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Effects of loss of the E2 conjugating enzymes *ubc-6* and *ubc-7* on the unfolded protein response and protein homeostasis in *C. elegans*

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Abstract

The endoplasmic reticulum associated degradation (ERAD) pathway ubiquitylates and removes misfolded proteins from the ER to prevent the accumulation of misfolded protein and activation of the ER Unfolded Protein Response (UPR^{ER}). We investigated the *C. elegans* ERAD E2 conjugase genes *ubc-6* and *ubc-7*. *Δubc-6* animals do not accumulate the misfolded protein CPL-1^{Y32AW35A}::YFP, but they upregulate the UPR^{ER} reporter gene, P*hsp-4*::GFP. *Δubc-7* animals accumulate significantly more CPL-1^{Y32AW35A}::YFP compared to wildtype, but do not hyper-activate the *hsp-4* reporter. Our findings suggest that *ubc-6* and *ubc-7* contribute differently to ER and protein homeostasis in *C. elegans*.

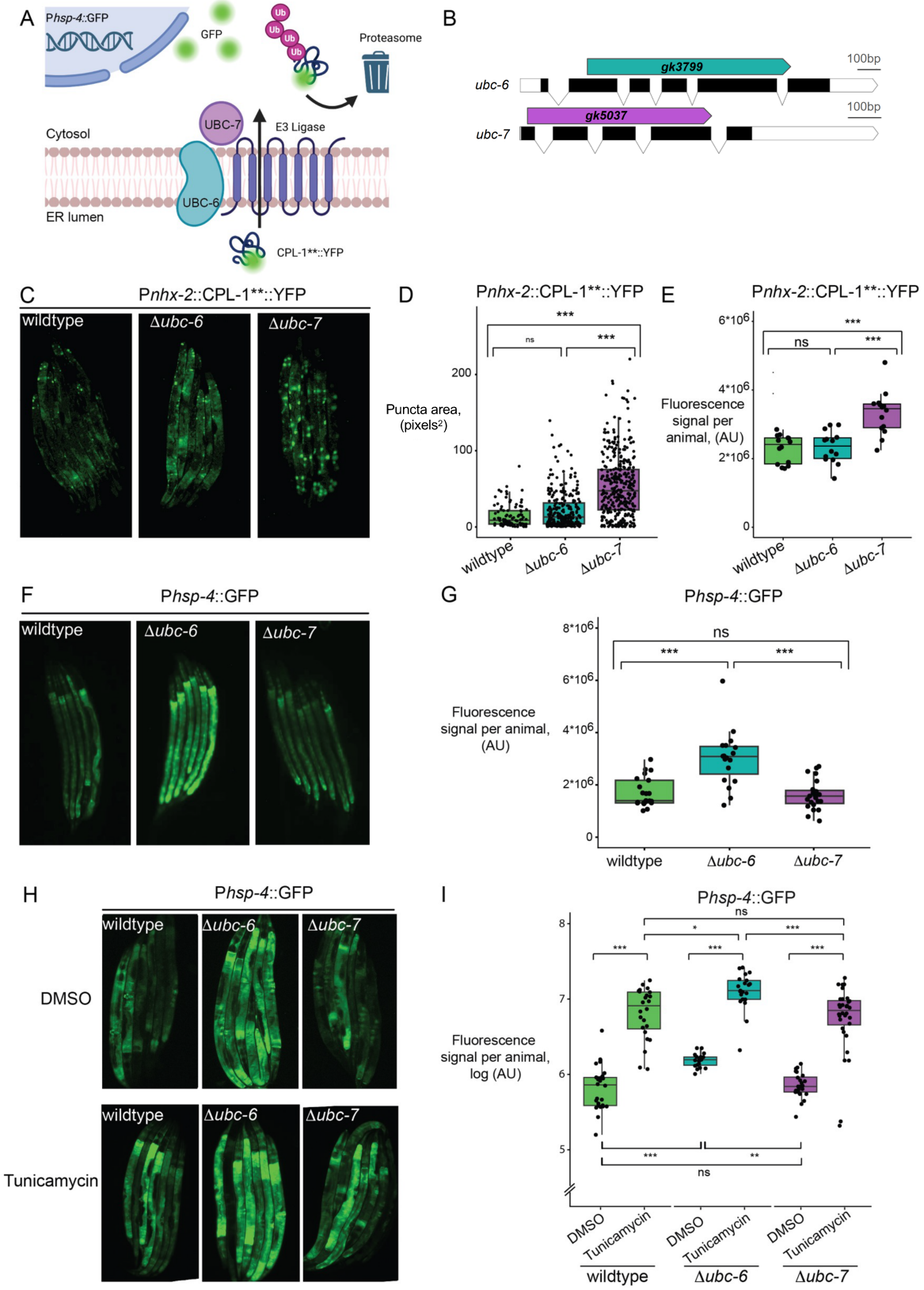


Figure 1. Model of ERAD and fluorescence imaging of *C. elegans* lacking *ubc-6* or *ubc-7*:

