Characterization of PUD-1 and PUD-2, Two Proteins Up-Regulated in a Long-Lived daf-2 Mutant

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Abstract

C. elegans PUD-1 and PUD-2, two proteins up-regulated in daf-2(loss-of-function) (PUD), are homologous 17-kD proteins with a large abundance increase in long-lived daf-2 mutant animals of reduced insulin signaling. In this study, we show that both PUD-1 and PUD-2 are abundantly expressed in the intestine and hypodermis, and form a heterodimer. We have solved their crystal structure to 1.9-Å resolution and found that both proteins adopt similar β-sandwich folds in the V-shaped dimer. In contrast, their homologs PUD-3, PUD-4, PUDL-1 and PUDL-2 are all monomeric proteins with distinct expression patterns in C. elegans. Thus, the PUD-1/PUD-2 heterodimer probably has a function distinct from their family members. Neither overexpression nor deletion of pud-1 and pud-2 affected the lifespan of WT or daf-2 mutant animals, suggesting that their induction in daf-2 worms does not contribute to longevity. Curiously, deletion of pud-1 and pud-2 was associated with a protective effect against paralysis induced by the amyloid β-peptide (1-42), which further enhanced the protection conferred by daf-2(RNAi) against Aβ.

Citation: Ding Y-H, Du Y-G, Luo S, Li Y-X, Li T-M, et al. (2013) Characterization of PUD-1 and PUD-2, Two Proteins Up-Regulated in a Long-Lived daf-2 Mutant. PLoS ONE 8(6): e67158. doi:10.1371/journal.pone.0067158

Editor: Jiyan Ma, Ohio State University, United States of America

Received April 27, 2013; Accepted May 14, 2013; Published June 14, 2013

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Funding: The Ministry of Science and Technology of China (863 grant 2007AA02Z1A7 and 973 grant 2010CB835203 to M-QD; 863 grant 2008AA022310 and 973 grant 2010CB835402 to KY) and the municipal government of Beijing funded this work. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

The insulin/insulin-like signaling pathway (IIS) negatively regulates lifespan in a variety of species from C. elegans, Drosophila, to mice [1,2]. Mutant C. elegans carrying a single missense mutation in daf-2, encodes the only insulin receptor in the worm, lives twice as long as the wild type (WT) [3]. The discovery that a single gene mutation can extend lifespan to such an extraordinary degree and that the lifespan regulation by IIS is conserved throughout evolution came as a surprise, because it was predicted that such genes would not exist based on a belief that the evolutionary pressure on any post-reproductive traits should be weak, if any [4]. While aging remains one of the last frontiers in biology, exceptionally long-lived mutants such as the daf-2 worms inspire excitement—they are likely the key to understanding the molecular mechanisms of aging. Logically, one of the first steps is to determine the changes in gene expression in the daf-2 mutants.

In C. elegans, most if not all insulin signaling transmits through a FOXO transcription factor called DAF-16 [1,2], thus, the downstream targets of DAF-16 are critical to the regulation of lifespan. A variety of experimental approaches have been applied to identifying DAF-16 targets, including microarrays, chromatin IP, quantitative mass spectrometry, and DamiD [5–10].

We previously looked for DAF-16 targets by quantitative proteomics [8]. Among the 86 proteins that are up- or down-regulated in daf-2, some are well-known DAF-16 targets such as SOD-3, while others are more or less unexpected. Of the 86 proteins, F15E11.13 (encoded by two genes F15E11.13 and Y19D10B.7) and F15E11.14 (also encoded by two genes F15E11.1 and F15E11.14) are up-regulated the most (at least 4-fold). We have given these genes new names, pud-1.1 (F15E11.13), pud-1.2 (Y19D10B.7), pud-2.1 (F15E11.1), and pud-2.2 (F15E11.14), in which pud stands for protein up-regulated in daf-2(3f). pud-1.1 and pud-1.2 are gene duplications with exactly the same DNA sequence, encoding the same protein PUD-1; and so are pud-2.1 and pud-2.2, both
encode PUD-2. They belong to an uncharacterized gene family, which we refer to as the PUD family. The other members of this gene family in *C. elegans* are *pud-3* (F15E11.15), *pud-4* (F15E11.12), *pudl-1* (Y54G2A.46), and *pudl-2* (Y54G2A.47), where *pudl* stands for *pud-like*.

A literature search finds that although these proteins or genes have not been characterized, they have appeared repeatedly in previous publications, often buried in the supplemental information as differentially expressed genes under various conditions. For example, a temperature shift of *glp-1* (e2144ts) mutant from 15 °C to 25 °C was accompanied with a remarkable increase of *PUD-1* and *PUD-2* [11]. A 7-fold increase of the *PUD-2* protein level was detected in the *pudl-2* mutant compared to WT cultured at the same temperature of 25 °C [12]. Moreover, the *PUD-1* protein was shown to build up conspicuously in transgenic *C. elegans* expressing a human 7-dehydrocholesterol reductase, an enzyme functioning in *de novo* biosynthesis of cholesterol [13]. Similarly, when the mRNA levels were measured, elevation of the *pud-1* and *pud-2* transcripts were ranked among the top eight in the *pept-1* (g601) mutant [14], and among the top 15 in *rde-1* (RNAi) and *rde-4* (RNAi) mutants [15]. Decreased transcript levels of these genes were also observed, in *mdt-15* (RNAi) worms [16], in the *glp-4* (bn2ts); *sek-1* (agt1) double mutant relative to the *glp-4* (bn2) single mutant [17], and in WT *C. elegans* exposed to high concentration of CO₂ [18]. It is worth noting that the *glp-1* and *pept-1* mutants, in which increased expression of these genes have been detected, have a longevity phenotype similar to that of *daf-2*.

