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

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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 (Schwarzstein 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.

Results

Reduced brood size in cdc-48.1 mutant worms

We have previously reported that 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 CDC-48.1 interacts with CUL-2 complexes and is involved in the regulation of TRA-1A levels. We propose that CDC-48.1 interacts with CUL-2 complexes and is involved in the regulation of TRA-1A levels.

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) qal57201[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 AX7203 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); cdc-48.2(tm659), 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
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...
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.

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

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. 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.

Fig. 4. The cdc-48.1 mutation suppresses the excess sperm phenotype of fbf-1(ok91) and fem-3(q20gf) 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.
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 times greater than in the wild-type.

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-ELC-1 were prepared in vitro, mixed 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 been characterized.
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);rpn-10(tm1180)* and *cdc-48.2(tm659);rpn-10(tm1180)* double 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 m and s). These results indicate
that oocyte development was not impaired, but sperm were not produced by the double mutant hermaphrodites of \( cdc-48.1(tm544); rpm-10(tm1180) \) and \( cdc-48.2(tm659); rpm-10(tm1180) \). 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-oocyte switch is regulated 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–UFD-1–NPL-4.1 mutant (Fig. 5). Taking these results together, we propose that \( p97/cdc-48 \), 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 Latcherek, 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 polyubiquitinated 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...
p97 regulates sperm-oocyte switch

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Materials and Methods

C. elegans strains

Nematodes were maintained on nutrient growth medium (NGM) agar plates, as described previously (Brenner, 1974). The deletion mutants of cdc-48.1(m544), cdc-48.2(m559) and rpn-10(tm1180) were reported previously (Sasagawa et al., 2007c). These mutations carry 688 bp, 639 bp and 357 bp deletions in their genes, respectively (Sasagawa et al., 2007c). Mutant worms JKI022 fub-1(ad12), JKI166 fem-3(e2006lf), CB3844 fem-3(e2006lf) and HTI593 unc-119(ed3) were provided by the Caenorhabditis Genetics Center. Male chores carrying mutations were generated from these deletion mutants and were used to transfer the mutation. We generated the following strains: fub-1(ad12), cdc-48.1(tm344), fem-3(e2006lf), cdc-48.2(m559), rpn-10(tm1180);cdc-48.1(tm344) and rpn-10(tm1180);cdc-48.2(m559); double deletion mutants were maintained as heterozygotes, and segregated double-homozygous worms were used for assays. Temperature-sensitive strains, namely, JKI61 fem-3(e2006lf), CB3844 fem-3(e2006lf) and fem-3(e2006lf);cdc-48.1(tm344), were maintained at 15°C. Other mutants were maintained at 20°C. Nematode experiments were performed at 20°C unless otherwise specified.

Constitution 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 (TakaraRa), yielding pHUNC1. The wild-type UNC-119 was used to rescue the unc-119(ed3) mutation. The cdc-48.1 gene with a 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 (Pratt, et al., 2001). Unc-rescued worms were obtained and transformed with FLAG::CDC-48.1, expressing transgenic lines were then screened by western blotting with anti-FLAG antibodies (Sigma, clone M2). The cdc-48.1 (tm344) mutation was transferred by mating. In this way, the following strains were prepared: XAT200 (unc-119(ed3) qats7200[unc-119(+)]), XAT201 (unc-119(ed3) qats7200[Pdc::48.1(FLAG::CDC-48.1, unc-119+)]), XAT202 (unc-119(ed3) cdc-48.1(m544) qats7200[unc-119(+)]), and XAT203 (unc-119(ed3) cdc-48.1(m544) qats7200[Pdc::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: trac-1Rv-XbaI+TRA-1A (nucleotides 3671–3676) and tra-1Rv-XbaI+Hoxa-5 (nucleotides 3671–3676) were inserted into the restriction sites XbaI and Hoxa-5 of GAP CATT ACC AAA ACC AAC CTA ACA C-3′. (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 levanum disolved in M9 buffer (2.2 mM KH2PO4, 4.2 mM Na2HPO4, 85 mM NaCl, 1 mM MgSO4). Gonads were dissected on poly-L-lysine-coated slides and fixed with 2% parafomaldehyde in PBSW (phosphate-buffered saline, 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, pH 7.4, containing 0.1% Tween20), followed by prechilled 100% dimethylformamide (10 minutes at −20°C). These slides were rehydrated with PBSW and blocked with 3% bovine serum albumin (BSA) in PBSW. 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 AAS, 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 PBSW 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 PBSW 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 CoolSnap HQ2 (Roper Scientific) color 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 (CA4), 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 cdcd-48.1, cdc-48.2, ufd-1, npl4-1, ubx-1, ubx-2, ubx-3, ubx-4, ubx-5, ubx-6, bal-2, rbx-1, ebl-1, ecl-1, fem-1, fem-2, fem-3 and tra-1 were amplified from ykl-corne or wild-type total RNA samples, and cloned into pGADT7 or pGBK7 plasmids. A plasmid to express mutant ELC-1 (L47DL49DDY887Y891D) 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 pGBK7 plasmids. Plasmids were verified by DNA sequencing. The two kinds of plasmid for production of G4l activation-domain fusion proteins and Gal4 DNA-binding-domain fusion proteins were cotransfected 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).

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