Caenorhabditis elegans p97 controls germline-specific sex determination by controlling the TRA-1 level in a CUL-2-dependent manner

Yohei Sasagawa[1,*,‡], Mieko Otani[2], Nahoko Higashitani[3], Atsushi Higashitani[3], Ken Sato[4], Teru Ogura[1] and Kunitoshi Yamanaka[1,*,§]

[1]Department of Molecular Cell Biology, Institute of Molecular Embryology and Genetics, Kumamoto University, 2-2-1 Honjo, Kumamoto 860-0811, Japan
[2]Faculty of Pharmaceutical Sciences, Kobe Gakuin University, 1-1-3 Minatojima, Chuo-ku, Kobe 650-8586, Japan
[3]The Graduate School of Life Sciences, Tohoku University, 2-1-1 Katahira, Aoba, Sendai 980-8577, Japan
[4]Laboratory of Molecular Traffic, Institute for Molecular and Cellular Regulation, Gunma University, 3-39-15 Showa-cho, Maebashi 371-8512, Japan
*These authors contributed equally to this work
‡Present address: Laboratory for Developmental Genomics, RIKEN Center for Developmental Biology, 2-2-3 Minatojima-minamimachi, Chuo-ku, Kobe 650-0047, Japan
§Author for correspondence (yamanaka@gpo.kumamoto-u.ac.jp)

Accepted 29 July 2009
Journal of Cell Science 122, 3663-3672 Published by The Company of Biologists 2009
doi:10.1242/jcs.052415

Summary

p97 (CDC-48 in *Caenorhabditis elegans*) is a ubiquitin-selective AAA (ATPases associated with diverse cellular activities) chaperone and its key function is to disassemble protein complexes. p97 functions in diverse cellular processes including endoplasmic reticulum (ER)-associated degradation, membrane fusion, and meiotic and mitotic progression. However, its cellular functions in development have not yet been clarified. Here, we present data that p97 is involved in the switch from spermatogenesis to oogenesis in the germline of the *C. elegans* hermaphrodite. We found that the *cdc-48.1* deletion mutant produced less sperm than the wild type and thus showed a decreased brood size. The *cdc-48.1* mutation suppressed the sperm-overproducing phenotypes of *fbf-1* and *fem-3*(*gf*) mutants. In addition, the p97/CDC-48–UFD-1–NPL-4 complex interacted with the E3 ubiquitin ligase CUL-2 complex via NPL-4 binding to Elongin C. Furthermore, TRA-1A, which is the terminal effector of the sex determination pathway and is regulated by CUL-2-mediated proteolysis, accumulated in the *cdc-48.1* mutant. Proteasome activity was also required for the brood size determination and sperm-oocyte switch. Our results demonstrate that the *C. elegans* p97/CDC-48–UFD-1–NPL-4 complex controls the sperm-oocyte switch by regulating CUL-2-mediated TRA-1A proteasome degradation.

Key words: AAA family, *C. elegans*, p97/Cdc48p, Sex determination, TRA-1, Ubiquitin ligase

Introduction

Caenorhabditis elegans occurs naturally in two sexes, self-fertilizing hermaphrodites and males. Males have a male soma and a germline that produces sperm continuously from the fourth larval stage (L4) throughout adulthood. By contrast, the hermaphrodite soma is female; its germline produces an average of 300 sperm during the L4 stage and then produces oocytes continuously throughout adulthood. The switching mechanism from spermatogenesis to oogenesis (i.e. the sex determination mechanism) involves many negative regulatory factors and is highly complex (Ellis and Schedl, 2006; Kimble and Crittenden, 2007). A regulatory hierarchy controls the activity of the master sexual regulator TRA-1 (Hodgkin, 1987; Zarkower and Hodgkin, 1992) (Fig. 3A). In the hermaphrodite germline, *tra-1* would initially be switched off (spermatogenesis) but, later in development, would be switched on (oogenesis). *tra-1* encodes two protein: TRA-1A (1109 amino acids, 135 kDa), which contains five zinc fingers and TRA-1B (287 amino acids, 37 kDa), which contains only the first two zinc fingers (Zarkower and Hodgkin, 1992; Zarkower and Hodgkin, 1993). TRA-1A belongs to the GLI family of transcription factors including mammalian proteins GLI and GLI3 and the *Drosophila* protein Cubitus interruptus (Ci). TRA-1A, but not TRA-1B, binds DNA in vitro and functions as a transcription regulator for *egl-1, mab-3,* and *fog-3* gene expression (Zarkower and Hodgkin, 1993; Conradt and Horvitz, 1999; Chen and Ellis, 2000; Yi et al., 2000).

Three *fem* genes located immediately upstream of TRA-1 in the genetic sex determination pathway promote the male fate by inhibiting TRA-1. FEM-1 contains Ankyrin repeats but its biochemical activity remains unknown (Spence et al., 1990); FEM-2 is a Type 2C protein Ser/Thr phosphatase (Pilgrim et al., 1995; Chin-Sang and Spence, 1996); and FEM-3 is a novel protein with no obvious motifs (Ahringer et al., 1992). Based on the fact that the loss of any of the FEM proteins causes TRA-1A accumulation, it was proposed that the role of FEM proteins in sex determination might be to stimulate TRA-1A degradation (Schvarzstein and Spence, 2006). Recently, it has been demonstrated that TRA-1 is regulated by ubiquitin-proteasome degradation mediated by a CUL-2 complex containing FEM proteins as a substrate recognition receptor (Starostina et al., 2007). CUL-2 belongs to the cullin family of E3 ubiquitin ligases and CUL-2 complexes consist of the following: CUL-2, which forms a rigid scaffold; the RBX-1 RING-H2 finger subunit that binds to the C-terminus of CUL-2; the adaptor protein ELC-1/Elongin-C, which binds to the N-terminus of CUL-2 and to the ubiquitin-like protein ELB-1/Elongin-B; and a variable substrate-recognition receptor that binds to ELC-1/Elongin-C (Kipreos, 2005; Petroski and Deshaies, 2005).

FEM-1 and FEM-3 proteins are targeted by the F-box protein SEL-10 and are proteolytically regulated (Jager et al., 2004). In addition, *fem-3* is regulated post-transcriptionally by FBF-1 and FBF-2 (*fem-3* mRNA binding factors), which are nearly identical regulators of the PUF (Pumilio and FBF) protein family (Zhang et al., 1997). Since *fbf-1 fbf-2* double mutants fail to switch from spermatogenesis to oogenesis, it has been proposed that FBF-1 and FBF-2 function redundantly to promote the sperm-oocyte switch (Zhang et al., 1997). As described above, many factors involved in this switch have been identified, and their genetic interactions have been well documented. However, the molecular mechanisms of regulation of their amounts and/or activities remain poorly understood.

p97 ATPase – also called valosin-containing protein (VCP), Cdc48p in yeast, or CDC-48 in *C. elegans* – is one of the best-characterized examples among AAA (ATPases associated with diverse cellular activities) family proteins (Patel and Latterich, 1998; Ogura and Wilkinson, 2001). It has been suggested that p97 is a ubiquitin-selective chaperone and that its key function is to disassemble protein complexes (Rape et al., 2001). p97 has important roles in ER-associated degradation (ERAD), membrane fusion, activation of transcription factors, spindle disassembly, DNA replication and other protein quality-control activities (Wang et al., 2004; Uchiyama and Kondo, 2005; Ye, 2006). Functional diversity of p97 is mainly determined by differential binding of distinct cofactors or adaptor proteins (Ye, 2006; Schuberth and Buchberger, 2008). It has also been demonstrated that the interaction between p97 and the E3 ubiquitin ligases gp78 and Hrd1 has an important role in ERAD (Zhong et al., 2004; Lilley and Ploegh, 2005; Ye et al., 2005).

p97 is encoded by a single gene in humans, mice and yeast, and is highly conserved. By contrast, *C. elegans* possesses two genes – *cdc-48.1*(*C06A1.1*) and *cdc-48.2*(*C41C4.8*) – encoding p97 with 88% identity over the entire protein (Yamanaka et al., 2004). We have previously reported that both homologues are essential in *C. elegans* and that their cellular functions are redundant (Yamanaka et al., 2004; Sasagawa et al., 2007b; Sasagawa et al., 2007c). Here, we report that the *cdc-48.1* deletion decreases the brood size to half, which results from decreased sperm production. Epistasis experiments, protein-protein interaction assays, and TRA-1A quantification revealed that CDC-48.1 interacts with CUL-2 complexes and is involved in the regulation of TRA-1A levels. We propose that *C. elegans* p97/CDC-48 has an important role in the switching processes from spermatogenesis to oogenesis.

Results

Reduced brood size in *cdc-48.1* mutant worms

We have previously reported that *C. elegans* possesses two p97 homologues, namely, CDC-48.1 and CDC-48.2, which are essential for embryogenesis, meiosis, ERAD and germline development (Yamanaka et al., 2004; Sasagawa et al., 2007b; Sasagawa et al., 2007c). The protein levels and expression patterns of these two p97 homologues are differentially regulated (Yamauchi et al., 2006). To elucidate the cellular functions of the two p97 homologues in more detail, we characterized the deletion mutants *cdc-48.1(tm544)* and *cdc-48.2(tm659)*, which have 668 bp and 639 bp deletions, respectively (Sasagawa et al., 2007c). Although the *cdc-48.1(tm544)* mutant grew slightly slower than the wild type and the *cdc-48.2(tm659)* mutant showed only a small delay, both mutant worms grew to adult without embryonic or larval lethal phenotypes. Morphology, motility, defecation cycles and sexual behavior of hermaphrodites and males were not affected by the deletion mutations (data not shown).

Interestingly, however, we found that the *cdc-48.1(tm544)* mutant produced less progeny than the wild type, which produces approximately 300 progeny on average (Fig. 1). Wild-type, *cdc-48.1(tm544)* and *cdc-48.2(tm659)* worms were synchronized and incubated from early L4 stage. At 24, 36, 48 and 60 hour time points, P0 worms were transferred to new plates, and eggs laid from these worms during these periods were counted. The total number of fertilized eggs laid from *cdc-48.1(tm544)* worms was 47% of that from wild-type and *cdc-48.2(tm659)* worms (Fig. 1B). Importantly, during the first 24 hours, *cdc-48.1(tm544)* worms produced almost the same number of fertilized eggs as wild-type and *cdc-48.2(tm659)* worms; however, in the later periods they produced progressively fewer fertilized eggs and finally did not produce any eggs at all after 48 hours (Fig. 1B). By contrast, wild-type and *cdc-48.2(tm659)* worms continued to produce fertilized eggs up to 72 hours. It should be mentioned that germline and germ cells, which were dissected and stained with DAPI, of *cdc-48.1(tm544)* worms were indistinguishable from those of wild-type worms (see DAPI-stained images in Fig. 1D and Fig. 3B). Furthermore, the *cdc-48.1(tm544)* mutant laid a similar number of eggs as the wild type overall; however, half were non-fertilized. These results suggest that the *cdc-48.1(tm544)* mutation does not affect germline formation and oocyte production in hermaphrodites.

