Biogerontology (2021) 22:345–367
https://doi.org/10.1007/s10522-021-09920-3

RESEARCH ARTICLE

Network analysis in aged *C. elegans* reveals candidate regulatory genes of ageing

Foteini Aktypi · Nikoletta Papaevgeniou · Konstantinos Voutetakis · Aristotelis Chatziioannou · Tilman Grune · Niki Chondrogianni

Received: 17 December 2020 / Accepted: 30 March 2021 / Published online: 19 April 2021
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Abstract Ageing is a biological process guided by genetic and environmental factors that ultimately lead to adverse outcomes for organismal lifespan and healthspan. Determination of molecular pathways that are affected with age and increase disease susceptibility is crucial. The gene expression profile of the ideal ageing model, namely the nematode *Caenorhabditis elegans* mapped with the microarray technology initially led to the identification of age-dependent gene expression alterations that characterize the nematode's ageing process. The list of differentially expressed genes was then utilized to construct a network of molecular interactions with their first neighbors/interactors using the interactions listed in the WormBase database. The subsequent network analysis resulted in the unbiased selection of 110 candidate genes, among which well-known ageing regulators appeared. More importantly, our approach revealed candidates that have never been linked to ageing before, thus suggesting promising potential targets/ageing regulators.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10522-021-09920-3.

F. Aktypi · N. Papaevgeniou · K. Voutetakis · N. Chondrogianni (✉)
Institute of Chemical Biology, National Hellenic Research Foundation, 48 Vassileos Constantinou Avenue, 116 35 Athens, Greece
e-mail: nikichon@eie.gr

N. Papaevgeniou
Nutrigenomics Section, Institute of Nutritional Sciences, Friedrich Schiller University of Jena, 07743 Jena, Germany

K. Voutetakis
Department of Biochemistry and Biotechnology, University of Thessaly, 41500 Viopolis Larissa, Greece

A. Chatziioannou
Center of Systems Biology, Biomedical Research Foundation of the Academy of Athens, 4 Soranou Ephessiou street, 115 27, Athens, Greece

A. Chatziioannou
e-NIOS Applications PC, 25 Alexandrou Pantou street, 176 71 Kallithea, Greece

T. Grune
Department of Molecular Toxicology, German Institute of Human Nutrition, Nuthetal, Germany

Keywords Ageing · Network analysis · *C. elegans* · Microarrays · Gene expression regulation

Introduction

Biological ageing is a universal process characterized by a gradual decline in the function and structure of cells and tissues that can eventually lead to mortality (Kirkwood and Austad 2000). The nematode *Caenorhabditis elegans* has emerged as an ideal experimental model organism to study ageing, due to its small size, relatively short lifespan, large brood size and its well-annotated and easily manipulated genome but most importantly due to significantly conserved genes governing ageing throughout species (Brenner 1974, C. elegans Sequencing Consortium 1998; Tissenbaum 2015). Since the identification of the *age-1* long-lived *C. elegans* mutants (Friedman and Johnson 1988), a number of genetic factors and pathways (Kenyon 2011) have been identified to critically affect its lifespan and to regulate the ageing process independently or in response to environmental cues (Russell and Seppa 1987; Van Voorhies and Ward 1999).

Study of the gene expression profile in the ageing nematode, can contribute to the identification of ageing biomarkers and regulators, for the design of gene therapies and drugs ultimately aiming to delay ageing and to prolong healthspan in humans. Previous time course studies that analyzed age-related gene expression changes, have provided a valuable insight into the ageing process. Lund et al. was the first to profile ageing gene expression changes in *C. elegans* using a microarray platform and proposed the measurement of physiological age utilizing molecular probes (Lund et al. 2002). Since then, a growing number of studies have focused on ageing biomarkers and the calculation of biological age (Eckley et al. 2013; Tarkhov et al. 2019), the association of distinct ageing phenotypes with distinct transcriptional profiles (Golden and Melov 2004; Golden et al. 2008; Eckley et al. 2018) and the identification of age-affected pathways and ageing regulators (Murphy et al. 2003; Budovskaya et al. 2008; Youngman et al. 2011; Inukai et al. 2018). There are several such regulatory factors in *C. elegans* that are conserved between species and manipulation of their activity can optimize gene expression and promote longevity (Martínez Corrales and Alic 2020). The ones that are most extensively studied include DAF-16 (FOXO in humans), HLH-30 (TFEB), PHA-4 (FOXA), HIF-1 (HIF1A), HSF-1 (HSF1) and SKN-1 (NRF2) which are all widely conserved transcription factors, as well as nuclear hormone receptors (Denzel et al. 2019).

The identification of ageing regulatory factors is a challenging task, since ageing is controlled through complex protein networks. While gene expression profiling can be very useful to monitor the overall transcriptional changes that cells, tissues or organisms undergo, the differentially expressed genes alone are not sufficient to methodically detect particular genes and proteins that are responsible for those changes. The basic idea of our approach considers the genes that are differentially expressed as the ones to be primarily regulated rather than being themselves the regulators. We based this assumption on the fact that activation or deactivation of transcriptional regulators may promote changes in gene expression levels of their targets but usually the regulators themselves are not necessarily differentially expressed. Therefore, by taking into account the interactions of the differentially expressed genes with other proteins or genes, we may detect central regulators that are significant for the ageing process.

In this study, we have combined microarray gene expression profiling of the wild type (wt) N2 nematodes during the progression of ageing with network analysis of the differentially expressed genes to detect regulatory genes and factors of pivotal importance in *C. elegans* ageing, an approach that has not been applied before for the detection of ageing regulators. Differential expression analysis with time course data has been previously performed in various studies in *C. elegans* but usually these studies include: (a) treatment with specific compounds or bacterial strains with anti-inflammatory properties (Grompone et al. 2012; Hou et al. 2016), (b) mutants for specific pathways (Lund et al. 2002; Tarkhov et al. 2019), or (c) animals at younger stages (Greer et al. 2010; Grompone et al. 2012). We used a popular Affymetrix microarray platform to quantify whole genome gene expression changes in three key time points (days 1, 6 and 12 of adulthood) in the wt nematodes lifespan. Along these selected time points, the population has spanned all stages from that of juvenile reproductive animals (day 1) through middle aged animals (day 6) that no longer

lay eggs (Lionaki and Tavernarakis 2013) to that of old animals which have undergone ageing changes, yet the majority of the population is still alive (day 12). To further validate our microarray analysis we compared our results with those of an already established dataset provided by Youngman et al. 2011 (GSE21784, stated as Youngman dataset onwards), which is the only one using a similar platform and overall design to our study. Subsequently, we focused on the common genes that are differentially expressed in both datasets, to construct a network of interactions between those ageing-related genes and their first neighbors. This network is based on the interactions provided by the WormBase database which is highly specific for *C. elegans* (Cho et al. 2018; Harris et al. 2020). The analysis of this network resulted not only in the detection of already known regulatory genes that influence ageing thus validating our approach, but most importantly, in the identification of candidate regulatory genes that have not been linked to ageing up to date.

Materials and methods

An overview of the pipeline followed in this study is described below and summarized in Fig. 1.

Caenorhabditis elegans strains and culture

We followed standard procedures for strain maintenance and culture. *C. elegans* strain N2 (wt Bristol isolate) was used during the course of the experiment, provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). Cultures were maintained at 20 °C in fresh nematode growth medium (NGM) plates, supplemented with streptomycin and nystatin and seeded with the OP50 strain of *E. coli*. Cultures were renewed every day by placing four L4 stage nematodes in freshly OP50 seeded plates.

RNA isolation during lifespan progression

Synchronization of nematodes was achieved by placing 30–40 gravid adults in freshly OP50 seeded plates to lay eggs for 2–3 h. Gravid adults were then removed and the plates were maintained at 20 °C until the L4 stage. Synchronized L4 stage animals (150–200 animals) were transferred to fresh NGM agar plates supplemented with antibiotics containing UV-killed bacteria (OP50). Day 1 of adulthood was set as t = 0. Food was replenished every 2 days. Alive animals were verified following provoked movement and pharyngeal pumping. RNA was extracted using the TRIzol® (Invitrogen) -Chloroform method from synchronized animals at days 1, 6 and 12 of adulthood.

