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Metabolome and proteome changes with aging in *Caenorhabditis elegans*

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Abstract

To expand the understanding of aging in the model organism *Caenorhabditis elegans*, global quantification of metabolite and protein levels in young and aged nematodes was performed using mass spectrometry. With age there was a decreased abundance of proteins functioning in transcription termination, mRNA degradation, mRNA stability, protein synthesis, and proteasomal function. Furthermore there was altered S-adenosyl methionine metabolism as well as a decreased abundance of the S-adenosyl methionine synthetase (SAMS-1) protein. Other aging-related changes included alterations in free fatty acid levels and composition, decreased levels of ribosomal proteins, decreased levels of NADP-dependent isocitrate dehydrogenase (IDH1), a shift in the cellular redox state, an increase in sorbitol content, alterations in free amino acid levels, and indications of altered muscle function and sarcoplasmic reticulum Ca²⁺ homeostasis. There were also decreases in pyrimidine and purine metabolite levels, most markedly nitrogenous bases. Supplementing the culture medium with cytidine (a pyrimidine nucleoside) or hypoxanthine (a purine base) increased lifespan slightly, suggesting that aging-induced alterations in ribonucleotide metabolism affect lifespan. An age-related increase in body size, lipotoxicity from ectopic yolk

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lipoprotein accumulation, a decline in NAD⁺ levels, and mitochondrial electron transport chain dysfunction may explain many of these changes. In addition, dietary restriction in aged worms resulting from sarcopenia of the pharyngeal pump likely decreases the abundance of SAMS-1, possibly leading to decreased phosphatidylcholine levels, larger lipid droplets, and ER and mitochondrial stress. The complementary use of proteomics and metabolomics yielded unique insights into the molecular processes altered with age in *C. elegans*.

Keywords

aging; *C. elegans*; metabolomics; proteomics; *Caenorhabditis*; lifespan; metabolome; proteome; pyrimidine; purine; S-adenosyl methionine; SAMS-1; SERCA; ascorbate; sorbitol; methylation; methyltransferase; methionine; nitrogenous bases

1. Introduction

The nematode *Caenorhabditis elegans* is used extensively as a model organism for investigating the molecular mechanisms of aging. The popularity of *C. elegans* as an aging model is largely due to its short lifespan (approximately 1.5 – 4 weeks depending on the temperature of incubation), and the ease at which it can be cultivated under laboratory conditions. *C. elegans* aging is characterized by a severe loss of muscle mass and function (sarcopenia), which gradually interferes with movement and the ingestion of food (Glenn et al. 2004). In addition aging results in an increased size of lipid droplets (LD) as egg yolk lipoproteins continue to be produced after reproduction ceases (Herndon et al. 2002), and the appearance of enlarged dysfunctional mitochondria within cells (Gerstbrein et al. 2005). Accordingly, aged *C. elegans* experience a dramatic decline in mitochondrial oxygen consumption, and ATP and ADP content, starting as early as the onset of adulthood (Brys et al. 2010; Houthoofd et al. 2005). Aged *C. elegans* have also been shown to experience a loss of proteostasis and an accumulation of protein aggregates (David et al. 2010; Labbadia and Morimoto 2014), as well as oxidatively damaged proteins and lipids (Ishii et al. 2002; Morcos et al. 2008). Macromolecular damage in the form of the non-enzymatic formation of advanced glycation end-products are a part of normal *C. elegans* aging, with the reactive molecule methylglyoxal likely playing a role in limiting the *C. elegans* lifespan (Morcos et al. 2008).

In 1988 the first genetic mutation resulting in increased longevity, the *C. elegans* phosphoinositide 3-kinase gene *age-1*, was identified (Friedman and Johnson 1988). Since that time, knowledge of genetic factors affecting the aging process has greatly expanded, and a search of the GenAge database (<http://genomics.senescence.info/genes/>) currently lists 741 *C. elegans* genes known to impact lifespan. Two conserved signaling pathways known to increase lifespan have been characterized in *C. elegans* (Cohen et al. 2006; Kenyon 2005; Leavy 2011; Murshid et al. 2013; Tissenbaum 2012); one involves disruption of the insulin/IGF-1 receptor, DAF-2 (abnormal dauer formation-2) signaling pathway, while the other involves activation of the NAD⁺-dependent histone deacetylase SIR-2.1. *C. elegans* loss-of-function mutations in *daf-2* have an extended lifespan, which is dependent on the activation of the transcription factor DAF-16 (homologous to human FOXO genes) (Apfeld and

Kenyon 1998; Gems et al. 1998; Tullet et al. 2008). Dietary restriction is one of the most consistent methods for producing lifespan extension in model organisms, activates DAF-16 through a decrease in PI3K/AKT signaling (Weinkove et al. 2006). Activation of SIR-2.1, the *C. elegans* homolog of human SIRT1 also results in lifespan extension through partially overlapping mechanisms, including activation of DAF-16 (Dali-Youcef et al. 2007).

The genetic dissection of the *C. elegans* aging process has progressed in part due to the widespread application of transcriptome analysis (Golden and Melov 2007) and through the ease with which RNAi gene knockdown experiments can be performed (Ni and Lee 2010). Despite these advancements in the field, there have been few global proteomic investigations of the *C. elegans* aging process, and even fewer metabolomic investigations of aging. Based on our previous findings regarding the role that dietary metabolites play in *C. elegans* lifespan extension and in an effort to add to the understanding of aging in this model organism (Edwards et al. 2015; Edwards et al. 2014; Edwards et al. 2013), we chose to employ a mass spectroscopy-based approach to global endogenous metabolite identification in young versus aged *C. elegans*. We also pursued a mass spectrometry-based heavy isotope labeling approach for global protein quantification, with the goal of contextualizing the results from both “-omic” investigations using the *C. elegans* aging literature.

2. Materials and Methods

2.1 Chemicals and Strains

The SLE1 HT115(DE3) *Escherichia coli* bacterial strain was used as described in (Larance et al. 2011). $^{15}\text{N}_4$ - $^{13}\text{C}_6$ -arginine was purchased from Thermo Scientific Pierce. $^{15}\text{N}_2$ - $^{13}\text{C}_6$ -lysine was purchased from Cambridge Isotope Laboratories, Incorporated.

2.2 *C. elegans* Culture

Between assays, *glp-4(bn2)* *C. elegans* (strain SS104) were grown at 15 °C on 10 cm NGM (nematode growth medium) plates containing a lawn of HT115(DE3) *E. coli*. Prior to use in the assays, several plates of *C. elegans* were washed into a 750 mL liquid culture of *glp-4(bn2)* *C. elegans* containing HT115(DE3) *E. coli* (6.9×10^9 CFU/mL) and grown at 15 °C using standard liquid culturing techniques (Stiernagle 1999). After becoming gravid adults, nematodes from this culture were used for experimentation.

2.3 Alkaline Bleach Synchronization

A liquid culture was chilled for 10 minutes on ice, and pelleted by centrifugation ($\sim 1150 \times g$ for 4 minutes). The majority of the supernatant was carefully decanted, leaving behind a concentrated slurry of nematodes. Eggs were collected by alkaline bleach synchronization as follows: 6% NaOCl (Clorox®) was combined with 5 M NaOH in a 2:1 ratio by volume, and added to the concentrated slurry in a ratio of 0.4 mL of alkaline bleach solution per 1 mL of slurry. The resulting *C. elegans* suspension was then shaken for 4–7 minutes until the carcasses dissolved, as monitored by microscopy, leaving behind only eggs. The egg-containing solution was then diluted 5-fold with 0.1 M NaCl, and centrifuged at $\sim 1,150 \times g$ for 2 minutes at room temperature. The supernatant was removed by aspiration, and the resulting pellet of eggs was washed 3 times by the addition of a similar volume 0.1 M NaCl,

and centrifuged at $\sim 1,150 \times g$ for 2 minutes at room temperature. The final pellet of eggs was then used to establish age-synchronized liquid cultures.

2.4 Metabolomics Culturing and Sample Preparation

Eggs obtained by alkaline bleach synchronization were used to start two 1.5 L liquid cultures, containing HT115(DE3) *E. coli* (6.9×10^9 CFU/mL), and grown at 25 °C to induce sterility in the *glp-4(bn2)* *C. elegans*. On the fourth and tenth days of culture, one of the 1.5 L cultures was filtered through a 10 micron filter to separate *C. elegans* from bacteria. The collected nematodes were then washed into 1.5 L of M9 buffer and shaken for 20 minutes to allow the *C. elegans* time to empty the contents of their intestinal tract. The *C. elegans* M9 suspension was then divided among 500 mL bottles and centrifuged at $\sim 1,100 \times g$ for 4 minutes at room temperature. The supernatants were aspirated and the resulting pellets were combined in 50 mL of M9 buffer. At this point, three 1 mL portions of the suspension were set aside for protein determination assays (described in a section below). The remaining suspension was divided among five 15 mL conical vials and centrifuged at $\sim 1,100 \times g$ for 4 minutes at room temperature. The supernatants were aspirated and the pellets were frozen in liquid nitrogen. The pellets were then each ground to a fine powder using a pre-cooled mortar and pestle. The individual ground pellets were suspended in 5 mL of 80% methanol, and the resulting slurries were sonicated on ice using a Heat Systems Ultrasonics W-380 sonicator (5-second pulses, 50% duty cycle, max power, 12 pulses total). Each slurry was centrifuged at $10,000 \times g$ for 30 minutes at 4 °C, and the resulting supernatant was saved on ice. The pellets were then washed twice more in a similar manner, once with 5 mL of HPLC grade water and once with 70% ethanol, and the resulting supernatants were combined with the 80% methanol supernatant on ice. The remaining pellets were then suspended in 5 mL of chloroform and shaken at 250 rpm for 15 minutes at 37 °C. A separate syringe fitted with a 0.45 micron filter was used to collect the bottom organic layer of each conical vial, which was then saved separately on ice. The five polar fractions (combined methanol, water, and ethanol supernatants), and the five nonpolar fractions (chloroform supernatants) were each dried in a centrifugal vacuum evaporator at 50 °C for 2.5 hours. The samples from days 4 and 10 (5 polar and 5 nonpolar samples each) were sent to the University of Illinois Biotechnology Center for metabolite identification using GC-MS, and normalization based on total protein content.

To guarantee that the *C. elegans* grown until day 10 did not deplete their bacterial food source or accumulate an unwanted amount of waste products in the culture media, on the sixth day the nematodes were pelleted by centrifugation at $\sim 1,100 \times g$ for 4 minutes at room temperature and the supernatant was removed by decanting and by aspiration. The *C. elegans* were then suspended in 1.5 L of fresh S-medium containing HT115(DE3) *E. coli* (6.9×10^9 CFU/mL), and returned to incubation at 25 °C.

2.5 Protein Assay

The total *C. elegans* protein content of each sample was assayed using a similar protocol as described by Braeckman *et al.* (Braeckman et al. 2002). Briefly, 1 mL of a sample was snap frozen in liquid nitrogen and stored at -10 °C until analysis. For the analysis, 500 μ L of the sample was transferred to a new 2 mL microcentrifuge tube and sonicated on ice using a

Heat Systems Ultrasonics W-380 sonicator (5-second pulses, 50% duty cycle, max power, 12 pulses total). A 1.5 mL portion of 1:1 ethanol:acetone was added, vortexed briefly, and incubated at 4 °C for 30 minutes to facilitate protein precipitation. The tube was then centrifuged at $15,000 \times g$ for 10 minutes at room temperature. The supernatant was decanted and the tube was left open and inverted on a paper towel for 5 – 10 minutes while the pellet dried. The pellet was then suspended in 180 μ L of 1 N NaOH, vortexed, and incubated at 70 °C for 25 minutes to degrade any remaining lipids within the sample. A 1.26 mL portion of deionized water was added to dilute the NaOH and 360 μ L of 10% sodium dodecyl sulfate (SDS) was added to facilitate the solubilization of the proteins. The tube was then mixed by inversion and centrifuged at $1,500 \times g$ for 2 minutes at room temperature. The protein content of the supernatant was then analyzed by the BCA protein assay (Pierce) according to the manufacturer's protocol, using bovine serum albumin protein standards with a similar pH and SDS content as the assayed samples.