A. Simplified model of the ERAD pathway that UBC-6 and UBC-7 participate in. A misfolded protein (CPL-1^{Y32AW35A}::YFP) is translocated to the cytosol where it is ubiquitylated and degraded by the proteasome. The UPR^{ER}

reporter, P*hsp-4*::GFP, is active at low levels in basal conditions and is upregulated in response to ER stress. Model created using BioRender.

B. Schematic diagram of the coding regions of the *ubc-6* and *ubc-7* genes and their deletion alleles, *gk3799* (*Δubc-6*, teal arrow) and *gk5037* (*Δubc-7*, magenta arrow). Black boxes indicate exons, separated by spliced introns, which are designated by black linkers. White boxes indicate untranslated sequences.

C. Fluorescence images of wildtype, *Δubc-6*, and *Δubc-7* animals expressing CPL-1^{Y32AW35A}::YFP under the control of the *nhx-2* promoter (CPL-1**::YFP).

D. Quantification of the size, in pixels^2, of puncta caused by CPL-1^{Y32AW35A}::YFP (CPL-1**::YFP) accumulation in wildtype, *Δubc-6*, *Δubc-7* animals.

E. Quantification of the total CPL-1^{Y32AW35A}::YFP fluorescence per animal in wildtype, *Δubc-6*, *Δubc-7* animals.

F. Fluorescence images of wildtype, *Δubc-6*, and *Δubc-7* animals expressing GFP under the control of the *hsp-4* promoter. All images were identical processed using the same parameters.

G. Quantification of the total GFP fluorescence per animal in wildtype, *Δubc-6*, *Δubc-7* animals.

H. Fluorescence images of wildtype, *Δubc-6*, and *Δubc-7* animals expressing GFP under the control of the *hsp-4* promoter in control (DMSO) and stress (2.5 ng/mL tunicamycin) conditions. Images for DMSO and tunicamycin treatments were processed separately to preserve visible differences.

I. Quantification of the total GFP fluorescence per animal in wildtype, *Δubc-6*, and *Δubc-7* animals treated with DMSO or tunicamycin.

*, p<0.05; **, p<0.001; ***, p<0.0001; ns, not significant. Tukey post-hoc test

Description

The endoplasmic reticulum (ER) regulates post-translational protein folding and maturation to maintain protein homeostasis and limit the accumulation of misfolded proteins. Accumulation of misfolded protein at the ER activates the ER Unfolded Protein Response (UPR^{ER}) and the downstream ER-associated protein degradation (ERAD) pathway, which alleviates the build-up of unfolded and/or unprocessed proteins (Christianson and Carvalho 2022). ERAD is facilitated by a protein complex that includes ubiquitin conjugating enzymes that target substrates on the cytosolic side of the ER membrane (Christianson and Ye 2014; Christianson et al. 2023). Ubiquitylated ERAD targets are retrotranslocated from the ER to the cytosol and degraded by the proteasome (Vembar and Brodsky 2008; Neal et al. 2018; Lopata et al. 2020; Christianson and Carvalho 2022). In *S. cerevisiae*, two ERAD E2 ligases, Ubc6 and Ubc7 have complementary roles in which they sequentially ubiquitylate misfolded proteins translocated from the ER lumen to the cytosol (Weber et al. 2016; Christianson and Carvalho 2022). Ubc6 adds the primary ubiquitin to lysines or hydroxylated amino acid residues (serine or threonine) on target proteins, while Ubc7 elongates the ubiquitin chain. Ubc7 has a more expansive role in ERAD than Ubc6 as it is also able to prime target proteins with ubiquitin on lysine residues prior to elongation of the ubiquitin chain (Weber et al. 2016). The mammalian orthologs to Ubc6 and Ubc7, Ube2j1/Ube2j2 and Ube2g1/Ube2g2, respectively, work with E3 conjugases to maintain ER protein homeostasis in a similar manner (Wang et al. 2009; Burr et al. 2013; Christianson and Ye 2014; Christianson and Carvalho 2022; Abdul Rehman et al. 2024).

