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APY-1, a Novel *Caenorhabditis elegans* Apyrase Involved in Unfolded Protein Response Signalling and Stress Responses

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Protein glycosylation modulates a wide variety of intracellular events and dysfunction of the glycosylation pathway has been reported in a variety of human pathologies. Endo-apyrases have been suggested to have critical roles in protein glycosylation and sugar metabolism. However, deciphering the physiological relevance of Endo-apyrases activity has actually proved difficult, owing to their complexity and the functional redundancy within the family. We report here that a UDP/GDPase, homologous to the human apyrase Scan-1, is present in the membranes of *Caenorhabditis elegans*, encoded by the ORF F08C6.6 and hereinafter-named APY-1. We showed that ER stress induced by tunicamycin or high temperature resulted in increased transcription of *apy-1*. This increase was not observed in *C. elegans* mutants defective in *ire-1* or *atf-6*, demonstrating the requirement of both ER stress sensors for up-regulation of *apy-1*. Depletion of APY-1 resulted in constitutively activated unfolded protein response. Defects in the pharynx and impaired organization of thin fibers in muscle cells were observed in adult worms depleted of APY-1. Some of the *apy-1(RNAi)* phenotypes are suggestive of premature aging, because these animals also showed accumulation of lipofuscin and reduced lifespan that was not dependent on the functioning of DAF-2, the receptor of the insulin/IGF-1 signaling pathway.

INTRODUCTION

From observations on human diseases and mutant mice, it has become clear that glycosylation plays a major role in metazoan development (Schachter, 2004). Given the numerous physiological roles of glycoproteins, which encompass many aspects of normal cellular function and survival, the structures of glycan moieties vary markedly providing the chemical diversity required to fulfill the vast range of their biological tasks. The diversity of natural sugar structures is ultimately derived from the activities of the enzymes responsible for sugar attachment, namely glycosyltransferases, which determine the feasibility of natural product enzymatic glycodiversification. Lumenal ecto-nucleoside tri- and diphosphohydrolases (E-NTPDases) of the secretory pathway of eukaryotes hydrolyzes the nucleoside diphosphates (NDPs) generated by glycosyltransferases. The resulting nucleoside monophosphates (NMPs) are weaker inhibitors of glycosyltransferases than NDPs. NMPs also serve as antiporters in the transport of nucleotide-sugars from the cytosol to the lumen of the endoplasmic reticulum (ER) and Golgi apparatus. Glycosylation plays an important role in the biogenesis of secreted and transmembrane proteins and an elaborate mechanism ensure that only properly folded and assembled proteins exit the ER, a process termed "quality control."

However, The ER's capacity to process proteins is relatively limited and the stress caused by accumulation of unfolded and misfolded proteins (ER stress) contributes to a number of important human diseases (Zhang and Kaufman, 2006). Cells respond to ER stress by activating the unfolded protein response (UPR), which limits new protein synthesis and promotes the expression of genes that enhance the organelle's capacity to process unfolded proteins (Patil and Walter, 2001). During this process UDP-glucose is transported from the cytosol into the lumen of the ER, where it serves as a substrate for reglucosylation of incompletely folded glycoproteins. A byproduct of this reaction is UDP, which, upon accumulation, can inhibit further progress of reglucosylation reactions (Trombetta and Helenius, 1999). Nucleoside diphosphatases (NDPases) are therefore though to play an essential role for efficient UPR process.

E-NTPases and E-NDPases or apyrases have been traditionally grouped into one of two families, with each family being distinguished from one another on the basis of sequence similarity. The first family of extracellular nucleotidases is the E-NTPDases (Zimmermann, 2000). At least six different members of the human E-NTPDases have been

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discovered (Kaczmarek *et al.*, 1996; Smith and Kirley, 1998; Wang *et al.*, 1998; Mateo *et al.*, 1999), each of which possesses different enzymatic properties and different physiological localizations. Each member of this family possesses amino acid sequence similarities in the extracellular region of the proteins, with five "apyrase-conserved regions" (ACR; Handa and Guidotti, 1996; Schulte *et al.*, 1997) shown to be essential for enzymatic activity (Smith and Kirley, 1998; Drosopoulos *et al.*, 2000). All reported members of the E-NTPDases possess these conserved phosphate-binding motifs consisting of invariant amino acids comprising the nucleotide-binding and hydrolysis sites. The second family of extracellular apyrases is relatively new in formation and consists of those apyrases cloned from a variety of hematophagous arthropods (Valenzuela *et al.*, 1998, 2001). Although analogous to the E-NTPDases in their enzymatic action, these enzymes are not similar to the E-NTPDase family with respect to their amino acid sequences, and it appears that these two families of apyrases are evolutionarily unrelated (Valenzuela *et al.*, 2001). Sequences related to the Ca^{2+}-dependent secreted apyrases from bloodsucking arthropods were also found in other metazoans from *Xenopus* to mammalian species (Failer *et al.*, 2002; Smith *et al.*, 2002; Devader *et al.*, 2006). The cDNA isolated from rat brain encodes a membrane-bound Ca^{2+}-dependent NDPase (Ca^{2+}-NDPase) that is targeted to the ER after heterologous expression.

C. elegans is an attractive model to study the relevance of intracellular E-NTPDases in alleviating ER stress and regulating protein and lipid glycosylation. Its genome encodes at least three proteins belonging to the former family of E-NTPDases with sequence similarity to the *Saccharomyces cerevisiae* Golgi apparatus (GA) Gda1p: UDA-1 related, in substrate specificity, to the yeast Gda1p, NTP-1 an apyrase related to the yeast Ynd1p (Abeijon *et al.*, 1993; Xiao-Dong *et al.*, 1999; Zhong and Guidotti, 1999; Uccelletti *et al.*, 2004) and a nucleotide diphosphatase functionally homologous to the yeast Ynd1p, encoded by the *mig-23* gene. Loss of MIG-23 function results in altered gonad morphogenesis, demonstrating the importance of this enzyme (Nishiwaki *et al.*, 2004). Transcription of *uda-1* but not *ntp-1* and *mig-23* was up-regulated by conditions causing ER stress and the accumulation of unfolded proteins such as tunicamycin, or high temperature; however, no relevant phenotypes were associated with loss-of-function mutation in *uda-1* obtained by RNA interference (RNAi; Uccelletti *et al.*, 2004).