2.6 Lifespan Assays

Following bleach synchronization, roughly 40 *C. elegans* eggs were placed in each of six 3 μ m cell culture inserts, which were placed in shaken 6-well microplates with liquid S-medium containing 9×10^{10} heat-killed HT115(DE3) *E. coli* per ml as described in (Edwards et al. 2014). 3 of the wells were treated from the beginning of the lifespan with either 10 mM cytidine ($n=163$ worms) or 10 mM hypoxanthine ($n=366$ worms) while the other 3 wells were left untreated as controls ($n=349$ worms for the hypoxanthine control and $n=112$ worms for the cytidine control). 400 μ M fluorodeoxyuridine was added at the L4 larval stage. Culture inserts were lifted out and culture media in the microplate wells was replaced every 3 days. Experiments were done in triplicate for hypoxanthine and in duplicate for cytidine and analyzed using Kaplan Meier Log-Rank analysis in Sigmaplot version 11.0.

2.7 Proteomics Culturing

Cultures of SLE1 HT115(DE3) *E. coli*, containing a pAG608 plasmid for dsRNA generation targeting the *orn-1* gene, were grown overnight with vigorous shaking at 37 °C in M9 media (12.8 mg/mL Na₂HPO₄, 3 mg/mL KH₂PO₄, 0.5 mg/mL NaCl, 1 mg/L NH₄Cl, 2 mg/mL glucose, 49.4 μ g/mL MgSO₄ • 7H₂O, 1.52 μ g/mL CaCl₂ • 2H₂O, 1 μ g/mL thiamine, 1 μ g/mL FeSO₄ • 7H₂O, and 100 μ g/mL of ampicillin). For bacteria grown for isotope labeling, 50 μ g/mL ¹⁵N₄-¹³C₆-arginine and 50 μ g/mL ¹⁵N₂-¹³C₆-lysine were incorporated into the buffer (final concentration), otherwise 50 μ g/mL unlabeled arginine and 50 μ g/mL unlabeled lysine were used. After ~16 hours (OD_{600 nm} = ~1), dsRNA expression was induced by the addition 1 mM IPTG (final concentration). The bacteria were then incubated for an additional 4 hours at 37 °C with shaking, and collected by pelleting at $3,000 \times g$ for 20 minutes.

For both the labeled and unlabeled samples, *C. elegans* were grown as follows: eggs were obtained by alkaline bleach synchronization and used to start a 100 mL liquid culture containing SLE1 HT115(DE3) *E. coli* (6.9×10^9 CFU/mL). Isotope label-incorporated *E. coli* was used exclusively for the *C. elegans* culture designated for label incorporation. The nematodes were grown at 15 °C until the culture contained primarily gravid adults, at which point eggs were obtained by alkaline bleach synchronization. This pattern was repeated

through two more generations to ensure adequate incorporation of $^{15}\text{N}_4$ - $^{13}\text{C}_6$ -arginine and $^{15}\text{N}_2$ - $^{13}\text{C}_6$ -lysine into the nematodes. Eggs from the fourth generation were grown at 25 °C. *C. elegans* containing $^{15}\text{N}_4$ - $^{13}\text{C}_6$ -arginine and $^{15}\text{N}_2$ - $^{13}\text{C}_6$ -lysine were harvested on the fourth day. *C. elegans* containing unlabeled arginine and lysine were harvested on the tenth day. As with the metabolomics assay, on the sixth day the S-medium and bacteria were replaced (see above).

2.8 Proteomics Sample Preparation and LC-MS/MS

Harvesting consisted of pelleting the *C. elegans* at $\sim 1,100 \times g$ for 4 minutes at room temperature, aspirating the supernatant, and then suspending the nematodes in M9 buffer. The *C. elegans* were washed twice more using this method and then suspended in ~ 1 mL of 6:4 M9 buffer/Percoll®. Living and dead nematodes were separated by carefully layering the *C. elegans* suspension on top of 10 mL of ice-cold 6:4 M9 buffer/Percoll®. The nematodes were then centrifuged at $1,500 \times g$ for 10 minutes at room temperature (living *C. elegans* pellet to the bottom of the solution; dead *C. elegans* stay suspended at the top). The supernatant (and dead nematodes) were aspirated, and the resulting pellet was washed twice in 10 mL of M9 buffer ($\sim 1,100 \times g$ for 4 minutes at room temperature). The pellet was then snap frozen in liquid nitrogen and ground into a fine powder using a pre-cooled mortar and pestle. The ground *C. elegans* were suspended in 10 mL of the following buffer: 50 mM ammonium bicarbonate at pH 8, 2% SDS, 100 mM dithiothreitol, 150 mM NaCl, and 1x Halt™ Protease Inhibitor (from Life Technologies). The slurry was then sonicated on ice using a Heat Systems Ultrasonics W-380 sonicator (5-second pulses, 50% duty cycle, max power, 12 pulses total). 1 mL samples were taken from each group (day 4 and day 10) for assaying total protein (see above; $n = 3$). The slurries from days 4 and 10 were combined in a 1:1 ratio based on total protein content.

A protein precipitation was performed on the slurry by the addition of 3 volumes of 1:1 ethanol:acetone (incubated for 30 minutes at 4 °C, followed by centrifugation at $15,000 \times g$ for 10 minutes at room temperature). The supernatant was decanted, and the sample was allowed to dry before being suspended in our buffer solution (described above). A 15 minute water bath sonication was performed to aid in solubilization of the precipitated proteins. The proteins were then digested using a filter-aided sample preparation (FASP) kit (from Protein Discovery), according to the manufacturer's instructions. Five separate digestions were performed, using approximately 100 µg of protein per digestion. The samples were digested with trypsin at a ratio of 1:100 w/w trypsin:protein, and incubated overnight at 37 °C. The resulting eluted peptides were de-salted using a vacuum manifold and Supelco Discovery® DSC-18 solid phase extraction columns. The de-salted peptides were then dried in a centrifugal vacuum evaporator at 50 °C, and suspended in 20 µL of 0.1% formic acid.

The peptides were pooled and then separated by high performance liquid chromatography (HPLC) using a Dionex Ultimate 3000 HPLC system and a strong cation-exchange (SCX) column loaded with 5 µm 300 angstrom polySULFOETHYL A-SCX material. Fractions were collected once per minute using a 1 hour gradient (5 mM to 500 mM ammonium formate, pH 3 to pH 6). Fractions selected for mass spectroscopy (LC-MS/MS) were mixed with 250 µL of 98% acetonitrile:2% formic acid and again dried using a centrifugal vacuum

evaporator at 50 °C. The dried peptides were then suspended in 200 µL of 50% acetonitrile: 2% formic acid. Tandem mass spectrometric analysis was then performed using a hybrid linear ion trap-Orbitrap instrument (LTQ Orbitrap XL, Thermo).

2.9 Analysis

Outliers of metabolite spectral counts were removed using the Tukey boxplot method, and the remaining counts were range scaled (van den Berg et al. 2006). Statistical significance between the values in the young and aged groups was determined using unpaired two-tailed *t*-tests followed by a Benjamini-Hochberg method to adjust the false discovery rate with a threshold *p*-value of 0.05. Metabolites with a *p*-value greater than the threshold were scored as unchanged. Principal component analysis was performed for the identified metabolites using XLSTAT. Metabolic pathways were assigned to each metabolite by manually searching the KEGG database. The pathway designated as “Metabolic Pathways” (map01100) was excluded since it encompasses most other pathway information. Proteomic results were checked for adequate incorporation of the labeled isotopes and RAW files were searched using MaxQuant version 1.4.1.2 (default settings) (Cox and Mann 2008) and *C. elegans* and *E. coli* Uniprot databases. Searched parameters included modifications of cysteine by carbamidomethylation and methionine oxidation as well as modifications corresponding to the weights of incorporated ¹⁵N₄-¹³C₆-arginine, ¹⁵N₂-¹³C₆-lysine, and ¹⁵N-¹³C₅-proline. Proteomic statistical analysis was performed using Perseus version 1.4.1.3. Log₂-fold change values were calculated for aged/young (unlabeled/labeled), and centered on a median of zero. Statistical significance was determined using the Significance B method (Benjamini-Hochberg corrected *p*-value; threshold = 0.05). Associated KEGG pathways were assigned to significantly up- and down-regulated proteins using the STRING database.

3. Results

3.1 Metabolomic Analysis of *C. elegans* Identifies Widespread Metabolite Changes with Age

One liter liquid cultures of *glp-4(bn2)* *C. elegans* were grown at 25 °C. This strain of *C. elegans* was chosen because it contains a temperature-sensitive mutant allele of *glp-4* (abnormal germ line proliferation-4), which completely restricts germ cell development when the nematodes are cultured 25 °C (Beanan and Strome 1992), maintaining the age-synchronized population throughout the course of the experiment. When cultured at 25 °C, adult *glp-4(bn2)* *C. elegans* contain ~12 prophase-arrested germ cell nuclei, as opposed to the 700 – 1000 non-arrested nuclei present in wild-type adults. Temperature is a major factor affecting developmental rate and lifespan in *C. elegans*, and in agreement with published findings we found that growth at 25 °C produced a mean liquid culture lifespan of ~10 days (Fig. S1) (Klass 1977). We chose to compare metabolite profiles of young adult nematodes at day 4 of their lifespan with aged nematodes at day 10 of their lifespan (the mean length of their lifespan). Our choice of ages was based on a desire to select an early time point of measurement that would exclude metabolite changes from larval development and a later time point of measurement for investigating metabolite changes from an organism near the

end of its lifespan, while still guaranteeing enough surviving *C. elegans* within the culture for adequate measurement.

The assay of metabolites from these two population pools was performed by gas chromatography-mass spectroscopy (GC-MS) and normalized to total protein content. The assay resulted in the successful identification of 186 metabolites within the samples. The data was analyzed by principal component analysis (PCA) to identify major factors affecting the distribution of metabolite levels. When graphed two dimensionally as a plane defined by the two largest factors the young and old samples separated along the primary (*F1*) axis showing that the majority of the underlying variation in metabolites levels was attributable to differences in age (Fig. 1A). When similarly graphed, the individual metabolites showed separation along the same age-related axis (Fig. 1B).

Log₂-fold change values were calculated for each metabolite by comparing metabolite levels on day 4 and day 10 and relevant affected pathways were identified by matching metabolites to known *C. elegans* enzymes using the KEGG (Kyoto Encyclopedia of Genes and Genomes) database (<http://www.genome.jp/kegg/>) (Kanehisa and Goto 2000). Pathways were assigned to a metabolite if that pathway contained a known *C. elegans* enzyme that either produces or consumes the metabolite (Table S1). The total observed change for all pathways was calculated as the sum of the absolute log₂-fold change of each metabolite associated with that pathway (Fig. 2 and Table S2).

3.2 Altered Amino Acid Pools with Age Reflect Changes in Cell Volume

The pathway found to be most changed with age was the biosynthesis of amino acids, primarily because of the large degree of overlap within that pathway with other metabolic processes, including the pentose phosphate pathway, S-adenosyl methionine metabolism, the urea cycle, and the citric acid cycle. Of the 24 metabolites identified within this pathway, 14 were significantly altered with age, including 7 amino acids. Of the 14 metabolites found to be altered in abundance, 11 were significantly decreased in older nematodes, giving this pathway a large negative total observed log₂-fold change.

Changes in the relative concentrations of identified free amino acids, including the two aromatic amino acids phenylalanine and tryptophan, which are associated with separate pathways, reflect known age-related changes in nematode cell-to-volume ratios. During larval development, the increase in body size greatly outpaces the increase in cell number. During this period, *C. elegans* body size increases ~32-fold, yet the total cell number increases by only ~3.5-fold (Sulston and Horvitz 1977; Sulston et al. 1983). By adulthood, all 959 somatic cells are postmitotic, but the *C. elegans* body size continues to increase (Byerly et al. 1976). Growth at this point is accomplished entirely by expanding cells volumes, which result in shrinking membrane-surface/cell-volume ratios for individual cells.

