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Characterization of C-terminal adaptors, UFD-2 and UFD-3, of CDC-48 on the polyglutamine aggregation in *C. elegans*

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ABSTRACT

CDC-48 (also called VCP or p97 in mammals and Cdc48p in yeast) is a AAA (ATPases associated with diverse cellular activities) chaperone and participates in a wide range of cellular activities including modulation of protein complexes and protein aggregates. UFD-2 and UFD-3, C-terminal adaptors for CDC-48, reportedly bind to CDC-48 in a mutually exclusive manner and they may modulate the fate of substrates for CDC-48. However, their cellular functions have not yet been elucidated. In this study, we found that CDC-48 preferentially interacts with UFD-3 in *Caenorhabditis elegans*. We also found that the number of polyglutamine (polyQ) aggregates was reduced in the *ufd-3* deletion mutant but not in the *ufd-2* deletion mutant. Furthermore, the lifespan and motility of the *ufd-3* deletion mutant, where polyQ40::GFP was expressed, were greatly decreased. Taken together, we propose that UFD-3 may promote the formation of polyQ aggregates to reduce the polyQ toxicity in *C. elegans*.

1. Introduction

CDC-48 (although it is also called VCP or p97 in mammals and Cdc48p in yeast, we use CDC-48 throughout this study) is a AAA (ATPases associated with diverse cellular activities) chaperone that converts the chemical energy generated from ATP hydrolysis into the mechanical force used for protein conformational changes such as the unfolding of proteins and disassembly of protein complexes. CDC-48 is involved in a wide variety of cellular processes, including the modulation of protein aggregation, cell-cycle control, organelle membrane fusion, endoplasmic reticulum-associated protein degradation and mitochondrial quality control [1–7]. It should be noted that two highly homologous CDC-48s, CDC-48.1 and CDC-48.2, exist in *Caenorhabditis elegans* and that their function is essential and redundant [8].

The functional diversity of CDC-48 is determined by a differential binding of a variety of adaptors. Several adaptors have been identified in *C. elegans*, e.g. NPL-4-UFD-1 and six different UBXN proteins, which bind to the N-terminal domain of CDC-48 [9], and UFD-2 and UFD-3, which bind to the C-terminal motif of CDC-48 [9–11]. In general, it is considered that N-terminal adaptors are involved in the determination of substrate proteins, whereas C-terminal adaptors may determine the fate of these substrate proteins [12,13]. UFD-2 is an E4 ubiquitin ligase, which extends the ubiquitin chains of ubiquitinated substrates to promote proteasomal degradation [13–15]. In contrast, neither enzymatic activity nor cellular function of UFD-3 is clarified yet. UFD-2 and UFD-3 reportedly bind to CDC-48 in a mutually exclusive manner [10,13].

Mutational expansion of CAG repeats encoding polyglutamine (polyQ) stretches is responsible for nine neurodegenerative diseases including Huntington's Disease, spinal and bulbar muscular atrophy, dentatorubral pallidoluysian atrophy and spinocerebellar ataxias types 1, 2, 3, 6, 7 and 17 [16,17]. PolyQ aggregates accumulate in nuclear or cytoplasmic inclusion bodies that are invariably associated with end-stage neurodegenerative disease in patients and model animals. It is previously considered that polyQ aggregates have cytotoxicity [18–22]. On the other hand, recently, it has been increasingly suggested that oligomeric forms of polyQ proteins are more toxic and polyQ aggregates may act as a cellular coping mechanism to sequester levels of potentially toxic soluble monomeric and oligomeric species of polyQ [23–25]. To develop a therapeutic tactics including drugs, it is quite important to know exactly what causes the cytotoxicity, but unfortunately it is still contradictory on this issue.

It has been reported that CDC-48 co-localizes with polyQ aggregates in cultured cells and with intraneuronal inclusions in

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several neurodegenerative diseases [26,27]. In addition, the *Drosophila* CDC-48 homologue was identified as a genetic modifier of polyQ-induced eye degeneration [28]. We have also reported that co-expression of either of CDC-48s with a polyQ protein partially suppressed the aggregation of the polyQ protein in *C. elegans* [8] and that CDC-48 bound Huntingtin fragments containing polyQ repeats directly, and retarded the aggregate formation of HttQ53 [29]. These results clearly suggest that CDC-48, a AAA chaperone, plays an important role in polyQ-associated diseases.

