of feminization of the germline in *rrf-1 (pk1417)* homozygotes that were treated with *rpn-10; ufd-2 (RNAi)*. Inset, Hoechst staining of dissected gonads show absence of sperm in the spermatheca. Bar, 10  $\mu$ m. (B) PCR verification of a double homozygote mutant, *rpn-10 (tm1349); rrf-1 (pk1417)*. (C) Results of a “rescue” experiment. RNAi of either *tra-1* or *tra-2* rescued the sterile phenotype of *rpn-10 (tm1349); rrf-1 (pk1417)* to produce viable embryos. Control vector RNAi did not influence the sterile phenotype.

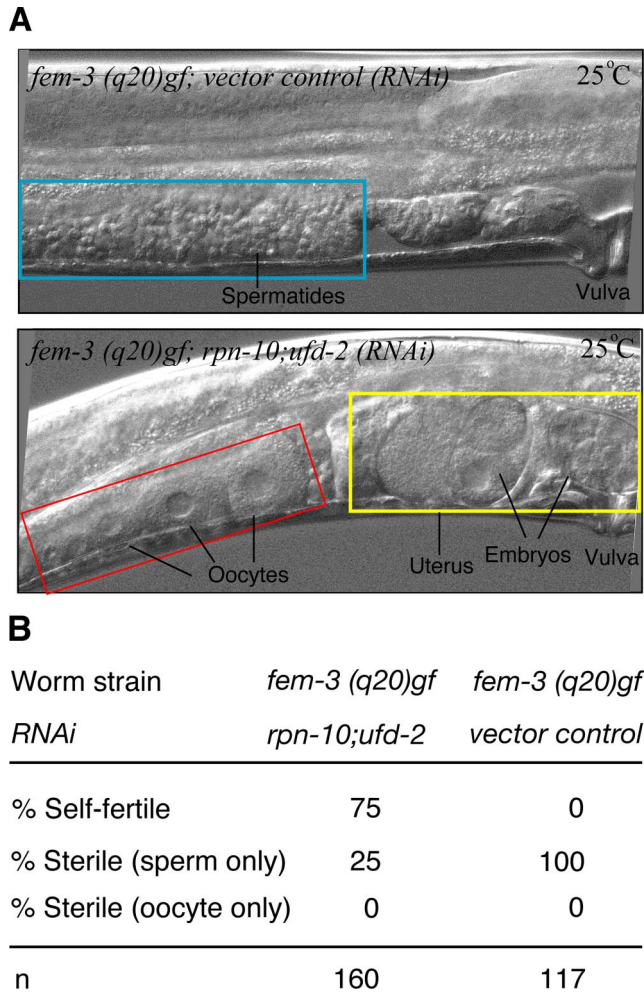
male or in any other cells of the hermaphrodite. We quantified the expression of *vit-2* transcript in individual males to determine whether knockdown of *rpn-10* influences the sexual fate of male somatic tissue. We found that *vit-2* transcript is reproducibly up-regulated in XO males treated with *rpn-10; ufd-2 (RNAi)* (Figure 9, B and C), although the amount was less than that in genuine hermaphrodites. In contrast, the expression of *msp-77*, a sperm marker, is down-regulated in these XO males (Figure 9B). We could not detect significant up-regulation of oogenesis-specific transcripts such as *rme-2* or *mex-3*. These results indicate that overall morphol-

ogy of XO males is apparently not influenced by knockdown of *rpn-10* but that it causes limited but reproducible XO intestinal feminization.

#### Defects in RPN-10 Result in Accumulation of TRA-2

To test whether RPN-10 is involved in the control of TRA-2 accumulation, we examined TRA-2 in *rpn-10*-defective worms. Semiquantitative RT-PCR analysis showed that the steady-state levels of *tra-2* transcript were similar in *rpn-10; ufd-2 (RNAi)* worms and N2 wild-type hermaphrodites (Figure 10A), indicating that the amount of *tra-2* transcript is not influenced by *rpn-10* knockdown. We then examined the accumulation of TRA-2 protein. The cellular behavior of endogenous TRA-2 protein is still enigmatic as a high titer antibody had not been available. To detect the endogenous TRA-2 ICD protein directly, we newly established an antiserum to the seventh intracellular domain of TRA-2 (TRA-2 ICD). As shown in Figure 10B, this antibody can specifically recognize TRA-2 ICD fragments expressed in mammalian cells and can detect antigens as efficiently as can high-performance anti-FLAG M2 antibody (Sigma-Aldrich). Furthermore, this antibody can detect proteins of 54 kDa as well as several lower-molecular-weight fragments in *C. elegans* ex-