This continuous change in cell surface-to-volume has been found to be associated with a specific change in protein content. In 2009, Swire *et al.*, performed H¹ NMR on young and old *C. elegans* to assay free amino acid pools and analyzed a previously published transcriptome dataset to determine age-related changes in membrane- and cytoplasm-associated genes (Jiang et al. 2001; Swire et al. 2009). Their conclusion was that

cytoplasmic genes showed a large and significant increase in expression during larval development compared to membrane-associated genes. The resulting relative increase in cytoplasmic protein production causes an increased incorporation of hydrophilic amino acid residues compared to hydrophobic residues, and the cellular supply of free amino acids should change to match the demand. Accordingly, their ^1H NMR analysis revealed a general age-related increase in the hydrophilic/hydrophobic ratio of free amino acids, with the extent of increase or decrease of individual amino acids corresponding approximately to their hydrophobicity. We found a similar correlation with the 9 free amino acids we identified to be significantly altered in abundance with age (Fig. 3), with a Pearson product-moment correlation coefficient of -0.665 (p -value = 0.05) using a glycine normalized scale of hydrophobicity (Monera et al. 1995). Overall, relatively hydrophobic free amino acids tended to decrease in concentration with age with methionine showing the largest decrease in abundance, and the few slightly more hydrophilic free amino acids detected tended to increase in concentration (or decrease to a lesser extent), in accordance with the requirements for cells increasing in size.

3.3 Purine Metabolite Levels Are Decreased in Older Nematodes

Purine metabolite levels showed the largest total decrease in $\log_2$ -fold change with age with adenine, guanine, adenosine, adenosine monophosphate, ribose, ribose 5-phosphate, hypoxanthine, and inosine (a nucleoside consisting of hypoxanthine bound to a ribose sugar) all showing decreased abundance by day 10. To validate these results, we chose to further assay hypoxanthine concentrations in young (day 5) and old (day 12) wild type N2 *C. elegans* grown at 20 °C. The selection of our nematode strain and growth conditions, which necessitated the use of 5-fluoro-2'-deoxyuridine (FUdR) to prevent egg-laying, was intended to expose any strain- or condition-specific effects present in our measurements. The administration of FUdR between the L4 and adult developmental stages is an alternative method to maintain the age-synchronous population. FUdR is a nucleotide analog and an inhibitor of DNA synthesis. Since only the germ cells are mitotically active in adult *C. elegans*, FUdR selectively restricts germ cell production. From our measurements, we determined that the hypoxanthine concentration decreased ~30% between days 5 and 12 (Fig. 4A), which matched our metabolomics results in the direction of change, but with a smaller magnitude. The smaller magnitude of change may possibly be due to an altered rate of degradation of purine deoxynucleotides following FUdR treatment when nuclear and mitochondrial DNA synthesis become limited by thymidine deoxynucleotide levels due to thymidylate synthase inhibition by FUdR. To determine if purine levels may be limiting lifespan, we supplemented hypoxanthine to the culture medium and monitored the lifespan. As shown in Fig. S2A, 10 mM hypoxanthine supplementation increased mean lifespan by 5%.

Pyrimidine metabolite levels were also largely decreased with age, however the nitrogenous base cytosine – the sixth most decreased metabolite overall – was not associated with pyrimidine metabolism using our method since *C. elegans* apparently lacks a cytidine phosphorylase enzyme to salvage cytosine bases, so no enzymes are directly associated with cytosine in the KEGG database. If cytosine had been associated with pyrimidine metabolism for calculations of total $\log_2$ -fold change calculations, it would have been the fourth most

decreased pathway and the fourth most changed pathway overall. To determine if pyrimidine levels may be limiting lifespan, we supplemented cytidine (the ribonucleoside of cytosine) to the culture medium. As shown in Fig. S2B, 10 mM cytidine increased mean lifespan by 11%.

The nitrogenous bases showed a distinct age-related decrease in abundance (Fig. 4B). Adenine, guanine, and cytosine as well as the nucleoside uridine were each decreased in the older *C. elegans*. Uracil and thymine barely failed to achieve significant *p*-values when evaluating young and old metabolite levels (*p*-values of 0.06 and 0.10, respectively), so they were scored as unchanged during analysis, but they also appeared to decrease with age (log₂-fold changes of -0.843 and -3.18, respectively). The mechanism through which the levels of the nitrogenous bases decreased with age remains unclear, but possibilities include 1) decreased de novo synthesis due to decreased S-adenosyl methionine (SAM) levels, 2) decreased ingestion due to the sarcopenic pharyngeal pump, 3) increased activity of nucleotide synthesis by the nucleotide salvage pathway draining nitrogenous base pools, and 4) a decreased rate of RNA and nucleotide degradation, or some combination of these mechanisms. But since supplementing back a purine or a pyrimidine to the culture medium extended mean lifespan, it is possible that the decreased purine and pyrimidine levels that occur with aging may limit lifespan. However, these results should be interpreted with caution as individual supplementation with many diverse cellular metabolites can extend lifespan (Edwards et al. 2015), and this may occur through augmentation of energy generating pathways through their catabolism.

3.4 S-Adenosyl Methionine and Altered Lipid Content

Four metabolites were identified as part of cysteine and methionine metabolism and all of these decreased in abundance with age. Of these, three were found to be directly associated with the SAM cycle; specifically, L-methionine and L-homocysteine, and the downstream product 5-methylthioadenosine. SAM is the universal methyl donor in eukaryotic cells, and it is capable of acting as a cofactor in the transfer of methyl groups to DNA, RNA, proteins, and lipids (Chiang et al. 1996). SAM is an important part of phospholipid metabolism, and is used during the synthesis of phosphatidylcholine (PC), which is a major component of cell membranes (Kent 1995). In bacteria, fungi, and mammals, PC is synthesized from choline by the *de novo* choline pathway, also known as the Kennedy pathway (Carman and Henry 1989; Lykidis and Jackowski 2000; Sohlenkamp et al. 2003). Yeast and mammalian liver cells use the CDP-diacylglycerol pathway, which begins with the conversion of phosphatidylserine into phosphatidylethanolamine (PE), and uses SAM to sequentially methylate the molecule into PC (Kanipes and Henry 1997; Vance et al. 1997). *Plasmodium falciparum* (one of the species of *Plasmodium* that cause malaria) and *C. elegans* have been shown to utilize an alternative method of PC synthesis, known as the phosphomethylethanolamine N-methyltransferase (PEAMT) pathway, which begins with the conversion of serine to phosphoethanolamine (Brendza et al. 2007; Palavalli et al. 2006; Pessi et al. 2004). *C. elegans* then use two phosphomethylethanolamine N-methyltransferase enzymes, PMT-1 and PMT-2, to sequentially methylate phosphoethanolamine into phosphocholine, which then enters the Kennedy pathway to yield PC.

Lipid droplets (LDs) have been identified within cells of a wide range of species, including humans. In general, LDs consist of a hydrophobic core composed of cholesterol esters and triacylglycerols, surrounded by a phospholipid monolayer that is primarily made of PC and PE (Bartz et al. 2007). The LD PC phospholipid monolayer appears to act as a surfactant that prevents LDs from coalescing, and the knockdown of genes involved in PC synthesis has been shown to produce larger LDs (Krahmer et al. 2011). Knockdown of either *C. elegans pmt-1* or S-adenosyl methionine synthetase (*sams-1*), which uses ATP and L-methionine to produce SAM, leads to decreased production of PC and increased LD size (Ehmke et al. 2014; Li et al. 2011). Furthermore, the resulting enlargement of *C. elegans* LDs was found to be associated with impaired reduction of LD size during starvation, presumably due to hindered accessibility of triacylglyceride-hydrolyzing lipases given the changed surface-to-volume ratio of the droplets (Ehmke et al. 2014). Large LDs have been shown to accumulate in *C. elegans* as they age (Herndon et al. 2002), and their ectopic accumulation has been theorized to contribute to the age-related dysfunction in the nematode (Ackerman and Gems 2012).

We found a decrease in monoacylglycerols with age and a concurrent increase in free fatty acids in older nematodes (Fig. 5 and Table S3). We also found an increased level of glycerol in the older nematodes ($\log_2$ -fold change = 1.948; p -value = 0.017) and a dramatic increase in the level of the ketone body β -hydroxybutyrate ($\log_2$ -fold change = 7.370; p -value = 0.003), which can form from excess acetyl-CoA under conditions of either fatty acid breakdown (β -oxidation) or fatty acid synthesis. Interestingly, citrate was also increased in abundance in older nematodes ($\log_2$ -fold change = 1.973; p -value = 0.0003). Mitochondrially derived citrate is exported into the cytoplasm and converted into acetyl-CoA by the enzyme ATP citrate lyase (Watson et al. 1969), which can then either be used for fatty acid synthesis or ketone body production. The increased levels of free fatty acids in the aged nematodes could be partially due to a decreased rate of fatty acid β -oxidation resulting from mitochondrial dysfunction, in addition to any increased rate of hydrolysis from monoacylglycerols. With this in mind, *C. elegans* contains two group VIA phospholipase A2 homologs IPLA-2 (F47A4.5) and IPLA-3 (W07A8.2) and a phospholipase B1 homolog (F13H8.11) that hydrolyze fatty acids from phospholipids that were shown to increase in abundance with aging in a previous report (Walther et al. 2015). These authors also showed that the lysosomal lipase LIPL-1 increased in abundance with age, while the intestinal lipase LIPL-5 increased in abundance from day 6 to day 12, all of which may contribute to the increased levels of free fatty acids in the aged nematodes.

3.5 D-sorbitol Increases in Aged *C. elegans*

Strikingly, fructose and mannose metabolism was identified as one of the most changed pathways with age solely due to an increase in a single age-related metabolite – sorbitol ($\log_2$ -fold change = 8.316; p -value = 0.001). Sorbitol is produced from glucose by aldose reductase (AR) in response to elevated glucose levels (Gabbay 1973; Lorenzi 2007; Oates 2002), and both increased sorbitol levels and increased AR activity have been associated with diabetes in multiple mammalian species and tissues (Asnaghi et al. 2003; Dagher et al. 2004; Szwergold et al. 1990). Sorbitol is strongly hydrophilic, does not readily cross cell membranes, and under conditions of high glucose availability, such as diabetes, it may

possibly accumulate to an adequate concentration to cause osmotic stress (Gabbay 1973). AR also uses reduced nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor, and increased metabolic flux through AR has been identified as a possible source of lowered cellular levels of NADPH (Barnett et al. 1986; Lorenzi 2007). Additionally, sorbitol is converted to fructose by sorbitol dehydrogenase, which uses nicotinamide adenine dinucleotide (NAD⁺) as a cofactor, and excess metabolic flux through sorbitol dehydrogenase has been associated with lowering the NAD⁺ level (Lou et al. 1988; Williamson et al. 1993). Taken together, the activities of AR and sorbitol dehydrogenase (collectively known as the polyol pathway) may affect intracellular redox state through changes in NAD(P)⁺/NAD(P)H ratios. Given that cellular redox state is a factor in protecting against oxidative stress, some evidence links the diabetic flux of glucose through the polyol pathway with diminished levels of the reduced antioxidants glutathione and ascorbate (Cameron et al. 2001; Lee and Chung 1999; Obrosova et al. 2005; Obrosova et al. 2002), increased lipid peroxidation, nitrosative stress (Lee and Chung 1999; Obrosova et al. 2005), and DNA damage as identified through the activation of poly(ADP-ribose) polymerase (Obrosova et al. 2005). Other sugars upregulated with age included glucose, galactose, and sucrose as well as glucose-6-phosphate, suggesting that glycolysis could be impaired in the older worms. To validate the observed increase in D-sorbitol levels, and to investigate whether the effect was strain or condition specific, we chose to assay D-sorbitol levels using the same conditions as stated above for the assay of hypoxanthine (wild-type N2 *C. elegans* grown at 20 °C). Under these conditions, we found a significant ~360% increase in D-sorbitol levels from day 5 to day 12 (Fig. 6A).