We report here the characterization of the *C. elegans* open reading frame (ORF) F08C6.6 coding for an NDPase similar to the apyrases of blood-sucking insects. The gene transcription was up-regulated by ER stress conditions under *ire-1* and *atf-6* control. Depletion of the enzyme by RNAi induced the activation of the UPR pathway and significantly shortened the lifespan of wild-type and *daf-2* mutant animals; multiple premature aging-like phenotypes were also present, including altered organization of actin-containing thin filaments in muscle cells and accumulation of lipofuscin.

MATERIALS AND METHODS

Media Strains and Reagents

Worms were cultured as described previously (Brenner, 1974) and grown at 16°C, unless otherwise indicated. The following *C. elegans* strains were used in this study: Bristol strain N2 as standard wild-type strain, SJ30 [*ire-1(zcI4)* II; *zcIs4* V], RB772 [*atf6* (ok551) X], RB545 [*pek-1* (ok275) X], SJ4005 [*zcIs4* [*hsp-4*::GFP] V], SU93 (*jcIs1*) IV, and CB1370 [*daf-2* (e1370) III], kindly provided by the *C. elegans* Genetics Center (CGC). The *S. cerevisiae* yeast strain *Scgda1*Δ G2-11 (*MATα, ura3-52, lys2-801 am, ade2-101 oc, trp1-Δ1, his3-Δ200, leu2-Δ1, gda1::LEU2*) was used for heterologous expression and was described previously (Abeijon *et al.*, 1993). Yeast cells were grown at 30°C in yeast extract/peptone/dextrose or SD medium supplemented with amino acids as needed. Transformations with plasmids were done by electroporation (Sherman *et al.*,1986). *Escherichia coli* strain DH5α (Invitrogen, Carlsbad, CA) was grown in LB medium with 100 μg/ml ampicillin when needed. Reagents for yeast media were obtained from Difco Laboratories (Detroit, MI). Unless otherwise stated, all other reagents were from Sigma (St. Louis, MO).

Characterization and Heterologous Expression of apy-1

Based on the nucleotide sequence of *F08C6.6* (Chromosome X: 7568212-7569664; WormBase), forward (CEf 5′-CAGAAGATCATGACACAAGAAAGTAACTC-3′) and reverse (Cer 5′-GGTTAGGCAAATGCAATTCCTTCCTTC-3′) primers were designed to amplify a *apy-1* cDNA fragment by PCR, which was performed with *C. elegans* mixed-stage cDNAs generated from total RNAs. The former ORF was sequenced and inserted appropriately into the L4440 double promoter vector (Timmons *et al.*, 2003) to generate a *apy-1* double-strand RNA (dsRNA) expression plasmid (L4440-*apy-1*) for RNAi assays and into the plasmid pRS426 (Mumberg *et al.*, 1995) to obtain p426-apy-1 for heterologous expression into yeast *gda1* mutant cells.

Drug Treatments

Mixed stage nematodes grown in liquid culture were treated with 5 μg/ml tunicamycin (Sigma) or 5% EtOH for 6 h and then collected.

Preparation of Membrane Fraction

Packed mixed-stage worms, grown in liquid culture, were suspended in equal volume of membrane buffer (0.8 M sorbitol, 1 mM EDTA, 10 mM triethanolamine/acetic acid, pH 7.2) supplemented with Protease Inhibitors mixture (Sigma) and disrupted several times (1 min each time) with 0.5-mm glass beads. The suspension was centrifuged at 700 × *g* for 3 min and then transferred to a Dounce homogenizer to disrupt the cells. The homogenate was centrifuged at 1500 × *g* for 10 min, and the resulting supernatant was centrifuged at 100,000 × *g* for 40 min at 4°C. The pellet was suspended in membrane buffer supplemented with protease inhibitor and stored in aliquots at −70°C.

Measurement of Nucleotidase Activities

Nucleotide phosphatase activity and substrate specificity were determined by incubating membrane fractions in solution containing 2 mM NDP/NTP/NMP, 2 mM $CaCl_2$ or $MnCl_2$ or $MgCl_2$, 0.1% Triton X-100, and 0.2 M Tris-HCl. The optimal pH range was determined using a Tris-HCl buffer ranging from pH 6.0 to pH 12. For measurement of P_i, samples were then assayed as previously described (Yanagisawa *et al.*, 1990).

Northern blot Analysis

Total RNA from mixed-stage worms, cultured on different media was isolated with Trizol reagent (Invitrogen), resolved on a 1% formaldehyde-containing gel, transferred to nylon membrane, and hybridized with ^{32}P-labeled cDNA probes using random priming kit (Roche Applied Science, Indianapolis, IN). The cDNA probes were obtained by amplification of cDNA template from RT-PCR reaction.

RNAi and Brood Size

Synchronized worms at L4 larval stage were placed onto IPTG-containing NGM plates seeded with bacteria (*E. coli HT115*[DE3] carrying the empty vector L4440 (pPD129.36) or the construct for RNAi by feeding of *apy-1*). Worms were allowed to lay eggs at 16°C, and all progeny was observed daily and counted with a Zeiss Axiovert 25 microscope (Thornwood, NY).

Life Span Assays

All life span assays were performed at 16°C starting when the L4 wild-type worms were fed on bacteria expressing *apy-1* RNAi or the empty vector as a control. Animals were transferred to fresh plates, monitored daily, and scored as dead when they no longer responded to gentle prodding with a platinum wire. Animals that crawled off the plates were not included in the analysis.

Pharyngeal Pumping Assay

Pharyngeal pumping was analyzed at 16°C under Zeiss Axiovert 25 microscope by counting the number of contractions (defined as backward grinder movements in the terminal bulb) on 40 animals for each treatment, during five periods of 30 s, starting when the L4 wild-type worms were fed on bacteria expressing *apy-1* RNAi or the empty vector as a control. The experiment was performed from the first day of adulthood and last for 14 consecutive days.

Body Bends Assay

We counted the body bends of worms moving forward continuously on an undisturbed plate or immediately after prodding with platinum pick (Koelle and Horvitz, 1996). For each animal, the number of body bends was counted by direct observation for a total of 9 min in 3-min blocks with 30 min between

each block. A body bend was scored each time a bend reached a maximum just posterior to the pharynx, either on the dorsal or ventral side of the animal. The experiment was performed when the L4 wild-type worms were fed on bacteria expressing *apy-1* RNAi or the empty vector as a control.