**Figure 6 (cont).** RNAi (*rpn-10; ufd-2*). This procedure ensures F1 sterility even in the absence of RNAi treatment at the F1 stage. From the young adult stage of P0 to F1 larval development, individuals were treated with second RNAi with a vector control, *sdsc-1 (RNAi)*, *tra-1 (RNAi)*, and *tra-2 (RNAi)*, respectively. The resulting number of F2 progeny per an F1 parent was counted. Second RNAi of *tra-2* and *tra-1* restored sperm formation. (F) RT-PCR analysis of the sperm-specific marker *msp-77*. Expression of *msp-77* is restored in *rpn-10; ufd-2; tra-2 (RNAi)* worms but not in *rpn-10; ufd-2; vector control (RNAi)* worms. *inf-1* transcript amplified under similar conditions was used as a control.



**Figure 8.** Co-knockdown of *rpn-10* and *ufd-2* overcomes the germline-masculinizing effect of *fem-3 (gf)*. P0 larvae of *fem-3 (gf)* XX homozygous mutants were treated with either *rpn-10; ufd-2 (RNAi)* or control vector (*RNAi*) at a permissive temperature (20°C), and resulting F1 larvae were synchronized and continued to treated with respective RNAi at a restrictive temperature (25°C). Resulting F1 adult worms (55 h after hatching) were observed (A). In control vector (*RNAi*) worms, complete germline masculinization was observed, whereas >70% of *rpn-10; ufd-2 (RNAi)*-treated F1 showed mutual suppression of germline-masculinizing phenotypes of *fem-3 (gf)*, resulting in self-fertility at a restrictive temperature (25°C) (B).

tracts. These signals are specific to endogenous TRA-2 protein because *tra-2 (e1095)* putative null mutant worms show no such signals (Figure 10C). We speculate that the 54-kDa band corresponds to the endogenous TRA-2 ICD fragment (Sokol and Kuwabara, 2000). We currently have no idea about the physiological significance of smaller fragments.

To examine the cellular behavior of TRA-2 ICD protein, we performed immunocytochemical experiments. We found that the nuclei of the hermaphrodite intestine, a female somatic tissue, were clearly immunostained with this anti-TRA-2 ICD antibody (Figure 11). The nuclear staining is specific to endogenous TRA-2 protein, as *tra-2 (RNAi)* on *fem-3 (e1996lf)* females greatly reduced the intensity of nuclear staining compared with that of the *fem-3 (e1996lf)* female itself (Figure 11A). Furthermore, there are essentially no nuclear signals in *tra-2 (e1095lf)* or wild-type males, whereas nuclei of *tra-2 (e2020gf)* were stained much stronger