To demonstrate that the reduced brood size is caused by the depletion of CDC-48.1, we first prepared transgenic worms expressing a FLAG::CDC-48.1 fusion protein driven by the authentic *cdc-48.1* promoter, and then transferred the *cdc-48.1(tm544)* mutation into the transgenic strain, yielding XA7203 (*unc-119(ed3)*; *cdc-48.1(tm544) qaIs7201*[*Pcdc-48.1*::FLAG::CDC-48.1, *unc-119(+)*]). As shown in Fig. 1C, the FLAG::CDC-48.1 fusion protein suppressed the reduction of brood size due to the *cdc-48.1(tm544)* mutation. In addition, RNA interference [*cdc-48.1(RNAi)*] of XA7203 worms partially, but significantly, cancelled this suppression effect of the FLAG::CDC-48.1 fusion protein (Fig. 1C). Furthermore, when *cdc-48.2(RNAi)* was applied to the XA7203 worms, the embryonic lethal phenotype caused by *cdc-48.1(tm544);cdc-48.2(RNAi)* was completely rescued by the FLAG::CDC-48.1 fusion protein. These results unambiguously demonstrate that the FLAG::CDC-48.1 fusion protein is fully functional and that the decreased brood size is caused by the *cdc-48.1(tm544)* mutation.

C. elegans hermaphrodites, in which sperm have been exhausted, can still produce progeny, if they are crossed with males, and sperm are supplied. To determine whether the decreased brood size in the *cdc-48.1(tm544)* mutant is due to an abnormality in oocytes or sperm, we performed mating experiments with normal males and mutant hermaphrodites. When *cdc-48.1(tm544)* mutant worms were co-cultivated with wild-type males, they produced a similar number of progeny as the wild type did, and almost all eggs laid were fertilized (wild type, 229.8±50.0, n=6; *cdc-48.1(tm544)*, 210.8±41.6, n=6). As described above, in the *cdc-48.1(tm544)* mutant, an embryonic-lethal phenotype and developmental defects were not observed except for the slight reduction in growth rate. Taken together, these results clearly indicate that oocytes of the *cdc-48.1(tm544)* mutant do not have a functional defect.

When experiments were performed at 20°C, only the *cdc-48.1(tm544)* mutant showed the defect in brood size described above. However, at 25°C, the brood size of *cdc-48.1(tm544)* (66.4±30.8, n=11) and *cdc-48.2(tm659)* (134.7±24.7, n=11) mutants decreased to 34% and 70% of the wild type (193.0±49.3, n=11), respectively. It should be mentioned that CDC-48.1 and CDC-48.2

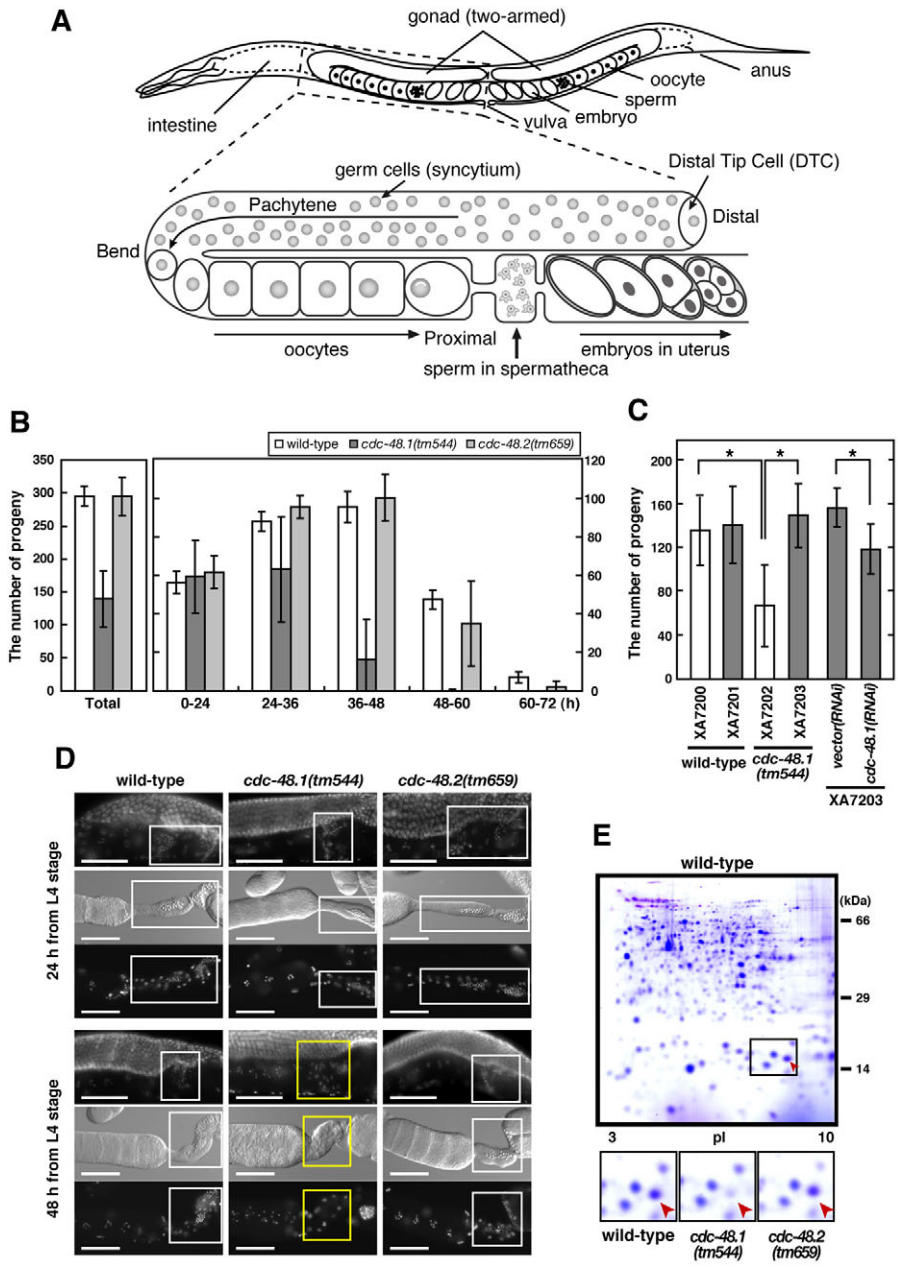

Fig. 1. Reduced progeny number in *cdc-48.1(tm544)* mutants results from lower sperm production. (A) Schematic drawing of *C. elegans* hermaphrodite. (B) The *cdc-48.1(tm544)* mutant shows a small brood size phenotype. N2 wild-type, *cdc-48.1(tm544)* and *cdc-48.2(tm659)* worms were synchronized and incubated from early L4 stage. F1 eggs laid from these worms were pooled and counted. Results are mean ± s.d. of at least ten animals. (C) Suppression of the reduced brood size by the expression of FLAG::CDC-48.1. Grey bars represent FLAG::CDC-48.1-expressing strains. **P*<0.001 (Student's *t*-test). Results are mean ± s.d. of at least ten animals. (D) Deletion of CDC-48.1 causes early exhaustion of sperm in hermaphrodites. Typical images are shown (frequency: 10/10). Wild-type, *cdc-48.1(tm544)* and *cdc-48.2(tm659)* worms were fixed and stained with DAPI after incubation for 24 or 48 hours from early L4 stage (upper rows). Each germline containing spermathecae was dissected on a poly-L-lysine-coated glass slide and stained with DAPI (middle rows, DIC; lower rows, DAPI). White and yellow boxed areas represent hermaphrodite spermathecae containing sperm or no sperm, respectively. Scale bars: 50 µm. (E) Top panel shows 2D gel electrophoresis of the total protein extract of wild-type worm 48 hours after early L4 stage. Boxed regions of wild-type, *cdc-48.1(tm544)* and *cdc-48.2(tm659)* extracts are shown enlarged in the bottom panels. Arrowheads indicate major sperm protein (MSP).

are highly homologous (Yamanaka et al., 2004) and that the amount of CDC-48.1 is almost double that of CDC-48.2 in *C. elegans* (Yamauchi et al., 2006). Therefore, the *cdc-48.2(tm659)* mutant would still contain enough CDC-48/p97 to maintain the normal brood size at 20°C. This also implies that the decreased brood size is not specific to the *cdc-48.1(tm544)* mutant, but common for *cdc-48.1(tm544)* and *cdc-48.2(tm659)* mutants.

cdc-48.1 mutant makes fewer sperm

Our results raised the possibility that the *cdc-48.1(tm544)* mutant produces fewer sperm or functionally defective sperm. *C. elegans* hermaphrodites produce sperm only in the early L4 stage and store them in the spermathecae to be used later to fertilize oocytes produced in the same germline after spermatogenesis is completed (Kimble and Crittenden, 2007). Therefore, to clarify whether the *cdc-48.1(tm544)* mutant produces fewer sperm or not, we observed the spermathecae of worms from the synchronized culture at the same intervals as in Fig. 1B. As shown in Fig. 1D, the *cdc-48.1(tm544)* mutant had a significant number of sperm until 24 hours from the early L4 stage, but sperm were almost exhausted by 48 hours. In addition, oocytes became stacked in the proximal gonad in *cdc-48.1(tm544)* worms at 48 hours (Fig. 1D), which is a characteristic phenotype of spermless worms. By contrast, wild-type and *cdc-48.2(tm659)* mutants had sperm even at 48 hours. We then counted the number of sperm in DAPI-stained young adults that contained oocytes, but no embryos. Single arms of gonads from wild-type, *cdc-48.1(tm544)* and *cdc-48.2(tm659)* worms contained an average number of sperm of 151 (range, 120-199; *n*=7), 96 (75-113; *n*=14) and 148 (98-186; *n*=6), respectively. These results clearly indicate that the *cdc-48.1(tm544)* mutant produces fewer sperm. To further confirm this, we resolved total lysates of worms on 2D PAGE gels and identified the spot corresponding to the major sperm protein (MSP), a sperm-specific protein, by MALDI-TOF analysis. As clearly shown in Fig. 1E, adult worms of *cdc-48.1(tm544)* contained