To understand the function of *PUD-1* and *PUD-2*, and the biological significance of their up-regulation in *daf-2* mutants, we set out to analyze their expression patterns, overexpression phenotype, loss-of-function phenotype, interacting proteins, and protein structures.

**Materials and Methods**

**Plasmids and *C. elegans* strains**

The plasmids and the worm strains used in this study are listed in Tables S4 and S5 in File S1, respectively. Bristol N2 was used as the wild type strain. Worms were cultured on NGM plates at 20 °C unless indicated otherwise. Transgenic strains were constructed through microinjection of plasmids, and transgenes were chromosomally integrated by gamma-irradiation. Single copy insertion of transgenes was generated as described [19]. All of the integrated transgenic strains and the mutant strains were backcrossed to N2 at least four times.

**RNAi**

RNAi by feeding was carried out at 20 °C as described [20]. For RNAi by injection, dsRNA was transcribed *in vitro* from linearized plasmid DNA using a kit (Promega), and the purified dsRNA was injected into the *C. elegans* gonad. The phenotype of the F1 progeny was analyzed.

**Quantitative RT-PCR**

Total RNA was extracted from synchronized young adults using TRIzol (Invitrogen), followed by the removal of contaminant DNA using DNase I. cDNAs were synthesized from the total RNA templates using a reverse transcription kit (Takara). Primers used for qPCR were *oMD241*[ACTATTACCGCATCCATCCACTGTT][oMD244*ATGTGCA CTGATCAAACCTCCAACCA*] for *pud-1*, *oMD246*[TGACGAACAATGGGCAAAAGTG]/*oMD247*[GCCGCTATACTGATGAAGCACCTGGA] for *pud-2*, and *actin-F*[TGCCGCTCTTTGTTGTAGACAATGG]/*actin-R*[TGACGTGGTCTTCTCCAGAATGGG] for *actin* as the internal standard. qPCR was carried out on an ABI 7500 Fast real-time PCR system using a Takara realtime PCR kit (SYBR Premix Ex Taq™ II).

**Mapping the boundaries of niDf209**

The precise boundaries of *niDf209* and *niDf207* in JU258 were determined by nested PCR followed by sequencing. Primers pairs were designed in 1-kb intervals to cover the annotated flanking sequences (wormbase.org). *niDf209* is a 15,979-bp deletion with a 1,608-bp insertion. *niDf207* is a 4072-bp deletion.

**Generating hq5 and hq6**

To help introduce *niDf209* (genetic position: -14.89) to the N2 background, *unc-60* (genetic position: -18.88) and *dpy-11* (genetic position: -0.02) were used to mark recombination events on Chr. V. JU258 was first crossed with DR181 *unc-60(m35) dpy-11(e224)*, and DpyNonUnc progeny with the recombination sites nearest to, and on the right side of *niDf209* were determined by PCR with oligos oMD307 and oMD308. Candidate alleles were crossed into DR35 *unc-60(m35)* and the resulting UncDpy progeny containing *niDf209* were selected for further validation. *hq6* was isolated using *niDf208* (16.7 Kbp upstream of *niDf209*) as a marker of recombination to the left of *niDf209*. *hq5* was isolated using another marker upstream of *niDf208*. The linked *unc-60(m35)* and *dpy-11(e224)* alleles were removed through recombination after repeated backcrosses to N2.

**Lifespan assay**

Unless indicated otherwise, all lifespan assays were carried out at 20 °C as described before [8]. Kaplan-Meier survival analyses were carried out using the SPSS software and the log-rank p values were reported.

**Heat tolerance assay**

For each strain, 30 animals were shifted from 20 °C to 35 °C at the young adult stage and examined for survival every two hours.

**PA14 fast killing assay**

The assay was performed as described previously [21].
Paralysis assay
On day 0, 100 L4s were transferred to fresh NGM plates seeded with OP50 (10 worms/plate) and moved to new plates every two days. Paralyzed worms that could only move their heads were counted every day. Paralysis assay was terminated after 7 days to avoid mistaking old worm as “paralyzed”.

Microscopy
All GFP and DIC images were taken using a Zeiss Axiolmager M1 microscope.

mRNA sequencing and data analysis
Total RNAs were extracted from the WT and hq6 day-1 adults using TRIzol (Invitrogen). The sequencing library was constructed by following the “mRNA sequencing sample preparation guide” provided by Illumina. The WT and hq6 mRNA sequencing libraries were bar-coded and mixed in equal amounts. The paired-end RNA sequencing data were acquired on an Illumina GA II instrument (in two lanes). The paired-end mRNA-seq 36 base pair reads were mapped to the C. elegans genome (WS220) using the TopHat software, allowing no more than two mismatches per sequencing read. In TopHat mapping, the expected (mean) inner distance between mate pairs was set to 100 bp, the standard deviation for the distribution on inner distances between mate pairs was set to 50 bp. Only uniquely mapped paired-end reads were extracted for subsequent analysis. Differential gene expression analysis was performed with the bioconductor package DESeq. Genes with reads number ≤ 10 in both samples were not included in the DESeq analysis. In total, the two samples produced 18 million uniquely mapped paired-end sequencing reads (9 million per sample). In a separate experiment, single-end RNA sequencing data were acquired for three independent WT N2 samples on Illumina HiSeq 2000.

Immunoprecipitation and Mass Spec analysis
A single-copy insertion strain expressing FLAG-tagged PUD-1 and the control N2 strain were cultured on High-growth (HG) plates and harvested for anti-FLAG IP. Proteins bound to the M2 beads (Sigma) were eluted using 0.5µg/µl 3xFLAG peptides. SDS-PAGE analysis followed by silver staining revealed three protein bands in the FLAG::PUD-1 IP but not in the control IP. These bands were cut from the gel, digested with trypsin, and subjected to LC-MS/MS analysis on LTQ-FT Ultra. The protein sequence of these bands were compared with the C. elegans protein database WS217 using Prolucid and filtering the search results with DTASelect 2.0.