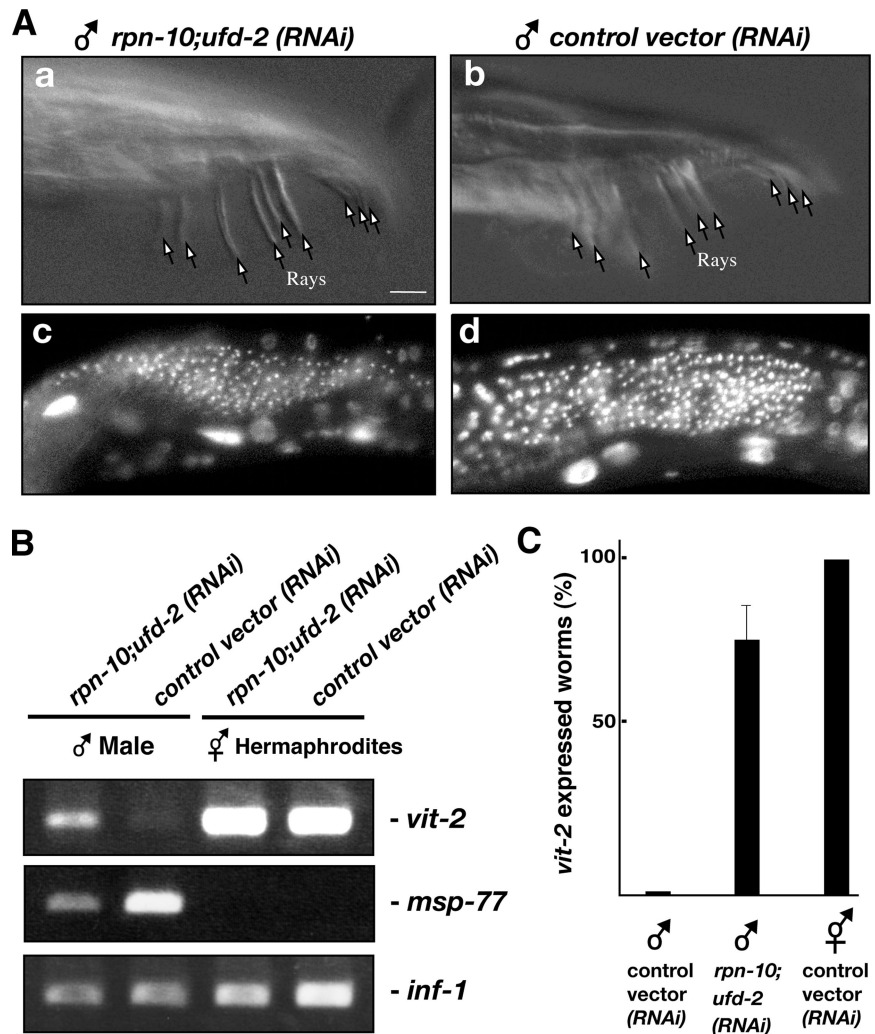
than that of wild-type (Figure 11B). For unknown reasons, immunosignals in the germline cells were difficult to detect under the experimental conditions we used. We speculate that the expression level of TRA-2 is much lower in the germline cells compared with that in intestinal cells. Thus, we currently have no immunological data of germline accumulation of TRA-2 protein.

Interestingly, we found that *rpn-10; ufd-2 (RNAi)* treatment significantly increased the nuclear staining in the intestine (Figure 11, A and B). We determined the intensities of nuclear TRA-2 signals in *fem-3 (e1996lf)* females. The quantified results show that TRA-2 signal was strengthened for ~2.8-fold in *rpn-10; ufd-2 (RNAi); fem-3 (e1996lf)* females compared with that in control females [*fem-3 (e1996lf)* with vector control (*RNAi*)]. These observations clearly support the notion that *rpn-10* regulates TRA-2 protein levels irrespective of the sex differentiation backgrounds. We confirmed that the immunosignal of TRA-2 was completely absorbed by competition with excess corresponding antigens, and preimmune serum did not stain the nuclei of the intestine from wild-type hermaphrodite and *rpn-10; ufd-2 (RNAi)* females. Finally, we found that both *rpn-10 (tm1180)* and *rpn-10 (tm1349)* show significantly increased signal of TRA-2 in the nuclei of the intestine, and these nuclear stainings were eliminated by *tra-2 (RNAi)* (Figure 12). Lum *et al.* (2000) reported that an exogenous GFP-tagged fragment of TRA-2c, overproduced from the heat shock promoter, localized predominantly to nuclei in some somatic cells, and caused completely somatic feminization of XO animals. Our observations support the idea that endogenous TRA-2 protein was overaccumulated in the nuclei of feminized cells, presumably as ICD fragments, in *rpn-10*-defective conditions and thus induces excess feminization; forced reduction of *tra-2* rescues this effect.