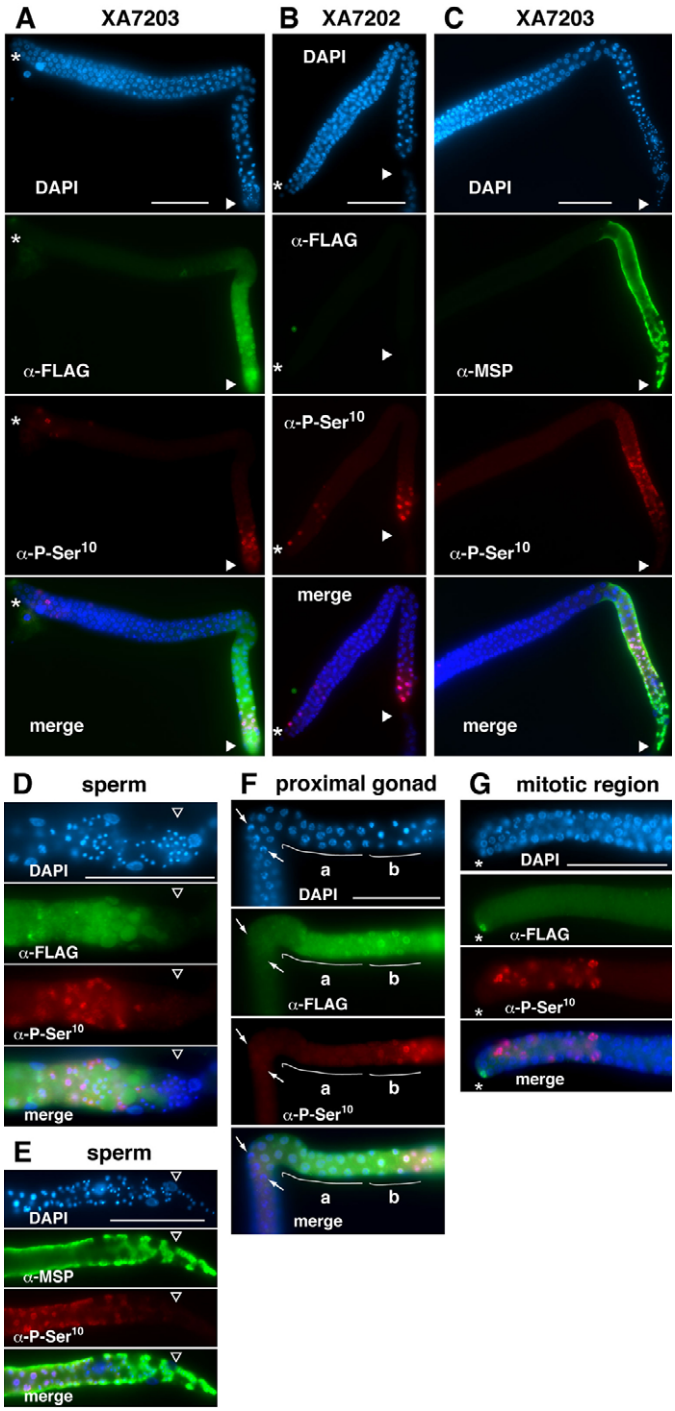

Fig. 2. Localization of FLAG::CDC-48.1 in the germline. Gonads were dissected from the early L4 stage (spermatogenesis) of the FLAG::CDC-48.1-expressing line XA7203 (A,C,D,E,F,G) and the control XA7202 (B). (A,B,D,F,G) Fixed gonads stained with DAPI (blue), anti-FLAG antibody (green) and anti-histone H3 phospho-Ser10 antibody (red). (C, E) Fixed gonads stained with DAPI (blue), anti-MSP antibody (green) and anti-histone H3 phospho-Ser^{10} antibody (red). Phosphorylation of histone H3 at Ser10 was used as an M-phase specific marker, and is detected in spermatocytes undergoing mitosis and mitotic nuclei (A-G), but not in sperm (D,E). MSP localizes to spermatogenic germ cells and sperm. FLAG::CDC-48.1 does not localize to sperm, which have no signal for phosphorylation of histone H3 at Ser10. Asterisks and arrowheads indicate distal tip cell and proximal gonad, respectively. Open arrowheads indicate sperm. Arrows indicate zygotene nuclei. Lines 'a' and 'b' show pachytene nuclei and spermatocytes, respectively. Scale bars: 50 μm.

only a trace amount of MSP compared with wild-type and *cdc-48.2(tm659)* worms. This is fully consistent with the results that sperm in *cdc-48.1(tm544)* worms were exhausted earlier, as shown in Fig. 1D. These results clearly demonstrate that the *cdc-48.1(tm544)* mutant produces fewer sperm than wild-type worms.

We observed the expression pattern of FLAG::CDC-48.1 in the early L4 stage, during which sperm are produced. FLAG::CDC-48.1 was expressed from the bend to the proximal gonad (Fig. 1A; Fig. 2A), although it was predominantly expressed at the pachytene stage and in spermatocytes (Fig. 2F). No FLAG signal was observed in control XA7202, which expressed no FLAG-tagged protein (Fig. 2B). The expression pattern of MSP, which is a spermatogenesis marker, was similar to that of FLAG::CDC-48.1 (Fig. 2C, compare with 2A). These results suggest that CDC-48.1 is required for the commitment to sperm production and are consistent with the above-mentioned results that the *cdc-48.1(tm544)* mutant produces fewer sperm. However, MSP was also localized in sperm, which have no signal of phosphorylation of histone H3 at Ser10 (Fig. 2E), whereas FLAG::CDC-48.1 was not localized in sperm (Fig. 2D). These results also indicate that CDC-48.1 is not involved in sperm maintenance. It should also be noted that FLAG::CDC-48.1 was expressed in the distal tip cell in the mitotic region (Fig. 2G, asterisk).

Next, we determined whether *cdc-48.1(tm544)* mutant males have defects in sperm formation or not. Males of the *cdc-48.1(tm544)* mutant were generated at a similar frequency to those of the wild type and *cdc-48.2(tm659)* mutant. Gonad structure of mutant males stained with DAPI appeared to be unaffected by the mutation and a significant number of sperm were observed, which were indistinguishable from wild-type worms (data not shown), suggesting that the processes of sperm formation in males are not affected by the *cdc-48.1(tm544)* mutation. Furthermore, mutant males were able to mate normally with hermaphrodites and the mutation was indeed transferred, implying that the sexual behavior of the *cdc-48.1(tm544)* mutant and the motility and fertility of their sperm are not defective. Given that oocytes and sperm themselves are not functionally impaired by the *cdc-48.1(tm544)* mutation and that the sperm of *cdc-48.1(tm544)* worms are exhausted earlier than those of wild-type worms, it appears likely that the switch from spermatogenesis to oogenesis (the sperm-oocyte switch) in the germline of *cdc-48.1(tm544)* hermaphrodites takes place at an earlier stage than in the wild type.

To confirm this idea, we analyzed expression of RME-2, which is an oogenesis marker (Grant and Hirsh, 1999), for 12 hours after L4 entry. We counted the numbers of RME-2-positive gonads and MSP-positive gonads at each time point (Fig. 3A). Typical gonads at each time point are shown in Fig. 3B. At 0 hour from L4 entry, no worm expressed RME-2 in gonads, but all worms expressed MSP as expected. In the wild type at 7 hours from L4 entry, only 6.3% of gonads faintly expressed RME-2 (type I expression). By contrast, in *cdc-48.1(tm544)* at the same time, 27% of gonads faintly expressed RME-2 (type I) and 48.6% of gonads expressed RME-2 in masses (type II expression). At 12 hours from L4 entry, all worms expressed RME-2 in gonads. These results strongly indicate that the sperm-oocyte switch in *cdc-48.1(tm544)* hermaphrodites takes place at an earlier stage than in the wild type.

The sperm-oocyte switch

To investigate whether CDC-48.1 is involved in the known sperm-oocyte switching process (Fig. 4A) or in a novel process, we

A

	RME-2 positive		MSP positive	
	N2 wild-type	*cdc-48.1(tm544)*	N2 wild-type	*cdc-48.1(tm544)*
0 hour from L4 entry	0/12	0/15	12/12	15/15
7 hours from L4 entry	2/32	28/37	32/32	37/37
12 hours from L4 entry	15/15	14/14	15/15	14/14

B

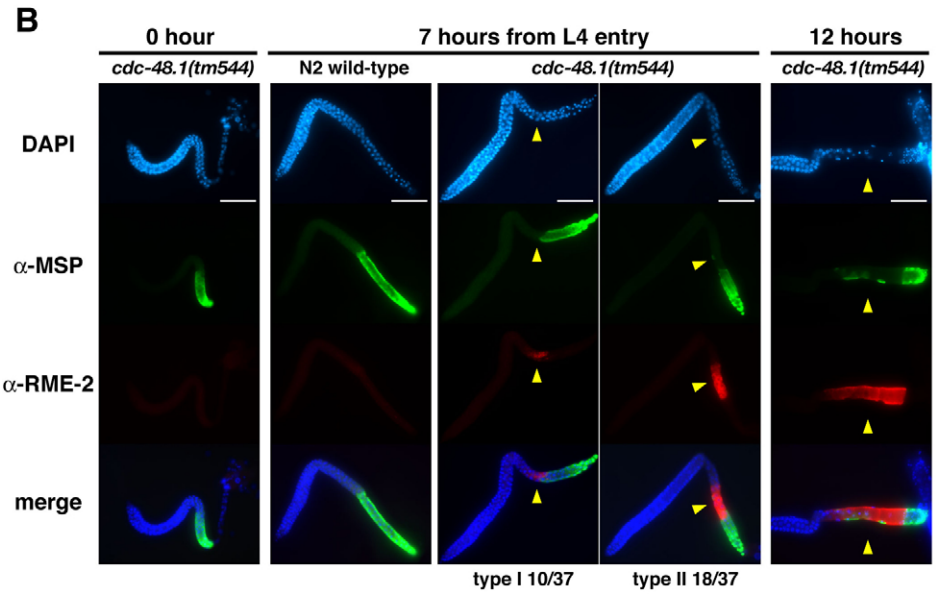

Fig. 3. The sperm-oocyte switch in *cdc-48.1(tm544)* hermaphrodites takes place at an earlier stage than in the wild type. Worms were first synchronized at the L3 stage, and incubated for several hours until they grew up to the L4 stage. Worms were analyzed from L4 entry for 12 hours. Gonads were dissected, fixed and stained with DAPI (blue), anti-MSP antibody (green) and anti-RME-2 antibody (red) at 0, 7 and 12 hours. RME-2 was used as an oogenesis marker (Grant and Hirsh, 1999). The numbers of RME-2-positive gonads and MSP-positive gonads were counted at each time point (A). Typical gonads at each time point are shown in (B). At 0 hour from L4 entry, no worm expressed RME-2 in its gonads. In the wild type at 7 hours from L4 entry, only 6.3% of gonads faintly expressed RME-2 (type I expression). In *cdc-48.1(tm544)* at 7 hours from L4 stage, 27% of gonads faintly expressed RME-2 (type I), and 48.6% of gonads highly expressed RME-2 (type II expression). At 12 hours from L4 entry, all worms expressed RME-2 in their gonads. Arrowheads indicate RME-2 signal. Scale bars: 50 μm.