In this study, we analyzed the interaction of CDC-48 with C-terminal adaptors and found that CDC-48 preferentially interacts with UFD-3 *in vivo*. We also analyzed whether UFD-2 and UFD-3 are involved in polyQ aggregate formation processes using the *C. elegans* system as a model of polyQ disease. We found that the number of polyQ40 aggregates was reduced in the *ufd-3* deletion mutant but not in the *ufd-2* deletion mutant and that the lifespan and motility of polyQ40-expressing worms were greatly decreased in the *ufd-3* deletion background. Taken together, we propose that UFD-3 may promote the formation of polyQ aggregates to reduce the polyQ toxicity.

2. Materials and methods

2.1. C. elegans strains and general methods

Worms were maintained using standard protocols as described previously [30]. The Bristol strain N2 was used as the wild-type strain. The deletion mutants of *cdc-48.1(tm544)*, *ufd-2(tm1380)* and *ufd-3(tm2915)* were obtained from Dr. Shohei Mitani (Tokyo Women's Medical University). XA7200 *unc-119(ed3) qaIs7200*[*unc-119(+)*] and XA7203 *unc-119(ed3); cdc-48.1(tm544) qaIs7201*[P*cdc-48.1*-FLAG::CDC-48.1, *unc-119(+)*] were reported previously [31]. To construct transgenic worms expressing expanded polyQ stretches, DNA fragment encoding 40 polyQ stretches fused to GFP (polyQ40::GFP) was cloned and microinjected as described previously [8]. Extrachromosomal DNA was integrated into a chromosome of *C. elegans* by UV irradiation as described previously [32]. To exclude unexpected additional mutations due to UV irradiation, integrated worms obtained were out-crossed 3 times, thus yielding XA7249 *qaIs7249*[P*unc-54*-polyQ0::GFP] and XA7250 *qaIs7250*[P*unc-54*-polyQ40::GFP]. Males carrying mutations were generated from mutants and were used to transfer the mutation. We generated the following strains: XA7251 *cdc-48.1(tm544) qaIs7201*, XA7252 *cdc-48.1(tm544) ufd-2*(*tm1380*) *qaIs7201*, XA7253 *cdc-48.1(tm544) ufd-3*(*tm2915*) *qaIs7201*, XA7260 *ufd-2*(*tm1380*) *qaIs7249*, XA7261 *ufd-3*(*tm2915*) *qaIs7249*, XA7262 *ufd-2*(*tm1380*) *ufd-3*(*tm2915*) *qaIs7249*, XA7263 *ufd-2*(*tm1380*) *qaIs7250*, XA7264 *ufd-3*(*tm2915*) *qaIs7250*, XA7265 *ufd-2*(*tm1380*) *ufd-3*(*tm2915*) *qaIs7250*. XA7200, XA7203 and strains carrying *qaIs7201* were maintained and analyzed at 25 °C. Others were maintained and analyzed at 20 °C.

2.2. Immunoprecipitation and immunostaining

Immunoprecipitation and immunostaining assays were performed as described previously [9,31].

2.3. Western blotting

Total lysates of worms and IP samples were resolved on 5–12% SDS gradient gels (Wako) or 10% or 12.5% SDS-PAGE gels, and then proteins were transferred to a nitrocellulose membrane. Prestained Protein Markers (Broad Range) (NACALAI TESQUE, INC.) or Dr. Western (Oriental Yeast CO., LTD.) was used as a Western blotting marker. Signals were detected with anti-GFP (Clontech; 1:1000), anti-UFD-2 (1:1000), anti-UFD-3 (1:1000), anti-CDC-48 (1:5000), anti-FLAG(M2) (Sigma; 1:1000), and anti-α-tubulin (Sigma; 1:1000) antibodies as a primary antibody. Secondary antibodies were anti-mouse IgG horseradish peroxidase–conjugated F(ab′)2 fragment (GE Healthcare; 1:5000) and anti-rabbit IgG horseradish peroxidase–conjugated F(ab′)2 fragment (GE Healthcare; 1:5000). Proteins were visualized using Western Lightning Chemiluminescence Reagent Plus (Perkin–Elmer Life Sciences). Chemiluminescent signals were detected with LAS-4000 mini. α-Tubulin was used as a loading control.

