Experimental Gerontology 113 (2018) 128–140

Contents lists available at ScienceDirect

Experimental Gerontology

journal homepage: www.elsevier.com/locate/expgero

Identification of key pathways and metabolic fingerprints of longevity in *C. elegans*

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ARTICLE INFO

Section Editor: Diana Van Heemst

Keywords:
C. elegans
Caloric restriction
Insulin/IGF-1 signaling
Transcriptomics
Metabolomics
Longevity

ABSTRACT

Impaired insulin/IGF-1 signaling (IIS) and caloric restriction (CR) prolong lifespan in the nematode *C. elegans*. However, a cross comparison of these longevity pathways using a multi-omics integration approach is lacking. In this study, we aimed to identify key pathways and metabolite fingerprints of longevity that are shared between IIS and CR worm models using multi-omics integration. We generated transcriptomics and metabolomics data from long-lived worm strains, i.e. *daf-2* (impaired IIS) and *eat-2* (CR model) and compared them with the wild-type strain N2. Transcriptional profiling identified shared longevity signatures, such as an upregulation of lipid storage and defense responses, and downregulation of macromolecule synthesis and developmental processes. Metabolomics profiling identified an increase in the levels of glycerol-3P, adenine, xanthine, and AMP, and a decrease in the levels of the amino acid pool, as well as the C18:0, C17:1, C19:1, C20:0 and C22:0 fatty acids. After we integrated transcriptomics and metabolomics data based on the annotations in KEGG, our results highlighted increased amino acid metabolism and an upregulation of purine metabolism as a commonality between the two long-lived mutants. Overall, our findings point towards the existence of shared metabolic pathways that are likely important for lifespan extension and provide novel insights into potential regulators and metabolic fingerprints for longevity.

1. Introduction

Average life expectancy has dramatically increased over the past few decades, resulting from improvements in healthcare systems in most economically developed countries (Salomon et al., 2012). Aging and life expectancy are affected by genetic predisposition and non-genetic and environmental risk factors, making the process of aging different between individuals (Lopez-Otin et al., 2013). Although the genetic and environmental causes of aging are not fully established, several processes have been identified as hallmarks of aging. Many of these hallmarks involve metabolism, such as deregulated nutrient sensing and mitochondrial dysfunction (Lopez-Otin et al., 2013). A prototypical example of how environmental factors are involved in the metabolic network of longevity is caloric stress. One example of this is caloric restriction (CR), a condition of 20–50% reduced caloric intake, that activates several metabolic pathways and thereby extends lifespan across species (Houtkooper et al., 2010). Besides environmental factors, genetics also play an important role in the metabolic control of aging. So far, several metabolic genes have been identified that influence longevity (Houtkooper et al., 2010). For instance, mammalian longevity is influenced by metabolic changes as a result of genetically determined insulin sensitivity and glucose handling (Riera and Dillin, 2015). Taken together, it is evident that both the environment and genetics play an important role in controlling metabolism and aging. However, it remains to be elucidated how both components interact to regulate metabolism and aging.

C. elegans is one of the most popular model organisms for aging studies due to its relatively short lifespan, fully annotated genome and its ease of manipulation (Gao et al., 2018). One of the first aging genes to be discovered was *daf-2*, which encodes a homolog of mammalian insulin/insulin-like growth factor (IGF), and its regulatory mechanisms involved in longevity are highly conserved across a wide range of species, including yeast, flies, mice and human beings (Kenyon, 2011). Lowered DAF-2 signaling leads to translocation of DAF-16, a fork-head transcription factor, that enters the nucleus and activates the expression of numerous genes involved in longevity, lipid metabolism, stress response and innate immunity (Kenyon, 2010). As a result, *daf-2* mutant animals can live twice as long as control animals (Kenyon et al., 1993).