performed genetic interaction analysis. FBF-1 (*fem-3* mRNA binding factor) binds to the 3′-untranslated region of *fem-3* mRNA and represses *fem-3* translation (Zhang et al., 1997). The *fbf-1(ok91)* mutant reportedly produces more sperm than the wild type (Crittenden et al., 2002). We first confirmed that the *fbf-1(ok91)* mutant had a larger brood size as reported (Fig. 4B). When the *cdc-48.1(tm544)* mutation was introduced into the *fbf-1(ok91)* mutant, the larger brood size was clearly suppressed, suggesting that *cdc-48.1* genetically interacts with *fbf-1*. Next, we investigated the genetic interaction between *cdc-48.1(tm544)* and *fem-3*. The *fem-3(q20gf)* hermaphrodites have been shown to produce only sperm at 25°C, but produce oocytes and excess sperm compared with the wild type at 15°C (Barton et al., 1987). The *fem-3(q20gf)* mutant was confirmed to have an extremely larger brood size at 15°C (Fig. 4C). As shown in Fig. 4C, the *cdc-48.1(tm544)* mutation again suppressed the larger brood size as a result of the *fem-3(q20gf)* mutation. These results suggest that CDC-48.1 has a role downstream or parallel to FEM-3 and affects the sperm-oocyte switch.

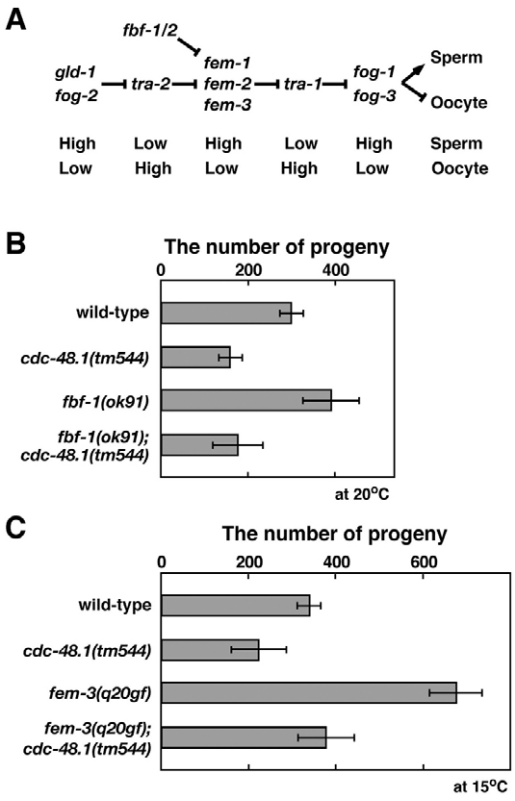

Fig. 4. The *cdc-48.1* mutation suppresses the excess sperm phenotype of *fbf-1(ok91)* and *fem-3(gf)* mutants. (A) Schematic diagram of the germline sex determination pathway in *C. elegans* (Ellis and Schedl, 2006). Arrow indicates activation and bars indicate inhibition. An indication of the level of expression is given for sperm and oocytes. (B,C) N2 wild-type and mutant worms were synchronized and incubated from early L4 stage. F1 eggs laid from these worms were pooled and counted. Experiments for *fbf-1(ok91)* (B) and *fem-3(q20gf)* (C) were carried out at 20°C and 15°C, respectively. Results are mean ± s.d. from at least five animals.

TRA-1A accumulation in *cdc-48.1* mutant worms

The zinc finger transcription factor TRA-1A is the terminal effector of the sex-determination pathway and is negatively regulated by FEM proteins (Chen and Ellis, 2000; Conradt and Horvitz, 1999; Schvarzstein and Spence, 2006; Yi et al., 2000; Zarkower and Hodgkin, 1992; Zarkower and Hodgkin, 1993).

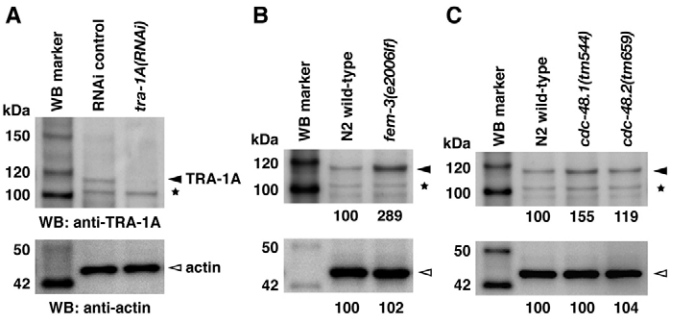

Fig. 5. Accumulation of TRA-1A in the *cdc-48.1* mutant. (A) Anti-TRA-1A antibody detects a 118 kDa TRA-1A protein. Extracts of the RNAi control and *tra-1A(RNAi)* worms were subjected to western blotting. The black arrowhead and asterisk represent TRA-1A and a nonspecific, unknown band, respectively. Anti-actin antibody was used as a loading control. White arrowhead indicates actin. (B) Extracts of N2 wild-type and *fem-3(e2006lf)* young adult worms incubated at 25°C were subjected to western blotting. TRA-1A and actin were quantified. TRA-1A accumulation in *fem-3(e2006lf)* was 2.9-fold greater than that of the wild type. (C) Extracts of N2 wild-type, *cdc-48.1(tm544)* and *cdc-48.2(tm659)* young adult worms incubated at 25°C were subjected to western blotting. TRA-1A and actin were quantified. TRA-1A accumulation in *cdc-48.1(tm544)* was 1.5 times greater than in the wild-type.

Recently, it has been shown that TRA-1A is regulated by degradation mediated by a CUL-2-based E3 ubiquitin ligase complex containing FEM-1, FEM-2 and FEM-3 as a substrate-recognition receptor and that TRA-1A accumulates in the feminized mutants, *fem-1, fem-2* and *fem-3*, compared with the wild type (Starostina et al., 2007). Given that *cdc-48.1* genetically interacts with *fem-3* as described above, TRA-1A is expected to accumulate in the *cdc-48.1(tm544)* mutant. To test this idea, we first prepared an anti-TRA-1A antibody. This antibody recognized a 118 kDa protein, which was markedly decreased in the *tra-1A(RNAi)* worms (Fig. 5A), suggesting that the 118 kDa protein is TRA-1A. TRA-1A was increased 2.9-fold in the *fem-3* loss-of-function mutant worms (Fig. 5B), which is consistent with a previous report (Starostina et al., 2007). By quantifying TRA-1A, we found that the TRA-1A level in the *cdc-48.1(tm544)* mutant was 1.5-fold that of the wild-type (Fig. 5C), suggesting that p97 regulates TRA-1A degradation.

p97 interacts with the CUL-2 complex via NPL-4 binding to ELC-1

To elucidate the connection between p97/CDC-48 and the CUL-2 complex, we carried out yeast two-hybrid analysis. Several functions of p97 are determined by distinct cofactors such as Ufd1, Npl4 and UBX-domain-containing proteins (Ye, 2006; Yamauchi et al., 2007; Schuberth and Buchberger, 2008). Ufd1 and Npl4 form a heterodimer, and the structural features of the Ufd1-Npl4 heterodimer and its interaction with p97 have been demonstrated (Pye et al., 2007). The p97-Ufd1-Npl4 complex (CDC-48–UFD-1–NPL-4.1 in worms) is required for the ERAD function of p97 (Bays et al., 2001; Ye et al., 2001). Components of the p97/CDC-48 complex including CDC-48.1, CDC-48.2, UFD-1, NPL-4.1, UBXN-1, UBXN-2, UBXN-3, UBXN-4, UBXN-5 and UBXN-6 were used as bait, whereas those of the CUL-2 complex including CUL-2, RBX-1, ELB-1, ELC-1, FEM-1, FEM-2, FEM-3, and TRA-1A (Petroski and Deshaies, 2005; Starostina et al., 2007) were used as prey in a yeast two-hybrid analysis. Among all of the combinations tested, only the combination of NPL-4.1 and ELC-1/Elongin C gave a positive signal (Fig. 6A). When ELC-1 was mutated, mutant ELC-1(L47D/L49D/Y88D/Y91D) did not interact with NPL-4.1 (Fig. 6A). Interestingly, L47 and L49 are in the α1 helix and Y88 and Y91 are in the α3 helix, and these helices are on the same side of ELC-1 (Yan et al., 2004). These results imply that NPL-4.1 recognizes and binds to the α1 and α3 helices of ELC-1. We next performed a pull-down assay. GST-fused NPL-4.1 and FLAG-tagged ELC-1 were prepared in vitro, mixed and pulled down with glutathione-Sepharose. As shown in Fig. 6B, NPL-4.1 weakly interacted with ELC-1. These results imply that CDC-48–UFD-1–NPL-4.1 interacts with the CUL-2 complex via NPL-4.1 binding to ELC-1/Elongin C. Taken together, these results indicate that CDC-48 is involved in TRA-1A degradation.