2.4. Measurement of polyglutamine aggregates

The number of polyQ::GFP aggregates in body wall muscle cells was counted under the Olympus SZX12 fluorescence microscope. Approximately 10 worms were analyzed per each experimental condition. Young adult worms were defined as Day 1.

2.5. Lifespan analysis

Synchronized young adults were treated with 0.4 mg/ml fluorodeoxyuridine (FUdR) for 24 h to prevent progeny production. Then worms were transferred to fresh 0.2 mg/ml FUdR-containing plates and started recording of survival every other day (at least 70 worms per strain). Lifespan assays were repeated at least two times. Worms were considered dead, when they did not move after repeated taps with a pick. The day when eggs were laid was considered Day 0. Data analysis was performed as described by Yang et al. [33], using the publicly available analysis suite OASIS (http://www.sbi.postech.ac.kr/oasis/).

2.6. Worm motility assay

Motility was measured as described previously [34].

3. Results and discussion

3.1. Preferential binding of CDC-48 to UFD-3 in C. elegans

UFD-2 and UFD-3 reportedly bind to CDC-48 in a mutually exclusive manner in yeast [10,13]. It was also reported that *C. elegans* UFD-2 and UFD-3 bound to the C-terminal motif of CDC-48.1 and CDC-48.2 (hereafter collectively CDC-48s) by using a yeast two-hybrid analysis [9,11] and we confirmed it (Supplemental Fig. S1). We then asked whether UFD-2 and UFD-3 can bind to CDC-48s in *C. elegans in vivo*. We first generated antibodies against *C. elegans* UFD-2 and UFD-3, and confirmed that they well recognized UFD-2 and UFD-3, respectively (Supplemental Fig. S2). Previously we have prepared the *C. elegans* strain XA7203, which expresses FLAG-tagged CDC-48.1 [31]. Lysates were immunoprecipitated with the anti-FLAG(M2) antibody and immunoprecipitates were analyzed by Western blotting by using anti-CDC-48, anti-UFD-2, and anti-UFD-3 antibodies. As shown in Fig. 1A, FLAG::CDC-48.1 was well precipitated. Both FLAG::CDC-48.1 and CDC-48.2 were detected, suggesting that FLAG::CDC-48.1 and CDC-48.2 can form a hetero-hexamer. Note that CDC-48 usually forms a hexameric ring-like structure [35]. UFD-3 was clearly co-precipitated with FLAG::CDC-48.1. It is interesting to mention, however, that UFD-2 was undetectable in the immunoprecipitates under the experimental conditions used, although UFD-2 existed in the total lysates. These results suggest that UFD-3 can form a stable complex with CDC-48 *in vivo*.

So, if UFD-2 and UFD-3 bind to CDC-48s in a mutually exclusive manner and UFD-3 forms a stable complex with CDC-48s, it might be possible that UFD-2 does not show any binding to CDC-48s.