We were able to use some of the identified metabolite levels to estimate a general age-related change in redox state. Ascorbic acid (vitamin C) is capable of behaving as an antioxidant in a cellular environment (Martin and Frei 1997; Michels and Frei 2013; Smith et al. 2002). In its fully oxidized form as dehydroascorbic acid (DHA), it can be reduced by glutathione back to ascorbate, thus making the ratio of dehydroascorbate to ascorbate an indicator of the available glutathione pool and the general cellular redox state (Meister 1994). Fig. 6B shows a shift from ascorbate to DHA in older *C. elegans*, with the ratio of DHA/ascorbate increasing 7.65 fold ($\log_2$ -fold change = 2.935; p -value = 0.0243). Furthermore, both erythronic acid (EA) and N-acetylglucosamine (NAG) were identified among the metabolites of young and old *C. elegans*. NAG is a peptidoglycan monomer and a component of chitin and hyaluronic acid. When oxidized, NAG has been found to degrade into EA (Jahn et al. 1999). Fig. 6B shows an increase in the ratio of EA to NAG in older nematodes. The EA/NAG ratio showed a dramatic ~17-fold change from day 4 to day 10 ($\log_2$ -fold change = 4.122; p -value < 0.0001). This data agrees well with previous data generated using fluorescent protein redox sensors that indicated an increased cytoplasmic oxidation state during aging in *C. elegans* (Kirstein et al. 2015).

3.6 Altered Ascorbate Metabolism and Redox Imbalance with Age

The metabolite in our dataset that decreased in abundance to the greatest extent with age was galactonic acid-1,4-lactone, which in plants is the direct precursor of ascorbate in the ascorbate biosynthesis pathway. However, it is unknown if *C. elegans* can convert galactonic acid-1,4-lactone into ascorbate as bioinformatic analysis did not detect an L-galactonic

acid-1,4-lactone dehydrogenase in the *C. elegans* genome (Patananan et al. 2015), but invertebrates may have evolved a unique, as of yet unidentified enzyme to catalyze this reaction. In support of this hypothesis *C. elegans* has been shown to synthesize both ascorbate and an unidentified hydrogenated lactone precursor to ascorbate, but the ascorbate synthesis pathway appears to be different from the pathways present in animals, plants, or protists, while ascorbate is not synthesized by *E. coli* (Patananan et al. 2015). In *C. elegans* ascorbate levels were highest in eggs, levels declined throughout larval growth, and were lowest in mixed age adults. In addition ascorbate levels were higher when worms were grown in liquid media compared to when they were cultured on agar plates, but ascorbate levels did not increase when worms were exposed to the free radical generator paraquat (Patananan et al. 2015). We predict that the decreased level of galactonic acid-1,4-lactone with age is partially due to its conversion into ascorbate, as we measured ascorbate and DHA levels to increase during aging. However, measurement of galactonic acid-1,4-lactone dehydrogenase activity in *C. elegans* extracts is needed to confirm this hypothesis.

Neither NAD^+ nor NADH were identified in our metabolomics screen, but we were able to use the ratio of pyruvate to lactate as an estimator of the ratio of NAD^+ to NADH, as the cellular levels of these metabolites are held in equilibrium by the activity of lactate dehydrogenase (Williamson et al. 1967). Fig. 6B shows that the pyruvate/lactate ratio as an estimator of the NAD^+/NADH ratio decreased approximately 69% from day 4 to day 10 ($\log_2$ -fold change = -1.672 ; p -value < 0.0001). To confirm this prediction, we directly measured NAD^+ and NADH levels in young and aged N2 worms using a commercially available kit (Fig. 6C). We measured a 3-fold decrease in NAD^+ levels and a 20% increase in NADH levels in the aged worms yielding over a 3-fold decrease in the NAD^+/NADH ratio with age (measured $\log_2$ -fold change = -1.90) (Fig. 6D), which matched fairly well with the estimated $\log_2$ -fold change in ratio (of -1.67) based on pyruvate and lactate levels. There was a substantial loss of total NAD^+ plus NADH levels with age that could either be a result of decreased pyridine nucleotide synthesis or increased pyridine nucleotide degradation with age. The decrease in the NAD^+/NADH ratio with age could be partly driven by the aging-related decline in mitochondrial electron transport chain complex I activity (Yasuda et al. 2006). A similar age-related decrease in the NAD^+/NADH ratio has been observed in heart, lung, liver and kidney from aging Wistar rats (Braidy et al. 2011), and the NAD^+ level was found to be negatively correlated with age in human brain (Zhu et al. 2015) and pelvic skin samples (Massudi et al. 2012). The decreased NAD^+/NADH could slow glycolysis, mitochondrial fatty acid beta-oxidation, and citric acid cycle function possibly explaining some of the metabolite level changes observed with age. Reductive stress could increase in specific organelles with age and contribute to aging phenotypes as the redox status of the ER lumen in *C. elegans* has recently been shown to become more reduced with age (Kirstein et al. 2015). It is possible that aging-induced redox changes in the mitochondrial matrix and ER lumen are partly responsible for the reduction of the NAD^+/NADH redox pair with age, although each of these organelles only takes up roughly 10% of the volume of a typical cell. We attempted to estimate the relative cellular ratios of $\text{NADP}^+/\text{NADPH}$ using identified levels of pyruvate and malate, respectively, based on the activity of NADP-malic enzyme (Veech et al. 1969). The resulting day 4 and day 10 ratios showed a decline, but the result lacked statistical significance (p -value = 0.1417).

3.7 Proteomic Investigation

To supplement our metabolomic analysis of young vs. aged *C. elegans*, we performed a global mass spectroscopy-based proteomic investigation under similar conditions (day 4 and day 10 liquid cultures of *glp-4(bn2)* *C. elegans* grown at 25 °C). To increase our capability for quantitation, we chose to follow the general ^{15}N - ^{13}C stable isotope labeling strategy outlined by Larance *et al.* (Larance et al. 2011). In short, we grew a lysine- and arginine-auxotrophic strain of *E. coli* (SLE1 HT115) in minimal media supplemented with $^{15}\text{N}_4$ - $^{13}\text{C}_6$ -arginine (heavy arginine) and $^{15}\text{N}_2$ - $^{13}\text{C}_6$ -lysine (heavy lysine). Three successive generations of *glp-4(bn2)* *C. elegans* were fed the heavy labeled bacteria, in order to ensure complete incorporation of the heavy isotopes into the nematodes. A potential complication with labeling using this approach is that *C. elegans* possesses a metabolic pathway including *orn-1* (ornithine transaminase 1) together with two other enzymes capable of converting arginine into proline. To prevent the accumulation of heavy-labeled proline from confounding the subsequent analysis, we fed *C. elegans* SLE1 *E. coli* expressing dsRNA targeting *orn-1*. Samples for mass spectroscopy were taken from the fourth generation of heavy isotope-labeled *glp-4(bn2)* *C. elegans*, grown to day 4 (young sample) in liquid culture at 25 °C. The older sample was taken from a similar RNAi-treated culture grown to day 10, but lacking the heavy isotopes. We calculated a heavy isotope labeling efficiency of 94.7%. A sample spectrum is shown in Fig. S3. Following high performance liquid chromatography (HPLC)-mass spectrometry of the samples and data analysis a total of 1,937 proteins were identified as present in both age groups (Table S4). After centering the median of the old/young $\log_2$ -fold change, a total of 54 proteins were determined to be significantly increased or decreased in abundance in the aged sample (Fig. 7 and Table 1). To help identify relevant connections to the metabolomics data set, we used the STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) online database (<http://www.string-db.org/>) to match significant proteins to KEGG database metabolites (Franceschini et al. 2013). We then calculated the total observed change for each pathway as the sum of the absolute $\log_2$ -fold change of each identified protein within that pathway (Fig. 8 and Table S5).

3.8 Proteins Showing the Largest Changes in Abundance with Age

The proteins showing the largest decline in abundance with age as shown in Table 1 were the circadian clock-regulating Timeless homolog and regulator of chromatid cohesion TIM-1 (Chan et al. 2003), the protein methyltransferase PRMT-3 (Takahashi et al. 2011), the glutathione-s-transferase GST-17, a mitochondrial prion-like glutamine/asparagine (Q/N)-rich domain-containing protein PQN-24, the poly(ADP-ribose) polymerase PME-1 (Gagnon et al. 2002), a CRAL/TRIO domain containing protein similar to retinal-binding proteins CTG-2, and a serine-arginine-rich splicing factor RSP-6. The proteins showing the largest increase in abundance with age were the kelch domain-containing protein KEL-3, the BTB/Kelch-associated domain containing protein C27D8.2, the essential sole Ras-like GTP-binding protein rhoA homolog in *C. elegans* RHO-1 (McMullan and Nurrish 2011), an uncharacterized protein F15D3.5, the ubiquitin conjugating enzyme UBC-20 (Jones et al. 2002), the glutathione-s-transferase GST-36, and a protein with homology to calmodulin CAL-4.

3.9 Changes Associated with Histone Methylation and Acetylation with Age

In agreement with the observed changes in methionine cycle metabolites, the level of SAMS-1, which catalyzes the synthesis of SAM from ATP and L-methionine, was found to be decreased in aged nematodes ($\log_2$ -fold change = -1.837 ; p -value = 1.8×10^{-5}). Since the levels of both substrates for SAMS-1, L-methionine and ATP also decline dramatically with age, it is likely that SAM levels decrease with age, although we did not detect SAM in our metabolomics analysis. In addition, the level of PRMT-3 (protein arginine methyltransferase 3) was dramatically decreased with age ($\log_2$ -fold change = -7.636 ; p -value = 1.8×10^{-20}) in contrast to a recent proteomics study where this protein was reported to increase with age (Walther et al. 2015). This protein possesses SAM-specific monomethyltransferase activity and is capable of transferring a methyl group from SAM onto arginine residues present in histone proteins (Takahashi et al. 2011). Evidence indicates that *C. elegans* lack methylated cytosine in DNA (Simpson et al. 1986), suggesting that methylation events primarily modulate gene expression either through adenine methylation (Greer et al. 2015) or histone modification.

Histone deacetylases are enzymes that remove acetyl groups from lysine residues within core histones. *C. elegans* histone deacetylase-1 (HDA-1), which is orthologous to human HDAC-1, affects gene expression during larval development in many tissues including neurons, muscles, hypodermis, intestines, and the extracellular matrix. Among its many genetic targets, HDA-1 appears to be a strong repressor of the cysteine protease inhibitor CPI-1 expression (homologous to human cystatin D), and RNAi knockdown of HDA-1 led to an 8.5-fold increase in CPI-1 levels (Whetstone et al. 2005). We found a ~6.4-fold (2.67 $\log_2$ -fold) increase in CPI-1 levels with age, along with a similar increase in several genes known to have a high confidence of co-expression with CPI-1 (correlation > 0.7 ; STRING database), including C53B7.2, Y62H9A.6, CPG-1, and TTR-51 supporting the hypothesis that HDA-1 activity may decline with age. A recent proteomics study indicated that HDA-1 protein levels increased with age (Walther et al. 2015), but the activity was not measured. Interestingly, a bacterial ortholog of TTR-51 was shown to play a role in the degradation of uric acid to allantoin as a part of purine catabolism (Eneqvist et al. 2003). Furthermore, we recently found β -hydroxybutyrate (β HB) to inhibit class I histone deacetylases to extend lifespan in *C. elegans*, and it is possible that the endogenous increase of β HB observed in older nematodes inhibits class I histone deacetylases to induce gene expression (Edwards et al. 2014), although the level of γ -hydroxybutyrate, another endogenous histone deacetylase inhibitor (Klein et al. 2009), declined with age.