## DISCUSSION

In the present study, we found that RPN-10, one of the proteasomal ubiquitin receptors, is required for appropriate sex determination in *C. elegans*. Knockdown of *rpn-10* by RNAi sexually transforms F1 hermaphrodites to females by eliminating hermaphrodite spermatogenesis. The results of our analysis in *rpn-10 (RNAi)* *C. elegans* indicate that there is no detectable defect in the 26S proteasome architecture under normal growing conditions (Figure 2, D and E). In *Drosophila*, it was also reported that deletion of the subunit S5a/RPN-10/p54 does not disturb the assembly of the 26S proteasome (Szlanka *et al.*, 2002). It has been reported that, in *Drosophila*, the deletion of the *rpn10* gene caused larval-pupal lethality, multiple mitotic defects, and accumulation of polyubiquitinated proteins that were associated with increased amount of 26S proteasome subunits. In contrast to *Drosophila*, we did not detect obvious defects in the viability, life time, behavior, embryonic development, and stress tolerance in *rpn-10 (RNAi)*-treated P0 and F1 worms except for defects in F1 germ cells. Furthermore, knockdown of the expressions of any other proteasome subunits to any extent did not result in similar sterility. These observations suggest that the overall defects in the proteasome-mediated degradation pathway cannot explain the specific phenotype found in *rpn-10 (RNAi)* worms. We think that most of the ubiquitinated substrates were normally metabolized in *rpn-10*-defective worms by an alternative pathway for the polyubiquitinated substrate recognition system.

Normally, the hermaphrodite germline begins sperm formation in late L4 from a set of sperm precursor cells set aside in each half of the gonad. After a brief period of spermatogenesis,

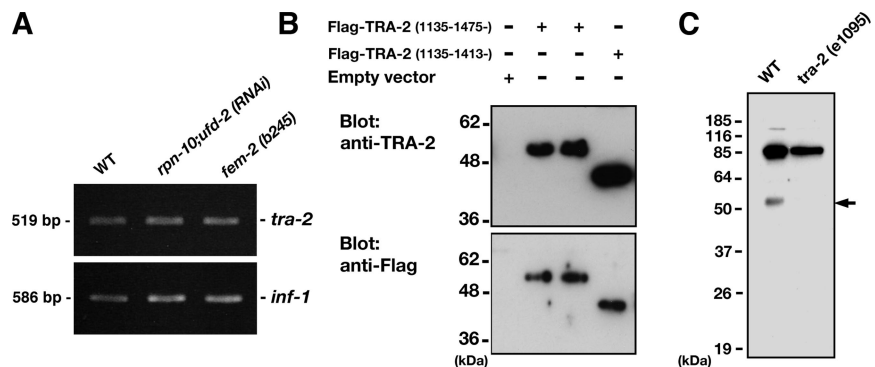


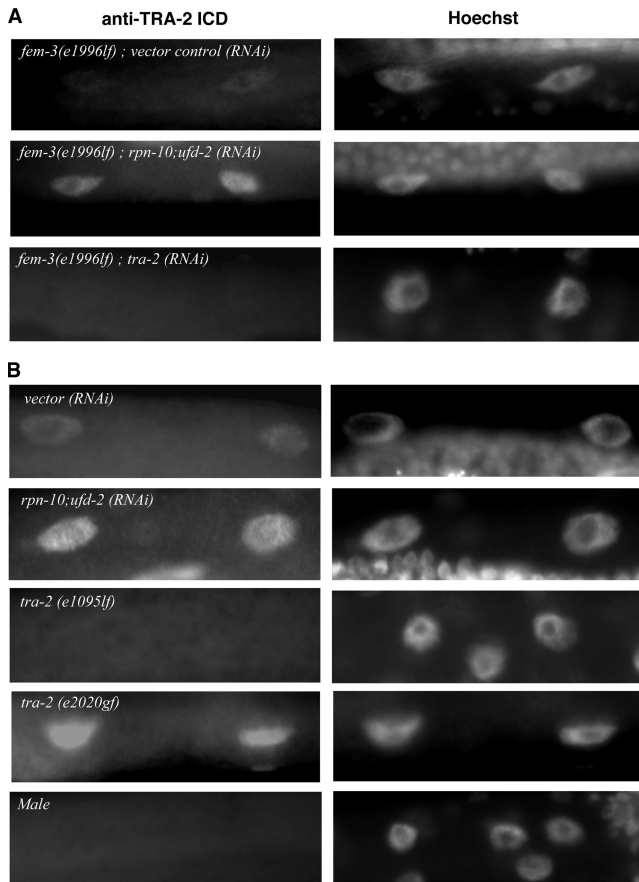
**Figure 9.** Co-knockdown of *rpn-10* and *ufd-2* cause weak XO intestinal feminization. (A) Tail morphologies of *rpn-10; ufd-2* (RNAi) and wild-type XO males (a and b). Hoechst staining of XO male gonads from *rpn-10; ufd-2* (RNAi) and wild-type worms (c and d). Bar, 10  $\mu$ m. (B) RT-PCR quantification of the somatic feminization marker *vit-2* (422 base pairs, exons 4 and 5) and the sperm-specific marker *msp-77* (360 base pairs) from a single individual worm. Note that the expression levels of *msp-77* signal in hermaphrodites were too low to detect under the conditions used in this experiment. *inf-1* transcript was used as an amplification control. (C) More than 70% individual males treated with *rpn-10; ufd-2* (RNAi) expressed increased amounts of *vit-2*, whereas no wild-type male express it.