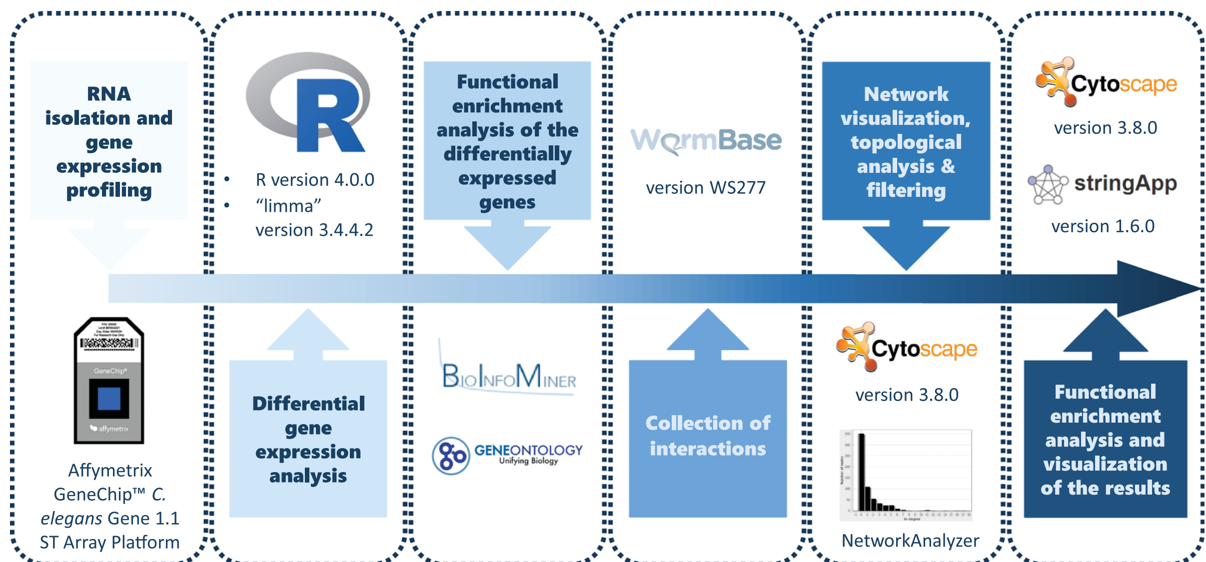


Fig. 1 Pipeline overview diagram

More specifically, 120 nematodes per sample were collected one-by-one, placed on M9, washed three times and centrifuged (6000 rpm, 30 s) to remove remaining bacteria. 1 ml of Trizol was added to the nematodes pellet. Three freeze (liquid nitrogen)-thaw (37 °C) cycles were performed before the addition of 200 µl chloroform. Following thorough mixing and 3 min incubation at room temperature, the mix was centrifuged (14,000 rpm, 15 min) and the aqueous phase was collected. RNA was precipitated with 500 µl isopropanol (overnight incubation at -20 °C followed by centrifugation at 14,000 rpm, 10 min). Total RNA was diluted in RNase-free water for injection. RNA quality and quantity was measured using NanoDrop 2000 (ThermoScientific, USA). RNA quality was evaluated using the 260/280 nm and 260/230 nm ratios. We profiled gene expression of the animals of each age group, in biological triplicates for young (day 1) and middle aged (day 6) ones and biological duplicates (due to an unforeseen problem during the RNA profiling that destroyed the third sample) for old animals (day 12) using the Affymetrix GeneChip™ *C. elegans* Gene 1.1 ST Array Platform.

Data pre-processing and differential expression analysis

The two datasets used in this study [our own dataset stated as Aktypi dataset onwards (GSE163253) and Youngman's dataset (GSE21784)], were analyzed individually using the same pipeline approach, since the platforms are of similar design. Sample pre-processing, plots and differential gene expression analysis was performed using R version 4.0.0 (R Core Team 2020) in R studio environment (version 1.3.1073). Background correction, quantile normalization and summarization, was performed using the RMA (Robust Multi-array Average) algorithm of Irizarry et al. (Irizarry et al. 2003), followed by the exclusion of control probes. We proceeded with probeset filtering based on the intensity values across the three age groups to remove probesets which are consistently not expressed in all groups. Data quality assessment was performed before and after RMA normalization by using plot distribution analysis via the generation of boxplots of gene expression and by conducting principal component analysis (PCA) of samples after the probeset filtering. The samples demonstrated an even distribution of the expression values after minimizing the technical errors making them directly comparable with statistical tests. Subsequently, the 'limma' R-package (Ritchie et al. 2015) version 3.4.4.2 was utilized to perform linear model fitting and moderated t-statistics, to determine the significantly differentially expressed genes. For each comparison of gene expression (for Aktypi dataset: day 6 compared to day 1, day 12 compared to day 6, day 12 compared to day 1 and for Youngman dataset: day 15 compared to day 6) the log2-fold change and adjusted p-value with Benjamini–Hochberg correction (Benjamini and Hochberg 1995) was calculated for each gene. A double cutoff of adjusted p-value < 0.05 and absolute value of log2-fold change > 0.5 was applied, to include as many differentially expressed genes as possible in the subsequent network analysis considering that even small changes in gene expression might be important.

Classification and functional enrichment analysis of the differentially expressed genes

We compared the gene expression levels between the following time points in the nematodes lifespan; at days 1, 6 and 12 of adulthood for Aktypi dataset and at days 6 and 15 of adulthood for the Youngman dataset to detect ageing-associated changes. The resulting differentially expressed genes were organized into upregulated and downregulated genes for each comparison of gene expression. Furthermore, Venn diagrams to visualize the extent of overlapping differentially expressed genes between the two datasets were created utilizing the online tool Venny 2.1 (Oliveros 2015).

To identify processes and pathways associated with the differentially expressed genes, we performed functional enrichment analysis using the BioInfoMiner platform (Koutsandreas et al. 2016) by exploiting the ontological terms of Gene Ontology (GO) (Ashburner et al. 2000). BioInfoMiner delivers unsupervised, fast and integrative interpretation of -omics experiments, with prioritization of detected systemic processes and genes deriving a compact gene signature, consisting of systemic processes and their driver genes. A cutoff of BioInfoMiner bootstrapping corrected p value < 0.05 was set to select the statistically significant altered GO biological processes.

Assembly of the network of interactions in ageing

The WormBase genome biology resource for *C. elegans* (Harris et al. 2020) was used to obtain all interactions between genes and gene products and to construct the network of physical interactions in the nematodes during ageing progression, based on the common results between the two distinct dataset. We specifically used WormBase interactions as this is a *C. elegans* specific resource, curated and regularly updated. Furthermore, the majority of these interactions refer to direct protein–protein, protein-DNA or protein-RNA interactions which are experimentally validated. Apart from the physical interactions, WormBase also provides regulatory and genetic interactions revealed following experimental interventions. Consequently, on top of the physical interactions, we have also included regulatory and genetic interactions resulting from experimental interventions, to incorporate as many of the differentially expressed genes as possible to our network for analysis. The interaction data were first filtered, with a script written in R, to establish the good data quality for the subsequent network analysis. Interactions with missing information, duplicated interactions and self-loops were removed.

To minimize the effects of reproduction and to focus exclusively on ageing, we based the construction of the network solely on the genes that are differentially expressed in both datasets in the following comparisons; at day 12 compared to day 6 of adulthood in our dataset and at day 15 to day 6 of adulthood in the Youngman dataset. These time points can sufficiently secure that eggs have almost no influence on the results and that worms have definitely undergone ageing changes throughout this time period while the majority of the population is still alive.

Visualization and analysis of the ageing network

The expanded network of differentially expressed genes in ageing was visualized and analyzed with Cytoscape v3.8.0, an open source software platform (Shannon et al. 2003) that has been specifically developed for the visualization of network interactions. We performed topological network analysis through the Cytoscape application NetworkAnalyzer (Assenov et al. 2008) to assess the parameters of the network and confirm that it has the characteristics of a biological network. Additionally, the whole network was analyzed as an undirected graph (Koutrouli et al. 2020) since only few interactions in WormBase are directed.

We then used the rounded value of the network parameter *“average degree”*, which is simply the average number of edges per node in the graph, as a guide to filter the network nodes and to retrieve candidate genes that influence the ageing process. The rounded *“average degree”* value that we used was equal to 7; the nodes that interact with at least 7 neighbors-genes that are differentially expressed in ageing were selected as candidate regulators. In our network, more than 50% of the nodes had a higher degree value than the *“average degree”* value and therefore could be selected as candidates if the criteria were met. We preferred this filtering method to avoid selecting nodes that albeit having a very high number of connections, they were not strongly related to our differentially expressed genes. Instead, we opted for nodes that would be connected to our differentially expressed genes even if they did not have a very high level of connections; regulatory genes with a lower degree value have been also shown to represent better drug targets (Feng et al. 2017). Subsequently, we created a subnetwork of the candidate ageing regulatory genes which we further explored for significantly overrepresented Gene Ontology (GO) terms and visualized the results.