Protein purification
The PUD family proteins were expressed in the Escherichia coli BL21(DE3) strain (Novagen) induced with 0.2 mM isopropyl β-D-1-thiogalactopyranoside normally for 8 h at 25 °C. PUDL-1 and PUDL-2 were expressed at 16 °C for 16 h. PUD-1 and PUD-2 were coexpressed and copurified as a dimer. The harvested cells were resuspended in buffer P500 (50 mM phosphate pH 7.6, 500 mM NaCl) and lysed using a high pressure cell disruptor (JNBI) followed by sonication. The cell lysates were clarified by centrifugation and passage through a 0.45-µm filter and loaded onto a 5-ml HisTrap column (GE healthcare). The column was washed by 50 mM imidazole in P500. The protein was eluted with 500 mM imidazole in P500 and incubated with ULP1 protease to cleave the His-SMT3 tag. After concentrating and dilution with 20 mM HEPES-K, pH 7.6, the sample was passed through a HiTrap column to remove the cleaved tag and uncleaved protein. The PUD-1/PUD-2 complex was further purified with a Superdex 200 column (GE healthcare) equilibrated in buffer E250 consisting of 5 mM HEPES-K, pH 7.6 and 250 mM KCl. The PUD-1/PUD-2 complex was labeled with seleno-methionine by inhibiting the methionine biosynthesis pathway and purified with the same procedure as unlabeled complex. The protein was concentrated by Amicon Ultra-15 units and stored at -80 °C.

Crystallization and structure determination
The Se-labeled full-length PUD-1/PUD-2 complex was crystallized by mixing 1 µl of protein sample (46 mg/ml in E250) with 1 µl of well solution consisting of 0.2 M NH₄H₂PO₄, 0.1 M Tris-HCl pH 8.5 and 40% 2-methyl-1,3 propanediol using the hanging drop vapor diffusion method at 20 °C. The crystal was flash frozen in liquid nitrogen without further cryo-protection. The diffraction data were collected at the wavelength of selenium peak at Shanghai Synchrotron Radiation Facility (SSRF) beamline BL17U and processed by HKL2000 [22]. The structure was solved by single-wavelength anomalous diffraction (SAD) method using a 3.6-Å dataset. The heavy atoms were located by SHELXD [23] and the phase calculation and density modification were conducted with SHARP [24]. The crystal belongs to space group P3,21 and contains two copies of PUD-1/PUD-2 heterodimer in the asymmetric unit. The structural model was built in COOT [25] and refined with Refmac to a free R-factor of 0.38 at 3.6 Å resolution [26]. The PUD-1 (9-151) and PUD-2 (7-152) complex (35 mg/ml in E250) was crystallized in 0.1 M HEPES-Na pH 8.5 and 4.3 M NaCl. The crystal was cryoprotected with 10% glycerol in liquid nitrogen. A dataset was collected at SSRF-BL17U to 1.9 Å resolution. The structure was solved by molecular replacement in PHASER using the initial heterodimeric structure of full-length complex as a search model [27]. The crystal belongs to space group P2₁2₁2₁ and contains one heterodimer in the asymmetric unit. The final model was refined with Refmac to 1.9 Å resolution and contains residues 9-151 of PUD-1, an N-terminal serine residue from the expression vector of PUD-1, residues 7-87 and 91-152 of PUD-2, one glycerol molecule, 10 chlorine ions and 326 waters. Analysis with RAMPAGE shows that 97.9% of the residues are in most favored regions and 2.1% in allowed regions [28]. Structural figures were prepared in PyMOL [29].

Size exclusion chromatography
All six C. elegans proteins of the PUD protein family were individually purified through one-step HisTrap chromatography with the His-SMT3 tag uncleaved. Each protein (500 µg) was
Results

The PUD-1 and PUD-2 protein abundance increase in daf-2 is independent of daf-16

The protein levels of PUD-1 and PUD-2 are greatly elevated in daf-2 mutants [8]. To find out whether this is due to activated gene expression by DAF-16, the key transcription factor normally inhibited by signaling from DAF-2 in WT C. elegans, we quantified their mRNA levels in WT, daf-2, daf-16, and daf-2 double mutants by quantitative PCR. The pud-1 and pud-2 mRNA levels increased in the daf-2(e1370) mutant relative to the WT, and this moderate increase required daf-16 (Figure S1a in File S1). Interestingly, at the protein level both the daf-2 mutant and the daf-2; daf-16 double mutant had higher amounts of PUD-1 and PUD-2 than WT (Figure S1b-c in File S1), even though the mRNA templates of the two proteins were reduced in the double mutant. We thus propose that these proteins are up-regulated in the daf-2 mutant mainly through a post-transcriptional mechanism independent of DAF-16.