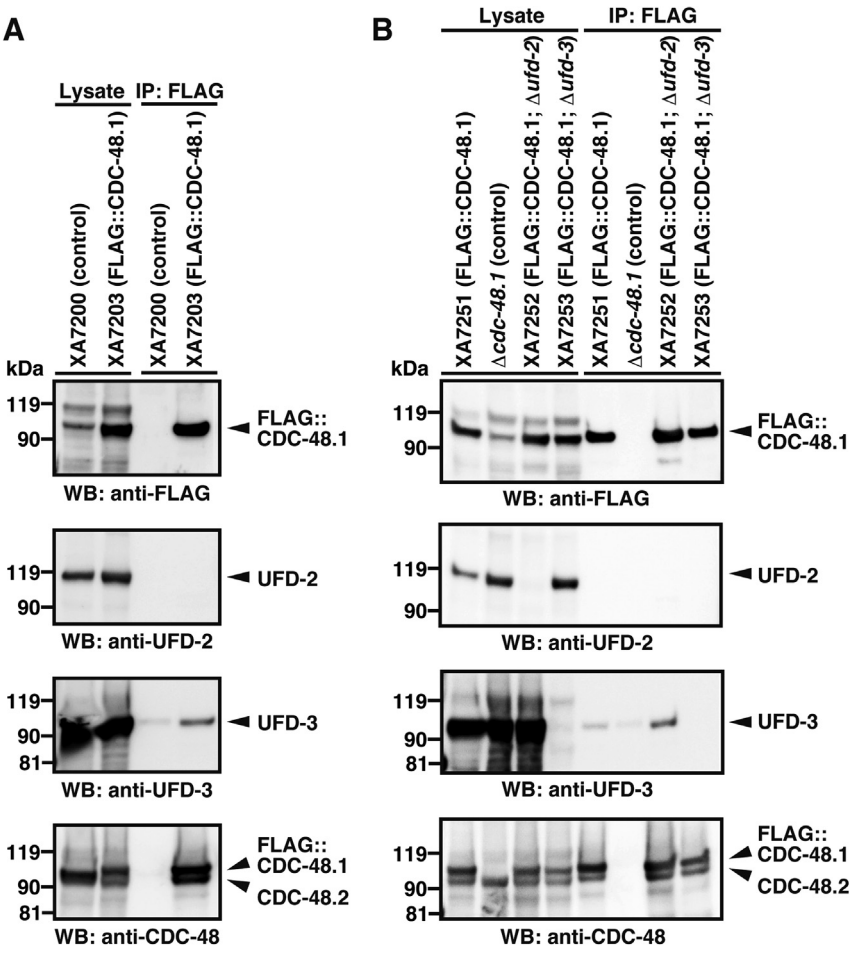


Fig. 1. Interaction between CDC-48 and its C-terminal adaptors, UFD-2 and UFD-3. Total lysates from mixed-staged worms of XA7200 and XA7203 (A), and the *cdc-48.1(tm544)* strain, XA7251, XA7252 and XA7253 (B) were used for immunoprecipitation analysis with anti-FLAG(M2) affinity gels. Immunoprecipitates were analyzed with Western blotting with anti-FLAG, anti-CDC-48, anti-UFD-2, anti-UFD-3 antibodies. XA7200 and the *cdc-48.1(tm544)* strain were used as negative controls. All strains were grown at 25 °C.

Therefore, we next asked whether UFD-2 can bind to CDC-48s in the *ufd-3* deletion mutant. For this purpose, we prepared the FLAG::CDC-48.1-expressing *C. elegans* strains in the *ufd-2* or *ufd-3* deletion background. As shown in Fig. 1B, UFD-3 was well co-precipitated with FLAG::CDC-48.1 in the *ufd-2* deletion background. However, again, UFD-2 was not detected even in the *ufd-3* deletion background. These results strongly suggest that at least a part of UFD-3, if not all, but not UFD-2, forms a stable complex with CDC-48s and that the interaction between UFD-2 and CDC-48s may be very weak or only transient *in vivo*. It seems possible that UFD-2 may interact with E3 ubiquitin ligase and ubiquitinated substrate and then recruit CDC-48 to extract the substrate, since UFD-2 reportedly possesses E4 ubiquitin ligase activity [13–15]. Indeed, it was reported that UFD-2 interacted with CHN-1, an E3 ubiquitin ligase, and assembled into a CDC-48-UFD-2-CHN-1 complex [11].