3.10 Poly(ADP-ribose) Polymerase Levels Decreased with Age

We found that the *C. elegans* poly(ADP-ribose) polymerase PME-1 decreased in abundance with age ($\log_2$ -fold change = -5.991 ; p -value = 2.9×10^{-13}) (Table 1). This contrasts with a proteomics study reporting an increase in PME-1 with age (Walther et al. 2015). *C. elegans* PME-1 is orthologous to human PARP1, which functions to detect DNA single-strand breaks and signals to proteins responsible for single-strand break repair (Dequen et al. 2005; Hegedüs and Virág 2014). As part of this process, PME-1/PARP-1 uses NAD^+ to attach ADP-ribose monomers to target proteins, forming chains of poly(ADP-ribose). Under conditions of genotoxic stress, PME-1/PARP-1 can significantly reduce cellular NAD^+

reserves (Dequen et al. 2005). Deletion of PARP-1 in mice has been shown to elevate NAD⁺ levels of brown adipose tissue and muscle leading to aerobic respiration and increased SIRT1 activity (Bai et al. 2011).

3.11 Decreased Levels of Enzymes Involved in Fatty Acid Synthesis and Breakdown with Age

Three key enzymes involved in fatty acid metabolism showed decreased abundance in aged *C. elegans*, cytosolic NADP⁺-dependent isocitrate dehydrogenase IDH-1, a mitochondrial acyl-CoA dehydrogenase ACDH-13, and a putative ER carboxylesterase C23H4.3 (Table 1). IDH-1 is homologous to human IDH1 and functions in the cytoplasm to convert isocitrate into α -ketoglutarate, reducing an NADP⁺ cofactor to NADPH in the process. We found decreased abundance of IDH-1 in older *C. elegans* (log₂-fold change = -2.349; *p*-value = 6.8×10^{-8}) consistent with a previous report (Walther et al. 2015). This may account for the increased citrate level found in the aged worms. Cytosolic aconitase is a regulator of fatty acid synthesis and is capable of converting citrate into isocitrate, which would otherwise be available for entering the fatty acid synthesis pathway through the activity of ATP citrate lyase (Zaidi et al. 2012).

C. elegans ACDH-13 (acyl CoA dehydrogenase 13) is one member of a family of long-chain-acyl-CoA dehydrogenase enzymes identified to function in the first steps of mitochondrial fatty acid β -oxidation. These enzymes act to form a trans double-bond in fatty acids, reducing the cofactor flavin adenine dinucleotide (FAD⁺) in the process. Our observed age-related decrease in ACDH-13 abundance (log₂-fold change = -4.318; *p*-value = 1.75×10^{-5}) may play a role in the increased levels of free fatty acids found in aged *C. elegans*. Interestingly, ACDH-13 is homologous to human acyl-CoA dehydrogenase 9 (ACAD9; BLAST *e*-value = $8.9e^{-28}$; % length = 69.7%). ACAD9 has been determined to be necessary for proper assembly of the mitochondrial electron transport chain complex I, although no such role has yet been investigated or identified in *C. elegans* (Nouws et al. 2010).

C. elegans C23H4.3 encodes an ortholog of human carboxylesterase-2, which is known to play an important role in xenobiotic metabolism, such as catalyzing the breakdown of amide and ester containing drugs. However, carboxylesterase-2 also plays an important role in lipid metabolism by hydrolyzing long-chain fatty acid esters and thioesters. C23H4.3 levels decreased with age (log₂-fold change = -4.487; *p*-value = 1.37×10^{-5}), so this enzyme does not likely play a role in the aging-induced increase in free fatty acids levels.

With aging, we also found decreased abundance of the RME-2 protein that functions as a yolk receptor on oocytes (Grant and Hirsh 1999). A previous proteomics study found increased abundance of this protein early in life followed by a slight decrease late in adulthood (Walther et al. 2015). It was unexpected to find this protein present in the *glp-4(bn2)* strain that contains greatly decreased numbers of oocytes. Therefore, we speculate that there might be a compensatory expression of RME-2 in other tissues in the *glp-4(bn2)* strain to take up excess yolk in the absence of egg production. Yolk is present in *C. elegans* as vitellogenin lipoprotein particles that carry high levels of cholesterol and other lipids (Ackerman and Gems 2012). Yolk is produced in the intestine and must be carried across the body cavity to the gonad, where it binds and provides nourishment for developing

oocytes (Kimble and Sharrock 1983). Following the reproductive period, extracellular yolk levels increase and become distributed throughout the body (Ackerman and Gems 2012), releasing fatty acids and other lipids that may damage tissues contributing to aging-induced physiological dysfunction. A decreased abundance of RME-2 with age would facilitate the non-specific distribution of lipid throughout the body resulting in lipotoxicity. Another surprising finding in our dataset was an aging-related increase in the PERM-2 eggshell-associated protein in the oocyte-deficient *glp-4(bn2)* strain.

3.12 Evidence for Muscle Dysfunction and Altered Ca^{2+} Homeostasis with Aging

Five distinct proteins known to be involved in muscle function were present at a lower abundance in aged nematodes in our analysis; SCA-1 (sarco-endoplasmic reticulum calcium ATPase 1, or SERCA), NRA-2 (nicotinic receptor associated protein 2), MLP-1 (muscle LIM protein 1), MLC-2 (myosin regulatory light chain 2), and AIPL-1 (actin interacting protein 1-like protein 1) (Table 1). The age-related changes in abundance we found for SCA-1, NRA-2, and MLC-2 are consistent with a previous report, while those we found for MLP-1 and AIPL-1 differ (Walther et al. 2015). The *C. elegans* protein SCA-1 is orthologous to the human SERCA protein ATP2A1, which utilizes the hydrolysis of ATP to pump cytosolic Ca^{2+} into the lumen of the sarcoplasmic reticulum in cardiac and slow twitch skeletal muscle (Prasad et al. 2004; Shaye and Greenwald 2011). In *C. elegans*, SCA-1 is expressed in all major muscle types, including body wall, pharyngeal, and uterine muscle (Zwaal et al. 2001). Reduction of SERCA function is associated with muscle dysfunction, and SERCA2 heterozygous mutant mice display deficits of muscle relaxation as a consequence of reduced rates of sarcoplasmic Ca^{2+} sequestration (Periasamy et al. 1999). Another protein involved in Ca^{2+} signaling, CAL-4, one of five *C. elegans* calmodulin homologs, was increased in abundance with age. This may occur in response to increased cytoplasmic Ca^{2+} levels resulting from lower SCA-1 activity.

The *C. elegans* protein NRA-2 (ortholog of human nicalin) is a transmembrane endoplasmic reticulum protein that functions as a molecular chaperone (Almedom et al. 2009). It contains an EF-hand motif, so it is likely regulated by changes in Ca^{2+} levels. Expression of NRA-2 occurs largely in neurons, body wall muscle, and pharyngeal muscle, where it helps regulate acetylcholine receptor subunit composition, and RNAi knockdown of NRA-2 expression has been shown to sensitize *C. elegans* touch receptor neurons to Ca^{2+} -mediated necrotic cell death (Kamat et al. 2014). The next protein decreased in abundance MLP-1 is a LIM domain-containing cysteine-rich protein (CRP) expressed in a wide range of cell types in both larva and adults (including intestine, spermatheca, gonad sheath, hypodermis, body wall muscle, pharynx, and neurons) (McKay et al. 2003; Meissner et al. 2011).

MLC-2 is expressed in body wall, vulval, and pharyngeal muscles and together with MLC-1 and MLC-4 make up the three *C. elegans* regulatory myosin light chains. These proteins function as regulators of myosin ATPase activity (Rushforth et al. 1998). In the pharyngeal muscles, MLC-2 is essential for proper muscle function, with 90% of MLC-2 mutants arresting at the L1 larval stage due to pharyngeal defects. Therefore the decreased levels of MLC-2 that occur with age may contribute to pharyngeal sarcopenia. AIPL-1 is a WD40 repeat-containing protein homologous to UNC-78 and functions with UNC-60B/ADF/cofilin

to regulate the ordered assembly of actin in muscle myofibrils necessary for proper larval development, but its expression was reported to diminish in adulthood (Ono et al. 2011) as we have confirmed here by measuring protein level changes.

3.13 Changes in RNA Metabolism and Translation with Age

From the proteomics data the pathway with the largest total observed changes with age was RNA degradation (Fig. 8 and Table S5), which is the result of the cumulative observed lower abundance of PAB-2 (poly(A) binding protein 2) and XRN-2 (5'-3' exoribonuclease 2 homolog)(Table 1). The abundance change we found for PAB-2 is consistent with a previous report, while the change we observed for XRN-2 differs (Walther et al. 2015). Most eukaryotic mRNAs of nuclear origin, including microRNAs, possess poly(A) tails, which consist of a series of adenosine nucleotides up to ~300 bases in length. Poly(A) binding proteins, such as PAB-2, bind along the length of this polyadenylated mRNA region and serve to regulate translation through both their interaction with the 5'-cap eukaryotic initiation factor complex eIF4F and their recruitment of the 40S and 60S ribosomal subunits (Kahvejian et al. 2005). Furthermore, depletion of the human poly(A) binding protein PABP decreases mRNA translation in vitro, suggesting that the decreased abundance of PAB-2 with age may decrease protein synthesis, perhaps in response to the reduced capacity of the protein-folding chaperone systems in aged worms.

The exonuclease XRN-2 is involved in transcription termination, where it is capable of dislodging RNA polymerase II from DNA following its binding to 5'-uncapped RNA and degrading the RNA until it reaches the polymerase (West et al. 2004). Furthermore, XRN-2 also degrades mature microRNAs regulating their cellular levels, and the activity of XRN-2 is necessary for proper larval molting and reproduction (Ackerman and Gems 2012; Cui et al. 2008; Kawano et al. 2000). Expression of *xrn-2* is ubiquitous and continues past the larval stages into adulthood. The decreased levels of XRN-2 in aged nematodes may prevent efficient transcription termination and/or RNA degradation resulting in decreased levels of nucleotides and nucleotide degradation products such as nitrogenous bases, possibly explaining the depleted nitrogenous base levels measured in the metabolome analysis.

The proteins C24H12.4 and ZK512.2 (ATP-dependent DEAD-box RNA helicase DDX56 and DDX55 homologs, respectively) and RSP-6 (splicing factor, arginine/serine-rich protein 6) were identified as being present at a significantly lower abundance with age (Table 1). The age-related changes in C24H12.4 and ZK512.2 abundance are consistent with a previous report (Walther et al. 2015). *C. elegans* RSP-6 is a splicing factor that contributes to nuclear premRNA processing (Cui et al. 2008; Kawano et al. 2000; Longman et al. 2000). However, not much is known regarding the functions of C24H12.4 and ZK512.2, but if their predicted functions as ATP-dependent RNA helicases hold, they may play roles in pre-mRNA splicing or the initiation of translation. The *C. elegans* 60S ribosomal subunit L11 (RPL-11.2) and the 40S ribosomal subunit (RPS-26) as well as DPH-2 (diphthamide biosynthesis protein 2) showed decreased abundance with age. The age-related abundance changes for RPL-11.2 and RPS-26 are consistent with a previous report, while the result with DPH-2 differs (Walther et al. 2015). Diphthamide is a non-standard amino acid found exclusively in translation elongation factor 2 (eEF2). The conversion of L-histidine to

diphthamide, in which DPH-2 participates, requires four SAM molecules and the transfer of three methyl groups, thus representing a direct link between protein translation and SAM (Liu et al. 2004).

The proteomics data also suggest that translation of mitochondrial DNA-encoded components of oxidative phosphorylation may be compromised in aged nematodes because there was a decreased abundance of PUS-1, pseudouridine synthase (Table 1). This is consistent with a previous report (Walther et al. 2015). PUS-1 is responsible for post-transcriptional modifications of mitochondrial and cytoplasmic tRNA essential for translation. Humans with PUS-1 mutations have mitochondrial myopathy and sideroblastic anemia (MLASA) (Patton et al. 2005).

3.14 Aging Decreased the Abundance of RPN-3, a Regulator of Proteasome Function

We found the RPN-3 protein to be present at lower levels in aged nematodes (Table 1). This contrasts with a previous study where slightly increased RPN-3 levels with age were reported (Walther et al. 2015). That study also reported increased levels of many proteasomal subunits and increased proteasomal activity with age. Proper RPN-3 levels are critical for proteasome function as either overexpression or deletion of *rpn-3* disrupted the function of the proteasome (Joshi et al. 2011). Irisflorentin, an isoflavone isolated from the roots of the traditional Chinese herbal, *Belamcanda chinensis*, was shown to specifically upregulate *rpn-3* expression, which directly increased the chymotrypsin-like activity of the proteasome (Chen et al. 2015). The increased proteasome activity led to decreased alpha-synuclein accumulation and decreased toxicity in a nematode model of Parkinson's disease.