Transgenic Animals Overexpressing apy-1::gfp

The reporter gene construct apy-1::gfp was obtained by a PCR fusion-based approach (Hobert, 2002). Genomic *apy-1*, with 2.86 kb immediately upstream of the start codon, was PCR-amplified from wild-type genomic DNA using Expand High Fidelity Taq (Roche Diagnostics, Penzberg, Germany). This product was then coamplified with a 1.8-kb PCR fragment encoding green fluorescent protein (GFP) from plasmid pPD95.75 (kindly provided by A. Fire, Stanford University, Stanford, CA). The resulting apy-1::gfp fragment was initially microinjected at 15–30 ng/μl into the syncytial gonad of 30 young wild-type adult hermaphrodites together with 130 ng/μl of the plasmid pRF4 (Mello and Fire, 1995) containing the dominant marker rol-6(su1006) (Mello and Fire, 1995). Because no transgenic progeny was obtained, two other microinjection experiments were performed decreasing the concentration of apy-1::gfp construct to 0.6–1 ng/μl.

Phalloidin Staining

Phalloidin staining was performed as described by Costa *et al.* (1997).

Electron Transmission Microscopy

Adults worms were used for ultrastructural analysis as reviewed by Hall (1985), with some minor differences. Briefly, worms were washed with M9 buffer and anesthetized in 8% alcohol in M9, fixed in 2.5% glutaraldehyde, 1% paraformaldehyde in 0.1 M sucrose, 0.05 M cacodylate on ice for 2 h. After three rinses in 0.2 M cacodylate on ice, worms were postfixed with 1% osmium tetroxide in 0.1 M cacodylate for 2 h on ice. After further rinses, worms were stained in 1% uranyl acetate for 1 h at room temperature, rinsed in distilled water, and pre-embedded in a thin 10% gelatin gel overnight. Gelatin small blocks were cut to have worms close to each other. Small pieces were dehydrated in an ethanol series and propylene oxide, infiltrated with a mixture of propylene oxide and Epon 812, embedded in pure Epon 812, and followed to polymerize at 60°C for 3 d. Transverse thin sections were cut, collected, and stained with uranyl acetate and lead citrate before viewing with a Philips CM10 electron microscope (Mahwah, NJ).

RESULTS

The ORF F08C6.6 Encodes for a Uridine Diphosphatase Up-regulated by ER Stress Conditions

We have previously identified and characterized the uridine diphosphatase (UDPase)/guanine diphosphatase (GDPase) UDA-1, involved in ER "quality control"; UDA-1 showed high similarity of sequence and of substrate specificity with *S. cerevisiae* Golgi Gda1p and with a human ER NDPase (Abeijon *et al.*, 1993; Trombetta and Helenius, 1999; Uccelletti *et al.*, 2004).

The presence in *C. elegans* ORFeome Database of the ORF F08C6.6 (hereinafter referred to as *C. elegans apy-1*), similar to a rat brain ER Ca^{2+}-NDPase (Failer *et al.*, 2002) and to human UDPase SCAN-1 (Smith *et al.*, 2002), prompted us to ask whether APY-1 was an NDPase too. To answer this point, we carried out a nucleotidase activity assay on membrane fractions from *apy-1(RNAi)* mixed-stage worms. Fractions derived from RNAi individuals showed a drastic reduction of UDPase activity with respect to control ones; GDP was hydrolyzed to a smaller extent but the activity inhibition was still evident. No significant differences were observed in the very low catalytic activity with ADP as substrate, and no activity was detected with NMPs (Figure 1A); these results are consistent with the hypothesis that APY-1 has NDPase activity.

To further confirm these data a heterologous approach was followed by expressing *apy-1* in a *S. cerevisiae gda1*Δ mutant strain, which has a low background in nucleotide phosphatase activities (Abeijon *et al.*, 1993). UDPase and GDPase activities were significantly increased in membranes from *gda1*Δ cells transformed with the vector containing *apy-1* cDNA compared with those transformed with the empty vector (Supplementary Figure S1).

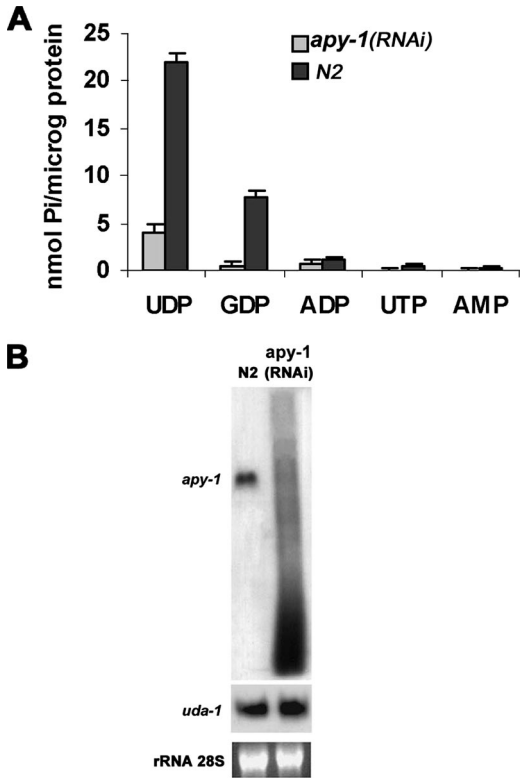

Figure 1. Results of *apy-1* RNAi on nucleotidase activity and gene expression in N2 strain. (A) Substrate specificity of membrane fractions from interfered (feeding) N2 worms. (B) Northern blot analysis of total RNA from N2 and N2 exposed to RNAi of *apy-1* animals. The values reported are averages of three experiments; error bars, SD.

To verify the *apy-1* mRNA effective degradation in RNAi experiments, a Northern blot analysis of *apy-1* transcript was performed on *apy-1* RNAi worms and, as a control, on worms fed with bacteria carrying the empty vector. As shown in Figure 1B, the *apy-1* mRNA from interfered individuals resulted completely degraded with respect to mock-treated, which had a normal profile. To verify the integrity of the mRNAs extracted from worms, the same samples as before were probed against *uda-1*, which showed an identical level of transcript between the mock- and the RNAi-treated animals (Figure 1B).

The expression of two different proteins involved in proteins folding and ER quality control with partially redundant catalytic activities may represent a safety margin. To verify a possible overlapping function of UDA-1 and APY-1 and to study transcriptional regulation of second one, we investigated if *apy-1* expression could be induced by ER homeostasis–altered conditions. Total RNA from N2 mixed-stage worms, untreated and treated with 5 μg/ml tunicamycin, 6% EtOH, or high temperature was isolated and hybridized with radiolabeled full-length cDNA of *apy-1* gene as a probe. Northern blot analysis revealed an evident increase of *apy-1*–hybridizing transcripts, supporting a possible involvement of *apy-1* in ER stress response mechanisms (Figure 2A).