3.2. CDC-48 co-localizes with UFD-3

Next, we investigated the localization of CDC-48 and its C-terminal adaptors, UFD-2 and UFD-3. We performed an immunostaining analysis using a dissected intestine from the FLAG::CDC-48.1-expressing worms. FLAG::CDC-48.1, UFD-2 and UFD-3 were detected by the anti-FLAG, anti-UFD-2 and anti-UFD-3 antibodies, respectively. FLAG::CDC-48.1 preferentially localized at the periphery of nuclei of intestinal cells (Fig. 2), which is consistent with the previous observation [9]. UFD-3 showed a similar localization pattern with FLAG::CDC-48.1 (Fig. 2B). However, UFD-2 showed a diffuse distribution throughout the intestinal cells, which is a different localization pattern of FLAG::CDC-48.1 and UFD-3 (Fig. 2A). These results also support that UFD-3, but not UFD-2, preferentially interacts with CDC-48s in *C. elegans*.

3.3. UFD-2 and UFD-3 may have a different role in the suppression of polyQ aggregate formation

We previously reported that when polyQ repeats are longer than 40, discrete cytoplasmic aggregates were formed and that the aggregate formation was partially suppressed by simultaneous overexpression of CDC-48s [8]. In addition, the purified CDC-48.1 promoted the formation of oligomers of the polyQ-containing fragment of Huntingtin *in vitro* [29]. These results imply that CDC-48 could have a protective role in polyQ aggregation. To decipher the roles of UFD-2 and UFD-3 on the aggregate formation, we constructed *ufd-2* and *ufd-3* deletion mutants, in which polyQ0::GFP or polyQ40::GFP was expressed. Expression of polyQ::GFP was directed to the body wall muscle cells by using the *unc-54* promoter and worms expressing polyQ40::GFP developed discrete cytoplasmic aggregates during adulthood (Fig. 3A) [8]. Worms were synchronized and young adults were defined as Day 1. We counted the number of polyQ40::GFP aggregates under the

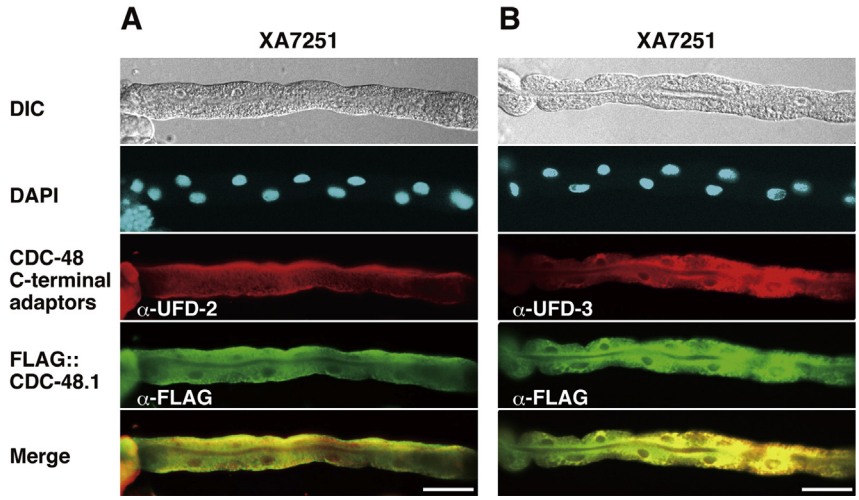


Fig. 2. Localization of CDC-48 and its C-terminal adaptors, UFD-2 and UFD-3. Dissected intestines prepared from the XA7251 strain at the young adult stage were stained with DAPI (blue), individual anti-UFD-2 (A) and anti-UFD-3 (B) antibodies (red) and the anti-FLAG antibody (green). XA7251 was grown at 25 °C. Bars represent 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

fluorescence microscope for 3 days (Fig. 3A and B). At Day 1, the number of polyQ40::GFP aggregates was very small and similar among all strains. We also analyzed the amount of polyQ40::GFP by Western blotting using the anti-GFP antibody (Fig. 3C). The amount of polyQ40::GFP protein in each strain was essentially the same. We also detected faint SDS-insoluble GFP signals, which may correspond to the polyQ40::GFP aggregates, at the gel top, which are indicated in Fig. 3C. As worms were aged, the number of aggregates and their amounts increased in all strains. The number of polyQ40::GFP aggregates and their amounts in the *ufd-2* deletion mutant were indistinguishable from those in the wild-type strain, suggesting that UFD-2 may not direct to the formation of