Overexpression of PUD-1 and PUD-2 did not extend lifespan

To find out if the increased PUD-1 and PUD-2 protein amounts contribute to the longevity of the daf-2 mutant, we overexpressed these proteins in WT animals to mimic their induction in daf-2. We generated two overexpression constructs, pYG2 and pWX1, in which the genomic sequences of pud-1 and pud-2 were fused to the GFP coding sequence, respectively (Figure S2a in File S1). Because pud-1 and pud-2 are adjacent genes transcribed from the same intergenic promoter region in opposite directions, un-tagged PUD-2 and a PUD-1::GFP fusion protein were expressed from the pYG2 transgene arrays hqls24 and hqls28, while un-tagged PUD-1 and PUD-2::GFP were expressed from the pWX1 transgene array hqls60. PUD-1::GFP and PUD-2::GFP are both expressed in the intestine and hypodermis, from either the multi-copy (Figure S2 in File S1) or the single-copy transgenes (Figure 1). None of the high-copy arrays altered the WT lifespan. Perplexingly, hqls28 and hqls60, but not hqls24, further extended the lifespan of daf-2(e1370) and daf-2(RNAi) animals by 27-38% (Figure S3 in File S1), and hqls60 animals also showed increased resistance to thermal stress (not shown). To clarify the overexpression phenotype, we made four additional transgene arrays (hqEx30, hqEx31, hqEx50, and hqEx52) from which untagged PUD-1 and untagged PUD-2 were co-expressed. None of them extended the C. elegans lifespan in either the WT or daf-2(RNAi) background; hqEx30, hqEx31, and hqEx52 even shortened lifespan somewhat (Figure 2A and data not shown), hqEx52 and another transgene hqEx47 that was made by co-injection of HA::pud-1::GFP and HA::pud-2::Cherry under their native promoters failed to alter the lifespan of daf-2(RNAi) or WT worms (not shown). We also examined, hqEx30, hqEx31, and hqEx47 animals for resistance to thermal stress at 35 °C and found that they were not different from WT worms (Figure 2B and data not shown). We thus conclude that overexpression of PUD-1 and PUD-2 does not extend the lifespan of WT or daf-2 animals. We suspect that the longevity phenotype associated with hqls60 and hqls28 may be due to independent background mutations linked to the transgenes. Because the transgenes were integrated into different chromosomes and each was backcrossed to WT at least four times, the chance for two mutations at different loci to cause the same phenotype should be extremely rare, but it appeared to be what had occurred in this case.

Deletion of pud-1 and pud-2 did not shorten the daf-2 lifespan

We next asked whether the longevity phenotype of the daf-2 mutant requires the gene activities of pud-1 and pud-2. Since knocking down of these genes using the RNAi-by-feeding method [20], either individually or in combination, failed to effectively reduce the fluorescence intensity of PUD-1::GFP or PUD-2::GFP in transgenic strains, we set out to obtain deletion alleles. Six members of the PUD gene family in C. elegans–pud-1.2, pud-2.2, pud-3, pud-4, pud-1.1 and pud-2.1 are next to each other on Chromosome V (Figure S4a in File S1) and the other two–pud-1.1 and pudl-2–are adjacent genes on Chromosome IV (Figure S4b in File S1). Fortunately, the six gene cluster on Chromosome V is deleted in JU258, a wild C. elegans strain isolated from the island of Madeira. We mapped the precise boundaries of this deletion, niDf209, and found that in addition to the PUD gene cluster, three flanking genes (Y19D10B.5, Y19D10B.6 and F15E11.11) are also deleted in niDf209 (Figure S4a in File S1). The three flanking genes each have at least one paralog in the C. elegans genome with 40% to 90% sequence similarity, so the deletion of them likely has little phenotypic consequence. Thus far, no phenotype of any kind has been associated with any of the three genes.

We transplanted niDf209 from JU258 to the standard WT N2 background by homologous recombination (Figure S5 in File S1). Briefly, JU258 was crossed to unc-60(m35) dpy-11(e224) (N2 background). The nonUnc Df Dpy recombinants were selected and backcrossed to N2 repeatedly until nonUnc Df nonDpy recombinants were obtained, and these recombinants were further backcrossed to N2 for five more times. We obtained two alleles, hq5 and hq6, that harbor niDf209 in the N2 background. hq5 has two deficiencies–niDf208 and niDf209–from JU258 whereas hq6 has only niDf209 (Figure S4a in File S1); the latter was used in most of the deletion phenotype analyses.

Both hq5 and hq6 are morphologically normal, their locomotion and egg production are also WT-like (data not shown). We found that neither hq5 nor hq6 affected the lifespan of WT or daf-2(RNAi) animals (Figure 2C). We also made a composite RNAi construct pYG17 in which 222-323 nucleotide sequences each targeting one or two PUD gene family members were concatenated together (Figure S6 in File S1). Injection of the dsRNA transcribed in vitro from pYG17 into
C. elegans (pud RNAi) reduced PUD-1::GFP and PUD-2::GFP levels by at least 50% in the next generation (data not shown). Consistent with the deletion alleles, pud RNAi did not change the lifespan of WT or daf-2(e1370) worms (Figure 2D).

hq6 conferred resistance to Aβ toxicity

Since daf-2 and many other IIS mutants are not only long-lived, but also resistant to various types of stress such as high temperature, pathogen, and Aβ toxicity [30-32], we asked if the deletion of pud-1 and pud-2 would affect stress tolerance. We found that the survival curves of hq5 and hq6 animals were similar to that of WT at 35 °C (Figure 2E). Likewise, in a fast killing assay using PA14, a pathogenic P. aeruginosa strain, we did not observe consistent difference between WT and hq6, or between daf-2 and daf-2; hq6 (Figure 2F). However, the hq6 allele protected WT animals from paralysis induced by Aβ(1-42) expressed in the body-wall muscles [33], and further enhanced the resistance of daf-2(RNAi) animals to the Aβ toxicity (Figure 3A). It also improved the brood size of Aβ transgenic worms (Figure 3B). The fact that hq6 and daf-2(RNAi) had a synergistic effect on Aβ resistance and that hq6 did not shorten the daf-2 lifespan both argue for the point

Figure 1. Expression of Ppud-1::GFP::pud-1 and Ppud-2::GFP::pud-2 from single-copy transgenes inserted into the C. elegans genome. PUD-1 (A, B, E) and PUD-2 (C, D, F) are both expressed the intestine (arrowheads) and hypodermis (long arrow). In the intestinal cells, PUD-1 (B) and PUD-2 (D) are distributed diffusely in the cytoplasm and the nucleoplasm, and largely excluded from the nucleolus (short arrow) expect for one or more nuclear puncta. The nucleolar GFP puncta are more distinct in high-copy transgenic strains (Figure S1). PUD-1 (E) and PUD-2 (F) are both localized in the hypodermal fibrous organelles with a characteristic circumferential orientation.