IRE-1 protein, and ATF-6 and PEK-1 in lower manner, are the most important folding sensors in the ER and their function is the up-regulation of many UPR genes, like *uda-1*, as a result of ER stress (Mori *et al.*, 1993, Urano *et al.*, 2002). We then analyzed *apy-1* transcriptional regulation in *ire-1*,

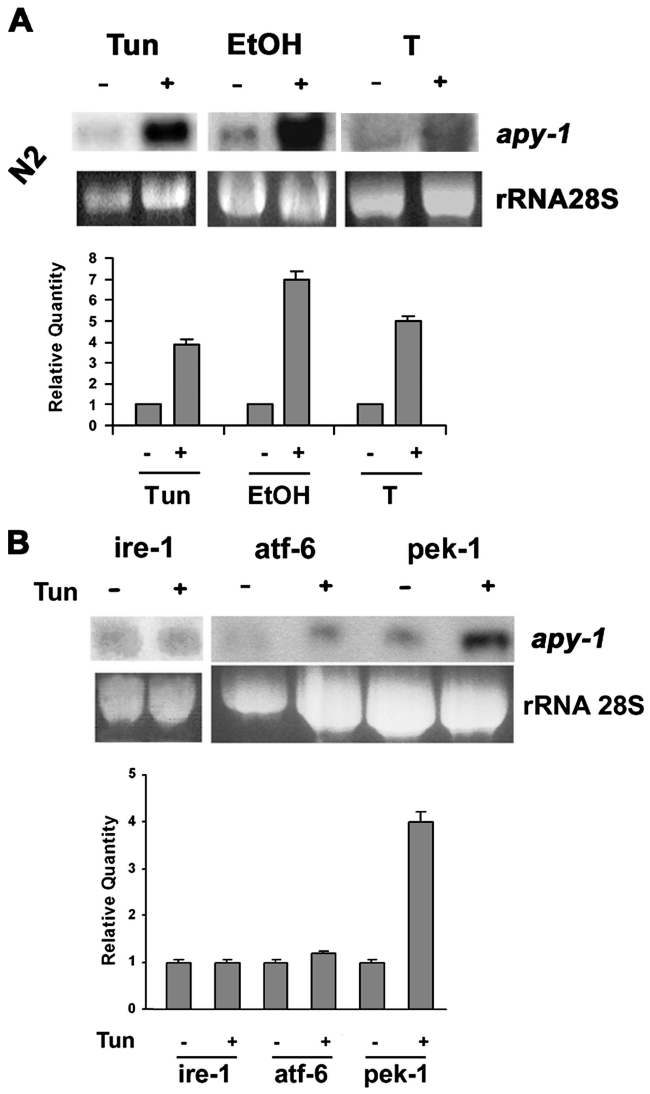

Figure 2. Transcriptional regulation of *apy-1* under stress conditions. (A) Northern blot analysis of total RNA from untreated and 5 μg/ml tunicamycin (Tun)- or 6% ethanol (EtOH)-, or 25°C (T)-treated N2 animals for 6 h. (B) Northern blot analysis of total RNA from untreated and 5 μg/ml tunicamycin (Tun)-treated animals of the indicated mutant strains, each lacking a different UPR activation pathway. Quantification of the radiolabeled signal on the blot is shown in bottom part of each panel. The hybridization signal for each strain in the untreated condition was set as 1. These data represent one of three independent experiments giving the same result; error bars, SD.

atf-6, and *pek-1* mutant strains. Total RNA from mixed-stage worms, treated and not with 5 μg/ml tunicamycin, was extracted and analyzed by Northern blot. Results showed a 3–4-fold increase of *apy-1* mRNA expression in the treated *pek-1* strain; this up-regulation was instead abolished, under the same conditions, in *ire-1* and *atf-6* mutant backgrounds (Figure 2B). Similar results were also observed in the other ER stress conditions used before (not shown). These experiments directly demonstrate a role for *ire-1* and *atf-6* genes in transcriptional regulation of *apy-1*.

Activation of UPR Takes Place in apy-1(RNAi) Animals

Because UDP, accumulated in ER as a by-product of reglucosylation reactions in protein folding control, might inhibit UDP-Glc:glycoprotein glucosyltransferase (GT; Parodi *et al.*, 1983), cells probably have NDPases in the ER to alleviate product inhibition. The impairment of UDP removal would probably impinge upon folding control and originate ER stress conditions. To examine the *apy-1* role in these circuits, we analyzed the expression of an ER stress reporter transgene, *hsp-4*, a *C. elegans* homologue of mammalian GRP-78/BiP. We used a transgenic strain (SJ4005) homozygous for a reporter gene (*zcIs4*), which consists of a fusion of the *hsp-4* promoter to GFP (*hsp-4*::GFP). The *hsp-4*::*gfp* expression is induced by ER stress conditions, such as high temperature or tunicamycin treatment (Calfon *et al.*, 2002). Inactivation of *apy-1* function by RNAi strongly increased GFP expression (fourfold with respect to control), which reflected an *hsp-4* up-regulation, indicating that loss of *apy-1* may effectively cause ER stress (Figure 3A). This induction was slightly further enhanced after tunicamycin exposure. To confirm the up-regulation of *hsp-4* expression, we performed a Northern blot analysis of *hsp-4* mRNA from *apy-1(RNAi)* worms in the N2 background; this showed a net increase of *hsp-4* transcription in comparison to the control preparation (Figure 3B). We then analyzed if this *hsp-4* up-regulation was dependent on the UPR regulatory system by knocking down *apy-1* in the SJ30 transgenic *C. elegans* strain, homozygous for *hsp-4*::GFP in a *ire-1* mutant background (Calfon *et al.*, 2002). In this case, induction of *hsp-4*::GFP by *apy-1(RNAi)* was not observed (Figure 3C), indicating that IRE-1 activity is required for the UPR induction resulting from *apy-1(RNAi)*. UDA-1 and APY-1 were, so far, the only nucleotidases to be up-regulated at transcriptional level by treatment of the animals with tunicamycin. Therefore, we asked whether also *uda-1(RNAi)* would be able to increase expression of *hsp-4*::GFP similar to *apy-1(RNAi)*; however, no UPR activation was observed in this case (Figure 3D).