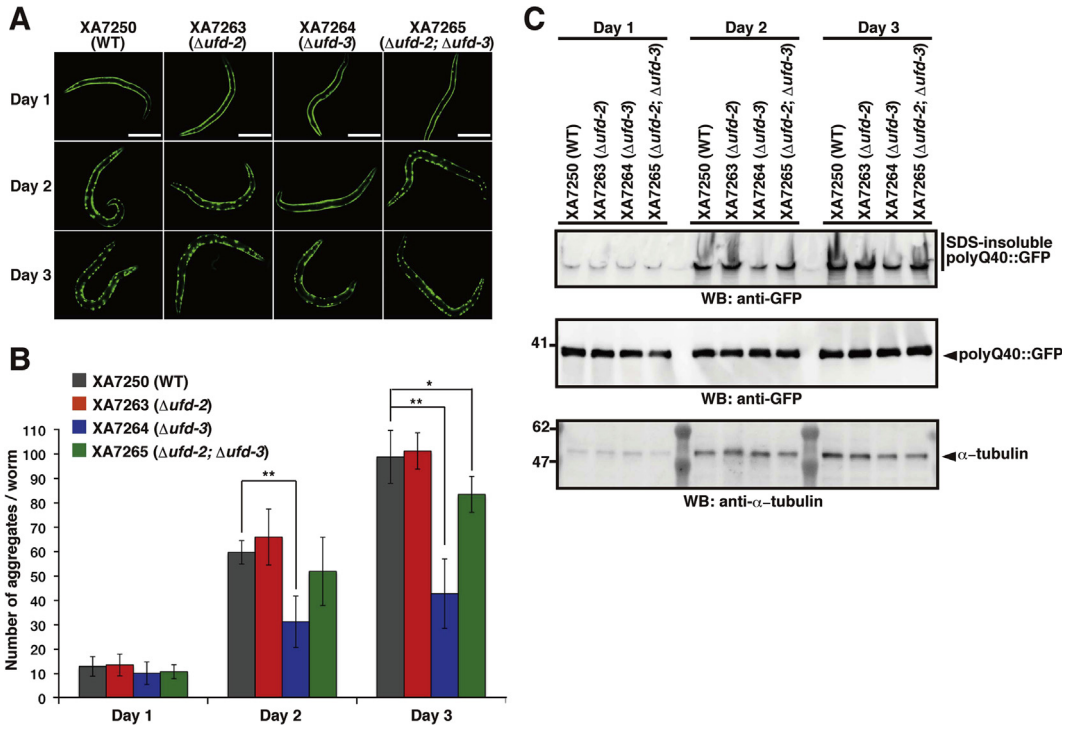
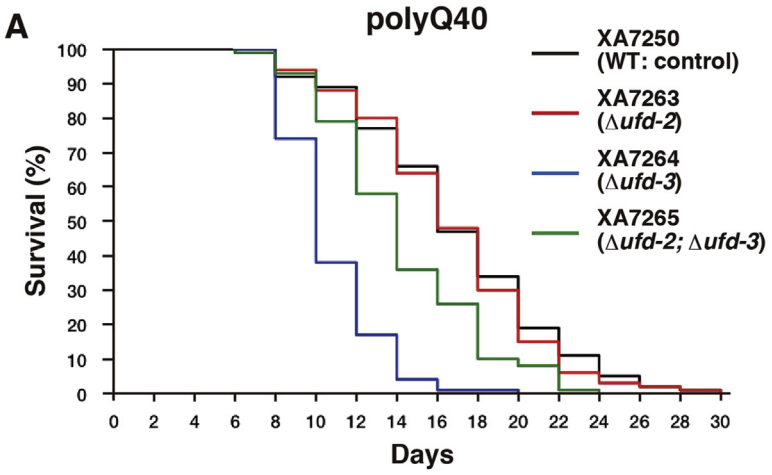