3.15 Verification of Select Hits from the Proteomics Data

As a partial verification of the proteomics data, we searched the Caenorhabditis Genetics Center (CGC) strain database and obtained six strains constructed so that expression of GFP is driven by a promoter for a gene, whose protein product was shown to be altered in abundance with age. Of the six strains, three contained promoters for genes whose protein products (KEL-3, CAL-4, and UBC-20) were shown to increase in abundance with aging, while three contained promoters for genes whose protein products (ZK512.2, RPL-11.2, and IDH-1) were shown to decrease in abundance with age. GFP fluorescence was monitored on days 5 and 12 of the lifespan. As shown in Fig. S4, the direction of change in GFP fluorescence mirrored the changes found in protein abundance in four of the six strains tested. There was no change in GFP fluorescence with aging in the strains where GFP expression was driven by the *ubc-20* and *idh-1* promoters. However, the aging-induced protein abundance changes we observed for these two proteins have been confirmed in a previous study (Walther et al. 2015), so these abundance changes likely occur as a result of post-transcriptional mechanisms.

4. Discussion

The metabolomics results indicate that aged *C. elegans* contain decreased levels of specific purine and pyrimidine metabolites, decreased levels of free hydrophobic amino acids, altered S-adenosyl methionine metabolism, increased sorbitol content, increased free fatty acid

levels, and a shift in cellular redox balance. Proteomic analysis of *C. elegans* showed decreased abundance of muscle proteins including SCA-1, the *C. elegans* SERCA homolog, suggesting muscle dysfunction. There was also decreased abundance of proteins involved in RNA stability and protein synthesis, as well as those involved in the regulation of fatty acid synthesis and breakdown. These two data sets yield important clues regarding the molecular mechanisms of aging-induced physiological dysfunction.

Several groups have investigated *C. elegans* metabolite and protein abundance changes with age. Liang *et al.* performed a mass spectrometry-based proteomic analysis with isotopically labeled protein samples using the iTRAQ (Isobaric Tags for Relative and Absolute Quantitation) technique to study young, middle-aged, and aged N2 *C. elegans* grown at 20 °C (approximately days 3.5, 9, and 14 counting from the first day of culture) (Liang et al. 2014). For a comparison, the mean lifespan of *C. elegans* grown at 25 °C, as was done in this report, is approximately 9-10 days, whereas the mean wild-type lifespan at 20 °C lies somewhere between 14 and 21 days with a large degree of variability. Still, there was a large degree of overlap in the altered pathways identified when comparing our results with those using the iTRAQ approach. Liang *et al.* identified a decrease in ribosomal proteins, poly(A) binding protein (PAB-1), myosin-related proteins, and fatty acid synthesis and degradation associated proteins (fatty acid CoA synthetase 2 and acyl CoA dehydrogenase 1) in aged nematodes. Furthermore, Pontoizeau *et al.* performed an NMR-based metabolomic study of *C. elegans* subjected to dietary restriction (DR) (a consistent method for extending lifespan in model organisms) and found that the phosphocholine level was significantly lowered by the DR condition (Pontoizeau et al. 2014). High phosphocholine levels were also found to be associated with shorter *C. elegans* lifespan. Similarly, high vitamin B12 (cobalamin) and folate levels have been found to shorten *C. elegans* lifespan (Virk et al. 2012; Watson et al. 2014). Notably, both dietary vitamin B12 and folate are required for the synthesis of tetrahydrofolate, which is a necessary part of the metabolic cycle that produces SAM.

Another group performed a proteomic and metabolic quantification analysis of a long-lived *eat-2* DR model (Yuan et al. 2012). The *eat-2* worms shifted their metabolism from largely glucose and long-chain fatty acid oxidation to short chain fatty acid oxidation. To accomplish this they upregulated the mitochondrial beta-oxidation enzyme ACDH-1 and downregulated the glycolytic enolase enzyme ENOL-1 and carnitine palmitoyltransferase CPT-1, which transports medium and long chain fatty acids to mitochondria. They further found that the *eat-2* worms greatly increased their citric acid cycle metabolism by measuring carbon dioxide production. Previous studies had found that *eat-2* worms have higher oxygen consumption than WT worms (Houthoofd et al. 2002), yet are smaller and not more active. Therefore the authors suggest that the *eat-2* worms somehow uncouple ATP synthesis from oxygen consumption. Consistent with this suggestion *eat-2* worms have been shown to generate more heat than WT controls (Houthoofd et al. 2002). This increased respiratory activity with partial uncoupling could increase AMP levels and the NAD/NADH ratio activating cytoprotective and lifespan extending AMP kinase and sirtuin longevity pathways. However others have found that DR induced by bacterial dilution decreased oxygen consumption and increased the proportion of respiration that is coupled to ATP synthesis (Moroz et al. 2014). PNC-1, an essential NAD⁺ salvage pathway enzyme and the sirtuin

SIR-2.1 were both required for lifespan extension highlighting the key role for increased NAD⁺ levels in both DR paradigms.

Most recently, the *C. elegans* N2 proteome was quantified in L4 larvae (day 0), and on days 1, 6, 12, 17, and 22 of adulthood (Walther et al. 2015). Over 5,000 proteins were profiled. Roughly one-third of the proteome were changed in abundance by at least 2-fold during the course of the lifespan. Notable findings include increases in extracellular proteins such as the 6 vitellogenin yolk proteins (that bind the RME-2 yolk receptor) as well as many transthyretin (TTR) proteins. DNA replication and repair proteins were also increased in abundance even though all adult *C. elegans* cells are postmitotic. Proteins that declined in abundance during aging included peroxisomal enzymes, nucleolar ribosome biogenesis factors, proteins involved in lipid glycosylation, and proteins involved in mitochondrial oxidative phosphorylation.

The oxidative phosphorylation proteins that declined with age in that study included subunits from all four electron transport chain (ETC) complexes and the ATP synthase complex as well as cytochrome c (Fig. S5, data taken from (Walther et al. 2015) and shown in Table S6). Interestingly there was a sharp decline in abundance of the vast majority of protein subunits between days 1 and 6, a slight increase in abundance between days 6 and 12 and then a decline again from day 12 until day 22. Several complex IV subunits deviated slightly from this pattern by increasing in abundance between days 1 and 6 in addition to between days 6 and 12 before declining in abundance from day 12 until day 22. Oxygen consumption steadily declines over the first 12 days of the lifespan (Houthoofd et al. 2002) indicating that the increased ETC complex protein abundance from days 6 to 12 does not prevent the overall decrease in ETC function. There were alternative (separate gene) isoforms to several subunits that, in contrast to the rest of the subunits in the complex, increased in protein abundance throughout the lifespan including complex I subunit NDUF-2.2 (alternative to and 93% identical to GAS-1), the complex II subunit SDHA-2 (alternative to and 83% identical to SDHA-1), the complex III subunit UCR-2.3 (alternative to and 39% identical to UCR-2.2), the ATP synthase subunit ASB-1 (alternative to and 71% identical to ASB-2), and the ATP synthase subunit ASG-1 (alternative to and 77% identical to ASG-2). It is of interest to determine how the presence or absence of these alternative subunits affects oxidative phosphorylation complex activity and lifespan. With this in mind, it has been shown that RNAi knockdown of *nduf-2.2* increases lifespan (Kim and Sun 2007), while a mutation in the alternative subunit *gas-1* decreases lifespan (Kayser et al. 2004).

Of the 54 proteins we found to be altered in abundance with age, 24 corroborated data reported in (Walther et al. 2015), with 8 being increased in abundance with age including RHO-1, UBC-20, GST-36, CPI-1, C10C5.1, PERM-2, TTR-51, and Y62H9A.6 and 16 being decreased in abundance with age including CTG-2, C24H12.4, MLC-2, C23H4.3, RAB-3, PUS-1, ZK512.2, C18H9.3, NRA-2, SCA-1, RPL-11.2, PAB-2, R09E10.5, IDH-1, SAMS-1, and RPS-26. We will primarily focus the proteomics portion of the discussion on these corroborated abundance changes. Of the 54 proteins we found with age-related abundance changes, 16 were found to change abundance in the opposite direction as previously found by the study by (Walther et al. 2015). These proteins include TIM-1, PRMT-3, PME-1, DPH-2, Y43F8B.1, RME-2, D1086.1, XRN-2, Y92H12BR.3, ZK1098.11,

AIPL-1, E04F6.4, DNC-2, RPN-3, MLP-1, and CPG-1. It is likely that the direction of change with age in these proteins is dependent upon the particular strain (N2 vs. *glp-4(bn2)*), temperature of growth (20 °C vs. 25 °C), or culture conditions (agar vs. liquid) used in the different studies.

Our study has several limitations. The most significant is likely the use of the *glp-4(bn2)* strain. Although it is commonly used for aging studies, it is known to contain a temperature sensitive mutation in a valyl tRNA synthetase gene *vars-2* (Rastogi 2012). Therefore, defects in translation likely lead to the loss of germ cells at the non-permissive temperature, but translation in somatic cells may also be slightly compromised even though there is no large effect on the rate of aging. These effects on translation could be minimal in the young worms and could grow progressively worse with age. Therefore some of the changes in the aged worms measured in this study could also be in part due to aging in a genetic background with a decreased rate of somatic translation. We chose to use the *glp-4(bn2)* strain because we could not grow up enough wild-type N2 nematodes for the metabolomics study without the use of fluorodeoxyuridine (FUDR) to sterilize the worms. FUDR is known to greatly perturb *C. elegans* metabolism and metabolomics analysis (Davies et al. 2012). In fact FUDR addition caused a much greater change in the metabolome than the *daf-2* mutation that activates the *daf-16* transcriptional regulator and results in large increases in lifespan. In addition FUDR addition can greatly decrease ATP levels starting in middle age (Rooney et al. 2014). Another commonly used temperature-sensitive sterile mutant is *glp-1*. However, *glp-1* mutants are long-lived (Wang et al. 2008) and so are not appropriate for studies of normal aging. Therefore future studies should focus on developing a better temperature-sensitive sterile worm strain for metabolic and metabolomics analysis of aging.

Another limitation brought about by the use of the *glp-4(bn2)* strain is the requisite temperature shift of the eggs from 16 °C to 25 °C to induce sterility. This shift could affect stress resistance. Stress resistance is often directly proportional to longevity (Lithgow et al. 1995). *C. elegans* cultured at a low temperatures between 15 °C to 25 °C live much longer than those grown at the higher temperatures (Klass 1977), so they should also have greater stress resistance. However, the response of worms to temperature shift is complex and several processes that oppose this general relationship can occur depending upon the developmental timing of a shift in temperature. During adulthood, cooler temperatures (16 °C) induce opening of the TRPA-1 ion channel, which induces DAF-16 activation to extend lifespan (Xiao et al. 2013). But during L1-L2 larval development, activation of DAF-16 by TRPA-1 at 16 °C activates a different set of genes to decrease lifespan (Zhang et al. 2015) and likely decrease stress resistance. Under our experimental conditions of switching the temperature from 16 °C to 25 °C at the L1 larval stage for the rest of the lifespan, TRPA-1 channels may only be able to transduce a small lifespan shortening signal to DAF-16 before it is deactivated by the temperature upshift and during adulthood the TRPA-1 channel is closed preventing DAF-16 activation and increased stress resistance. But it has also been shown that the protective HSF-1 heat shock factor not only gets activated by heat shock (30°C and above in *C. elegans*), but also by an upshift in temperature from 15 °C to 25 °C (Sugi et al. 2011). This would likely generate a transient pro-longevity stress resistance signal in the L1 larvae. But overall, the *C. elegans* switched from 16 °C to 25 °C

at an early larval stage would likely have decreased stress resistance and lifespan comparable to worms cultured at 25 °C from conception.