Because *uda-1* and *apy-1* transcripts resulted in up-regulation under ER stress, we investigated whether some nucleotidase activities in *C. elegans* increased under similar conditions. To this aim, total membrane fractions from N2 mixed-stage worms treated and untreated with 5 μg/ml tunicamycin were obtained and assayed for nucleotidase activity and substrate specificity. In the presence of calcium as a cofactor, membranes from worms treated with tunicamycin showed a 75% increase of UDPase activity with respect to control ones. The GDPase activity, although significant, was not enhanced by this treatment as well as the ADPase activity (Supplementary Figure S2A). To correlate the increased UDPase activity with ER responses originated by unfolded proteins, we performed the same experiment as before in the *ire-1* mutant background, where the UPR pathway is not induced (Calfon *et al.*, 2002). The UDPase activity of membranes from treated worms, in this case, increased only of ~20% compared with the control (Supplementary Figure S2B).

The assay revealed an optimal pH between 7 and 8 when UDP was used as substrate and incubated with N2 membrane fractions (Supplementary Figure S2C). Moreover, a strictly Ca^{2+}-UDPase activity was observed in the same fractions, because neither magnesium nor manganese were able to reveal any activity when utilized as cofactors (Supplementary Figure S3D). Identical results in terms of cation requirements were obtained with GDP, ADP, UTP, and ATP as substrates (not shown).

APY-1 Plays a Role during Development and Aging

To investigate phenotypes linked to *apy-1* loss-of-function, we observed RNAi worms to check for morphological and developmental defects and analyzed their brood size and

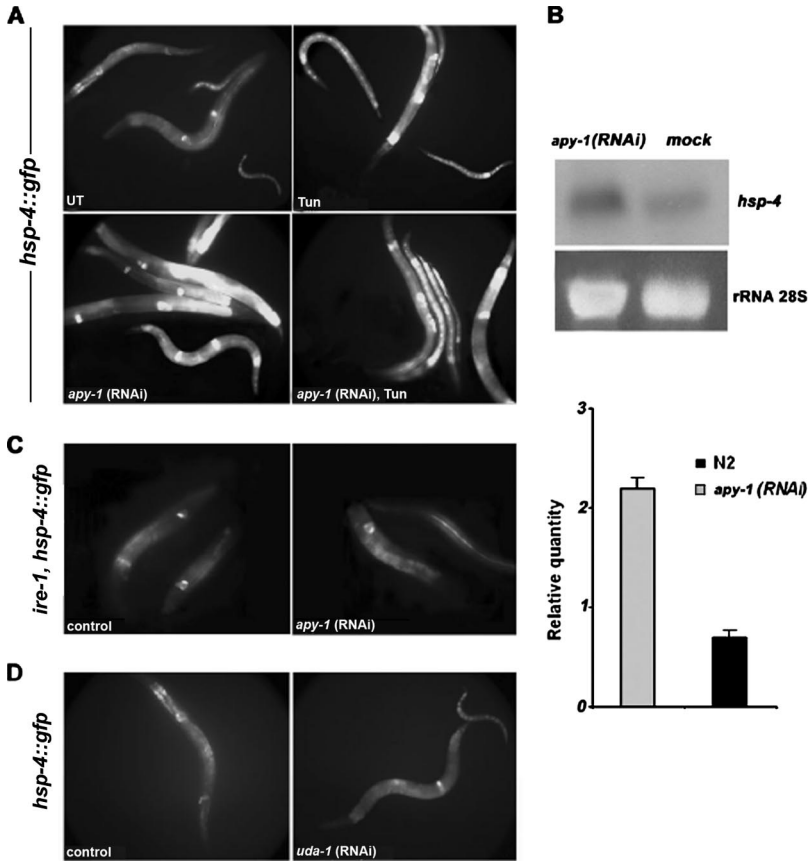

Figure 3. Induction of an ER stress reporter gene by *apy-1* RNAi treatment. (A) Fluorescence micrographs of SJ4005 worms, containing an integrated copy of hsp-4::gfp, a green fluorescent protein transcriptional reporter driven by the *hsp-4* promoter, untreated (UT), tunicamycin-treated (Tun; 5 μg/ml), or *apy-1(RNAi)* by feeding animals. Fluorescent images were obtained using the same exposure for all treatments. (B) Northern blot analysis of total RNA from N2 and *apy-1(RNAi)* worms. The blot was hybridized with *hsp-4* probe that detects the endogenous gene. Quantification of the radiolabeled signal is shown in the bottom part of the panel. These data represent one of three independent experiments giving similar results; error bars, SD. (C) Fluorescence micrographs of SJ30 worms, containing in a ire-1 background an integrated copy of hsp-4::gfp, fed with bacteria carrying the empty vector (control) or fed with bacteria expressing *apy-1(RNAi)*. (D) Fluorescence micrographs of SJ4005 worms fed with bacteria carrying the empty vector (control) or fed with bacteria expressing *uda-1(RNAi)*. Fluorescent images were obtained using the same exposure for all treatments.

growth rate of the progeny. The brood sizes of *apy-1* worms were significantly decreased, on average a 50% reduction of progeny number compared with the wild type (Figure 4). In the daily progeny count *apy-1* worms had significantly lower number of laid eggs compared with N2. In addition, most of the interfered animals showed slow growth, and the development to progressive larval stages was retarded with respect to control worms: in general, the RNAi worms took more than 20 h to reach adulthood (data not shown).

We have also analyzed the impact of APY-1 depletion on the life span by interfering only the L4 stage animals and

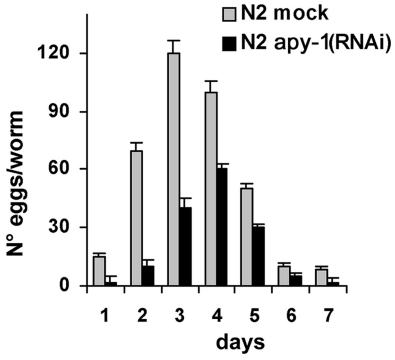

Figure 4. Effects of *apy-1(RNAi)* inactivation on brood sizes. Average brood sizes per worm of N2 and *apy-1* (RNAi by feeding) animals was reported. Worms were allowed to lay eggs at 16°C and all progeny was counted daily. The histogram represents four experiments; the values reported are averages of a minimum of 25 animals; error bars, SD.

then monitoring the survivors in order to avoid the developmental problems that could cause general sickness as observed in the F1 progeny. Indeed, *apy-1(RNAi)* individuals exhibited a reduced life span: half-life of 14 d, compared with 20 d for N2 worms (Figure 5A). This prompted us to monitor the accumulation of intestinal autofluorescence in adult animals. Intestinal autofluorescence, which is caused by lysosomal deposits of lipofuscin, accumulates over time in aging animal and is an established marker for aging (Garigan *et al.*, 2002). In agreement with short life span, the *apy-1(RNAi)* animals accumulated intestinal autofluorescence more rapidly than the control worms (Figure 5B). In fact, at day 4 of treatment the RNAi animals started to accumulate intestinal fluorescence, whereas in the mock-treated animals, no fluorescence was detectable. At day 8 as well as at day 12 a strong difference in accumulated fluorescence was observed between the RNAi-treated and the mock-treated worms (Figure 5B).