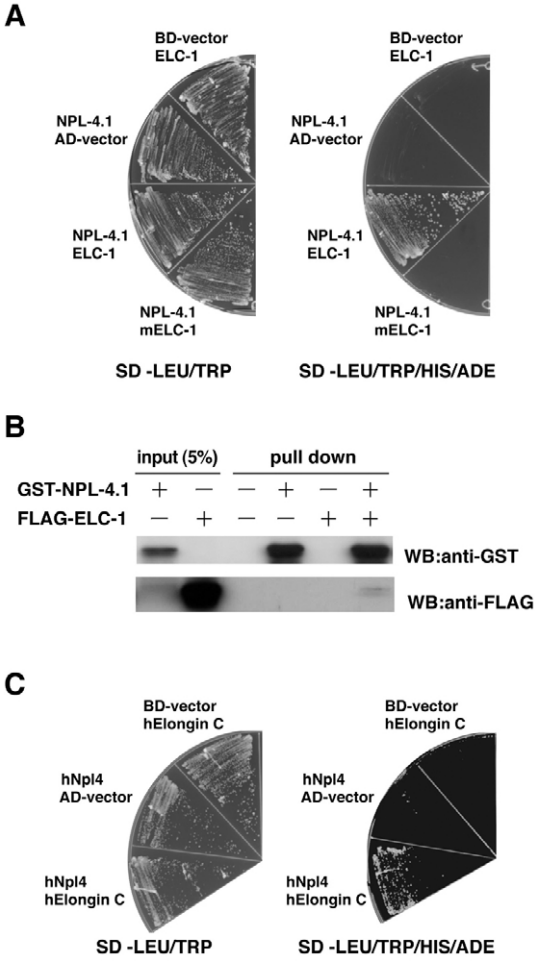

Fig. 6. Interaction between NPL-4.1 and Elongin C. (A) Yeast two-hybrid assay with BD-NPL-4.1 and AD-ELC-1. Mutant ECL-1 (mECL-1) carries L47D-L49D-Y88D-Y91D mutations. Transformants were tested for growth on SD-LEU/TRP and SD-LEU/TRP/HIS/ADE plates at 30°C for 3 days. (B) GST-NPL-4.1 and FLAG-ELC-1 were prepared and mixtures were incubated and pulled down with glutathione-Sepharose, and analyzed with anti-GST and anti-FLAG antibodies. (C) Yeast two-hybrid assay with human Npl4-BD and human Elongin-C-AD. Transformants were tested for growth on SD-LEU/TRP and SD-LEU/TRP/HIS/ADE plates at 30°C for 3 days.

It is known that p97 and the Cul2 complex are well conserved in eukaryotes (Kamura et al., 2004; Ye, 2006; Schuberth and Buchberger, 2008). It is also interesting to mention that human FEM1B, a homologue of *C. elegans* FEM-1, has been shown to be a component of the Cul2 complex, although its function has not

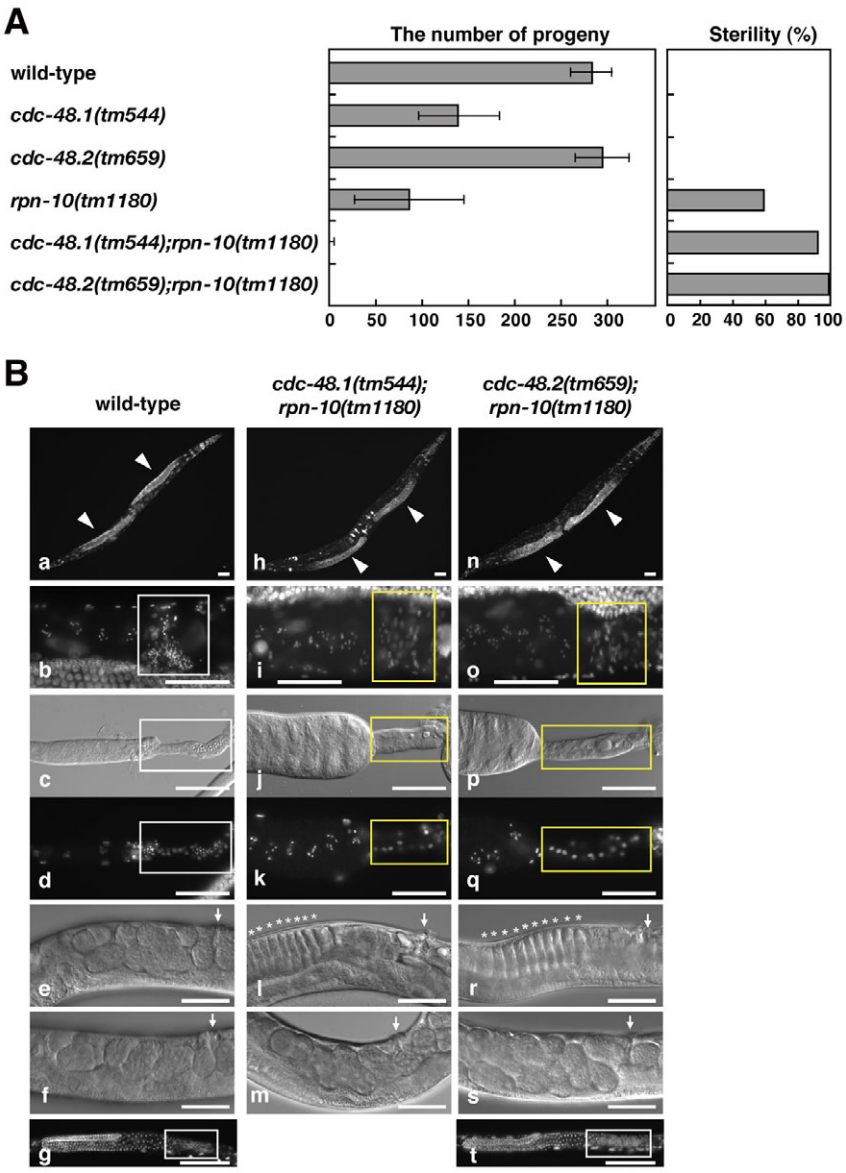

Fig. 7. p97/CDC-48 is involved in RPN-10-dependent gametogenesis. (A) Effects of *rpn-10(tm1180)* on the number of progeny and sterility of *cdc-48.1(tm544)* and *cdc-48.2(tm659)* mutants. The total number of progeny was counted in at least 15 worms of each genotype. Means ± s.d are shown. Sterility of the F1 generation was also counted. *cdc-48.1(tm544);rpn-10(tm1180)* and *cdc-48.2(tm659);rpn-10(tm1180)* double deletion mutants were maintained as heterozygotes and segregating double homozygotes were used. (B) Germline feminization phenotype was observed in *cdc-48.1(tm544);rpn-10(tm1180)* and *cdc-48.2(tm659);rpn-10(tm1180)* double mutants. a-g, N2 wild-type; h-m, *cdc-48.1(tm544);rpn-10(tm1180)*; and n-t, *cdc-48.2(tm659);rpn-10(tm1180)*. Chromosomal DNA was stained with DAPI after whole-mount fixation (a,b,g,h,i,n,o,t). Germline development was normal in *cdc-48.1(tm544);rpn-10(tm1180)* (h) and *cdc-48.2(tm659);rpn-10(tm1180)* (n) double mutants, similarly to the wild type (a). Arrowheads indicate normally elongated gonad arms. The part of the gonad containing the spermathecae shown in a, h and n was enlarged and is shown in b, i and o, respectively. Each germline containing a spermatheca was dissected on a poly-L-lysine-coated slide and stained with DAPI (c,j,p; d,k,q; DAPI). White and yellow boxed regions represent hermaphrodite spermathecae containing sperm and no sperm, respectively. Worms were observed without fixation before (e,l,r) and after (f,m,s) mating. Arrows and asterisks indicate vulva and stacked oocytes, respectively. Males of wild type (g) and *cdc-48.2(tm659);rpn-10(tm1180)* (t) were fixed and stained with DAPI. Boxed regions indicate spermathecae. Scale bars: 50 μm.

yet been clarified (Kamura et al., 2004). To elucidate whether the interaction between the p97-Ufd1-Npl4 complex and Cullin 2-type E3 ubiquitin ligase is specific for *C. elegans* or common in eukaryotes, we carried out a yeast two-hybrid assay using human Npl4 and Elongin C. As shown in Fig. 6C, Npl4 clearly interacted with Elongin C, suggesting that the interaction between p97-Ufd1-Npl4 and the Cul2 complex through Npl4 binding to Elongin C is well conserved in eukaryotes.

Proteolytic activity is probably involved in determination of brood size

It has been reported that proteasomal ubiquitin receptor RPN-10 controls sex determination in *C. elegans* and that double knockdown of *rpn-10* and *ufd-2* overcomes the germline-musculinizing effect of *fem-3(gf)* (Shimada et al., 2006). As mentioned above, we found that the *cdc-48.1(tm544)* mutation overcame the sperm-overproducing phenotype of *fem-3(gf)* (Fig. 4C). Therefore, we investigated the effect of *rpn-10* mutation on brood size. Approximately 60% of *rpn-10(tm1180)* worms showed a sterile phenotype and the remaining 40% had a markedly decreased brood size (Fig. 7A), which is consistent with a previous report (Shimada et al., 2006). Given that *rpn-10* encodes a subunit of the 26S proteasome, proteolytic activity is probably involved in the process of determining brood size. When the *rpn-10(tm1180)* mutation was introduced into the *cdc-48.1(tm544)* and *cdc-48.2(tm659)* mutants, almost all worms became sterile (Fig. 7A). As shown in Fig. 7B, although development and formation of the germline of these double mutant worms appear to be normal (compare Fig. 7B, panels h and n, with panel a), there were no sperm in the spermathecae, no embryos in the uterus, and stacked oocytes, which is a characteristic phenotype of spermless worms (Fig. 7B). However, when these mutant worms were crossed with wild-type males, they then produced progeny (Fig. 7B, compare panels m and s with l and r). Furthermore, the gonad structure of double mutant [*cdc-48.2(tm659);rpn-10(tm1180)*] males stained with DAPI appeared to be unaffected and a significant number of sperm were observed, making them indistinguishable from wild-type worms (Fig. 7B, compare panels and g). These results indicate