doi: 10.1371/journal.pone.0067158.g001
Figure 2. Neither overexpression nor deletion of pud-1 and pud-2 extended lifespan in WT or daf-2 animals. (A) hqEx30 and hqEx31, two extra-chromosomal transgenes expressing untagged PUD-1 and PUD-2, slightly shortened lifespan of WT and daf-2(RNAi) worms ($p < 0.014$ between hqEx30 or hqEx31 and WT with or without daf-2 RNAi, N>77, 20 °C). (B) hqEx30 and hqEx31 animals were as sensitive to thermal stress as the WT. Stress resistant daf-2(e1370) served as a positive control (35 °C, N>27). (C) hq5 and hq6, two deletion alleles that removed all the gene copies of pud-1 and pud-2, did not affect the lifespan of WT or daf-2(RNAi) animals (N>63). (D) Knocking down pud-1 and pud-2 by heritable RNAi (via injection of dsRNA into parent worms) had no effect on WT or daf-2(e1370) lifespan (N>79). (E-F) hq5 and hq6 did not affect the survival of C. elegans at 35 °C (E) (N>27, representative of two experiments) or the pathogenic bacteria P. aeruginosa PA14 (F) (N >39, representative of three repeats).

doi: 10.1371/journal.pone.0067158.g002
that PUD-1 and PUD-2 are not connected to the longevity of daf-2 animals.

In addition to hq5 and hq6, we also isolated tm5053 and tm6107 on chromosome IV. In tm5053, part of the coding sequence of pudl-1 and much of the intergenic region between pudl-1 and pudl-2 are deleted. In tm6107, both pudl-1 and pudl-2 are deleted (Figure S4b in File S1). No obvious defects were detected in tm5053 or tm6107 worms. The tm6107;hq6 and tm5053; hq6 double mutants showed similar resistance to Aβ toxicity as the hq6 single mutant (Figure 3C-D).

The PUD family members are likely positive regulator of a subset of collagen genes and a subset of sperm protein genes

To find out the functions of pud-1 and pud-2 in a non-biased way, we compared the gene expression profiles of the WT and hq6 animals using next-generation mRNA sequencing. At 5% false discovery rate and excluding the genes deleted in the hq6 allele, only 21 genes were differentially expressed in hq6 animals compared with the WT (Table 1), and 19 of them were down-regulated in hq6. Most of these genes had a modest mRNA abundance change in hq6 except for haf-6 (2.5-fold decrease) and srbc-15 (no transcripts detected). Significantly enriched in this short list of genes are the ones encoding collagens (col-39, col-125, col-133, col-147, col-149, and
Table 1. Differentially expressed genes in hq6 compared to wild type.

| Gene Name     | Fold Change  | Adjusted P-value | Gene Description (Concise)                                      |
|---------------|--------------|------------------|-----------------------------------------------------------------|
| col-133       | 0.37         | 1.0E-07          | Collagens (type IV and type XIII), and related proteins         |
| col-147       | 0.30         | 1.0E-07          | Collagens (type IV and type XIII), and related proteins         |
| hat-6         | 0.17         | 2.1E-06          | A half-molecule ATP-binding cassette (ABC) transporter          |
| col-39        | 0.37         | 3.8E-05          | Collagens (type IV and type XIII), and related proteins         |
| ssp-34        | 0.45         | 2.1E-04          | Sperm Specific family, class P                                  |
| C15C6.2       | 0.39         | 2.6E-04          | Unnamed protein                                                 |
| ssq-1         | 0.55         | 3.3E-04          | Sperm-Specific family, class Q                                  |
| col-149       | 0.49         | 4.5E-04          | Collagens (type IV and type XIII), and related proteins         |
| gipc-2        | 0.49         | 2.4E-03          | GIPC (RGS-GAIP Interacting Protein C) homolog                   |
| col-179       | 1.69         | 5.7E-03          | Collagens (type IV and type XIII), and related proteins         |
| F08H9.2       | 0.48         | 5.7E-03          | Unnamed protein                                                 |
| nspd-7        | 0.53         | 5.7E-03          | Nematode Specific Peptide family, group D                       |
| nspd-1        | 0.57         | 6.1E-03          | Nematode Specific Peptide family, group D                       |
| ssq-2         | 0.59         | 7.0E-03          | Sperm-Specific family, class Q                                  |
| rpl-39        | 1.57         | 1.2E-02          | Large ribosomal subunit L39 protein                             |
| Y57G11A.2     | 0.46         | 1.4E-02          | Unnamed protein                                                 |
| Y45F10C.4     | 0.50         | 1.9E-02          | Unnamed protein                                                 |
| arbc-15       | 0.00         | 4.3E-05          | Serpentine Receptor, class BC (class B-like)                    |
| col-125       | 0.32         | 2.4E-02          | Collagens (type IV and type XIII), and related proteins         |
| msp-77        | 0.54         | 4.0E-02          | Major Sperm Proteins (MSPs)                                    |
| ssq-4         | 0.61         | 4.7E-02          | Sperm-Specific family, class Q                                  |

PUD-1 and PUD-2 form a heterodimer in vitro and in vivo

We also tried to infer the functions of PUD-1 and PUD-2 from their binding proteins. Using the MosSCI technique [19], we generated a single copy transgene expressing FLAG-tagged PUD-1 under its native promoter (Pud-1:FLAG::pud-1). Using an anti-FLAG antibody, we immunoprecipitated (IP) FLAG::PUD-1 and analyzed the protein bands in the IP by mass spectrometry. Endogenous PUD-2 and HSP-1, a constitutively expressed Hsc70 protein that is also inducible upon heat shock [36], were found associated with FLAG::PUD-1 (Figure 4A). The intensity of the silver-stained protein bands suggested that PUD-1 and PUD-2 have a 1:1 stoichiometry, while HSP-1 is substoichiometric. In vitro cross-linking and gel-filtration experiments using purified recombinant PUD-1 and PUD-2 indicated that they form a heterodimer (Figure 4B–D). With this information, we revisited the protein and mRNA quantitation results and realized that indeed, they had similar fold-change values in the mutants relative to the WT (Figure S1 in File S1 and [8]), and their expression patterns were the same (Figure 1 and Figure S2 in File S1). Together, our results demonstrate that PUD-1 and PUD-2 form a heterodimer in vivo and suggest that their expressions are regulated as a single unit.