Longevity in *C. elegans* is regulated by the insulin-like signaling pathway that includes the gene *daf-2*, encoding a protein most closely related to vertebrate insulin and insulin-like growth factor (IGF)-I receptors (Kenyon *et al.*, 1993; Kimura *et al.*, 1997). Mutations in *daf-2* result in a two- to threefold increase in adult lifespan; in order to determine whether *apy-1* interacts with the insulin pathway, *daf-2* worms were interfered with *apy-1* as was done for the N2 animals. The long living *daf-2* animals showed, upon *apy-1* interference, a reduction in their lifespan to the same extension that previously reported for the N2 background (S3A). The above data suggest that the role of APY-1 in maintaining a normal lifespan is independent of the DAF-2 activity.

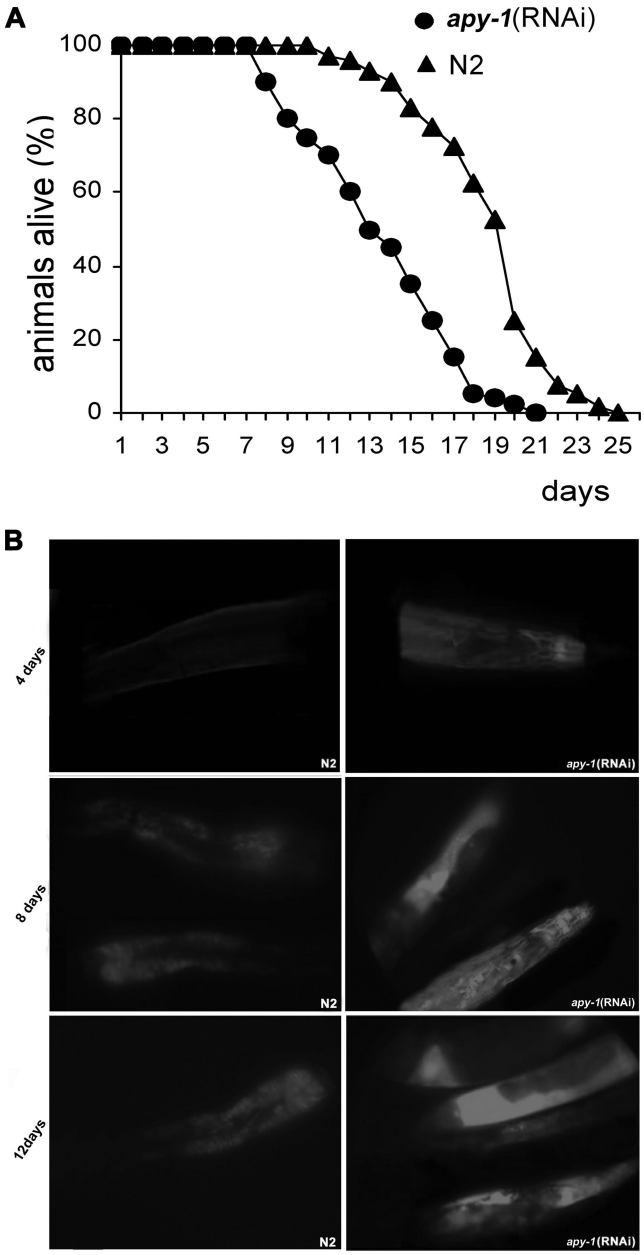

Figure 5. Effects of *apy-1(RNAi)* inactivation on lifespan and aging. (A) Lifespan analysis of *apy-1(RNAi)* and N2 mock-treated worms. Survival is plotted against days of adulthood, where 0 is the L4 larvae phase. Error bars, SD; n = 60 for each data point of single experiment. The results are the mean of three independent experiments. (B) Accumulation of lipofuscin autofluorescence with age. N2 mock-treated and *apy-1(RNAi)* worms at 4 and 8 d of adulthood were photographed under identical conditions.

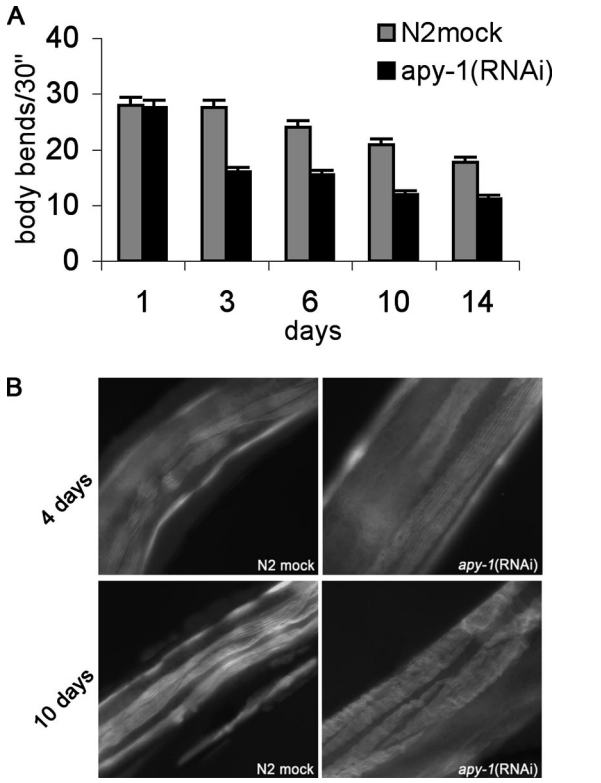

Figure 6. Muscle structure in *apy-1*(RNAi) animals. (A) Mean of body bends in *apy-1(RNAi)* animals and N2 mock-treated animals treated for the indicated period of time. Error bars, SD; n = 60 for each treatment. The histogram shows the mean of three different experiments. (B) Muscle structural integrity was examined by phalloidin staining of actin in body wall muscle at days 4 and 10 of adulthood in N2 mock- and RNAi-treated worms.