Network functional enrichment analysis and visualization

To identify statistically significant enriched biological terms in our subnetwork, we retrieved functional enrichment information for our nodes based on the Gene Ontology (GO) terms using the Cytoscape stringApp (Doncheva et al. 2019). The stringApp performs redundancy filtering in the list of enriched terms that are sorted by FDR-corrected p value (< 0.05) and removes terms that are too similar to any of the previous. The redundancy filtering is based on the similarity between two terms, measured by the Jaccard index (Jaccard 1901) of the sets of genes annotated by the two terms. Finally, our results were visualized in Cytoscape utilizing the Cytoscape Layout Tools and Algorithms (Shannon et al. 2003).

Identification of known ageing regulators within the list of candidates

To obtain an overview of the extent of predicted genes that are known lifespan regulators in *C. elegans*, we compared our list with the GenAge resource list that includes already established longevity regulators in *C. elegans* (de Magalhães and Toussaint 2004; Tacutu et al. 2018). GenAge incorporates gene mutation findings from humans and various model organisms that either delay or accelerate ageing. Furthermore, we utilized SynergyAge (Bunu et al. 2020), a more recent, comprehensive, manually curated database for ageing-associated genes and synergistic or antagonistic effects of genetic interventions on *C. elegans* lifespan. We thus examined whether any of the predicted candidate genes directly affects lifespan or enhances the effect of known longevity regulating genes. A literature review on the predicted candidate regulatory genes further led to their categorization into three groups; the 1st group includes genes that have been found to affect lifespan, the 2nd group includes candidates that have been studied in various other physiological processes and the 3rd group includes candidates that are relatively unstudied (found in maximum two studies in PubMed). Finally, we converted the predicted candidate genes to their orthologous HGNC human gene symbols with OrthoList2, a collection of *Caenorhabditis elegans* genes and their human orthologs (Kim et al. 2018).

Results

Differentially expressed genes during ageing progression in *C. elegans*

The principal component analysis performed on filtered data (scaled log2 values) showed that the samples clustered together based on the age of the individuals, with only minor variations between the independent biological replicates (Fig. 2). Middle aged animals (day 6) were more similar to young adult animals (day 1) than to the old ones (day 12), evident also in the distances of the respective samples along PC2. In the Youngman dataset, the principal component analysis performed on filtered data (Online Resource 1) revealed that day 6 animals were more similar to day 15 animals and very different from L4 which are animals still undergoing development.

Statistical analysis resulted in 4588 unique differentially expressed genes in all different time points examined in our dataset. More specifically, the comparison between middle aged and young animals resulted in 1385 differentially expressed genes, the comparison between old and middle aged animals resulted in 2632 differentially expressed genes while the comparison between old and young animals resulted in differences in 3885 differentially expressed genes. In the Youngman dataset, the comparison between day 15 and day 6 animals resulted in 5982 differentially expressed genes with an equal number of genes being upregulated and downregulated (Table 1, Online Resource 2).

To find common differentially expressed genes in both datasets, we created Venn diagrams of the differentially expressed genes at day 12 to day 6 comparison (for our dataset) and at day 15 to day 6 comparison (for Youngman dataset) which represent the more comparable time points between the two datasets. We found 1131 genes differentially expressed in both comparisons; 240 genes were upregulated in both datasets, 516 genes were downregulated in both datasets and 375 genes exhibited opposite expression pattern with the majority of which being upregulated at day 12 to day 6 comparison in our dataset but appeared downregulated at day 15 to day 6 comparison in the Youngman dataset (Fig. 3, Online Resource 3).

A heatmap representation of all the differentially expressed genes revealed that most of the genes are either upregulated or downregulated as the population ages (Fig. 4). Moreover, fewer transcriptional alterations occurred between day 6 and day 1 of adulthood compared to day 12 and day 6 of adulthood and even more differences became apparent in the day 12 vs day 1 comparison. The comparison between day 15 (that represents animals in more advanced age) and day 6 in the Youngman dataset resulted in even more differentially expressed genes.

The genes resulting from each comparison of gene expression were further divided into upregulated and downregulated (Online Resource 2) as described in the Materials and Methods section. Table 1 summarizes the number of differentially expressed genes in each case, coupled with the BioInfoMiner functional enrichment analysis results. The 5 most

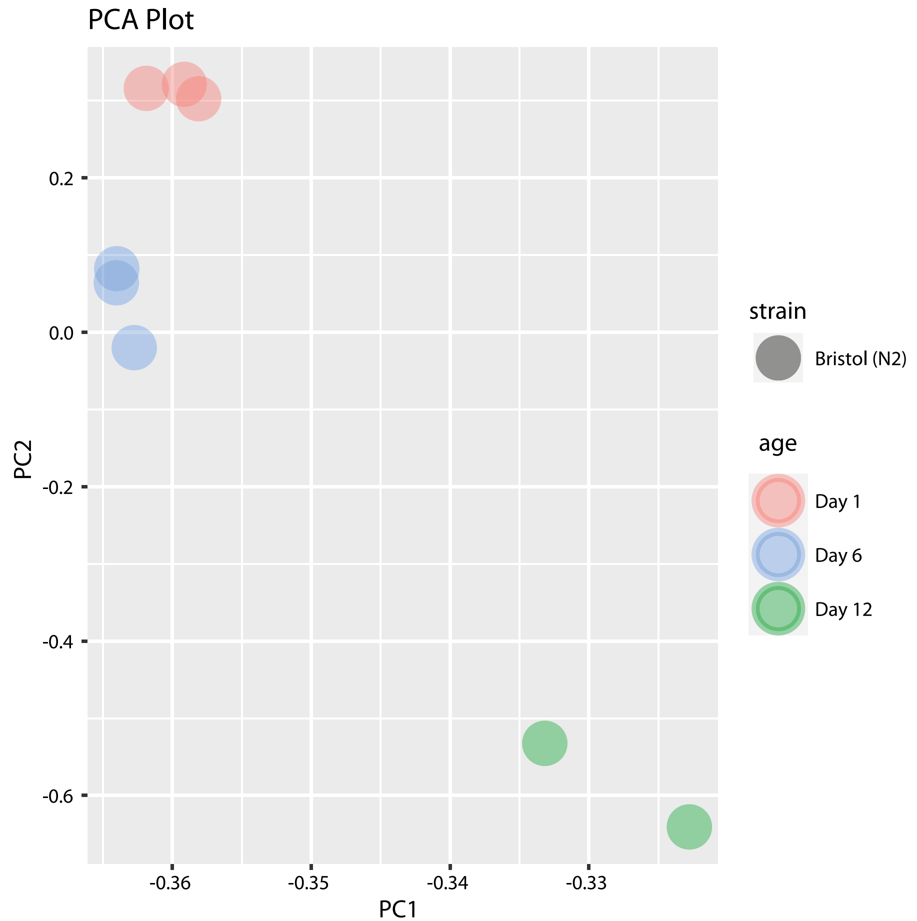


Fig. 2 Principal component analysis (PCA) of individual samples in our dataset. Our data suggest that ageing is a characteristic transcriptomic drift along a single direction while PC1 and PC2 accounted for 98.2% of variance observed

representative, statistically significant enriched GO terms for the biological processes component are shown for each group of genes. BioInfoMiner complete results for the enriched GO Biological Processes can be found in Online Resource 4.

Mapping of the network of interactions

To address the need for a more systematic approach, we constructed and analyzed the network of interactions of differentially expressed genes in the nematodes during the ageing progression. To exclude transcriptional alterations related to egg production rather than ageing, we focused on post-reproductive changes between days 12 vs day 6 of adulthood in our dataset and between days 15 vs day 6 of adulthood in Youngman dataset. We started with a total of 1131 WormBase gene ids that were found differentially expressed in both datasets to collect interactions between them and their first neighbors. The resulting network incorporates 676 out of the 1131 genes ($\sim$ 60% of the common differentially expressed genes between the two datasets) and consists of 2971 nodes and 21,892 interaction edges (Online Resource 5 and Supporting data 1). For the remaining differentially expressed genes there were not any reported interactions in WormBase.