The association of PUD-1 and PUD-2 with HSP-1 is intriguing. Although no sequence homology can be found between the PUD family proteins and any heat shock proteins, PUD-1 and PUD-2 appear to be regulated like heat shock proteins. For example, they are markedly induced by an increase of temperature from 20 °C to 27 °C (Figure S2d-e in File S1) or by exposure to cadmium [37]. Moreover, from an analysis of genes differentially regulated by the C. elegans RB protein LIN-35, a non-E2F binding element was found in the promoter regions of the small heat shock protein genes, and it was also found in the promoters of pud-1 and pud-2 [38]. We tested if the purified PUD-1/PUD-2 heterodimer displayed any chaperone activity in an in vitro assay [39], and found that it did not (data not shown). There is a possibility that this heterodimer may act as a co-chaperone of HSP-1, but more likely is a substrate of HSP-1.

The crystal structure of the PUD-1 and PUD-2 heterodimer

Next, we asked if these proteins have structural homologs that could give us clues about their function. The structure of the PUD-1/PUD-2 complex was first determined at 3.6 Å resolution for full length proteins by Se-phasing. The initial model shows that a few terminal residues are disordered. We then prepared a fragment of PUD-1 with residues 9-151 and a fragment of PUD-2 with residues 7-152 and obtained high quality crystals for the slightly truncated complex. The structure of the truncated complex was determined to 1.9 Å resolution with an Rwork/Rfree of 0.192/0.225 and is discussed below (Figure 5, Table S1 in File S1).

In the structure, both PUD-1 and PUD-2 adopt similar β-sandwich folds that further associate with each other into a heterodimer (Figure 5A). As expected from 54% sequence similarity between PUD-1 and PUD-2, the two subunit
structures can be well superimposed with a root mean square deviation (RMSD) of 1.277 Å over 118 Cα pairs (Figure 5B).

The β-sandwich fold of PUD-1 and PUD-2 is composed of 9 major β-strands arranged into two sheets. One sheet is formed by strands β3, β2, β5, β6 and β9 in an antiparallel manner, whereas the other sheet is composed of strands β4, its extension β4’, β1, β7, β8 and β9 in a mixed manner, where all adjacent strands are aligned in antiparallel except β1 and β7 that are aligned in parallel. The strand β9 pairs with both sheets and closes one side of the sandwich. In addition, strands β5 and β6 project out from the sandwich body and form a prominent protrusion involved in dimerization.

The sandwich structures of PUD-1 and PUD-2 contact each other in a face-to-back manner with an inclination angle of 30°, forming a V-shaped dimer. The dimerization buries 1081 Å² of solvent accessible surface area per subunit and is mediated by two interfaces. In the first interface, one end of the PUD-1 sandwich, which is composed of the β7-β8 loop, the β1-β2 loop and the exposed regions of strands β5 and β6, packs against the β3-β9 sheet of PUD-2 (Figure 5C). This interface is stabilized by hydrophobic and polar interactions. The hydrophobic interactions involve residues M80, F105, F131, P132, H136 of PUD-1 and residues L31, L39, A41, W44, H76, I78, A102 and H152 of PUD-2. A number of polar and water-

Figure 4. PUD-1 and PUD-2 form a heterodimer. (A) FLAG IP followed by MS analysis identified HSP-1 and PUD-2 as binding proteins associated with FLAG-PUD-1. Shown is the silver stained gel of the IP products before MS analysis. (B) Purified recombinant PUD-1 and PUD-2 can be cross-linked together and the cross-linked species seems to be a heterodimer. (C) The PUD-1 and PUD-2 heterodimer, each with a SMT3 tag, was also observed by gel filtration. (D) Gel filtration analysis of the C. elegans PUD gene family proteins. SMT3 tagged proteins were loaded onto a Superdex-200 10/300 column individually or in indicated combinations.

doi: 10.1371/journal.pone.0067158.g004
Figure 5. Structure of the PUD-1 and PUD-2 heterodimer. (A) Ribbon representation of the PUD-1 and PUD-2 heterodimer in cross-eye stereoview. PUD-1 is green and PUD-2 is cyan. The β-strands are labeled with numbers and the N and C termini are indicated. (B) Structural superposition of PUD-1 and PUD-2 subunit. (C) Interactions at the dimer interface. For clarity, only residues at the interface are shown for PUD-1. Oxygen is red, nitrogen is blue, sulfur is orange and carbon is green for PUD-1 and cyan for PUD-2. Hydrogen bonds are shown as yellow dashed lines. (D) Conserved surface. The ribbon and surface representations of PUD-1 structure are shown side-by-side and in two opposite orientations. The residues conserved in 100% and 80% of the 31 homologs of PUD-1 and PUD-2, as shown in Figure S7, are colored orange and yellow, respectively, for side chain atoms. (E) Sequence alignment of the PUD family proteins in *C. elegans*. Residues with 100% and 80% conservation are shaded with black and grey, respectively. The secondary structures observed in the crystal structure are shown on the top for PUD-2 and at the bottom for PUD-1. Residues whose surface area is buried by 10-30 Å² and at least 30 Å² upon dimerization are denoted by open and solid circles, respectively.

do: 10.1371/journal.pone.0067158.g005
mediated interactions are present at more peripheral regions. The second dimer interface is constituted by the extensions of β5 and β6 of both PUD-1 and PUD-2 (named β5' and β6' in PUD-2). They form an intermolecular antiparallel 4-stranded sheet with PUD-1 β5 aligned with PUD-2 β6' (Figure 5A).