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https://doi.org/10.1016/j.exger.2018.10.003
Received 18 February 2018; Received in revised form 16 August 2018; Accepted 4 October 2018
Available online 06 October 2018

In addition, *C. elegans* also serves as a tractable model to study molecular mechanisms underlying CR. Distinct fashions of CR exist, including genetic (i.e. *eat-2* mutants) and dietary interventions (e.g. diluting bacterial food) (Lakowski and Hekimi, 1998; Greer and Brunet, 2009). *eat-2* mutant animals have a dysfunctional pharynx, which slows down food intake, and results in a lifespan increase of approximately 50% (Gao et al., 2017a). Previous studies on *daf-2* and *eat-2* mutants have outlined the essential biological processes involved in the regulatory networks of aging, and have opened up novel avenues for treatments and biomarkers for aging-related diseases (Kenyon, 2010). In fact, longevity mediated mechanisms of either CR or impaired insulin/IGF-1-like signaling (IIS) seem to be partially overlap in terms of the downstream regulatory processes, including activation of autophagy, and inhibition of the target of rapamycin pathway (TOR) (Kenyon, 2010; Panowski et al., 2007; Walker et al., 2005; Yen and Mobbs, 2010). Although the IIS pathway, especially the activation of DAF-16, is associated with CR-induced longevity, it is dispensable for the longevity phenotype observed in *eat-2* mutants (Kenyon, 2010; Lakowski and Hekimi, 1998).

In recent years, studies using high-throughput "omics" approaches have gained commendation as they have provided comprehensive information on the genomic, metabolomic, and proteomic changes that occur during the aging process (Wan et al., 2017). Among all the omics analyses, metabolomics or profiling of metabolites represents possibly the most diverse and complex level of biological regulatory processes, and is intimately linked to phenotype. For instance, metabolite profiles facilitate the generation of phenotypic data of complex metabolic alterations that incorporate messages from multiple levels of systemic regulation, including the genome, the transcriptome, the proteome, the environment and their interactions (Chan et al., 2010). Technological advancements in metabolomics have enabled numerous discoveries of perturbations in physiological networks and have further expanded our knowledge of longevity mechanisms underlying specific biological functions in different organisms, including worms, mice and humans (Houtkooper et al., 2011; Cheng et al., 2015; Gao et al., 2017b).

Although distinct age-related metabolic signatures have been reported in *C. elegans*, the relationship between gene transcription and metabolite levels remains to be fully elucidated. In this study, we aim to identify key metabolic signatures of longevity. We directly compared two long-lived mutant strains, namely *daf-2(e1370)* and *eat-2(ad465)* (from here on called *daf-2* and *eat-2* mutants) by whole-genome microarray and mass spectrometry-based platforms (Gao et al., 2017b). Next, we superimposed these two platforms in a cross-omics approach to visualize the actual changes at two regulatory levels, and identified shared modes of metabolic regulation of longevity.

2. Materials and methods

2.1. C. elegans strains and bacterial feeding strains

The *C. elegans* N2 (wild-type strain, Bristol), *daf-2(e1370)* and *eat-2(ad465)*, and *E. coli* OP50 and HT115 strains were obtained from the *Caenorhabditis* Genetics Center (CGC). Both mutant strains were backcrossed three times and sequenced before use.

2.2. Worm growth conditions and worm pellet collection

C. elegans were routinely grown and maintained on nematode growth media (NGM) at 20 °C. Worms of each strain were cultured on plates seeded with *E. coli* OP50, then eggs were obtained by alkaline hypochlorite treatment of gravid adults and placed onto plates seeded with *E. coli* HT115. At the young adult stage, each strain was collected by washing them off the culture plates with M9 buffer and the worm pellet was washed with dH_2O for three times before collection in a 2 mL Eppendorf tube, snap-frozen in liquid N_2 and lyophilized overnight. For RNA extraction, 500 worms/tube was collected in four replicates per worm strain; for polar metabolite extraction, 2000 worms/tube was collected in five replicates for both long-lived mutants and seven for N2 strain; for fatty acid and amino acid extraction, 2000 worms/tube was collected in four replicates.