Network topological analysis was performed with Cytoscape 3.8.0 and the NetworkAnalyzer. The network's node degree distribution follows the power-law, meaning that it is a scale-free network (Barabási and Oltvai 2004). The "*average number of neighbors*" is 14.764 and the *"average degree"* is 7.368 meaning that on average a node in our network has $\sim$ 7 first neighbors. The network's "*density*" value is 0.005, which shows that it is a sparse network (Leclerc 2008). The metric "*clustering coefficient*" has a value of 0.175 higher than that of a random network which is indicative of the network's capability to be divided into clusters while the "*characteristic path length*" is

Table 1 Summary table of the number of resulting differentially expressed genes along with the statistically significant enriched GO biological processes of each gene set

Dataset	Comparison of gene expression	# Genes	GO term	Description	Corrected p-value
	GSE163253 (Aktypi)	day 6 vs day 1 ↗	811	GO:0009605	Response to external stimulus
0.001					
			GO:0006955	Immune response	0.0108
			GO:0048856	Anatomical structure development	0.0164
			GO:0032502	Developmental process	0.0244
			GO:0010628	Positive regulation of gene expression	0.0301
	day 6 vs day 1 ↘	574	GO:0019752	Carboxylic acid metabolic process	0.0007
			GO:0055114	Oxidation–reduction process	0.0156
			GO:0006631	Fatty acid metabolic process	0.0173
			GO:0030239	Myofibril assembly	0.0231
			GO:0050801	Ion homeostasis	0.0426
	day 12 vs day 6 ↗	1798	GO:0007399	Nervous system development	0.0012
			GO:0030154	Cell differentiation	0.0078
			GO:0030030	Cell projection organization	0.0228
			GO:0001708	Cell fate specification	0.0374
			GO:0010628	Positive regulation of gene expression	0.0409
	day 12 vs day 6 ↘	834	GO:0055114	Oxidation–reduction process	0.0032
			GO:0045087	Innate immune response	0.0069
			GO:0006629	Lipid metabolic process	0.0183
			GO:0006631	Fatty acid metabolic process	0.046
			GO:0006006	Glucose metabolic process	0.0497
	day 12 vs day 1 ↗	2614	GO:0007399	Nervous system development	0.0009
			GO:0030154	Cell differentiation	0.005
			GO:0000902	Cell morphogenesis	0.0144
			GO:0009605	Response to external stimulus	0.0347
			GO:0045893	Positive regulation of transcription, DNA-templated	0.0413
	day 12 vs day 1 ↘	1271	GO:0044281	Small molecule metabolic process	0.0011
			GO:0055114	Oxidation–reduction process	0.0048
			GO:0006629	Lipid metabolic process	0.0148
			GO:0006955	Immune response	0.0294
			GO:0046034	ATP metabolic process	0.0493
	GSE21784 (Youngman)	day 15 vs day 6 ↗	2955	GO:0043412	
	Macromolecule modification	0.0015			
			GO:0006468	Protein phosphorylation	0.014
			GO:0022604	Regulation of cell morphogenesis	0.0189
			GO:0006396	RNA processing	0.0368
			GO:0042127	Regulation of cell population proliferation	0.0481

Table 1 continued

Dataset	Comparison of gene expression	# Genes	GO term	Description	Corrected p-value
	day 15 vs day 6 ↘	3027	GO:0006952	Defense response	0.0007
			GO:0055114	Oxidation–reduction process	0.0087
			GO:0019752	Carboxylic acid metabolic process	0.0138
			GO:0044255	Cellular lipid metabolic process	0.0399
			GO:0055001	Muscle cell development	0.045

The genes are organized in gene sets based on their pattern of expression as described in Materials and methods. Arrows facing upwards indicate gene upregulation while arrows facing downwards show downregulation. The corrected enrichment p-values appear in the last column of the table.

3.139 (Mao and Zhang 2013) that is lower than that of a random network. All these values are indicative of a network with small-world properties (Wang and Chen 2003).

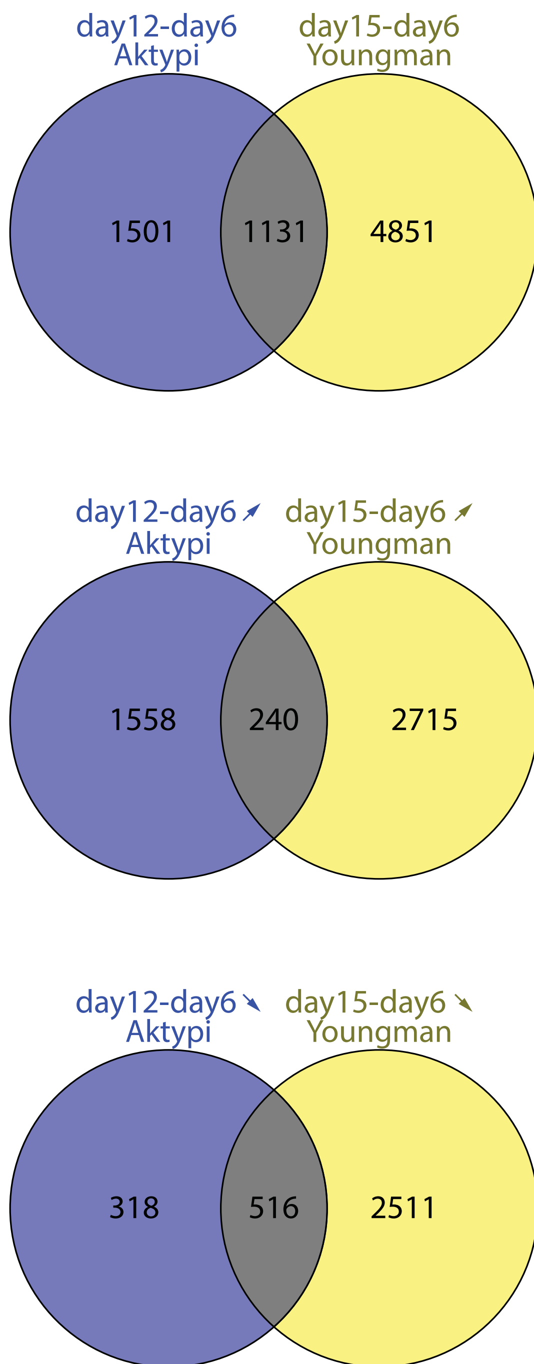
To detect critical players for the ageing process, we applied a specific topology filter in our network that resulted in the selection of 110 genes as candidate regulators. More specifically, each selected candidate regulator had at least 7 neighbors that were differentially expressed in our analysis. This filtering threshold is the rounded integer value of the metric "*average degree*". This threshold permitted an even chance of selection for at least 50% of the nodes in the network while it prevented accidental selection. The subnetwork of those 110 genes with 544 interaction edges (Supporting data 1 and Online Resource 6) was visualized (Fig. 5) and functional enrichment analysis of the genes followed utilizing the Cytoscape StringApp (Doncheva et al. 2019).

Interestingly, the GO term *"developmental process"* appeared as the most enriched term (FDR: 1.15E-50), whereas many of the candidate regulatory genes are transcription factors (Fig. 5; genes in ◇). Other important GO biological processes are related to "*cellular response to stress"*, "*reproductive behavior"*, "*muscle organ development", "positive regulation of cell death"*, "*regulation of neuron death", "regulation of cellular catabolic process"* and *"mitochondrial unfolded protein response"*. Table 2 summarizes the non-redundant enriched GO biological processes where only terms with Jaccard similarity index < 0.15 and FDR-corrected p-value < 0.05 appear (complete table of results before the redundancy filtering can be found in Online Resource 7).

Detection of known longevity regulating genes among the candidate genes

We used the GenAge database (de Magalhães and Toussaint 2004) to identify already established longevity regulating genes within our list of candidate genes; we retrieved 31 known ageing regulators. Furthermore, querying of the SynergyAge database (Bunu et al. 2020) resulted in 6 more genes in our list that are known ageing regulators and 3 additional ones that have been found to affect lifespan in a synergistic manner through enhancement or inhibition of the effects of known regulators. In combination with literature review, we identified 42 genes that are known to affect *C. elegans* lifespan. This number represents 38.2% of the genes in our candidates list.

Thereafter, these 110 genes were divided into 3 groups based on our current knowledge on their potential effects on the lifespan and healthspan of *C. elegans* and were also converted to their human orthologs utilizing OrthoList2 (Kim et al. 2018) (Table 3). The 1st group includes 42 genes strongly associated with ageing in the nematodes, that have been shown to affect its lifespan. The most known transcription factors such as DAF-16 and PHA-4 appear in this list (Hsu et al. 2003; Sheaffer et al. 2008). These known ageing regulators validate our network and microarray analysis. The rest of the candidate genes in our list were categorized in two additional groups based on the number of PubMed ids that are returned when we query PubMed for those particular genes (https://pubmed.ncbi.nlm.nih.gov/). The 2nd group includes 46 genes that are mostly known to affect development and other physiological processes (e.g. stress, reproduction, cell death and cell division). The 22 genes in the 3rd group are relatively



◀**Fig. 3** Venn diagrams of the common differentially expressed genes in wt *C. elegans* during ageing progression between the two datasets used in this study. The comparison of gene expression was performed between day 12 vs day 6 animals in our dataset (number of differentially expressed genes in blue circles) and between day 15 vs day 6 animals for Youngman dataset (number of differentially expressed genes in yellow circles). In each case, the number of overlapping differentially expressed genes between the two comparisons is displayed. Arrows facing upwards indicate upregulated genes while arrows facing downwards show downregulated genes

unknown (with maximum two PMIDs returned for each gene in PubMed) and have not been associated with ageing. These 22 genes are the candidates that our analysis proposes as potential novel ageing regulators.