When studying the *glp-4* mutant strain in WT N2 nematodes, there was shown to be a 6-fold increase in mtDNA levels from L4 larvae to adulthood (Tsang and Lemire 2002). This is due to mtDNA replication in oocytes as oocyte-deficient strains do not show this increase. There were 3-4 fold declines in mtDNA levels during this time period in *glp-1* and *glp-4(bn2)* germ cell-deficient strains, but no loss of mtDNA during this time period in the temperature-sensitive *fem-1* or *fem-3* nematode strains that lack either just sperm or just eggs at the non-permissive temperature. *glp-1* and *glp-4(bn2)* show increased activation of DAF-16 (TeKippe and Aballay 2010), which may lead to TOR inhibition and mitophagy, which may decrease mitochondrial number and thus mtDNA levels. It is unknown why *glp-4* mutants are not long-lived when grown on live *E. coli* (Arantes-Oliveira et al. 2002; TeKippe and Aballay 2010) when DAF-16 is activated, but it is likely due to increased bacterial pathogenicity as the *glp-4(bn2)* strain has been shown to be long-lived when fed heat-killed *E. coli* (TeKippe and Aballay 2010). The likely decreased number of mitochondria in the *glp-4(bn2)* mutant strain, as inferred from the measured decrease in mtDNA levels, at the whole nematode level likely leads to decreased electron transport chain function and a decreased rate of fatty acid oxidation, in part, leading to the increased lipid content. This increase in lipid content may induce ER stress, alter lipid droplet dynamics and organelle distribution of PC, and likely plays a major role in the data generated in this report.

In the following paragraphs we will speculate on how the proteomics changes we observed with age may interact with known phenotypes of aging to result in the observed metabolomics changes. Phenotypically, *C. elegans* aging is defined by many factors including sarcopenia (loss of muscle mass and function) (Glenn et al. 2004), the accumulation of ectopic fat as yolk lipoprotein production continues beyond the cessation of egg production (Herndon et al. 2002), and a dramatic collapse of aerobic respiration and ATP content as enlarged dysfunctional mitochondria accumulate within cells (Gerstbrein et al. 2005). In mammals, free fatty acid accumulation outside of adipocytes has been associated with ER stress (Bosma et al. 2014), insulin resistance (Griffin et al. 1999; Shulman 2000), and cardiomyopathy (Chiu et al. 2001; Yagyu et al. 2003). Evidence suggests that the accumulation of fatty acids as triglycerides sequestered within LDs acts as a preventative measure against lipotoxicity (Ackerman and Gems 2012; Bosma et al. 2014; Listenberger et al. 2003). Deficiencies in SAM lead to decreased PC levels and enlarged LDs. These enlarged LDs sequester fatty acids and likely protect against lipotoxicity extending *C. elegans* lifespan. However, decreased PC levels are not a prerequisite for enlarged LDs or an extended lifespan as several long-lived *C. elegans* strains have enlarged LDs with increased PC:phosphatidylethanolamine (PE) ratios (Shi et al. 2013). However, increased PE levels have been linked with longevity as well (Rockenfeller et al. 2015), so the specific subcellular localization of these phospholipids may likely control their effects on lifespan.

We also found an aging-related decline in the E04F6.4 protein, the *C. elegans* homolog of phospholipase D3, an ER membrane protein that hydrolyzes PC into choline and phosphatidic acid. However, this result contrasts with the aging-induced increase in

abundance reported previously (Walther et al. 2015). In addition we found an aging-related decline in abundance in CTG-2, a SEC14 homolog confirming results in the previous report (Walther et al. 2015). CTG-2 likely functions in the transport of phosphatidylinositol and PC between organelles. A meta-analysis of metabolomics data from several long-lived *C. elegans* mutants found alterations in PC and niacin levels as a common theme (Patti et al. 2014). Additionally, PC accounts for about one-third of the phospholipids in the mitochondrial inner membrane. Research is lacking regarding a direct relationship between PC levels and mitochondrial size, but it would be interesting to determine if the same dynamics of surface-to-volume ratio that result in larger LDs when PC is low would also contribute to the accumulation of enlarged mitochondria that might become damaged and resist normal degradation through the autophagosomal/lysosomal pathway to produce high amounts of reactive oxygen species contributing to the aging process.

The connection between aging-induced changes in lipid metabolism and ER stress is also especially pertinent. The ER stress response declines with age, which may contribute to the aging process and the development of aging-related disease (Brown and Naidoo 2012). The ER stress response is normally activated under conditions of increased protein misfolding, but it can also be induced by decreased PC levels in the ER induced by *sams-1* RNAi (Hou et al. 2014). Part of the ER stress response is a down-regulation of protein synthesis, which gradually decreases the total protein content of the ER, increasing the number of free chaperones. Accordingly, the down-regulation in the activity of the translation machinery that is consistently observed in aged *C. elegans* (Syntichaki et al. 2007) may be partially induced by an aging-related decline in SAMS-1 level, which decreases PC levels in the ER, which induces ER stress and the ER stress response pathway to slow the rate of protein synthesis.

The finding by Liang *et al.* of an age-related decrease in the levels of myosin-related proteins, as well as our identification of protein changes associated with decreased muscle function seem to fit well with the observations of sarcopenia in aged *C. elegans*. In addition to body wall muscle, the *C. elegans* pharyngeal pump is greatly affected by sarcopenia. In *C. elegans*, food is consumed through the rhythmic contraction of a set of 20 muscle cells and 20 neuronal cells, termed the pharyngeal pump located between the mouth and the start of the intestine. As *C. elegans* age, the pumping rate gradually slows, and bacteria will often form a plug within the structure, blocking the passage of food to the intestine. The muscle cells of the pharyngeal pump also lose their shape and organization and by late in the *C. elegans* lifespan the loss of structure and function can be rather extreme (Chow et al. 2006). The most common *C. elegans* model of dietary restriction is the *eat-2* mutant, which has slowed pharyngeal pumping resulting in a reduced rate of food intake (Lakowski and Hekimi 1998). Dietary restriction appears to work partially through the inhibition of SAMS-1, which down-regulates the expression of the eIF4H gene *drm-2* (Ching et al. 2010), and RNAi knockdown of SAMS-1 fails to further extend the lifespan of *eat-2* mutants (Hansen et al. 2005). Given that vitamin B12 and folate are both necessary for SAM metabolism and are both obtained by *C. elegans* only through diet, loss of pharyngeal pump function in aged *C. elegans* may result in a late-life dietary restriction phenotype. This sarcopenia-induced dietary restriction in aged worms may contribute to lowering SAM and PC levels, and increasing the size of LDs in intestine, hypodermis, and muscle (Herndon et

al. 2002; Palgunow et al. 2012). LDs are known to increase in size in aged human muscle as well, perhaps in response to increased fatty acid levels from decreased mitochondrial beta-oxidation rates due to aging-induced mitochondrial electron transport chain dysfunction (Crane et al. 2010). It is of interest to determine if similar mechanisms exist to regulate LD size in humans and nematodes.

The age-related decrease in monoacylglycerols and increase in free fatty acids and the ketone body β HB have at least two possible non-exclusive explanations. First, the increase in free fatty acids and β HB may be the byproduct of increased LD-directed autophagy, up-regulated either as a mechanism for dealing with increased LD accumulation, or as an attempt to meet energy demands under conditions of dietary restriction. Or second, an up-regulation of fatty acid synthesis may result in an increased conversion of citrate into acetyl-CoA (a fatty acid precursor), which is converted into β HB when present in excess, and the increased citrate levels observed in older *C. elegans* may be indicative of this. The mitochondrial enzyme aconitase can divert the metabolic flux away from fatty acid synthesis by converting citrate into isocitrate, which is then converted into α -ketoglutarate by isocitrate dehydrogenase. Notably, aconitase is highly susceptible to impairment by reactive oxygen species (ROS) (Chen and Zweier 2014; Walden 2002). Increases in ROS production or decreased antioxidant capacity within mitochondria could lead to an increase in mitochondrial citrate, which would then be exported to the cytoplasm and made available for fatty acid synthesis. Furthermore, the decreased abundance in the cytoplasmic enzyme, IDH-1, that we observed could help to promote the use of cytosolic citrate for fatty acid synthesis, since the alternate conversion of citrate to isocitrate and α -ketoglutarate in the cytoplasm would be restricted. However, fatty acid synthesis relies on NADPH, which may be in short supply when IDH-1 protein levels decline with age. We also previously reported that the dietary supplementation of citrate to *C. elegans* produced ER stress without affecting lifespan (Edwards et al. 2015).

The mechanisms behind our finding of decreased abundance of the poly(ADP-ribose) polymerase PME-1 with aging are unknown. In a previous report PME-1, PME-2, and PME-3 levels were shown to be increased with aging, while PME-5 levels decreased with aging (Walther et al. 2015). In *C. elegans* there are six members of the PME family of poly(ADP-ribose) polymerases. Our proteomic analysis only identified two of these proteins, the second of which (PME-5) was unchanged with age, but interestingly poly(ADP-ribos)ylation capacity in PBMCs declines with age in humans and rodents (Beneke et al. 2010). In addition poly(ADP-ribosyl)ation capacity was shown to be directly proportional to lifespan when comparing 13 species (Grube and Burkle 1992) and was also shown to be very high in human centenarians (Muiras et al. 1998). Therefore much evidence links poly(ADP-ribosyl)ation capacity and lifespan. This data is somewhat surprising given that high poly(ADP-ribose) polymerase levels can deplete cells of NAD^+ , which may limit lifespan. However, it is possible that cells modulate their poly(ADP-ribose) polymerase levels in direct proportion to cellular NAD^+ levels.

The increased level of sorbitol with aging is striking in that its accumulation, along with an increased flux through the polyol pathway, is associated with diabetes in mammals (Asnaghi et al. 2003; Dagher et al. 2004; Gabbay 1973; Szwergold et al. 1990). Sorbitol is likely

formed as a result of increased glucose levels when glycolysis is slowed due to low NAD⁺ levels or possibly due to oxidative damage to glycolytic enzymes such as GAPDH (Samson et al. 2014) or enolase (Butterfield and Lange 2009). The low NAD⁺ levels in aged cells would likely slow the conversion of sorbitol to fructose and re-entry into glycolysis, leading to sorbitol build up. As mentioned previously, ectopic fat and lipotoxicity have been associated with the onset of insulin resistance (Griffin et al. 1999; Shulman 2000), suggesting that the observed alterations in fat metabolism and the accumulation of sorbitol may be linked. However, in some yeasts, fungi, and insects, as well as in mammalian kidney cells, sorbitol has been identified as an osmolyte for adjusting the intracellular environment to osmotic stress (Burg and Ferraris 2008; Garcia-Perez and Burg 1991; Kiewietdejonge et al. 2006; Salvucci et al. 1998). The age-related increase in sorbitol may be a cellular strategy for combating water loss, or simply to balance the dilution of cellular contents that would occur as *C. elegans* increase their cell volume as the animals grow in size.

5. Conclusions

We observed an age-related increase in free fatty acid levels, as well as decreased levels of many purine and pyrimidine metabolites, a decreased abundance of free hydrophobic amino acids, signs of altered SR Ca²⁺ homeostasis and muscle dysfunction, a decreased abundance of SAMS-1 and altered SAM metabolism, many indications of a cellular redox shift including drastically decreased NAD⁺ levels, a decreased abundance of proteins involved in protein synthesis, and a large increase in sorbitol content. We hypothesize that the ectopic accumulation of lipids possibly through the accumulation of vitellogenin yolk proteins that occurs after the cessation of egg-laying contributes to lipotoxicity that may account for some of the changes that were observed with age. We further hypothesize that the age-related reduction in SAM synthesis may be a consequence of dietary restriction caused by age-related pharyngeal pump dysfunction and may act to counter some of the effects of the lipotoxicity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Decreased nitrogenous base levels with age suggest altered nucleotide metabolism
- Age-related increases in fatty acid levels suggest possible lipotoxicity
- Increased sorbitol levels in older *C. elegans* suggest altered glucose metabolism
- Decreased NAD⁺ levels and S-adenosyl methionine metabolism in aged *C. elegans*
- Proteomics data suggest age-related muscle dysfunction and altered Ca²⁺ homeostasis

Fig. 1. Principal component analysis of metabolites shows separation of young and aged samples (A) When graphed as the 2D relationship between the two top explanatory factors ($F1 = 59.92\%$ of the variation; $F2 = 10.20\%$ of the variation), the scaled young day 4 samples ($n = 5$) and the scaled aged day 10 sample ($n = 5$) are separated primarily linearly along the $F1$ axis, showing that the major variation in the distribution of metabolite levels is explained by the difference in ages between the groups. (B) When similarly graphed using the same two factors, the identified metabolites ($n = 186$) visibly congregate at two poles along the $F1$ axis.