2.3. Microarray

Total RNA was extracted using the standard Trizol method, followed by phenol-chloroform purification and further processing by OakLabs Gmbh (Germany). In short, RNA quality and quantity was assessed after DNase clean-up using a 2100 Bioanalyzer (Agilent Technologies). All samples had a RNA integrity value of ≥7.2. RNA was amplified and labeled using a Low Input QuickAmp Labeling Kit (Agilent Technologies) and hybridized using the Agilent Gene Expression Hybridization Kit (Agilent Technologies). An ArrayXS-068300 with WormBase WS241 genome build (OakLabs) was used and fluorescence signals were detected by the SureScan microarray Scanner (Agilent Technologies). Data of all samples were quantile normalized using the ranked median quantiles as described previously (Bolstad et al., 2003). The most significant coding-gene isoform was selected to represent expression for mentioned genes. Fold-change of gene expressions in the mutant strains was characterized as $\log_2$ (expression$_{daf\text{-}2\ or\ eat\text{-}2\ mutant}$/expression$_{N2}$), and the significance (p-value) was calculated using a Student's t-test. The expression of a certain gene in each long-lived mutant with a p-value < 0.05 and a fold-change either less than -1 or higher than 1 was considered as a significant compared to N2 and selected for further analysis. The overlapping up- or down-regulated genes in the long-lived mutants were selected using Venny diagram 2.1.0 (Oliveros, n.d.). KEGG pathway analysis was performed with David (Huang et al., 2009). GO term enrichment analysis was performed with David and ReviGo (Supek et al., 2011). The result from ReviGO was downloaded as an R script and images were imported into Illustrator (Adobe) for further editing.

2.4. Metabolomics analysis: polar metabolite measurements

500 μL MQ water, 500 μL MeOH and 1 mL chloroform was added to the dry worm pellet. The following internal standards were added to the homogenate: a mixture of amino acids ((Cambridge Isotope Laboratories, UK) containing 68 nmol DL-alanine-2,3,3,3-D_4, 44 nmol DL-glutamic acid-2,4,4-D_3, 40 nmol L-leucine-5,5,5-D_3, 28 nmol L-phenylalanine-ring-D_5, 34 nmol L-valine-D_8, 34 nmol L-methionine-(methyl-D_3), 26 nmol L-tyrosine-ring-D_4, 22 nmol L-tryptophan-(indole-D_5), 46 nmol DL-serine-2,3,3-D_3, 48 nmol proline-D_7, 24 nmol L-arginine-2,3,3,4,4,5,5-D_7, 28 nmol L-glutamine-2,3,3,4,4-D_5, 32 nmol L-lysine-4,4,5,5-D_4, 26 nmol L-citrulline-ureido-^{13}C, 28 nmol L-ornithine-3,3,4,4,5,5-D_6, 42 nmol L-isoleucine-D_{10}, and 46 nmol DL-aspartic acid-2,3,3-D_3 (Gao et al., 2017b)), $^{13}C_3$-pyruvate, $^{13}C_6$-glucose, $^{13}C_6$-fructose-1,6-biphosphate, $^{13}C_6$-glucose-6-phosphate, adenosine-$^{15}N_5$-monophosphate and guanosine-$^{15}N_5$-monophosphate (5 μM). Thereafter, the worm suspension was homogenized using a TissueLyser II (Qiagen) for 5 min at a frequency of 30 times/s. After centrifugation at 4 °C, ~800 μL of the "polar" top layer was transferred to a new 1.5 mL Eppendorf tube and dried in a vacuum concentrator at 60 °C (GeneVac). Dried residues were dissolved in 100 μL MeOH:MQ water (6:4, v/v). For polar metabolite measurements, we used a Thermo Scientific ultra-high pressure liquid chromatography system (Waltman, MA, USA) coupled to a Thermo Q Exactive (Plus) Orbitrap mass spectrometer (Waltman, MA, USA). The auto-sampler was kept at 10 °C during the analysis and 5 μL sample was injected on the analytical column. The chromatographic separation was established using a Sequant ZIC-cHILIC column (PEEK 100 × 2.1 mm, 3.0 μm particle size, Merck, Darmstadt, Germany) and held at 15 °C. The flow rate was kept at 0.250 mL/min. The mobile phase was composed of (i) acetonitrile:water (9:1, v/v) with 5 mM ammonium acetate; pH = 6.8 and (ii) acetonitrile:water (1:9, v/v) with 5 mM ammonium acetate; pH = 6.8, respectively. The LC gradient program was: start with 100% (i) hold 0–3 min; ramping 3–20 min to 36% (i);