In *C. elegans*, there are two predicted orthologs of ER-associated E2 conjugating enzymes, UBC-6 and UBC-7 (Figure 1A). We investigated the roles of the *ubc-6* and *ubc-7* genes in *C. elegans* by characterizing the effect of deletion alleles on the UPR^{ER} and ERAD. We used genetic reporters to measure accumulation of misfolded proteins and activation of the UPR^{ER} in strains with CRISPR-Cas9-targeted deletion alleles of either *ubc-6 (gk3799)* (hereafter Δ*ubc-6*) or *ubc-7 (gk5037)* (Δ*ubc-7*) (Figure 1B), which we crossed into two different reporter lines. To measure the accumulation of misfolded proteins, we used P*nhx-2*::CPL-1^{Y32AW35A}::YFP (*hhIs113*), which serves as a reporter for ERAD function because CPL-1^{Y32AW35A}::YFP is engineered to misfold, but is normally cleared from the ER lumen via ERAD (Miedel et al. 2012; Efstathiou et al. 2022). In order to observe the activation of the UPR^{ER}, we measured the expression of GFP under the control of the *hsp-4* promoter P*hsp-4*::GFP (*zcIs4*), whose activity increases in response to heat shock and other forms of ER stress (Ye et al. 2004; Walter and Ron 2011; Bar-Ziv et al. 2020). Using the Δ*ubc-6* and Δ*ubc-7* alleles, we observed how misfolded protein accumulation and regulation of a UPR^{ER} reporter depend on each gene in *C. elegans*.

We used fluorescence microscopy to determine how the loss of the E2 conjugases *ubc-6* and *ubc-7* affect the accumulation of CPL-1^{Y32AW35A} and the levels of *hsp-4* expression. We measured CPL-1^{Y32AW35A}::YFP accumulation by quantifying the size of CPL-1^{Y32AW35A}::YFP puncta in Δ*ubc-6* and Δ*ubc-7* animals. Compared to wildtype, the average puncta area

of CPL-1^{Y32AW35A}::YFP that accumulated was statistically unchanged in Δ*ubc-6* animals (21.7 pixels^2 compared to 14.5 pixels^2 in wildtype), whereas Δ*ubc-7* animals had larger average puncta size (53.8 pixels^2 compared to 14.5 pixels^2 in wildtype and 21.7 pixels^2 in Δ*ubc-6* animals) (Figure 1C, D). The overall signal intensity correlated with the puncta area since wildtype and Δ*ubc-6* animals had essentially the same overall fluorescence, while Δ*ubc-7* animals were, on average 1.45 times brighter (Figure 1E). Immunoblot analysis (not shown) confirmed these observations. This result aligns with previous results from yeast, in which Ubc6 mutants accumulate less misfolded protein in the ER lumen compared to Ubc7 mutants (Weber et al. 2016). Because Δ*ubc-7* animals have high accumulation of CPL-1^{Y32AW35A}::YFP, we expected that they would have a similarly high expression of the *Phsp*-4::GFP reporter, compared to wildtype and Δ*ubc-6* animals. However, animals lacking *ubc-6* exhibited 1.75 times higher GFP expression compared to the wildtype, whereas animals lacking *ubc-7* had similar GFP levels as wildtype (Figure 1 F,G). These results were also confirmed by immunoblotting (not shown). To determine if either mutant was more sensitive to an acute ER stressor, we treated animals with tunicamycin, a compound that prevents protein glycosylation in the ER, or DMSO as a vehicle control. All animals were affected by tunicamycin as measured by P*hsp-4*::GFP activation, which shows that Δ*ubc-7* animals are able to respond to ER stress. We also found that animals lacking *ubc-6* had significantly higher P*hsp-4*::GFP activation in response to tunicamycin compared with both wild-type and Δ*ubc-7* animals (Figure 1, H, I).