Despite strong similarity between PUD-1 and PUD-2 structures, the dimer interface is asymmetric and engages nonequivalent faces of each subunit. Sequence alignment shows the residues at the dimer interface are dissimilar in PUD-1 and PUD-2, which accounts for the specificity of the dimerization mode (Figure 5E).

Functional implication of the PUD-1/PUD-2 structure

A BLAST search in a non-redundant protein database of NCBI shows that PUD-1 and PUD-2 homologs have a very limited distribution across species and can only be identified in three worm species, five fungi and two bacteria (Figure S7 in File S1). Mapping the residues conserved in these sequences on the PUD-1 structure reveals a conserved surface patch at one end of the β-sandwich, opposite to the dimerization end (Figure 5D). The patch is mainly composed of residues from β6-β7 loop, β4'-β5 loop and the N-terminal part of β2-β3 loop. This region is likely important to the function of the PUD family proteins.

A DALI search shows that the β-sandwich of PUD-1/PUD-2 bears some topology similarity with proteins of diverse function, such as pore-forming cytotoxins, transporter transthyretin and chaperone FaE involved in pilus assembly (Z score to 6.9). However, the functional insight provided by these structural homologs is vague.

To summarize, after the genetic, genomic, biochemical, and structural analyses described above, much has been learned about the PUD-1 and PUD-2 proteins, but the significance of their biological function remains unclear.

Characterization of other C. elegans homologs of PUD-1 and PUD-2

We determined the expression patterns of all the PUD gene family members in C. elegans by GFP fusion proteins, and the result is summarized in Table S2 in File S1. As described above, pud-1 and pud-2 are expressed strongly and uniformly in the intestine, and less strongly in the hypodermis (Figure 1 and Figure S2 in File S1). Besides, their GFP fusion proteins were both seen in fibrous organelles, the hypodermal hemidesmosome structures that fasten muscles to the cuticle (Figure 1E-F). At the subcellular level, both proteins were found distributed diffusely in the cytoplasm and the nucleus, and largely excluded from the nucleolus except for one or more nucleolar puncta (Figure S2b-c in File S1).

The P_{pud-3::pud-3}::GFP transgene was expressed in the nuclei of hyp7, the largest hypodermal cell that wraps around most of the worm body. It was also expressed in the pharyngeal muscle pm3, rectal gland cells, and less frequently, in the intestine and a few neuron-like cells in the head (Figure 6A).

The expression of P_{pud-4::pud-4}::GFP was detected most strongly in the hyp7 nuclei and sporadically in the intestine (Figure 6B).

Also concentrated in the hyp7 nuclei were PUDL-1::GFP and PUDL-2::GFP. Elsewhere, PUDL-1::GFP was seen in a head neuron and PUDL-2::GFP in the pharyngeal muscle pm5 (Figure 6C-D).

Judging by GFP intensity, pud-1::GFP and pud-2::GFP are the strongest expressers. From mRNA sequencing data of WT day-1 adults (Table S3 in File S1), the sequence reads of pud-1 and pud-2 are 10-fold higher than those of pud-3 and pud-4, and about 1000-fold higher than those of pudl-1 and pudl-2. We suspect that pudl-1 and pudl-2 are minimally expressed in young adult worms under normal conditions. In contrast, pud-1 and pud-2 are constitutively expressed at high levels, and can be induced further. The nuclear localization of the PUD-1/2/3/4 proteins suggests that they might regulate the transcription of the collagen genes whose mRNA levels were altered in the hq6 mutant (Table 1).

As PUD-1 and PUD-2 form a dimer, we asked whether the rest of the worm homologs are structural counterparts and can also form a dimer. To assess their oligomeric state in solution, we performed analytical gel filtration experiments (Figure 4C-D). Since these proteins alone have a low solubility, they were analyzed with a fused solubility-enhancing SMT3-tag. Separately, SMT3-PUD-1 and SMT3-PUD-2 eluted in a position corresponding to a monomeric species. Combining them led to the appearance of a new peak corresponding to a dimeric species, which is expected for the PUD-1/PUD-2 heterodimer (Figure 4C). When either SMT3-PUD-1 or SMT3-PUD-2 was mixed with other four homologs, the elution peak stayed the same at the monomeric position (Figure 4D). This indicates that the four homologs cannot form a dimer with either PUD-1 or PUD-2, or with one another. Mixing of all six PUD family proteins led to a dimeric species that can be ascribed to the PUD-1/PUD-2 dimer (Figure 4D). We thus conclude that PUD-3, PUD-4, PUDL-1, and PUDL-2 are monomeric, in contrast with the heterodimeric structure of PUD-1 and PUD-2. The sequence alignment shows that the residues at the dimer interface of PUD-1 and PUD-2 are not well conserved in other homologs (Figure 5E), corroborating the different oligomeric states of these proteins.

Discussion

There is no doubt that the downstream targets of DAF-16 are responsible for the remarkable longevity of daf-2, age-1, and other IIS mutants. However, a concrete and comprehensive list of DAF-16 targets has remained elusive despite the efforts of multiple research groups [40]. Yet, even more difficult are the tasks of verifying the functions of candidate targets. Here we find that PUD-1 and PUD-2, two proteins displaying the most notable abundance increase in a quantitative proteomics analysis of daf-2, appear to have no contribution to longevity (Figure 2A–D), tolerance of stress (Figure 2E-F), and dauer formation (not shown). This is similar to sod-3, which encodes an inducible mitochondrial superoxide dismutase and is a bona fide DAF-16 target once believed to be beneficial to longevity. Despite its marked increase of expression in daf-2 mutants [41], independent reports recently showed that deletion of sod-3 had no effect on either WT or daf-2 lifespan [42-44]. In
Figure 6. Expression patterns of the paralogs of PUD-1 and PUD-2 in C. elegans. (A-D) Distributions of the PUD-3, PUD-4, PUDL-1, and PUDL-2 translational GFP fusion proteins, respectively.

doi: 10.1371/journal.pone.0067158.g006
fact, all five *C. elegans* *sod* genes are dispensable for WT lifespan [45].