Discussion

Profiling of gene expression alterations throughout lifespan provides a better understanding of the ageing process and reveals how the transcriptional age-related changes correlate with the observed age-related phenotypic alterations in *C. elegans*. Here, we combined the findings of ageing-related gene expression changes in wt nematodes with network analysis. This approach enabled the identification of 110 promising candidate regulators of longevity in *C. elegans*. Twenty two of these genes have never been linked to ageing and their identification paves the way to future studies for novel ageing regulators. Given the high levels of evolutionary conserved genes between the nematodes and humans with regard to ageing (Yanai et al. 2017), our results might suggest potential regulators of human ageing as well, although further experimentation is needed.

We observed changes in the gene expression of 1385 genes between animals at day 6 and day 1, while those differences were doubled (2632 differentially expressed genes) when comparing animals at day 12 and day 6. This correlates well with the end of the animals' fertility period around day 6 of adulthood (Lionaki and Tavernarakis 2013) and the additional massive physiological alterations until day 12. For example, locomotion becomes irregular during this timeframe and eventually individuals begin to die around day 10 of adulthood (Herndon et al. 2018). In the Youngman dataset, where more distant time points were examined, more genes were found differentially expressed in the comparison between day 15 and day 6 of adulthood (5982 differentially expressed genes). Furthermore, 1131 genes (43% of differentially expressed genes) in the comparison between day 12 and day 6 animals in our dataset were also

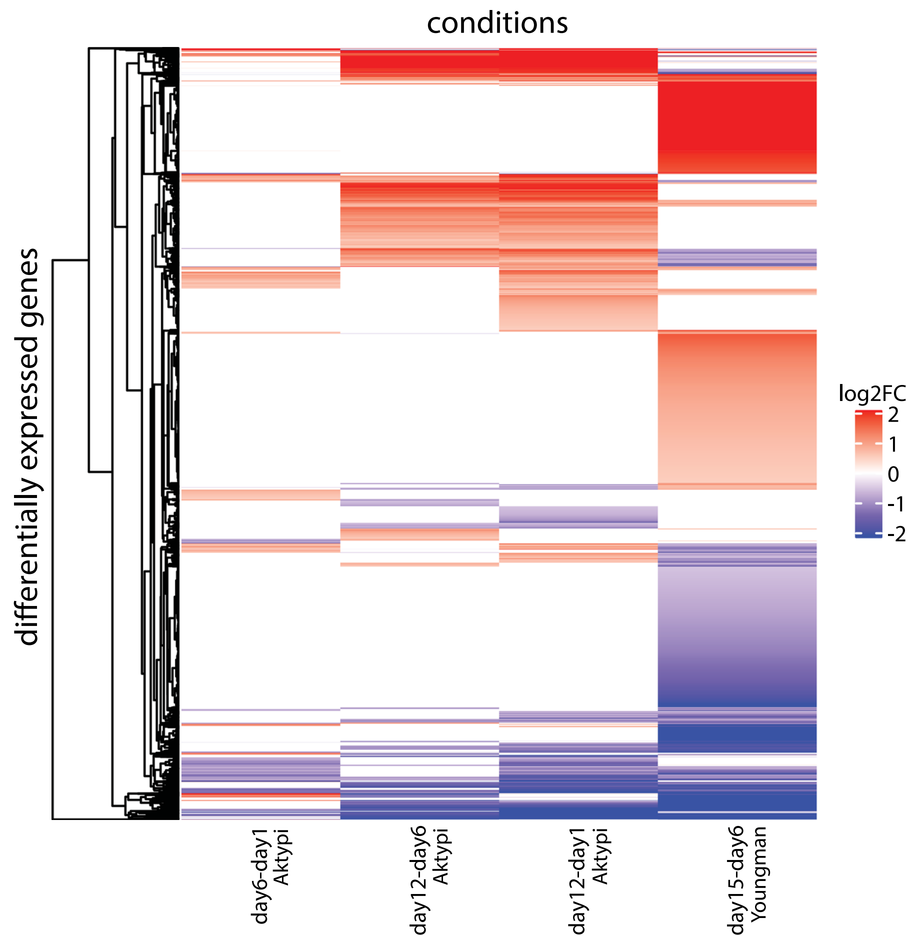


Fig. 4 Heatmap of the differentially expressed genes in wt *C. elegans* during ageing progression. Columns show the type of comparison of gene expression between the different time points and each row represents a differentially expressed gene (clustering distance Euclidean, Complete linkage algorithm). Red and blue represents gene upregulation and downregulation, respectively

differentially expressed in the comparison between day 15 and day 6 animals in Youngman dataset. Taking into consideration that *C. elegans* gene expression profiles are sensitive (Portman 2006) and that the time points examined between the two datasets are not identical, the overlap of differentially expressed genes between the two datasets is significant. Moreover, only 375 of those genes display opposite expression patterns that could however be attributed to the different time points compared (day 12 and day 15).

We observed decreased expression of genes that are mostly associated with oxidation–reduction metabolic processes, muscle contraction, lipid and fatty acid metabolism and mitochondrial metabolism in both datasets (Table 1). These observations highly correlate with the ageing progression where reproductive senescence, locomotor decline and mitochondrial dysfunction occur (Herndon et al. 2018). In accordance with previous studies, we also found decreased expression of various collagen genes (*col-101, col-122, col-154, col-155, col-157, col-179, col-184, col-92, col-93*) in old animals (Golden et al. 2008) as well as of various immune response genes (Youngman et al. 2011). Interestingly, in our dataset we also reported the downregulation of the *bcat-1* gene during physiological ageing which has been found to be consistently downregulated in multiple species (Mansfeld et al. 2015).

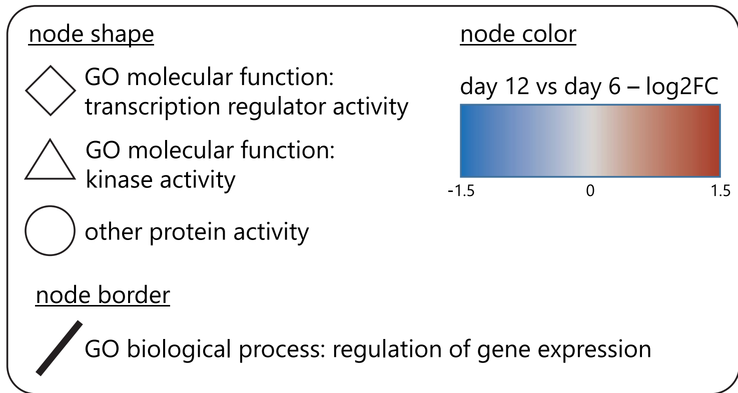
Intriguingly, the majority of genes that were upregulated in our dataset are strongly related to development and more specifically to the nervous system development (Table 2), albeit the lack of eggs at this time point. An upregulation in genes of the nervous system with ageing, has previously been noted by Lund et al. but this increase was not further evaluated, as it was considered an artifact due to the method of data normalization (Lund et al. 2002).

Y69H2.3 sem-5 let-60 par-2 lin-36 mel-47 arcp-1 hsp-4 let-413 unc-29 cfp-1 pid-3 rrc-1 tost-1 smk-1 let-756 egl-9 erh-2 lec-1 pas-4 sel-12 cdc-42 dhc-1 let-7 sacy-1 lin-12 sip-1 hsp-16.2 nmad-1 lgg-1 lgl-1 dep-1 igdb-2 dsh-1 alp-1 sod-3 gpdh-1 dve-1 sgt-1 glh-1 daf-10 ztf-1 par-3 T04H1.2 hsp-6 itsn-1 abu-15 pat-12 pgl-1 zen-4 vps-45 akir-1

lin-53 smo-1 let-711 clk-1 dcr-1 spe-39 gld-1 rde-8 atx-2 wdr-5.1 mrg-1 ztf-2 swsn-2.2 mab-5 csr-1 ain-1 daf-7 unc-62 lin-41 mig-5 hmg-4 rde-10 lst-3 eri-1 cdc-48.2 alg-1 ced-3

skn-1 unc-30 mab-3 elt-2 daf-16 nhr-49 dmd-8 mec-3 sbp-1 nhr-111 glp-1 lin-39 vab-3 unc-86 hbl-1 elt-1 eya-1 pha-4 daf-12 pop-1 daf-3 bar-1

daf-2 let-23 mbk-2 src-1 pmk-1 ckb-2 dlg-1 ire-1 egl-15



◀**Fig. 5** Topological network of the 110 candidate ageing regulators. The shape and color of the nodes is explained in the visual legend box. Nodes with bold borders are associated with the GO biological process "*regulation of gene expression*" (GO:0010468)

Nevertheless, experimental findings on the nervous system of the aged nematodes have reported neuronal outgrowths as well as beading along the axonal length that appear in middle aged animals and become even more visible in old individuals (Toth et al. 2012; Chew et al. 2013). Thus, the observed increase in transcript abundance of neuronal genes could also be related to the spontaneous age-related neuronal branching as a result of the effort of the organism to compensate for damaging events but further experimentation is needed. Furthermore, we have also found upregulated a number of genes associated with programmed cell death and development such as *ces-2, crn-2, egl-1, pmk-1* and *F54B8.4* in the post-reproductive animals, indicative of apoptotic and necrotic cell death with advanced age in accordance with data from Golden and colleagues (Golden et al. 2008). Additionally, the collagen-regulating genes *dpy-17* and *sqt-3*, previously reported to be upregulated in older animals (Golden et al. 2008; Herndon et al. 2017), were also upregulated in our dataset.