Our results show that loss of *ubc-7* has a detrimental effect on the ability of cells to clear the misfolded protein CPL-1^{Y32AW35A}::YFP, which suggests that it is particularly important for maintaining ER protein homeostasis. Despite the increase in protein CPL-1^{Y32AW35A}::YFP accumulation, loss of *ubc-7* does not lead to an upregulation of the *Phsp-4::GFP* reporter over wildtype levels, which suggests that either Δ*ubc-7* animals are unable to mount a higher UPR^{ER} response via *hsp-4*, or that a redundant pathway is activated but not detected in our assays. Our quantification of P*hsp-4*::GFP expression also shows that loss of *ubc-6* leads to the activation of the UPR^{ER} reporter above the wildtype levels in control and stress (tunicamycin) conditions. Therefore, although Δ*ubc-6* animals do not accumulate significantly more CPL-1^{Y32AW35A}::YFP than wildtype animals, the loss of *ubc-6* induces an elevated UPR^{ER} response. In conclusion, while *ubc-6* and *ubc-7* are necessary for ERAD and activation of the UPR, they seem to have independent, though related, functions. These experiments were carried out in single mutants, but it will be interesting to determine the epistatic relationship of *ubc-6* and *ubc-7*, especially because Ubc6 and Ubc7 deletions in yeast can have additive effects on stress responses (Owutey et al. 2024). In addition, future work will help determine if UBC-6 and UBC-7 share the biochemical features of their yeast and mammalian orthologs, such as preferences for different amino acids and addition of primary ubiquitin versus chain elongation.

Methods

Animal maintenance and crossing

Animals were reared at 20°C on NGM. Genetic crosses were performed using established methods and genotypes were confirmed as outlined below.

Genotyping

Worms were lysed in a buffer of 50mM KCl, 10mM Tris, 2.5mM MgCl2, 0.45% Tween 20, and 0.45% NP-40; proteinase K was added in 200µg/mL final concentration. Lysate was used in PCRs with Apex 2.0X Taq RED Master Mix (1.5mM) from Genesee Scientific according to manufacturer directions (annealing: 55 C, 30 seconds; 90 second extension) with the following primers.

Primer	Sequence
ubc-6 FWD	5'-ATGAGTGAGCAGT-3'
ubc-6 REV	5'-ACTCGGAACCGTC-3'
ubc-7 FWD	5' gacaggtaccaaaaaaTGGAGCAATCCTC -3'*
ubc-7 REV	5'-gacggaattcTCATTCTTCTTGACTTCTGCG -3'*

* lowercase sequence indicates exogenous sequence used for unrelated subcloning experiments.

Tunicamycin treatment

L4 stage *C. elegans* were incubated in 2.5 ng/ul tunicamycin or DMSO as vehicle control (both Fisher Scientific) for 4 hours with microfuge lids open at 20 degrees C. After incubation, animals were settled by microcentrifugation at 2000 rpm for 30 seconds and liquid was removed from the worm pellet.

Fluorescence widefield microscopy

Animals from all conditions for each experiment were imaged on the same day over several imaging sessions. Slides were prepared using 1% levamisole in 1% agarose in H2O. L4 worms were collected from plates and placed into 1% levamisole in M9 onto agarose slides. Using an eyebrow pick, worms were moved into stacks (arranged side by side in a row) on the slides. Images were collected on the Leica iDM6000 widefield microscope at 10X, 1 gain, and 200 ms exposure.

Quantification/Statistical analysis

Microscope images were processed in ImageJ (FIJI). To quantify GFP expression under the control of the *hsp-4* promoter (P*hsp-4*::GFP), the perimeter of each animal was traced, and mean fluorescence brightness was recorded and subtracted from corresponding background. To quantify CPL-1^{Y32AW35A}::YFP puncta, images of animals with the Pnhx-2::CPL-1^{Y32AW35A}::YFP transgene were enhanced in FIJI using the difference of Gaussians algorithm. Images were then thresholded using the IsoData algorithm with a lower intensity limit of 10 and upper intensity limit of 225. A watershed was applied to isolate overlapping puncta and the area of each punctum was calculated. Stacks of worms were traced to exclude surrounding noise. Significance ($p < 0.05$) was determined using an ANOVA followed by a Tukey post-hoc test. Statistical tests and graphs were done in Rstudio version 4.5.3.

Reagents

C. elegans **strains**

Name	Genotype	Purpose	Source
N2	Wildtype	Control for no YFP expression	CGC
PP1386	*hhIs113* (P*nhx-2*::CPL-1^{Y32AW35A}::YFP; P*myo-2*::mCherry)	Control for baseline level of CPL-1^{Y32AW35A}::YFP accumulation	CGC, T. Hoppe lab
SJ4005	*zcIs4* (P*hsp-4*::GFP)	Control for expression of *hsp-4*	CGC
CLD159	*ubc-7* (*gk5037*); *hhIs113*	Experimental strain for CPL-1^{Y32AW35A}::YFP accumulation with *ubc-7* deletion	This manuscript
CLD160	*ubc-6* (*gk3799*); *hhIs113*	Experimental strain for CPL-1^{Y32AW35A}::YFP accumulation with *ubc-6* deletion	This manuscript

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