Fig. 3. Effects of the *ufd-3* deletion on polyQ40 aggregate formation. (A) Fluorescence micrographs of deletion mutants of UFD-2 and UFD-3 expressing polyQ40::GFP in body wall muscle cells. Strains XA7250 *qaIs7250*[P*unc-54*-polyQ40::GFP], XA7263 *ufd-2*(*tm1380*) *qaIs7250*, XA7264 *ufd-3*(*tm2915*) *qaIs7250*, and XA7265 *ufd-2*(*tm1380*) *ufd-3*(*tm2915*) *qaIs7250* were used. Young adult worms were defined as Day 1. Bars represent 250 μm. (B) The number of polyQ40::GFP aggregates of each mutant as shown in (A) was counted. Approximately 10 worms were scored per experimental group in each experiment. We performed three independent experiments and similar results were obtained in each experiment. Statistical significance was assessed by Student's *t* test; *, $P < 0.05$; **, $P < 0.001$. Error bars indicate standard deviation. (C) Total lysates of each mutant were boiled with SDS-containing sample buffer and resolved on 12.5% SDS-PAGE gel. Proteins were transferred to the nitrocellulose membrane and polyQ40::GFP proteins were detected by the anti-GFP antibody. Nine worms' lysates were loaded each lane. α-Tubulin was used as a loading control.

aggregates. In contrast, the number of polyQ40::GFP as well as the amount of SDS-insoluble polyQ40::GFP signal were significantly lower in the *ufd-3* deletion mutant than those in the wild-type strain. These results suggest that UFD-3 may direct to the formation of aggregates. Defects in the *ufd-3* deletion mutant were partially suppressed by the *ufd-2* deletion (Fig. 3). It should be noted that few aggregate was observed in all strains throughout the experimental period when polyQ0::GFP-expressing worms were analyzed and that no SDS-insoluble polyQ0::GFP signal was detected at the gel top (Supplemental Fig. S3).



B

Strain	*N*	Mean lifespan days ± S.E. (95 % C.I.)	Maximum days	*P* value
polyQ40				
XA7250 (WT)	105	16.92 ± 0.48 (15.99~17.86)	30	
XA7263 (Δ*ufd-2*)	105	16.59 ± 0.43 (15.73~17.45)	30	> 0.05
XA7264 (Δ*ufd-3*)	76	10.71 ± 0.28 (10.17~11.25)	20	< 0.01
XA7265 (Δ*ufd-2; Δufd-3*)	105	14.17 ± 0.37 (13.45~14.90)	24	< 0.01