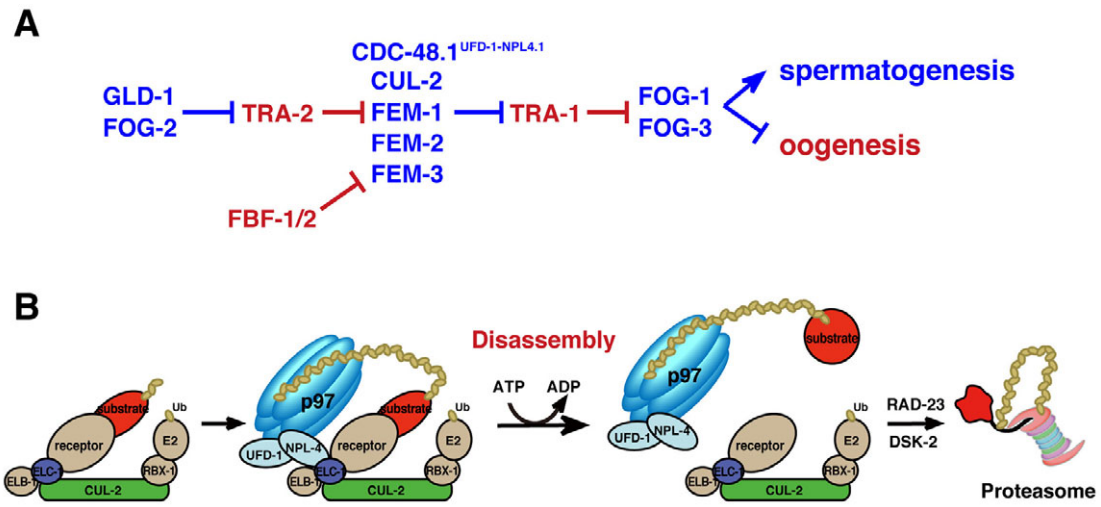

Fig. 8. Model for p97 involvement in regulation of substrate fate. (A) CDC-48.1–UFD-1–NPL-4.1 is involved in the regulation of TRA-1 degradation in combination with CUL-2 ubiquitin ligase containing FEM-1,FEM-2 and FEM-3 as a substrate recognition receptor. TRA-1 degradation is not efficient in the *cdc-48.1(tm544)* mutant and thus switching from spermatogenesis to oogenesis occurs earlier than in the wild type. Proteins that promote spermatogenesis are blue, and those that promote oogenesis are red. (B) Proposed model of p97 requirement for disassembly of the ubiquitylated substrate from the CUL-2 ubiquitin ligase complex. p97 might disassemble the substrate by changing its conformation using ATP-hydrolyzing energy.

that oocyte development was not impaired, but sperm were not produced by the double mutant hermaphrodites of *cdc-48.1(tm544);rpn-10(tm1180)* and *cdc-48.2(tm659);rpn-10(tm1180)*. This implies that these double mutants show a gonad feminization phenotype. Taking these results into account, TRA-1A is proteolytically regulated in concert with CDC-48–UFD-1–NPL-4.1 and the CUL-2-based ubiquitin ligase complex and controls the sperm-oocyte switch.

Discussion

In the present study, we found that the brood size was decreased in p97/CDC-48 mutants (Fig. 1). The number of sperm produced was also decreased; sperm were exhausted earlier than in the wild type; no defect was observed in oocyte production; and the sperm-overproducing phenotype of *fbf-1(ok91)* and *fem-3(q20gf)* mutants was suppressed by the *cdc-48.1(tm544)* mutation (Figs 1 and 4). These results suggest that the decreased brood size resulted from decreased sperm production. Furthermore, the RME-2 signal in the *cdc-48.1(tm544)* mutant appeared earlier than in the wild type (Fig. 3). Therefore, we conclude that p97/CDC-48 is involved in the sperm-oocyte switching process. It is noteworthy to mention that the onset of spermatogenesis is governed by the post-transcriptional regulation of *tra-2*, whereas the sperm-oocyte switch is regulated by the post-transcriptional control of *fem-3* (Ahringer and Kimble, 1991; Puoti et al., 2001; Shimada et al., 2006). Determination of the precise targeting process of p97/CDC-48 remains to be addressed.

We also found that CDC-48–UFD-1–NPL-4.1 interacts with the CUL-2 complex through the interaction between NPL-4.1 and ELC-1/Elongin-C (Fig. 6). It has been shown that TRA-1A is regulated by degradation mediated by a CUL-2-based ubiquitin ligase complex containing FEM-1, FEM-2 and FEM-3 as a substrate-recognition receptor (Starostina et al., 2007). Here, we indeed demonstrated that TRA-1A accumulates in the *cdc-48.1(tm544)* mutant (Fig. 5). Taking these results together, we propose that p97, an AAA chaperone, with UFD-1 and NPL-4 has an important role in regulating the TRA-1A level, in combination with the CUL-2 complex containing FEM-1, FEM-2 and FEM-3, and is required for the appropriate operation of the sperm-oocyte switch (Fig. 8A).

The CDC-48–UFD-1–NPL-4.1 complex also has important roles in ERAD and DNA replication in *C. elegans* (Mouysset et al., 2006; Sasagawa et al., 2007c; Mouysset et al., 2008). Phenotypes observed in *ufd-1(RNAi)* and *npl-4(RNAi)* were similar to those observed in p97-depleted worms. Unfortunately, we were not able to examine the effects of *ufd-1(RNAi)* and *npl-4(RNAi)* on brood size, because *ufd-1(RNAi)* and *npl-4(RNAi)* cause severe defects in germline formation (Sasagawa et al., 2007c).

AAA proteins including p97 are thought to be an unfoldase or a segregase (Patel and Latterich, 1998). Furthermore, it has been suggested that p97 is a ubiquitin-selective chaperone and that its key function is to disassemble protein complexes (Rape et al., 2001). Thus, p97 probably functions as a segregase to disassemble polyubiquitylated proteins from the CUL-2 complex in an ATP-dependent manner and delivers them to the proteasome containing RPN-10 in combination with shuttle factors such as Rad23 and Dsk2 (Richly et al., 2005) (Fig. 8B). It should be emphasized that the interaction of p97 and E3 ubiquitin ligases is not only required for the degradation of ERAD substrates (Zhong et al., 2004; Lilley and Ploegh, 2005; Ye et al., 2005), but also soluble cytoplasmic substrates. Therefore, in the p97 mutants, more TRA-1A, the master sexual regulator, accumulated than in the wild type, which in turn caused the sperm-oocyte switch to occur earlier than in the wild type. It is interesting to mention that the defect in the brood size of *cdc-48.1(tm544)* is severer than that of *cdc-48.2(tm659)*. We have previously reported that the amount of CDC-48.1 is double that of CDC-48.2 (Yamauchi et al., 2006). These results appear to support the above-mentioned notion that the chaperone activity of p97 controls the efficiency of CUL-2-mediated TRA-1A degradation by the proteasome. It should also be mentioned that the interaction between p97 and the Cul2 complex is not restricted to *C. elegans*, but might be conserved in eukaryotes (Fig. 6C). Therefore, Cul2-mediated degradation of substrate proteins generally occurs with the aid of p97 chaperone activity.

It has been reported that in *C. elegans*, CUL-2 is required for several key processes in cell division and embryonic development, including

cyclin-dependent kinase (CDK) inhibitor CKI-1 degradation, meiotic progression, PAR-6 degradation and the establishment of anterior-posterior polarity, cyclin B1 degradation, chromatin condensation, mitotic progression, proper cytoplasmic organization and the degradation of CCCH-finger polarity proteins such as PIE-1 (Feng et al., 1999; DeRenzo et al., 2003; Liu et al., 2004; Sonneville and Gonczy, 2004; Sasagawa et al., 2005; Sasagawa et al., 2007a; Pacquelet et al., 2008). Importantly, some of the phenotypes observed in the *cul-2* mutant, including defects in meiotic progression, chromosome condensation, cyclin B1 degradation, and establishment of anterior-posterior polarity, have also been observed in p97-depleted worms (Sasagawa et al., 2007b) (and our unpublished results). Therefore, it can be reasonably assumed that p97 is globally involved in CUL-2-dependent protein degradation. It is important to identify substrates and substrate recognition receptors to decipher the detailed cellular functions of p97. Moreover, although the levels of TRA-2 were reported to be proteolytically regulated in an RPN-10-dependent manner (Shimada et al., 2006), E3 ubiquitin ligase and the substrate recognition receptor have not yet been clarified. In addition, SEL-10 is a WD40-repeat-containing F-box protein and might serve as a substrate-recognition component that targets substrate proteins including FEM-1 and FEM-3 for ubiquitin-mediated proteolysis by the proteasome (Jager et al., 2004). It will thus also be interesting to investigate whether p97 is involved in processes performed by other E3 ubiquitin ligases, such as CUL-1 and the anaphase-promoting complex/cyclosome (APC/C), in the future.

Materials and Methods

C. elegans strains

Nematodes were maintained on nutrient growth medium (NGM) agar plates using standard protocols, as described previously (Brenner, 1974). The deletion mutants of *cdc-48.1*(*tm544*), *cdc-48.2*(*tm659*) and *rpn-10*(*tm1180*) were reported previously (Sasagawa et al., 2007c). These mutations carry 668 bp, 639 bp and 357 bp deletions in their genes, respectively (Sasagawa et al., 2007c). Mutant worms JK3022 *fbf-1(ok91)*, JK816 *fem-3(q20gf)*, CB3844 *fem-3(e2006lf)* and HT1593 *unc-119(ed3)* were provided by the *Caenorhabditis* Genetics Center. Males carrying mutations were generated from these deletion mutants and were used to transfer the mutation. We generated the following strains: *fbf-1(ok91);cdc-48.1(tm544)*, *fem-3(q20);cdc-48.1(tm544)*, *rpn-10(tm1180);cdc-48.1(tm544)*, and *rpn-10(tm1180);cdc-48.2(tm659). rpn-10(tm1180);cdc-48.1(tm544)* and *rpn-10(tm1180);cdc-48.2(tm659)* double deletion mutants were maintained as heterozygotes, and segregated double homozygotes were used for assays. Temperature-sensitive strains, namely, JK816 *fem-3(q20gf)*, CB3844 *fem-3(e2006lf)*, and *fem-3(q20gf);cdc-48.1(tm544)*, were maintained at 15°C. Other mutants were maintained at 20°C. Nematode experiments were performed at 20°C unless otherwise specified.

Construction of transgenic line expressing FLAG-tagged CDC-48.1

We constructed the plasmid to express FLAG::CDC-48.1 and wild-type UNC-119. First, the wild-type *unc-119* gene was cloned into pHSG298 (TaKaRa), yielding pHUNC1. The wild-type UNC-119 was used to rescue the *unc-119(ed3)* mutation. The *cdc-48.1* gene with a 1 kb 5′-upstream region and a 1 kb 3′-untranslated region was PCR amplified from the wild-type genomic DNA, and then cloned into pHUNC1. Finally, the DNA sequences encoding the FLAG tag were inserted immediately upstream of the translation initiation codon of *cdc-48.1* to produce an N-terminal fusion protein. The plasmid was bombarded into *unc-119(ed3)* mutant worms using the BioRad Biolistic PDS-1000/He particle delivery system, as described previously (Praitis et al., 2001). Unc-rescued worms were obtained and FLAG::CDC-48.1-expressing transgenic lines were then screened by western blotting with anti-FLAG antibodies (Sigma, clone M2). The *cdc-48.1(tm544)* mutation was transferred by mating. In this way, the following strains were prepared: XA7200 (*unc-119(ed3) qaIs7200*[*unc-119(+)*]), XA7201 (*unc-119(ed3) qaIs7201*[*Pcdc-48.1*::FLAG::CDC-48.1*, unc-119(+)*]), XA7202 (*unc-119(ed3);cdc-48.1(tm544) qaIs7200*[*unc-119(+)*]), and XA7203 (*unc-119(ed3);cdc-48.1(tm544) qaIs7201*[*Pcdc-48.1*::FLAG::CDC-48.1*, unc-119(+)*]).