genesis, the germline of each half gonad switches from sperm to oocyte production. Based on the phenotype of gain-of-function alleles of *tra-2*, Doniach (1986) suggested that the activity of *tra-2* is thought to be transiently repressed in the hermaphrodite germline (Doniach, 1986), thus allowing a burst of spermatogenesis to occur. In hermaphrodites,

the TRA-2 activity is controlled by two posttranscriptional pathways. One post-transcriptional control of *tra-2* activity is mediated through *tra-2* 3' UTR binding proteins, such as GLD-1, which control *tra-2* translation (Francis *et al.*, 1995; Goodwin *et al.*, 1997; Graves *et al.*, 1999; Jan *et al.*, 1999). The other is involved in regulation of TRA-2 activity by a post-

**Figure 10.** Establish of anti-TRA-2 ICD antibody. (A) RT-PCR-based expression analysis of *tra-2* transcript (top; 519 base pairs, exons 22 and 23). *inf-1* transcript (586 base pairs, exons 3–5) amplified under similar conditions was used as a control (bottom). (B) FLAG-tagged TRA-2 protein (fragments of amino acids 1135–1475 and 1135–1413, respectively) was expressed in COS7 cells, and the extracts were blotted with either an antibody against the C-terminal fragment (ICD) of TRA-2 or anti-FLAG M2 antibody (Sigma-Aldrich). (C) Fifty individual animals showing an appropriate phenotype were picked up and boiled with SDS sample buffer and then subjected to immunoblot experiments. The anti-TRA-2 ICD antibody can detect the endogenous 54-kDa band (indicated by an arrow) as well as several lower-molecular-weight fragments in the extracts. A *tra-2* (*e1095*) putative null mutant was used as a negative control. Note that the 85-kDa signal is not specific because preimmune control serum stained the same band as well.