Because aging is usually also related to reduced motility, we then examined whether *apy-1(RNAi)* had any effects on motility by measuring the frequency of the body bends. The body-bending movements gradually decreased in mock- and RNAi-treated worms during aging (Figure 6A). However, the reduction in the body bends was more pronounced in *apy-1(RNAi)* than in the mock-treated animals. The frequency of body bends per 30 s was reduced by ~60% in RNAi-treated worms compared with mock-treated worms along all the period analyzed. Similar results were also obtained in *daf-2* strain (Supplementary Figure S3C).

The reduced motility strongly suggests that the depletion of APY-1, during adulthood, reduces the rate of muscle contraction. On the other hand, aging in *C. elegans* is accompanied by sarcopenia, the progressive deterioration of muscle tissue (Huang *et al.*, 2004). We then examined muscle structure during a period of adulthood. To this aim, we stained animals with phalloidin to visualize the actin-containing thin filaments of the body wall muscles. Phalloidin-stained muscles at adult days 4 of RNAi-treated worms showed patched or wrinkled sarcomeres, whereas the mock-treated animals exhibited straight and evenly stained filaments, as expected for intact muscle cells. These differences between the RNAi-treated and the control animals became even more evident at day 10 of treatment (Figure 6B).

To further characterize APY-1, we attempted to construct transgenic animals carrying *apy-1::gfp* by microinjection technique. A first round of microinjections at standard concentrations of the constructs failed to generate transgenic progeny, suggesting a toxic effect of *apy-1* overexpression. Therefore, we reinjected the product at 15–30-fold dilution and obtained 80 F1 transformants over two independent assays. Only one of these animals gave rise to a stable transgenic line. The slight percentage of heritable transformation reinforces the idea of toxicity of the *apy-1* gene product when expressed at higher levels than in native conditions. GFP expression pattern was analyzed in a portion of F1 roller animals and in the APY-1::GFP stable transgenic

line. Fluorescence intensity was very faint, making difficult any conclusion about its localization. In animals showing a perceptible signal, it was localized mainly to the pharynx as a network pattern (data not shown). The experiments performed on the transgenic line showed a lifespan reduction compared with the wild-type counterpart (half-life of 10 d, compared with 18 d for N2 worms), further reinforcing the idea of the toxicity of APY-1 when over expressed, although we cannot rule out the possibility that the observed toxicity is a result of the APY-1::GFP fusion instead of a higher level of APY-1.

Altered Pharynx Morphogenesis Take Place in apy-1(RNAi) Animals

The *C. elegans* pharynx is regarded as a model system to study organ development; pharyngeal activity is one prominent characteristic of adult worms, and its functionality declines with age; to look for phenotypes in feeding behavior possibly correlated with depletion of APY-1, we visualized the pharyngeal region of the digestive tube using a transgenic strain carrying *ajm-1::gfp*, a fusion protein that localizes to epithelial cell junctions located at the most apical portion of their lateral membranes (Mohler *et al.*,1998). It was possible to observe an irregular and discontinuous distribution of fluorescence signals at the beginning and at the end of procorpus and at level of entire isthmus of *apy-1(RNAi)*–treated worms with respect to the more complete and organized appearance observed in the same regions of the control animals (Figure 7); this may suggest alterations in the morphology of the pharynx in animals depleted of APY-1.

Furthermore, analysis of pharyngeal pumping revealed a reduction in pumping rate of *apy-1(RNAi)* worms when compared with control (Figure 8A). The mean pumping rate in *apy-1(RNAi)* animals was about 70% compared with mock-treated worms along all the period analyzed. We also analyzed the morphology of the pharynx of both the wild type and the mutant *C. elegans* at ultrastructural level. Pharyngeal morphology appeared normal in wild-type worms, with the typical three-radiate shape, surrounded by marginal and myoepithelial cells (Figure 8B). The lumen of the pharynx appeared normal (Figure 8B), and no intact *E. coli* cells accumulated in the pharyngeal lumen, suggesting an efficient pharyngeal function for feeding and pumping. On the other hand, cross-sections of mutant worms showed the typical shape of the pharynx with a striking morphological feature: the loss of a loop from the three-radiate shape of the pharynx (Figure 8C, arrow). Moreover, a very tight lumen was evident (Figure 8C, lumen); also in this case no intact *E. coli* cells were observed. These observations, together with the pumping results, suggest that mutant worms probably had a still adequate food transport through the pharynx, although a notably retarded pumping function was also present.

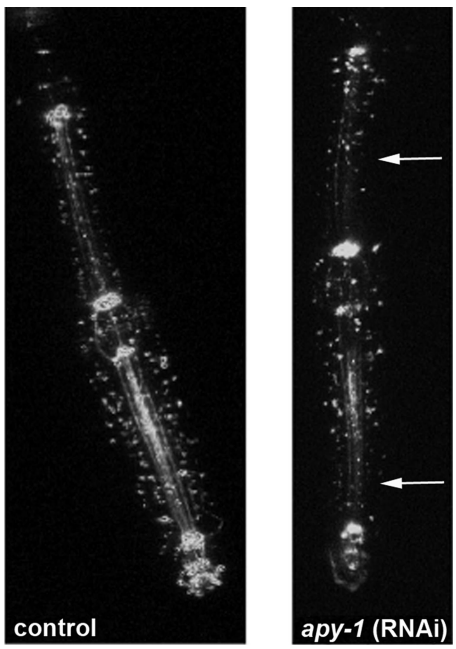

Figure 7. Pharynx analysis in *apy-1(RNAi)* animals. Fluorescence micrographs of pharyngeal region of the digestive tube from SU93 adult worm, containing an integrated copy of *ajm-1::gfp*, a marker for the epithelial apical junctions, treated and untreated with *apy-1(RNAi)* for 3 d. An irregular and reduced distribution of fluorescence signal was observed along the pharynx of *apy-1(RNAi)* worms (arrows).