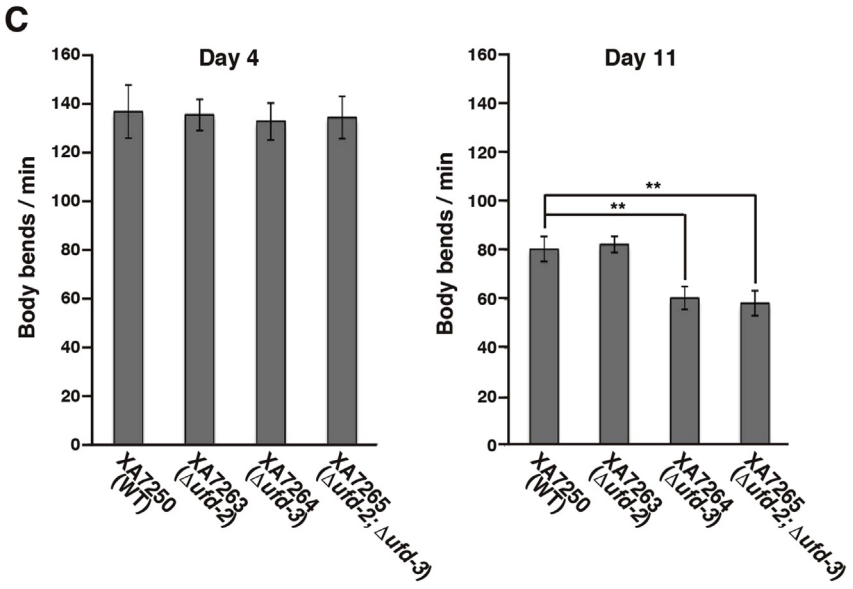


Fig. 4. Effects of deletion of CDC-48 C-terminal adaptors on the lifespan and motility of polyQ40::GFP-expressing worms. (A) Kaplan–Meier survival curves for one representative survival experiment for polyQ40::GFP worms with deletion mutations of CDC-48 C-terminal adaptors. Strains XA7250 *qaIs7250*[P*unc-54*-polyQ40::GFP], XA7263 *ufd-2*(*tm1380*) *qaIs7250*, XA7264 *ufd-3*(*tm2915*) *qaIs7250*, and XA7265 *ufd-2*(*tm1380*) *ufd-3*(*tm2915*) *qaIs7250* were used. (B) Mean lifespan and maximum lifespans of each mutant are shown. Data analysis was performed by using OASIS (http://www.sbi.postech.ac.kr/oasis/) [33]. (C) Motility of each mutant was measured at Day 4 and Day 11 of panel (A). Approximately 10 worms were scored in each experiment. We performed three independent experiments and similar results were obtained in each experiment. Statistical significance was assessed by Student's *t* test; **, $P < 0.001$. Error bars indicate standard deviation.

3.4. The ufd-3 deletion has a negative effect on lifespan and motility

It is known that ageing plays an important role in the process of late onset neurodegeneration, and indeed it is observed that the time before polyQ pathology manifests is approximately correlated with lifespan of organisms [16]. Therefore, we analyzed effects of *ufd-2* and *ufd-3* deletions on the lifespan of worms expressing polyQ0::GFP (Supplemental Fig. S4) or polyQ40::GFP (Fig. 4). In polyQ0::GFP-expressing worms, the *ufd-3* deletion mutant has a slightly shorter lifespan, suggesting that lack of UFD-3 negatively affects the lifespan determination process to some extent directly or indirectly. On the other hand, in polyQ40::GFP-expressing worms, the lifespan of the *ufd-3* deletion mutant was greatly decreased (Fig. 4A and B). Lifespan of the *ufd-2* deletion mutant was indistinguishable from that of the wild-type strain, and the *ufd-2* deletion partially suppressed the defect of the *ufd-3* deletion as similar to the observation shown in Fig. 3.

To analyze cytotoxicity of polyQ40::GFP aggregates, we then measured worm motility. As shown in Fig. 4C, at the young adult stage (Day 4), motility was not affected by any mutations. However, at Day 11, motility was significantly reduced in the *ufd-3* deletion mutant, while the wild-type and the *ufd-2* deletion strains showed the similar activity. The *ufd-2* deletion did not show suppression of the defect of the *ufd-3* deletion in this case. This could be due to that the timing of measurement was too late to show the suppression. Providing that the aggregate formation was decreased in the *ufd-3* deletion mutant (Fig. 3), these results shown in Fig. 4 suggest that polyQ40 aggregates may have low toxicity and that UFD-3, but not UFD-2, may promote the formation of polyQ40 aggregates to reduce the toxicity of the intermediate oligomers. Therefore CDC-48 and UFD-3 might prevent toxicity of polyQ-expanded proteins by modulating its oligomeric states. Our results seem to support the notion that oligomeric forms of polyQ proteins are more cytotoxic [23–25].

3.5. Perspective

In this study, we found that CDC-48 preferentially interacts with UFD-3 in *C. elegans* under the physiological condition. We also demonstrated that the CDC-48-UFD-3 complex acts as a promoter for the polyQ aggregate formation and reduces polyQ cytotoxicity.

Besides CDC-48, a AAA chaperone, it has been reported that chaperonin TRiC/CCT modulates the polyQ aggregation and suppresses its toxicity [36–38]. Furthermore, several chaperones, including Hsp70, Hsp104, Hsp110 together with Hsp40, and small heat shock proteins, reportedly suppressed polyQ-induced cell death in model organisms [39–42]. Therefore, modulating chaperone activity would be a promising strategy for polyQ disease therapy.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.02.088.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.02.088.

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