The ageing theory of antagonistic pleiotropy suggests that genes with an advantageous effect for the organismal development, which are normally silenced after a certain developmental stage, might have adverse late-life effects if they become dysregulated (Martin 2007; Pincus and Slack 2008). In support of this theory, Budovskaya et al. described a dysregulation in the GATA transcription factors circuit in aged animals which normally play a role in development (Budovskaya et al. 2008). Furthermore, many known longevity regulators are highly associated with developmental processes; genetic perturbations targeting developmental genes have resulted in the identification of longevity regulators (Curran and Ruvkun 2007; Budovsky et al. 2009; Tacutu et al. 2012). Consequently, our own observations might be a result of the modulation of the expression of development-related genes with age, integrating adaptive and maladaptive cues.

Previous studies have started with gene lists of known longevity regulators to create interaction networks and to identify novel longevity regulators (Witten and Bonchev 2007; Li et al. 2010; Tacutu et al. 2012). In our study, we followed a different approach. We focused on the gene expression changes observed in ageing after analyzing two similar ageing datasets of wt nematodes undergoing ageing and constructed the network of differentially expressed genes and their first neighbors in ageing using the interactions provided in the WormBase database (Harris et al. 2020). Analysis and filtering of this network resulted in the selection of 110 candidate regulators; the chosen nodes had to interact with at least 7 genes that were differentially expressed in our dataset. Notably, the most enriched term associated with the candidate genes was the GO biological process "*developmental process*" which as briefly discussed, correlates with the antagonistic pleiotropy theory. Consequently, it is possible that the related candidate genes might have a significant role in ageing regulation (Tacutu et al. 2012). Such genes may be the ones that have a distinct role during development but also during age-related diseases such as *swsn-2.2*, a chromatin remodeling factor required for proper cell divisions during early development (Ertl et al. 2016) that has been proposed to regulate cellular senescence in humans (He et al. 2017). Other genes include the *atx-2* gene with a role in development in early life (Kiehl et al. 2000) and neurodegeneration later in life (Rosas et al. 2020) and the heterochronic gene *lin-41* (Tocchini et al. 2014; Azzi et al. 2020) that is also associated with age-related diseases (Niwa et al. 2008).

Forty two of these candidate regulators (which is 38.2% of candidate genes) already have an established role in ageing or have been shown to affect the nematode's lifespan (1st group) either directly (39 genes) or in a synergistic way (3 genes: *gpdh-1*, *sacy-1*, *hsp-16.2*). The transcription factors *daf-16, skn-1, pha-4, nhr-49* (Hsu et al. 2003; Van Gilst et al. 2005; Bishop and Guarente 2007; Panowski et al. 2007) together with chromatin remodeling factors *wdr-5.1* and *lin-53* (Greer et al. 2010; Müthel et al. 2019), the apoptosis controlling protease *ced-3* (Curran and Ruvkun 2007), ubiquitin related genes *lgg-1* and *smo-1* (Tóth et al. 2008; Princz et al. 2020), the important factor for post transcriptional gene silencing *dcr-1* (Mori et al. 2012) and the stress response proteins *sip-1, smk-1* (Hsu et al. 2003; Samuelson et al. 2007) are included in this group validating our microarray data and network analysis. It is also worth

Table 2 Functional enrichment analysis results of the 110 genes identified as candidate regulators of ageing utilizing the Cytoscape StringApp

GO term	Description	FDR	Genes
GO.0032502	Developmental process	1.15E-50	*abu-15, ain-1, alg-1, alp-1, atx-2, bar-1, cdc-42, cdc-48.2, ced-3, cfp-1, clk-1, csr-1, daf-12, daf-16, daf-2, daf-3, daf-7, dcr-1, dep-1, dhc-1, dlg-1, dve-1, egl-15, elt-1, elt-2, eri-1, eya-1, gld-1, glh-1, glp-1, hbl-1, hsp-4, ire-1, let-23, let-413, let-60, let-711, let-756, lgg-1, lin-12, lin-36, lin-39, lin-41, lin-53, mab-3, mab-5, mbk-2, mec-3, mel-47, mig-5, mrg-1, nhr-49, par-2, par-3, pat-12, pgl-1, pha-4, pmk-1, pop-1, sbp-1, sel-12, sem-5, sip-1, skn-1, smo-1, spe-39, src-1, unc-30, unc-62, unc-86, vab-3, wdr-5.1*
GO.0061063	Positive regulation of nematode larval development	1.48E-12	*bar-1, clk-1, daf-10, daf-12, daf-16, daf-3, dep-1, let-23, let-60, lin-39, pop-1*
GO.0010608	Posttranscriptional regulation of gene expression	5.05E-12	*ain-1, alg-1, csr-1, dcr-1, eri-1, gld-1, let-711, lin-41, pha-4, rde-10, rde-8, skn-1, unc-62*
GO.0051293	Establishment of spindle localization	5.71E-11	*atx-2, bar-1, cdc-42, dhc-1, let-711, lgl-1, mig-5, par-2, par-3, src-1*
GO.0033554	Cellular response to stress	5.63E-10	*abu-15, bar-1, cdc-48.2, daf-16, dve-1, hmg-4, hsp-4, hsp-6, ire-1, nhr-49, pmk-1, skn-1, smk-1, sod-3*
GO.0009266	Response to temperature stimulus	1.30E-08	*atx-2, daf-12, daf-16, daf-2, daf-7, hsp-16.2, sel-12, skn-1, sip-1*
GO.0009952	Anterior/posterior pattern specification	1.98E-08	*csr-1, lin-39, mab-5, mbk-2, par-3, pgl-1, src-1, vab-3*
GO.0060795	Cell fate commitment involved in formation of primary germ layer	3.96E-07	*eya-1, mig-5, pop-1, skn-1, src-1, unc-62*
GO.0034329	Cell junction assembly	1.18E-06	*bar-1, dlg-1, let-413, lst-3, pat-12*
GO.0019098	Reproductive behavior	1.22E-05	*bar-1, daf-12, egl-15, eya-1, hbl-1, lin-12, sel-12*
GO.0007517	Muscle organ development	1.26E-05	*egl-15, glp-1, let-60, pop-1, sem-5*
GO.0032880	Regulation of protein localization	1.66E-05	*ain-1, atx-2, daf-2, dhc-1, egl-9, let-60, pmk-1*
GO.0048806	Genitalia development	2.16E-05	*alg-1, bar-1, let-23, lin-53, sem-5*
GO.0031329	Regulation of cellular catabolic process	3.93E-05	*daf-2, gld-1, lgg-1, mbk-2, pha-4*
GO.0010942	Positive regulation of cell death	9.01E-05	*ced-3, daf-16, eya-1, ire-1, lgg-1*
GO.0071363	Cellular response to growth factor stimulus	1.50E-04	*daf-3, daf-7, egl-15, let-756*
GO.0042127	Regulation of cell population proliferation	1.80E-04	*atx-2, gld-1, glp-1, mab-5*
GO.0007059	Chromosome segregation	2.80E-04	*atx-2, csr-1, eri-1, mel-47, smo-1, zen-4*
GO.0036293	Response to decreased oxygen levels	4.50E-04	*egl-9, ire-1, sbp-1*
GO.0009967	Positive regulation of signal transduction	5.20E-04	*erh-2, ire-1, mig-5, sel-12, sem-5*
GO.0006366	Transcription by RNA polymerase II	5.40E-04	*elt-1, elt-2, glp-1, mec-3, sbp-1*
GO.1901214	Regulation of neuron death	5.80E-04	*ced-3, pmk -1, skn-1*

Table 2 continued

GO term	Description	FDR	Genes
GO.0047496	Vesicle transport along microtubule	9.60E-04	*dhc-1, zen-4*
GO.0008544	Epidermis development	0.0012	*elt-1, lin-41, mig-5*
GO.0006796	Phosphate-containing compound metabolic process	0.0033	*ckb-2, clk-1, daf-2, dep-1, dlg-1, egl-15, erh-2, eya-1, gpdh-1, ire-1, let-23, mbk-2, pmk-1, src-1*
GO.0034514	Mitochondrial unfolded protein response	0.0042	*dve-1, hsp-6*
GO.0060290	Transdifferentiation	0.0048	*cfp-1, wdr-5.1*
GO.0045807	Positive regulation of endocytosis	0.0284	*cdc-42, itsn-1*

The resulting enriched GO biological processes have an FDR value < 0.05 and were filtered for redundant terms with a maximum Jaccard similarity index < 0.15

mentioning that when we lower the filtering threshold for selection (i.e. requiring fewer interactions with differentially expressed genes), more known longevity regulators such as *hif-1* (Zhang et al. 2009), *elt-6* (Budovskaya et al. 2008), *daf-4* (Shaw et al. 2007), *jun-1* (Uno et al. 2013), *atp-2* (Tsang et al. 2001) gradually appear in our list. Nevertheless, the stricter filtering threshold was applied, to highlight genes with a higher probability to represent important regulators.