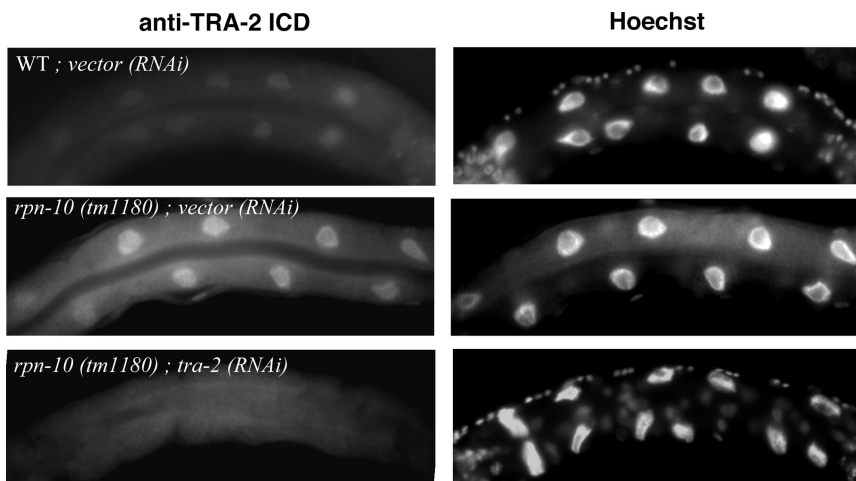




**Figure 11.** TRA-2 protein accumulates in RPN-10-defective worms. (A) *fem-3* (*e1996lf*) is a putative null allele and developed as a true female regardless of whether *tra-2* gene was knocked down or not. Even in a background of *fem-3* (*e1996lf*), *rpn-10;ufd-2* (*RNAi*) enhanced the nuclear accumulation of TRA-2, whereas *tra-2* (*RNAi*) completely remove the staining. (B) The intestinal nuclei of *rpn-10;ufd-2* (*RNAi*)-treated worms as well as gain-of-function mutant *tra-2* (*e2020*) were stained more strongly than those of wild-type worms (vector *RNAi* control), whereas nuclei of loss-of-function mutant *tra-2* (*e1095*) and wild-type male were scarcely immunostained with anti-TRA-2 ICD antibody. Note that all photographs were taken with identical exposure time. Bar, 10  $\mu$ m.

translational mechanism, identified as *tra-2* (*mx*) (*mixed* character) mutations (Lum *et al.*, 2000; Wang and Kimble, 2001). The regulation of TRA-2 pathway at the proteolytic level might allow further precise control of protein dosage. In this report, we suggest an additional layer of precise control over the activity of TRA-2 in XX hermaphrodites mediated by the RPN-10-dependent ubiquitin pathway.

Loss-of-function alleles of *fog-2* and *rpn-10;ufd-2* (*RNAi*) have similar feminization effects on the sexual phenotype of the hermaphrodite germline. The fact that both *rpn-10;ufd-2* (*RNAi*), *fog-2* (*lf*) and *tra-2* (*gf*) suppress *fem-3* (*gf*) to generate self-fertile hermaphrodites (Barton *et al.*, 1987; Schedl and Kimble, 1988; this study) implies a common molecular pathway in these gene products. We also found that knockdown of *rpn-10;ufd-2* results in potential somatic feminization in XO males. It was reported that overproduction of the intracellular domain of TRA-2 induced the expression of a vitellogenin reporter gene (*vit-2::gfp*) in transgenic XO animals (Lum *et al.*, 2000). Although we found that *vit-2* expression increased in *rpn-10;ufd-2* (*RNAi*) XO animals, we also found that no increase in *vit-2* expression was evident in *tra-2* (*e1095*) null animals after *rpn-10;ufd-2* (*RNAi*) treatment. Therefore, somatic feminizing activity induced by *rpn-10* knockdown, although weak, was dependent on *tra-2* activity. In addition, in XX hermaphrodite, the feminization phenotype of *rpn-10* (*tm1349*) is rescued by knockdown of *tra-2* or its downstream target *tra-1*, indicating that the TRA-2-mediated sex determination pathway is crucial for the *rpn-10* (*tm1349*)-induced sterile phenotype. In accordance with this assumption, we detected the accumulation of TRA-2 by its specific antibody in *rpn-10;ufd-2* (*RNAi*) worms as well as *rpn-10*-defective mutants. These observations are consistent with the view that the primary defect induced by RPN-10 removal is dysregulation in the TRA-2-mediated sex determination pathway; In larvae of hermaphrodite, timely inactivation of TRA-2 protein would lead to a lower TRA-2/FEM-3 ratio, resulting in transient spermatogenesis, whereas compromise of *rpn-10* activity might abolish the appropriate down-regulation of TRA-2 and thus lead to feminization. Because of the intricacies of the germline sex determination pathway in *C. elegans*, the possibility that *rpn-10* regulates another factor(s) in addition to TRA-2 remains. Among various known negative regulators of *tra-2*, *fog-2* and *rpn-10*



**Figure 12.** *rpn-10* mutant worm *tm1180* shows significantly increased immunosignal of TRA-2 in nuclei of the intestine. The nuclear staining was eliminated by potent *tra-2* (*RNAi*) treatment. Note that worms used in this experiment were fixed at the late larval stage (L4 to young adult). All photographs were taken with identical exposure time. Bar, 10  $\mu$ m.

defects have a phenotype similar to that of *tra-2(gf)* mutants: XX mutants are transformed into females, and XO mutants are apparently normal males (Schedl and Kimble, 1988; this study). In contrast, the other *tra-2* repressor has distinct phenotypes; *her-1* functions only in XO males (Trent *et al.*, 1991). These findings indicate that the RPN-10–dependent ubiquitin pathway plays a role mainly in the onset of transient spermatogenesis to modulate sex-specific germ cell differentiation in hermaphrodites.