Fig 2. Top 10 changed pathways based on metabolome analysis

The total observed change for each identified pathway was calculated as the total of the absolute log₂-fold change for each identified metabolite found within the pathway ($n = 50$ pathways). The top 10 changed pathways are shown in this figure. The width of each pie wedge is equivalent to the percent change of that pathway out of the total absolute log₂-fold change of all pathways. Numbers shown on each pie wedge are the total absolute log₂-fold change for that pathway.

Fig. 3. Age-related changes in free amino acid levels as they correspond to amino acid hydrophobicity

The levels of the more hydrophobic amino acids decreased and the levels of the more hydrophilic amino acids increased on day 10 compared to day 4 (Pearson correlation -0.67 ; p -value = 0.05). The data point for Leu is obscured by the data point for Trp. At pH 7.0 the relative hydrophobicity (on a scale from -100 to $+100$ normalized to glycine) is as follows: Met = 74, Phe = 100, Leu = 97, Trp = 97, Val = 76, Ile = 99, Thr = 13, Gly = 0, Ala = 41.

Fig. 4. Age-related decreases in hypoxanthine and nitrogenous base levels

(A) By measuring hypoxanthine levels of N2 nematodes treated with FUDR using a commercially available kit (Xanthine/Hypoxanthine assay kit, BioVision, Inc.), we found the concentration of hypoxanthine fell from a mean of 100.37 pmol/mg of protein (SEM $\pm$ 3.48) on day 5 (n = 6 samples; ~100 nematodes/sample), to a mean of 70.22 pmol/mg of protein (SEM $\pm$ 2.65) on day 12 (n = 6 samples; ~100 nematodes/sample; unpaired two-tailed t-test p -value = 4.2×10^{-5}). (B) The metabolomics data indicated that nitrogenous base levels decreased with age.

Fig. 5. Age-related changes in monoacylglycerol and fatty acid levels

Monoacylglycerols primarily decreased with age, while fatty acids primarily increased. For brevity, we've only listed fatty acids with a log₂-fold change above 1 or below -1. For a complete list, see Table S3. *16-methylheptadecanoic acid.

Fig. 6. Age-related changes in D-sorbitol content and nematode redox state

(A) D-sorbitol levels in FUDR-treated N2 nematodes increased ~360% from day 5 to day 10 (day 5 mean = 63.28 nmol/mg protein, SEM $\pm$ 8.29, n = 4 samples, ~100 nematodes/sample; day 12 mean = 229.95 nmol/mg protein, SEM $\pm$ 6.60, n = 4 samples, ~100 nematodes/sample; unpaired two-tailed t -test p -value = 4.19×10^{-6}) as measured by a commercial D-sorbitol assay kit (Megazyme, Inc.). (B) Aged/young log₂-fold changes for pyruvate/lactate (log₂-fold change = -1.67; young vs. aged unpaired two-tailed t -test < 0.0001), dehydroascorbic acid/ascorbic acid (DHA/AA; log₂-fold change = 2.94; young vs. aged unpaired two-tailed t -test = 0.024) and erythronic acid (EA)/ N-acetylglucosamine (NAG). (C) Both NAD⁺ and NADH were determined in 5- and 10-day-old wild-type N2 *C. elegans* using a fluorescence-based assay (NAD/NADH Ratio Assay Kit, eEnzyme, LLC) after growth in liquid culture at 20 °C in the presence of FUDR added from the L4 larval stage onward. Fluorescence was normalized to total protein content at each age. NAD⁺ content significantly decreased and NADH content significantly increased in the older nematodes (p -value < 0.05). (D) There was a strong decrease in the NAD⁺/NADH ratio with age as calculated from the data in panel C.

Fig. 7. Proteomic analysis of young vs. aged *C. elegans* using stable isotope labeling

The y -axis represents the $\log_2$ of the detected abundance, as determined by adding together the total detected intensities of both the heavy ($^{15}\text{N}_4$ - $^{13}\text{C}_6$ -arginine and $^{15}\text{N}_2$ - $^{13}\text{C}_6$ -lysine) labeled proteins (young day 4 nematodes) and the light non-labeled proteins (aged day 10 nematodes). The x -axis indicates the relative fold-change in abundance of individual proteins from the aged nematodes as compared to the young nematodes. The unfilled circles represent proteins with a significantly changed abundance, as determined by the Significance B method (Benjamini-Hochberg corrected p -value) with a threshold of 0.05. The dark gray circles are proteins without a significant change in abundance.

Fig 8. The top 10 altered pathways with age based on proteomic analysis

The total observed change for each identified pathway was calculated as the total of the absolute log₂-fold change for each identified protein found within the pathway ($n = 20$ pathways). The top 10 changed pathways are shown in this figure. The width of each pie wedge is equivalent to the percent change of that pathway out of the total absolute log₂-fold change of all pathways. Numbers shown on each pie wedge are the total absolute log₂-fold change for that pathway.

Table 1
Proteins showing a significant increase or decrease in abundance with age

Significance was determined using Perseus version 1.4.1.3 based on the Significance B method (Benjamini-Hochberg corrected *p*-value; threshold = 0.05). Log₂-fold change values (aged/young) are sorted from lowest to highest.

Log2-fold change (old/young)	Significance B <i>p</i> -value	Protein IDs	Protein name/description	Gene names
-10.045	4.314E-34	G5EDN3	Regulator of chromatid cohesion	tim-1
-7.636	1.787E-20	O02325	Protein methyltransferase	prmt-3
-6.851	1.119E-11	Q93699	Ortholog of human glutathione S-transferase alpha 3	gst-17
-6.324	1.409E-14	O01876	Mitochondrial glutamine/asparagine (Q/N)-rich domain protein	pqn-24
-5.991	2.990E-13	Q9N4H4	Poly(ADP-ribose) polymerase pme-1	pme-1
-5.675	4.693E-12	Q22467;B7WN92	CRAL/TRIO and GOLD domain containing protein	ctg-2
-5.440	1.244E-33	Q18409	Probable splicing factor, arginine/serine-rich 6	rsp-6
-5.363	8.971E-08	Q09454	Diphthamide biosynthesis protein 2	dph-2
-5.262	1.385E-10	Q2HQL8	Uncharacterized protein Y43F8B.1	Y43F8B.1
-5.246	1.708E-07	O17157;Q95X81	DEAD-box helicase required for larval development	C24H12.4
-4.828	3.740E-09	O44191	Yolk receptor required for yolk uptake during oogenesis	rme-2
-4.682	5.375E-06	G5ECP5	Uncharacterized protein T02E9.6	T02E9.6
-4.635	3.963E-06	P19626	Myosin regulatory light chain 2 required for pharyngeal pump	mlc-2
-4.592	1.170E-24	O17726	Uncharacterized protein D1086.1	D1086.1
-4.487	1.373E-05	Q93254	Ortholog of human carboxylesterase 2	C23H4.3
-4.345	1.551E-05	Q95QV3;Q94986	Ras-related protein Rab-3 involved in synaptic transmission	rab-3
-4.318	1.754E-05	H2KYN3	Acyl-CoA dehydrogenase domain-containing protein	acdh-13
-4.259	1.882E-07	Q20199	Ortholog of human sperm autoantigenic protein 17	F39H12.3
-4.134	6.792E-05	Q9XXN0;B1V8I7	Pseudouridine synthase, mitochondrial and cytoplasmic	pus-1
-4.111	7.504E-05	Q9U299	5'→3' exoribonuclease 2 involved in transcription termination	xrn-2
-3.907	1.776E-04	Q09488	Ser/thr-protein kinase sma-6 orthologous to TGF-beta receptors	sma-6
-3.734	3.566E-04	P34640;H2FLF4	Probable ATP-dependent RNA helicase DDX55 homolog	ZK512.2
-3.463	2.112E-05	Q09237	Ortholog of human GRB10 interacting GYF protein 2	C18H9.3
-3.424	2.602E-05	Q9BPN8	Uncharacterized protein Y92H12BR.3	Y92H12BR.3
-3.352	3.803E-05	A5JYX8;O18033	Ortholog of human nicalin; localized to the ER	nra-2
-3.230	1.382E-03	G5EEK8;A8WI68	SR Ca ²⁺ ATPase homolog required for muscle function	sca-1
-3.206	8.032E-05	Q19162	Large ribosomal subunit L11 protein	rpl-11.2
-3.131	1.166E-04	O62416	One of ten lysozyme genes involved in immune function	lys-2
-2.896	3.564E-04	G5EE42	Uncharacterized protein ZK1098.11	ZK1098.11
-2.803	5.432E-04	Q19579;Q19581	Homolog of human polyadenylate-binding protein 1 (PABP 1)	pab-2
-2.689	8.960E-04	Q21874	Ortholog of human sushi domain containing 2 protein	R09E10.5
-2.657	1.028E-03	O17939	Functions in assembly of actin filaments in embryonic muscle	aipl-1
-2.623	1.187E-03	Q19056	Ortholog of human phospholipase D3; ER membrane protein	E04F6.4
-2.349	6.810E-08	H9G2T4;Q21032	Isocitrate dehydrogenase [NADP-dependent]	idh-1
-1.837	1.804E-05	O17680	Probable S-adenosylmethionine synthase 1	sams-1

Log2-fold change (old/young)	Significance B <i>p</i> -value	Protein IDs	Protein name/description	Gene names
-1.794	2.731E-05	Q09248	Probable dynactin subunit 2 involved in spindle assembly	dnc-2
-1.515	3.295E-04	Q04908	26S proteasome non-ATPase regulatory subunit 3	rpn-3
-1.454	5.424E-04	Q9GP96;H2KZV8	LIM domain-containing protein; member of the MLP/CRP family	mlp-1
-1.363	1.107E-03	O45499	40S ribosomal protein S26	rps-26
2.093	9.552E-04	Q9N5N3	Extracellular protein; <i>C. elegans</i> SCP/TAPS domain family member	scl-12
2.249	3.600E-04	Q9XWT3	Uncharacterized protein Y62H9A.6	Y62H9A.6
2.390	6.855E-04	Q18805	Putative secreted TIL-domain protease inhibitor	C53B7.2
2.553	4.445E-05	O62289	TransThyretin-Related family domain-containing protein	ttr-51
2.571	3.896E-05	O44145	PERMeable eggshell	perm-2
2.622	2.669E-05	B9WRT3;O01260	Ortholog of human piezo-type mechanosensitive ion channel component 2	C10C5.1
2.674	1.802E-05	G5EDZ9	Homolog of cysteine protease inhibitors (cystatins)	cpi-1
2.719	1.275E-05	Q17802	Chondroitin proteoglycan 1	cpg-1
2.749	1.009E-05	G5EF93	One of five predicted <i>C. elegans</i> calcium-binding calmodulin homologs	cal-4
2.774	8.287E-06	Q09607	Glutathione S-transferase-36; ortholog of human prostaglandin D synthase	gst-36
2.874	1.550E-04	Q9TZ69	Ortholog of human ubiquitin-conjugating enzyme E2K	ubc-20
3.191	2.759E-05	Q9XVQ3	Uncharacterized protein F15D3.5	F15D3.5
3.577	2.716E-06	Q22038	Ras-like GTP-binding protein rhoA	rho-1
4.191	2.369E-06	Q93289	Uncharacterized protein C27D8.2	C27D8.2
5.679	6.513E-21	Q8WQC4;018147	Ortholog of human kelch-like family member 18	kel-3