Genes in the 2nd group are quite well characterized, essential for the animals physiology that have been mostly associated with important physiological processes such as stress resistance and immune response [*eya-1* (Wang et al. 2014), *lec-1* (Takeuchi et al. 2013)], DNA damage and epigenetic changes [*mrg-1* (Miwa et al. 2019), *itsn-1* (Edifizi et al. 2017)], reproduction [*gmeb-4* (Kulkarni et al. 2012), *fkh-6* (Chang et al. 2004), *nmad-1* (Wang et al. 2019), *spe-39* (Zhu and L'Hernault 2003)], cell death [*cdc-42* (Neukomm et al. 2014)], cell division [*par-3* (Feldman and Priess 2012)] and cell communication [*let-413* (Liu et al. 2018), *pat-12* (Hetherington et al. 2011), *dlg-1* (Firestein and Rongo 2001)]. Most of these genes are also linked to development and cell fate and therefore, they represent significant candidates that need to be further considered in the context of ageing.

The 3rd group features the most interesting set of genes as they have not been extensively studied nor have been linked to ageing before. These are the genes that our analysis proposes as potential ageing regulators. More specifically, the transcription factors *dmd-8* and *nhr-111*, along with the putative transcription factors *ztf-1 and ztf-2*, genes encoding for uncharacterized proteins in *C. elegans* such as *T04H1.2, Y69H2.3* and *abu-15* appear in our list and are qualified for further experimentation. Finally, interesting genes linked to environment sensing such as the chromatin remodeling protein *swsn-2.2* (Ertl et al. 2016), absence of which has been linked to increased embryonic lethality (Large and Mathies 2014), genes associated with the development of the nervous system such as the recently characterized dendritic scaffolding protein *arcp-1* (Beets et al. 2020) and the members of the newly identified PETISCO complex *pid-3*, *erh-2*, *tofu-6* and *tost-1* (Cordeiro Rodrigues et al. 2019) are also highlighted by our analysis.

Interestingly, human orthologs of several of these genes are associated with processes related to ageing while others are interesting candidates for future studies in humans. The proteasome subunits PSMA7, PSMA8 are parts of the 20S core proteasome complex with an established role in protein quality control and ageing (Chondrogianni et al. 2019). Similarly, the gene SGTA encodes for a co-chaperone that binds to misfolded proteins and it is also important for protein quality control (Benarroch et al. 2019). The ZNF655 gene is characterized as a transcription factor and has been recently associated with Alzheimer's disease in a whole exome sequencing study (Bis et al. 2020), while the ALKBH4 dioxygenase is believed to act as a regulator of actomyosin-processes during cell division and migration, processes with a distinct role in ageing

Table 3 Sorting of the resulting 110 candidate genes into groups according to their current association with ageing in *C. elegans*

	Symbol	WormBase ID	Pubmed ID	HGNC symbol
1st Group	ain-1	WBGene00015547	18006689	
	bar-1	WBGene00000238	15905404	CTNNB1, JUP
	ced-3	WBGene00000417	17411345	CASP2, CASP3, CASP7, CASP14, CASP9, CASP1, CASP5, CASP4, CASP12, CASP8
	clk-1	WBGene00000536	12709403	COQ7
	daf-10	WBGene00000906	10617200	IFT122
	daf-12	WBGene00000908	16626392	NR1H4, NR1H3, RARB, NR1H2, RARA, THRB, RARG, NR1I2
	daf-16	WBGene00000912	7789761	FOXO3, FOXO1, FOXO4, FOXO6
	daf-2	WBGene00000898	17411345	IGF1R, INSRR, INSR
	daf-3	WBGene00000899	17900898	SMAD4
	daf-7	WBGene00000903	17900898	GDF11, MSTN, INHBA, INHBB
	dcr-1	WBGene00000939	22958919	DICER1
	dve-1	WBGene00022861	21215371	
	egl-9	WBGene00001178	19372390	EGLN1, EGLN3, EGLN2
	elt-1	WBGene00001249	18662544	GATA3, GATA1, GATA2, TRPS1, GATA4, GATA6
	elt-2	WBGene00001250	18662544	TRPS1
	eri-1	WBGene00001332	18006689	ERI1
	gld-1	WBGene00001595	30125273	QKI
	glp-1	WBGene00001609	11799246	NOTCH3, NOTCH2, NOTCH1, NOTCH4, EYS, NOTCH4, NOTCH4, NOTCH4, JAG1, DLL4, CRB1, CRB2, JAG2, DLL1, SNED1, FBN2, FBN3, FBN1, NOTCH4, NOTCH4, NOTCH4
	gpdh-1	WBGene00009824	30247515	GPD1L, GPD1
	hmg-4	WBGene00001974	23144747	SSRP1
	hsp-16.2	WBGene00002016	12845331	CRYAB, HSPB6, HSPB1, HSPB8, CRYAA, HSPB3, HSPB2, HSPB2-C11orf52
	hsp-4	WBGene00002008	16741121	HSPA5, HSPA8, HSPA2, HSPA6, HSPA1B, HSPA1A, HSPA1L, HSPA1B, HSPA1A, HSPA1B, HSPA1B, HSPA1B, HSPA1A, HSPA1A, HSPA1A, HSPA1L, HSPA1L, HSPA1L, HSPA1L
	hsp-6	WBGene00002010	11959102	HSPA9
	ire-1	WBGene00002147	20460307	ERN1, ERN2
	let-23	WBGene00002299	20497132	ERBB3, ERBB2, EGFR, ERBB4
	let-60	WBGene00002335	16164423	KRAS, NRAS, HRAS, HRAS, ERAS, RRAS2
	let-711	WBGene00002845	21723504	CNOT1
	lgg-1	WBGene00002980	19469880	GABARAP, GABARAPL1, GABARAPL2
	lin-53	WBGene00003036	31397537	RBBP4, RBBP7
	nhr-49	WBGene00003639	15719061	HNF4A, HNF4G
	pgl-1	WBGene00003992	11799246	
	pha-4	WBGene00004013	18804378	FOXA1, FOXA2, FOXA3
	pmk-1	WBGene00004055	20520844	MAPK14, MAPK11, MAPK13, MAPK12
	sacy-1	WBGene00019245	18006689	DDX41
	sem-5	WBGene00004774	17411345	GRB2, GRAP, GRAPL
	sip-1	WBGene00004798	12750521	CRYAA, HSPB6, HSPB1, CRYAB, HSPB8, HSPB3, HSPB2, HSPB2-C11orf52