RNAi

To construct RNAi feeding bacteria, the cDNA fragment for *tra-1A* (nucleotides 835–2133) was amplified using an RT-PCR kit (Qiagen) with total RNA from N2 adult hermaphrodites as a template. The following primer sets were employed: tra-1Fw+BamHI 5′-TAC TGA GGA TCC CGA TAC GGT TGT CGA GGC TCA ACG-3′ (forward) and tra-1Rv+XhoI 5′-TAT CTC GAG TCA AAT CTC ATT ATC ACC ACC AAA ACC AAC CTC ACA C-3′ (reverse). Note that the amplified fragment is specific for *tra-1A* but not for *tra-1B*. The cDNA fragment was cloned into the plasmid LITMUS 28 (New England BioLabs). The dsRNA was prepared in vitro using T7 RNA polymerase, and *tra-1A* dsRNA (1 mg/ml) was microinjected. The RNAi construct for *cdc-48.2* was described previously (Sasagawa et al., 2007b). Plasmids were transformed into *Escherichia coli* strain HT115 (DE3). We used Optimal Protocol I for feeding RNAi methods, as described previously (Kamath et al., 2001).

Immunofluorescence and microscopic observation

We fixed whole worms using fixation solution (60% methanol, 30% acetic acid and 10% chloroform) at 4°C for 30 minutes. Unfixed whole worms were mounted on 1% agarose pads in 1 mg/ml levamisole diluted in M9 buffer (2.2 mM KH_2PO_4, 4.2 mM Na_2HPO_4, 85 mM NaCl, 1 mM $MgSO_4$). Gonads were dissected on poly-L-lysine-coated slides and fixed with 2% paraformaldehyde in PBSTw (phosphate-buffered saline, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4, containing 0.1% Tween20), followed by prechilled 100% dimethylformamide (10 minutes at –20°C). These slides were rehydrated with PBSTw and blocked with 3% bovine serum albumin (BSA) in PBSTw. The slides were incubated with primary antibodies, anti-FLAG antibody (Sigma, 1:500 dilution), anti-histone H3 phospho-Ser10 antibody (Millipore, 1:1000 dilution), anti-MSP antibody (clone 4A5, 1:40 dilution), and anti-RME-2 antibody (1:500 dilution) (Grant and Hirsh, 1999) in antibody solution (1% BSA, 0.5% Triton X-100 and 0.05% sodium azide in PBS). The slides were washed with PBSTw six times for 10 minutes each, and subsequently incubated with secondary antibodies, anti-mouse Alexa Fluor 488 (Invitrogen, 1:1000 dilution) and anti-rabbit Alexa Fluor 568 (Invitrogen, 1:1000 dilution) antibodies in antibody solution for 3 hours at 25°C. The slides were finally washed with PBSTw six times for 10 minutes each, and mounted with 1 μg/ml DAPI. These samples were observed under an Olympus Power BX51 microscope equipped with a CoolSnapHQ (Roper Scientific) CCD camera. MetaMorph software (Universal Imaging) was used to control the camera and to process acquired images. Sperm were counted in DAPI-stained young adults that contained oocytes, but no embryos (Lamont and Kimble, 2007).

Western blotting analysis

TRA-1A (residues 278-1110) protein was expressed in *E. coli*, purified and used to immunize rabbits to obtain the anti-TRA-1A antibody. Total lysates of 100 young adult worms were resolved on 5-20% SDS-PAGE and transferred to a nitrocellulose membrane. Signals were detected with the antibodies against TRA-1A and actin (mouse anti-actin monoclonal antibody (C4), Millipore, 1:5000 dilution), and quantified using ImageJ (National Institutes of Health, Bethesda, MD). The amount of actin was detected as a loading control.

Yeast two-hybrid assay

Full-length cDNA fragments derived from *cdc-48.1*, *cdc-48.2*, *ufd-1*, *npl-4.1*, *ubxn-1*, *ubxn-2*, *ubxn-3*, *ubxn-4*, *ubxn-5*, *ubxn-6*, *cul-2*, *rbx-1*, *elb-1*, *elc-1*, *fem-1*, *fem-2*, *fem-3* and *tra-1* were amplified from yk-clone or wild-type total RNAs, and cloned into pGADT7 or pGBKT7 plasmids. A plasmid to express mutant ELC-1(L47D/L49D/Y88D/Y91D) was prepared using the QuikChange II XL Site-Directed Mutagenesis kit (Stratagene). Full-length cDNA fragments for human Npl4 and Elongin C were also amplified from a human cDNA library and cloned into pGADT7 or pGBKT7 plasmids. Plasmids were verified by DNA sequencing. The two kinds of plasmid for production of Gal4 activation-domain fusion proteins and Gal4 DNA-binding-domain fusion proteins were cotransformed into *Saccharomyces cerevisiae* AH109 strain. Leu^+ Trp^+ transformants were selected and then incubated on test plates lacking Leu, Trp, His and Ade at 30°C for 3 days.

Pull-down assay

GST-NPL-4.1 and FLAG-ELC-1 were prepared using the TNT SP6 High-Yield Protein Expression System (Promega). Mixtures of GST-NPL-4.1 and FLAG-ELC-1 were incubated and pulled down with glutathione-Sepharose (GE Healthcare Bioscience), and then subjected to western blotting with anti-GST (GE Healthcare Bioscience) and anti-FLAG (Sigma) antibodies.

Two-dimensional gel electrophoresis and protein identification by MALDI-TOF MS

Two-dimensional (2D) gel electrophoresis of total protein extracts of *C. elegans* and the subsequent identification of proteins by MALDI-TOF MS were performed as described previously (Yamauchi et al., 2008).

We thank Yuko Saito-Sasagawa for constructing FLAG::CDC-48.1 expressing transgenic worms, Shohei Mitani (Tokyo Women's Medical University) and the *Caenorhabditis* Genetic Center for *C. elegans* mutants, Yuji Kohara (National Institute of Genetics) for *C. elegans* cDNA clones, Barth Grant (Rutgers University) for antibodies against

RME-2, David Greenstein (University of Minnesota) for anti-MSP antibody, Asako Sugimoto (RIKEN CDB, Kobe, Japan) for suggestions, and Masumi Shimada and Hiroyuki Kawahara (Tokyo Metropolitan University) for valuable comments. We also thank Yoshiko Okubo, Chiyome Ichinose and Yuki Kawata for assistance. This work was supported in part by grants from the Ministry of Education, Culture, Science, Sports and Technology, Japan.

References

Ahringer, J. and Kimble, J. (1991). Control of the sperm-oocyte switch in *Caenorhabditis elegans* hermaphrodites by the *fem-3* 3′ untranslated region. *Nature* **349**, 346-348.

Ahringer, J., Rosenquist, T. A., Lawson, D. N. and Kimble, J. (1992). The *Caenorhabditis elegans* sex determining gene *fem-3* is regulated post-transcriptionally. *EMBO J.* **11**, 2303-2310.

Barton, M. K., Schedle, T. B. and Kimble, J. (1987). Gain-of-function mutations of *fem-3*, a sex-determination gene in *Caenorhabditis elegans. Genetics* **115**, 107-119.

Bays, N. W., Wilhovsky, S. K., Goradia, A., Hodgkiss-Harlow, K. and Hampton, R. Y. (2001). HRD4/NPL4 is required for the proteasomal processing of ubiquitinated ER proteins. *Mol. Biol. Cell* **12**, 4114-4128.

Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics* **77**, 71-94.

Chen, P. J. and Ellis, R. E. (2000). TRA-1A regulates transcription of *fog-3*, which controls germ cell fate in *C. elegans*. *Development* **127**, 3119-3129.

Chin-Sang, I. D. and Spence, A. M. (1996). *Caenorhabditis elegans* sex-determining protein FEM-2 is a protein phosphatase that promotes male development and interacts directly with FEM-3. *Genes Dev.* **10**, 2314-2325.

Conradt, B. and Horvitz, H. R. (1999). The TRA-1A sex determination protein of *C. elegans* regulates sexually dimorphic cell deaths by repressing the *egl-1* cell death activator gene. *Cell* **98**, 317-327.

Crittenden, S. L., Bernstein, D. S., Bachorik, J. L., Thompson, B. E., Gallegos, M., Petcherski, A. G., Moulder, G., Barstead, R., Wickens, M. and Kimble, J. (2002). A conserved RNA-binding protein controls germline stem cells in *Caenorhabditis elegans*. *Nature* **417**, 660-663.

DeRenzo, C., Reese, K. J. and Seydoux, G. (2003). Exclusion of germ plasm proteins from somatic lineages by cullin-dependent degradation. *Nature* **424**, 685-689.

Ellis, R. and Schedl, T. (2006). Sex determination in the germ line. In *WormBook* (ed. The *C. elegans* Research Community). http://www.wormbook.org.

Feng, H., Zhong, W., Punkosdy, G., Gu, S., Zhou, L., Seabolt, E. K. and Kipreos, E. T. (1999). CUL-2 is required for the G1-to-S-phase transition and mitotic chromosome condensation in *Caenorhabditis elegans. Nat. Cell Biol.* **1**, 486-492.

Grant, B. and Hirsh, D. (1999). Receptor-mediated endocytosis in the *Caenorhabditis elegans* oocyte. *Mol. Biol. Cell* **10**, 4311-4326.

Hodgkin, J. (1987). A genetic analysis of the sex-determining gene, *tra-1*, in the nematode *Caenorhabditis elegans. Genes Dev*. **1**, 731-745.

Jager, S., Schwartz, H. T., Horvitz, H. R. and Conradt, B. (2004). The *Caenorhabditis elegans* F-box protein SEL-10 promotes female development and may target FEM-1 and FEM-3 for degradation by the proteasome. *Proc. Natl. Acad. Sci. USA* **101**, 12549-12554.

Kamath, R. S., Martinez-Campos, M., Zipperlen, P., Fraser, A. G. and Ahringer, J. (2001). Effectiveness of specific RNA-mediated interference through ingested double-stranded RNA in *Caenorhabditis elegans. Genome Biol.* **2,** RESEARCH0002.