In yeast, it was recently proposed that there are at least two independent but closely related substrate delivery routes to the 26S proteasome: one route is the Rpn10p pathway that is more biased toward recognition of heavily ubiquitinated proteins, and the other route is the Ufd2p-Cdc48p-Rad23p(Dsk2p) route, which works through generation and recognition of proteins with a smaller number of ubiquitin molecules (Bazirgan and Hampton, 2005; Richly *et al.*, 2005). Cdc48 seems to be particularly adapted to act on membrane proteins. For example, Cdc48 is crucial for the degradation of ER membrane proteins, because it seems to extract these proteins from the membrane after their ubiquitination and before their encounter with the proteasome (Elsasser and Finley, 2005). The lack of an overhanging structure, so-called Lid, may allow Cdc48 to unfold bulky ubiquitinated proteins more readily, converting them into a state that is amenable for degradation by the proteasome. Many proteins that require Cdc48 to be degraded also require Ufd2 and Rad23 (or Dsk2, a Rad23 paralogue), suggesting that Rad23 links ubiquitinated targets of Ufd2 to the proteasome.

In this pathway, Rad23 binding to Ufd2 is critical for delivering targets from their site of ubiquitination to the proteasome. The transcription factor Spt23, many ERAD substrates, and the model substrate Ub-Pro- $\beta$ -Gal all seem to be degraded by this pathway (Chen and Madura, 2002; Richly *et al.*, 2005). In *C. elegans*, it has also been reported that UFD-2 and CHN-1 directly bind to UNC-45 and regulate UNC-45 protein level (Hoppe *et al.*, 2004). In this study, we found that impairment of RPN-10 specifically induces F1 sterility, whereas knockdown of either *ufd-2*, *rad-23*, or *dsk-2* or any combination of these genes did not, indicating for the first time the biological relevance of RPN-10 in *C. elegans*, and the RPN-10–mediated ubiquitin pathway is a major route for controlling sex determination. The fact that codepletion of *ufd-2* with *rpn-10* is required for the most penetrant effect suggests that UFD-2 (with RAD-23 and/or DSK-2) possesses a function partly overlapping the RPN-10–mediated route. It should be noted that even in the condition of *rpn-10;ufd-2 (RNAi)* (efficacy verified by RT-PCR and Western blot), there are no apparent defects in the P0 or F1 worm except for strictly restricted F1 reproductive abnormality. It could be explained that sex-determining pathway are highly sensitive to the partial retardation of ubiquitin–proteasome pathway, but this possibility seems unlikely as global and manifold abnormalities were induced by only a partial retardation of general 26S proteasome activity without preceded abnormality in reproduction (Figure 1 and Supplemental Tables SI and SII). We suggest the possible existence of alternative, as yet unidentified ubiquitinated substrate delivery route(s) to the 26S proteasome in *C. elegans* that is functionally distinct to the RPN-10–mediated ubiquitin recognition. This notion gives rise the interesting possibility that, in the ubiquitin-dependent pathway of higher eukaryotes, specificity of substrate discrimination is achieved not only by well-appreciated E3 ubiquitin–ligase diversity but also by an additional layer of the machineries that recruit the specific substrates to the proteasome by

multiple ubiquitin receptors. It should be noted that knock-down of expression of RAD-23 and DSK-2, the other nonessential proteasomal ubiquitin receptors, did not induce reproductive defects, indicating that the induced sterility was specific to knockdown of *rpn-10*. These findings support the previously reported notion that the UFD pathway substrate Ub-Pro- $\beta$ gal is remarkably stabilized in Rpn10p-deleted yeast, although the half-life of many short-lived proteins is unaffected (van Nocker *et al.*, 1996; Fu *et al.*, 1998). Interesting challenges for the future will be to determine the number of existing receptor pathways, sort out the mechanisms underlying the allocation of substrates to different receptor pathways, and determine whether individual receptor pathways are differentially regulated to modulate the repertoire of proteins degraded by the ubiquitin–proteasome system in response to specific cells. Accordingly, clarification of the substrate–recognition diversity by the RPN-10 family and other ubiquitin–recognition proteins is the future prospect.

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