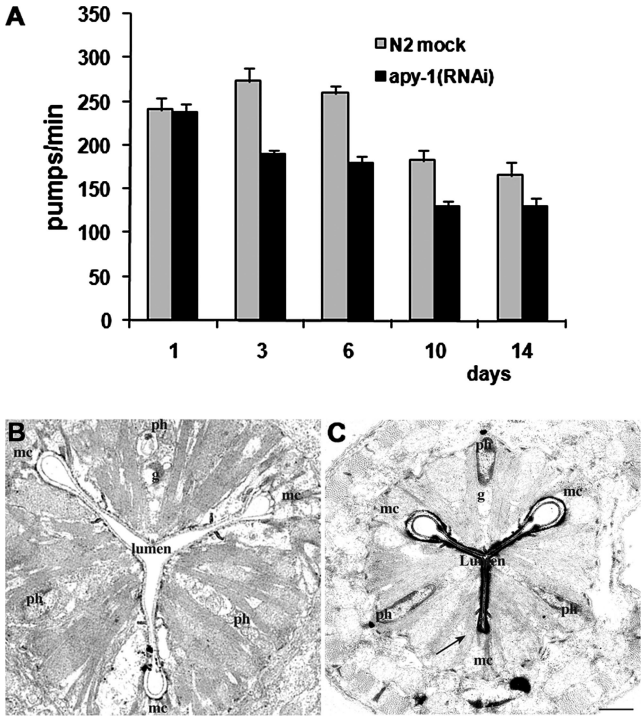

Figure 8. Pharynx functionality in *apy-1(RNAi)* worms. (A) Mean pharyngeal pumping rate of *apy-1(*RNAi*)* animals and N2 mock-treated animals for the indicated period of time. Error bars, SD; n = 60 for each treatment. The histogram shows the mean of three different experiments. (C and B) Ultrastructural analysis of pharynx in *apy-1(*RNAi*)* animals and N2 mock-treated animals after 3 d of treatment in sample electronic photographs from transverse section. Mar1–3 represents three marginal cells; Ph1–3 represents three pharyngeal myoepithelial cells. The lumen is the internal part of the pharynx. Arrow points to the failing loop of the pharynx. Scale bar, 1 μm.

DISCUSSION

We have identified and named APY-1, a *C. elegans* homologue of the apyrase first found in the saliva of blood-sucking insects (Valenzuela *et al.*, 1998; Charlab *et al.*, 1999;

Valenzuela *et al.*, 2001); closely related enzymes have now been described in humans (Smith *et al.*, 2002) and rats (Failer *et al.*, 2002). The substrate specificity of APY-1 is very similar to that of the purified E-NDPase SCAN-1 of human and of the rat Ca^{2+}-NDPase. It utilizes as a substrate mainly UDP and, to a minor extent, GDP. Only a small activity was detected with ADP, the preferred substrate of the apyrases from blood-sucking insects. Gene expression data (available on the World Wide Web at http://nematode.lab.nig.ac.jp/db2/index.php) indicate that the transcript for *apy-1* (F08C6.6) can be detected in all developmental stages of the nematode. As mentioned above, NDPs are generated as by-products of glycosylation reactions and can inhibit glycosyltransferases if allowed to accumulate. NDP accumulation is normally prevented by the action of an NDPase that also generates the NMPs needed for the import of new nucleotide sugars (Hirschberg *et al.*, 1998). This transport of nucleotide sugars into the Golgi lumen is necessary for subsequent addition of the corresponding sugars to proteins. NDPases are then though to play an essential role for efficient glycosylation process. Therefore, the "quality control" mechanisms, which ensure that only properly folded and assembled proteins exit the ER, rely on NDPase activity for the proper functioning of glycosyltransferases. Thus, we hypothesized that APY-1 could be required for an efficient organelle capacity to process unfolded proteins. The results presented in this article show that *apy-1* is up-regulated under conditions that induce the UPR. Moreover such increase is dependent on *ire-1* and *atf-6* signaling resident in the ER. Consistently, the lack of APY-1 results in increased expression of the chaperone *hsp-4*, a signature of UPR activation, suggesting that worms with impaired *apy-1* function are continuously under stress. Thus, the up-regulation of *apy-1* in conditions that trigger the UPR response suggests that NDPases play an important role in protein quality control. Knocking down APY-1 correlated with some progeria-like phenotypes, as indicated by accumulation of lipofuscin, and, possibly, by reduction in body bends accompanied by alteration in the muscle cells. On the other hand, overexpression of APY-1 was toxic; a fine-tuning of APY-1 level appears therefore of physiological relevance. Numerous diseases are caused by defective UPR signaling (Schroder and Kaufman, 2006). There is evidence suggesting the existence of age-related deficit of the UPR signaling as a common pathogenic mechanism in several neurodegenerative disorders (Zhao and Ackerman, 2006), and several studies suggest that protein damage can be at least as important as DNA damage in the aging phenotype (Sierra, 2006). We might speculate that a lower efficiency in the glycosylation process, due to impairment of UDPase activity, could result in an increased fraction of misfolded or damaged proteins and premature aging phenotypes. Glycosylation changes have been observed, on the basis of DSA lectin binding, for progeria fibroblasts (Clark and Weiss, 1995), and other progeria cells display different, as yet uncharacterized, changes in glycosylation (Quentin *et al.*, 1990). In this respect, elevated levels of the glycoprotein gp200 were consistently observed in all progeria fibroblast strains examined, and indeed gp200 was identified through glycan detection (Clark and Weiss, 1995). In any case, it should also be mentioned that it is possible that some of the premature aging phenotypes observed could be rather related to general sickness conditions of the individuals experiencing UPR stress.

Finally, underlying the complexity of the phenotype originated by *apy-1* loss-of-function, we found that worms depleted of APY-1 showed structural and functional pharyngeal alterations. As a matter of fact, several mutants defective in proteoglycan synthesis exhibit pharyngeal defects; these include *sqv-1* and *sqv-8* mutants, defective in synthesis of chondroitin and heparin sulfate proteoglycans (Herman and Horvitz, 1999; Bulik *et al.*, 2000), as well as *pyr-1* animals defective in pyrimidine biosynthesis (Franks *et al.*, 2006). These recurrent phenotypes highly suggest that impaired proteoglycan synthesis someway underlies the pharyngeal abnormalities seen in all of these mutants. Although protein glycosylation modulates a wide variety of intracellular events, the defect induced by the *apy-1* loss-of-function has a particular effect on the pharynx development. A similar specific influence was observed with mutations of MIG-23, an NDPase that affects gonad morphogenesis through abnormal glycosylation of the MIG-17 ADAM protease (Nishiwaki *et al.*, 2004). Thus it is possible that, beside the role in the UPR signaling, defects in *apy-1* activity may affect a certain set of glycoproteins, resulting in cell type– or tissue-specific defects.

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