Kamura, T., Maenaka, K., Kotoshiba, S., Matsumoto, M., Kohda, D., Conaway, R. C., Conaway, J. W. and Nakayama, K. I. (2004). VHL-box and SOCS-box domains determine binding specificity for Cul2-Rbx1 and Cul5-Rbx2 modules of ubiquitin ligases. *Genes Dev.* **18**, 3055-3065.

Kimble, J. and Crittenden, S. L. (2007). Controls of germline stem cells, entry into meiosis, and the sperm/oocytes decision in *C. elegans. Annu. Rev. Cell Dev. Biol.* **23**, 405-433.

Kipreos, E. T. (2005). Ubiquitin-mediated pathways in *C. elegans*. *WormBook* (ed. The *C. elegans* Research Community). http://www.wormbook.org.

Lamont, L. B. and Kimble, J. (2007). Developmental expression of FOG-1/CPEB protein and its control in the *Caenorhabditis elegans* hermaphrodite germ line. *Dev. Dyn.* **236**, 871-879.

Lilley, B. N. and Ploegh, H. L. (2005). Multiprotein complexes that link dislocation, ubiquitination, and extraction of misfolded proteins from the endoplasmic reticulum membrane. *Proc. Natl. Acad. Sci. USA* **102**, 14296-14301.

Liu, J., Vasudevan, S. and Kipreos, E. T. (2004). CUL-2 and ZYG-11 promote meiotic anaphase II and the proper placement of the anterior-posterior axis in *C. elegans*. *Development* **131**, 3513-3525.

Mouysset, J., Kahler, C. and Hoppe, T. (2006). A conserved role of *Caenorhabditis elegans* CDC-48 in ER-associated protein degradation. *J. Struct. Biol.* **156**, 41-49.

Mouysset, J., Deichsel, A., Moser, S., Hoege, C., Hyman, A. A., Gartner, A. and Hoppe, T. (2008). Cell cycle progression requires the CDC-48$^{UFD-1/NPL-4}$ complex for efficient DNA replication. *Proc. Natl. Acad. Sci. USA* **105**, 12879-12884.

Ogura, T. and Wilkinson, A. J. (2001). AAA^{+} superfamily ATPases: common structure-diverse function. *Genes Cells* **6**, 575-597.

Pacquelet, A., Zanin, E., Ashiono, C. and Gotta, M. (2008). PAR-6 levels are regulated by NOS-3 in a CUL-2 dependent manner in *Caenorhabditis elegans. Dev. Biol.* **319**, 267-272.

Patel, S. and Latterich, M. (1998). The AAA team: related ATPases with diverse functions. *Trends Cell Biol.* **8**, 65-71.

Petroski, M. D. and Deshaies, R. J. (2005). Function and regulation of cullin-RING ubiquitin ligases. *Nat. Rev. Mol. Cell Biol.* **6**, 9-20.

Pilgrim, D., McGregor, A., Jackie, P., Johnson, T. and Hansen, D. (1995). The *C. elegans* sex-determining gene *fem-2* encodes a putative protein phosphatase. *Mol. Biol. Cell* **6**, 1159-1171.

Praitis, V., Casey, E., Collar, D. and Austin, J. (2001). Creation of low-copy integrated transgenic lines in *Caenorhabditis elegans*. *Genetics* **157**, 1217-1226.

Puoti, A., Pugnale, P., Belfiore, M., Schlappi, A. C. and Saudan, Z. (2001). RNA and sex determination in *Caenorhabditis elegans. EMBO Rep*. **2**, 899-904.

Pye, V. E., Beuron, F., Keetch, C. A., McKeown, C., Robinson, C. V., Meyer, H. H., Zhang, X. and Freemont, P. S. (2007). Structural insights into the p97-Ufd1-Npl4 complex. *Proc. Natl. Acad. Sci. USA* **104**, 467-472.

Rape, M., Hoppe, T., Gorr, I., Kalocay, M., Richly, H. and Jentsch, S. (2001). Mobilization of processed membrane-tethered SPT23 transcription factor by CDC48$^{UFD1/NPL4}$, a ubiquitin-selective chaperone. *Cell* **107**, 667-677.

Richly, H., Rape, M., Braun, S., Rumpf, S., Hoege, C. and Jentsch, S. (2005). A series of ubiquitin binding factors connects CDC48/p97 to substrate multiubiquitylation and proteasomal targeting. *Cell* **120**, 73-84.

Sasagawa, Y., Kikuchi, K., Dazai, A. and Higashitani, A. (2005). *Caenorhabditis elegans* Elongin BC complex is essential for cell proliferation and chromosome condensation and segregation during mitosis and meiotic division II. *Chromosome Res.* **8**, 357-375.

Sasagawa, Y., Sato, S., Ogura, T. and Higashitani, A. (2007a). *C. elegans* RBX-2-CUL-5- and RBX-1-CUL-2-based complexes are redundant for oogenesis and activation of the MAP kinase MPK-1. *FEBS Lett.* **581**, 145-150.

Sasagawa, Y., Yamanaka, K., Nishikori, S. and Ogura, T. (2007b). *Caenorhabditis elegans* p97/CDC-48 is crucial for progression of meiosis I. *Biochem. Biophys. Res. Commun.* **358**, 920-924.

Sasagawa, Y., Yamanaka, K. and Ogura, T. (2007c). ER E3 ubiquitin ligase HRD-1 and its specific partner chaperone BiP play important roles in ERAD and developmental growth in *Caenorhabditis elegans. Genes Cells* **12**, 1063-1073.

Schuberth, C. and Buchberger, A. (2008). UBX domain proteins: major regulators of the AAA ATPase Cdc48/p97. *Cell Mol. Life Sci.* **65**, 2360-2371.

Schvarzstein, M. and Spence, A. M. (2006). The *C. elegans* sex-determining GLI protein TRA-1A is regulated by sex-specific proteolysis. *Dev. Cell* **11**, 733-740.

Shimada, M., Kanematsu, K., Tanaka, K., Yokosawa, H. and Kawahara, H. (2006). Proteasomal ubiquitin receptor RPN-10 controls sex determination in *Caenorhabditis elegans*. *Mol. Biol. Cell* **17**, 5356-5371.

Sonneville, R. and Gonczy, P. (2004). *zyg-11* and *cul-2* regulate progression through meiosis II and polarity establishment in *C. elegans*. *Development* **131**, 3527-3543.

Spence, A. M., Coulson, A. and Hodgkin, J. (1990). The product of *fem-1*, a nematode sex-determining gene, contains a motif found in cell cycle control proteins and receptors for cell-cell interactions. *Cell* **60**, 981-990.

Starostina, N. G., Lim, J., Schvarzstein, M., Wells, L., Spence, A. M. and Kipreos, E. T. (2007). A CUL-2 ubiquitin ligase containing three FEM proteins degrades TRA-1 to regulate *C. elegans* sex determination. *Dev. Cell* **13**, 127-139.

Uchiyama, K. and Kondo, H. (2005). p97/p47-mediated biogenesis of Golgi and ER. *J. Biochem.* **137**, 115-119.

Wang, Q., Song, C. and Li, C. C. H. (2004). Molecular perspectives on p97-VCP: progress in understanding its structure and diverse biological functions. *J. Struct. Biol.* **146**, 44-57.

Yamanaka, K., Okubo, Y., Suzaki, T. and Ogura, T. (2004). Analysis of the two p97/VCP/Cdc48p proteins of *Caenorhabditis elegans* and their suppression of polyglutamine-induced protein aggregation. *J. Struct. Biol.* **146**, 242-250.

Yamauchi, S., Yamanaka, K. and Ogura, T. (2006). Comparative analysis of expression of two p97 homologues in *Caenorhabditis elegans*. *Biochem. Biophys. Res. Commun.* **345**, 746-753.

Yamauchi, S., Sasagawa, Y., Ogura, T. and Yamanaka, K. (2007). Differential expression of UBX family genes in *Caenorhabditis elegans*. *Biochem. Biophys. Res. Commun.* **358**, 545-552.

Yamauchi, S., Higashitani, N., Otani, M., Higashitani, A., Ogura, T. and Yamanaka, K. (2008). Involvement of HMG-12 and CAR-1 in the *cdc-48. 1* expression of *Caenorhabditis elegans*. *Dev. Biol.* **318**, 348-359.

Yan, Q., Kamura, T., Cai, Y., Jin, J., Ivan, M., Mushegian, A., Conaway, R. C. and Conaway, J. W. (2004). Identification of Elongin C and Skp1 sequences that determine cullin selection. *J. Biol. Chem.* **279**, 43019-43026.

Ye, Y. (2006). Diverse functions with a common regulator: ubiquitin takes command of an AAA ATPase. *J. Struct. Biol.* **156**, 29-40.

Ye, Y., Meyer, H. H. and Rapoport, T. A. (2001). The AAA ATPase Cdc48/p97 and its partners transport proteins from the ER into the cytosol. *Nature* **414**, 652-656.

Ye, Y., Shibata, Y., Kikkert, M., van Voorden, S., Wiertz, E. and Rapoport, T. A. (2005). Recruitment of the p97 ATPase and ubiquitin ligases to the site of retrotranslocation at the endoplasmic reticulum membrane. *Proc. Natl. Acad. Sci. USA* **102**, 14132-14138.

Yi, W., Ross, J. M. and Zarkower, D. (2000). *mab-3* is a direct *tra-1* target gene regulating diverse aspects of *C. elegans* male sexual development and behavior. *Development* **127**, 4469-4480.

Zarkower, D. and Hodgkin, J. (1992). Molecular analysis of the *C. elegans* sex-determining gene *tra-1*: a gene encoding two zinc finger proteins. *Cell* **70**, 237-249.

Zarkower, D. and Hodgkin, J. (1993). Zinc fingers in sex determination: only one of the two *C. elegans* Tra-1 proteins binds DNA in vitro. *Nucleic Acids Res.* **21**, 3691-3698.

Zhang, B., Gallegos, M., Puoti, A., Durkin, E., Fields, S., Kimble, J. and Wickens, M. P. (1997). A conserved RNA-binding protein that regulates sexual fates in the *C. elegans* hermaphrodite germ line. *Nature* **390**, 477-484.

Zhong, X., Shen, Y., Ballar, P., Apostolou, A., Agami, R. and Fang, S. (2004). AAA ATPase p97/valosin-containing protein interacts with gp78, a ubiquitin ligase for endoplasmic reticulum-associated degradation. *J. Biol. Chem.* **279**, 45676-45684.