IIS controls many biological processes. Some of them are relevant to aging and some probably not. This study and the efforts of others trying to elucidate whether and how some of the DAF-16 targets contribute to longevity have highlighted the complexity of aging regulation. Initially, many of the genes that had been tested because their mRNA levels increased or decreased in *daf-2(-)* vs. *daf-2(+) backgrounds appeared to contribute to *daf-2* longevity [10]. However, as more genes were examined, it became clear that not all changes in *daf-2* were in the direction of extending lifespan [8]. In fact, some protein abundance changes in *daf-2* such as the increase of ACO-2 may serve to shorten lifespan, possibly as part of a compensation mechanism acting to restore the WT status [8]. *daf-2* mutants are resistant to Aβ toxicity and have increased levels of the PUD-1/PUD-2 heterodimer. Our finding that mutant worms lacking *pud-1* and *pud-2* were more resistant to paralysis caused by Aβ seems to provide another example (Figure 3).

**Supporting Information**

**File S1.** Figure S1. Increase of the PUD-1 and PUD-2 protein abundance in the *daf-2* mutant is mostly due to post-transcriptional regulation independent of *daf-16.*

(a) Quantitative RT-PCR of *pud-1* and *pud-2* in WT, *daf-16(mu86), daf-2(e1370),* and *daf-16(mu86); daf-2(e1370)* worms. Data are shown as mean ± s.e.m. of three independent experiments, each with duplicate measurements. (b) Anti-PUD-1 and anti-PUD-2 western blots showing the protein levels in whole-worm lysates of the indicated strains. The anti-tubulin signals control for loading. (c) Summary of the densitometry measurements of three independent experiments including the one shown in (b), expressed as mean ± standard deviation. *p < 0.01 vs. the wild type N2. Figure S2. PUD-1::GFP and PUD-2::GFP expressed from transgene arrays under the control of their native promoters. (a) GFP expression constructs of PUD-1 (pYG2) and PUD-2 (pWX1). (b-c) Both are strongly expressed in the intestine and less strongly in the hypodermis. The inset shows that PUD-1::GFP or PUD-2::GFP is expressed in the nucleoplasm of intestinal cells, largely excluded from the nucleolus except for one or more puncta. (d-e) A temperature shift from 20 °C to 27 °C stimulated the expression of PUD-1::GFP (d) and PUD-2::GFP (e). Figure S3. hqls28 and hqls80, but not hqls24, extended *daf-2* lifespan. hqls28 and hqls80 extended the lifespan of *daf-2(e1370)* (a) or *daf-2(RNAi)* (b) mutants, whereas hqls24 (c) did not. Figure S4. The distribution of the PUD gene family members in the *C. elegans* genome and the location of *niDf209.* (a) *pud-1.2, pud-2.2, pud-3, pud-4, pud-1.1* and *pud-2.1* are next to each other on Chromosome V. *pud-1.2* is a perfect duplicate of *pud-1.1* and *pud-2.2* is a perfect duplicate of *pud-2.1.* The boundary of *niDf209* as annotated in the wormbase.org is not precise. The actual endpoints of *niDf209* are indicated. The flanking sequences are GTACTGTAGGCC [15979 bp deletion] [1667 bp insertion] TGTAAATCCAGC. The 1667 bp insertion consists of a 67-bp sequence "TACTGATGATTACTGATGGATTTCAGCCTTGAAAGGATTGCAGAAGATTAG" (indicated by a very short blue segment right before an orange arrow) followed by an inverted duplicate of a 1600-bp fragment from Y19D10B.5 "TGAGAATCTC....ACGGAACGTT" (orange arrows). (b) *pudl-1* and *pudl-2* are adjacent genes on Chromosome IV. Figure S5. Transplanting *niDf209* from JU258 to the N2 background by homologous recombination. Figure S6. pYG17, an RNAi construct for knocking down multiple members of the PUD gene family. Figure S7. Sequence alignment of the PUD gene family proteins. Sequences were aligned by MUSCLE program and slightly adjusted. The abbreviation of species name and Genebank ID are indicated for each sequence. The secondary structures of PUD-2 are indicated above the alignment. Shading of black, grey and light grey represent 100%, 80% and 60% conservation, respectively. Table S1. Data collection and refinement statistics. Table S2. Endogenous promoter-driven expression of GFP translational fusion proteins. Table S3. mRNA levels of *pud* family genes. Table S4. Constructs. Table S5. *C. elegans* Strains.

**Acknowledgements**

We thank Dr. Christopher D. Link (University of Colorado) for providing the Aβ transgenic strain CL2006 and Dr. F. Ulrich Hartl for help with the chaperone activity assay. Some strains were provided by the CGC. We thank the staff in Shanghai Synchrotron Radiation Facility beamline BL17U for assistance in data collection.

**Author Contributions**

Conceived and designed the experiments: M-QD Y-HD Y-XL SL. Contributed reagents/materials/analysis tools: SY KK SM. Wrote the manuscript: M-QD Y-HD KY. Performed the experiments: Y-HD Y-GD SL T-ML XW. Analyzed the data: Y-HD Y-XL SL. Contributed reagents/materials/analysis tools: SY KK SM. Wrote the manuscript: M-QD Y-HD KY.

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