Table 3 continued

	Symbol	WormBase ID	Pubmed ID	HGNC symbol
	skn-1	WBGene00004804	17538612	NFE2L3, NFE2L1, NFE2L2, NFE2
	smk-1	WBGene00018285	16530049	PPP4R3A, PPP4R3B, PPP4R3CP
	smo-1	WBGene00004888	32968203	SUMO1, SUMO3, SUMO2
	sod-3	WBGene00004932	12845331	SOD2
	unc-62	WBGene00006796	17411345	MEIS2, MEIS1, MEIS3, PKNOX1, PKNOX2
	wdr-5.1	WBGene00006474	22012258	WDR5, WDR5B
2nd Group	akir-1	WBGene00017088	31767636	AKIRIN2, AKIRIN1
	alg-1	WBGene00000105	31073019	AGO2, AGO1, AGO3, AGO4
	alp-1	WBGene00001132	19261811	LDB3, PDLIM5, PDLIM7, PDLIM1
	atx-2	WBGene00000231	30989774	ATXN2L, ATXN2
	cdc-42	WBGene00000390	27861585	CDC42
	cdc-48.2	WBGene00008053	16647269	VCP, SPATA5L1
	cfp-1	WBGene00009924	31602465	CXXC1
	csr-1	WBGene00017641	29579041	
	dep-1	WBGene00009717	15901674	PTPRB, PTPRO, PTPRH, PTPRJ
	dhc-1	WBGene00000962	30262881	DYNC1H1, DYNC2H1, DNAH9, DNAH6, DNAH7, DNAH5, DNAH11, DNAH1, DNAH8, DNAH3, DNAH12, DNAH2, DNAH14, DNAH17, DNAH10
	dlg-1	WBGene00001006	22510987	DLG1, DLG4, DLG2, DLG3
	dsh-1	WBGene00001101	26460008	DVL2, DVL1, DVL3, DIXDC1
	egl-15	WBGene00001184	11689700	FGFR3, FGFR2, FGFR1, FGFR4
	eya-1	WBGene00001377	25108328	EYA1, EYA2, EYA4, EYA3
	glh-1	WBGene00001598	32245789	DDX4
	hbl-1	WBGene00001824	31597658	REST, ZNF142, ZNF462, ZNF827, ZNF513, ZNF507, ZSCAN10
	itsn-1	WBGene00006405	18298590	ITSN1, ITSN2
	lec-1	WBGene00002264	23935187	LGALS9, LGALS9B, LGALS9C, LGALS4, LGALS8, LGALS4, LGALSL, LGALS3, LGALS7B, LGALS7, GRIFIN
	let-413	WBGene00002632	10878806	SCRIB, LRRC1, LRRC7, ERBIN
	let-7	WBGene00002285	26399619	
	let-756	WBGene00002881	16672054	FGF1, FGF2, FGF10, FGF20, FGF14, FGF9, FGF12, FGF13, FGF5, FGF7, FGF3, FGF16
	lgl-1	WBGene00018987	23536568	LLGL2, LLGL1
	lin-12	WBGene00003001	29950427	NOTCH3, NOTCH2, NOTCH1, NOTCH4, NOTCH4, NOTCH4, EYS, NOTCH4, NOTCH4, NOTCH4, NOTCH4, JAG1, DLL4, CRB1, CRB2, JAG2, DLL1, SNED1, FBN2, FBN3, FBN1
	lin-36	WBGene00003021	10393123	
	lin-39	WBGene00003024	27475488	HOXA5, HOXB5, HOXD4, HOXA4, HOXC4, HOXC5, HOXC6, HOXB4, HOXA6, HOXB6, HOXA7, HOXB7
	lin-41	WBGene00003026	26764090	TRIM71
	mab-3	WBGene00003100	20498281	DMRT1, DMRTB1
	mab-5	WBGene00003102	26863303	HOXA5, HOXB5, HOXD4, HOXA4, HOXC4, HOXA7, HOXB7, HOXB4, HOXA3, HOXB3, HOXD3, PDX1, GSX1, GSX2, HOXA6, HOXB6, HOXC5, HOXC6, HOXC8, HOXB8

Table 3 continued

	Symbol	WormBase ID	Pubmed ID	HGNC symbol
	mbk-2	WBGene00003150	14634695	DYRK2, DYRK3, DYRK4
	mec-3	WBGene00003167	9735371	
	mig-5	WBGene00003241	16899238	DVL2, DVL1, DIXDC1, DVL3
	mrg-1	WBGene00003406	22212480	MORF4L1, MORF4L2
	par-2	WBGene00003917	31636075	
	par-3	WBGene00003918	28615321	PARD3B, PARD3, LNX1, PDZD2, LNX2, IL16
	pat-12	WBGene00003936	21130760	
	pop-1	WBGene00004077	31740621	TCF7L2, TCF7L1, TCF7, LEF1
	rde-10	WBGene00021634	22508728	
	sbp-1	WBGene00004735	28379943	SREBF1, SREBF2
	sel-12	WBGene00004769	12413907	PSEN1, PSEN2
	src-1	WBGene00005077	16024786	FYN, YES1, SRC, FGR, HCK, BLK, LCK, LYN, FRK
	unc-29	WBGene00006765	9221782	CHRNA3, CHRNA4, CHRNE, CHRNB4, CHRNA2, CHRND, CHRNA1, CHRNB3, CHRNA6, CHRNB2, CHRNA5, CHRNB1, CHRNG
	unc-30	WBGene00006766	11517269	PITX2, PITX1, PITX3
	unc-86	WBGene00006818	21656875	POU4F3, POU4F1, POU4F2
	vab-3	WBGene00006870	30890567	PAX6
	vps-45	WBGene00016643	17235359	VPS45
	zen-4	WBGene00006974	9693365	KIF23
3rd Group	abu-15	WBGene00004099		KRTAP9-1
	arcp-1	WBGene00009375	31757604	
	ckb-2	WBGene00000512	12758145	CHKB, CHKA, CHKB-CPT1B
	dmd-8	WBGene00020708		
	erh-2	WBGene00009444	31147388	ERH
	igdb-2	WBGene00007736	28803967	
	lst-3 (ccar-1)	WBGene00003085	30003683	CCAR1, CCAR2
	mel-47 (tofu-6)	WBGene00017132	31147388	
	nhr-111	WBGene00003701	22690911	
	nmad-1	WBGene00017304	31283754	ALKBH4
	pas-4	WBGene00003925	24563851	PSMA7, PSMA8
	pid-3	WBGene00021270	31147388	
	rde-8	WBGene00022620	25635455	
	rrc-1	WBGene00009800	17434147	ARHGAP33, ARHGAP32, ARHGAP31, ARHGAP30
	sgt-1	WBGene00019893	18187053	SGTA, SGTB
	spe-39	WBGene00004975	19109425	VIPAS39
	swsn-2.2	WBGene00015971	26739451	SMARCD1, SMARCD3, SMARCD2, ASIC2, ASIC1
	T04H1.2	WBGene00011449		GTPBP1, GTPBP2
	tost-1	WBGene00016449	31147388	
	Y69H2.3	WBGene00013481		
	ztf-1	WBGene00018833		
	ztf-2	WBGene00008762		ZNF655

The resulting genes are either known regulators of *C. elegans* lifespan (1st Group), studied and associated with other physiological processes (2nd Group) or relatively unstudied genes that have not been linked to ageing (3rd Group)

and cellular senescence (Li et al. 2013). Furthermore, intriguing candidate orthologs such as the KRTAP9-1 gene which is associated with keratinization (Shimomura and Ito 2005) and VIPAS39 that takes part in post-translational modification of proteins in the endosomes (Tornieri et al. 2013) have been revealed; further experimentation is needed.

In our study, we have used one of the best performing and more popular Affymetrix microarray platforms (Irizarry et al. 2005; Liu et al. 2012). Even though RNA sequencing is now becoming a more popular technology as it enables the identification of unknown transcripts and provides a wider quantitative range of expression changes, microarrays technology remains much easier to use, without the need for large-scale sequencing and it allows the simultaneous quantification of thousands of genes from multiple samples. Moreover, it was thought to be the best option to quantify gene expression changes and compare them with already existing datasets (Portman 2006; Golden and Melov 2007). The rather small sample size of our dataset is an important limitation in our study. Nevertheless, the filtering of genes with low expression values across the samples and the use of an empirical Bayes method utilizing “limma” for the differential expression analysis that borrows information across genes, helped attenuating this limitation (Ritchie et al. 2015), that however should be taken into consideration.

In conclusion, our study provides a better insight on the genes and molecular processes that change with age in the wt nematodes. The combination of the microarray analysis data with network analysis enabled the detection of regulators that might be pivotal ageing regulators. With the unbiased selection of these candidate regulators in ageing, based solely on the transcriptional analysis and the network interactions from WormBase, we provide candidate targets for future experimental studies.

Acknowledgements We acknowledge financial support from the European Union and Greek national funds through the Operational Program Competitiveness, Entrepreneurship and Innovation, under the call RESEARCH—CREATE—INNOVATE (project code: T1EDK-01610) to NC. BioInfoMiner is a product developed by e-NIOS Applications PC, for the automated functional interpretation of various omics data streams.

Author contributions FA, KV and NC contributed to the study conception and design. Material preparation and data collection were performed by NP. Data analysis was performed by FA. NC, KV and AC supervised the research. FA and NC wrote the manuscript, review and editing were performed by all authors. All authors read and approved the final manuscript.

Funding Financial support was received from the European Union and Greek national funds through the Operational Program Competitiveness, Entrepreneurship and Innovation, under the call RESEARCH—CREATE—INNOVATE (project code: T1EDK-01610) to NC. BioInfoMiner is a product developed by e-NIOS Applications PC, for the automated functional interpretation of various omics data streams.

Data availability The dataset supporting the conclusions of this article is available in the GEO repository, under the accession number GSE163253.

Code availability The BioInfoMiner platform is available online at the website https://bioinfominer.com. Cytoscape is a free software available for download at https://cytoscape.org/download.html and all of its plugin applications are available at the Cytoscape App store https://apps.cytoscape.org/. R is available for download at the website https://cran.r-project.org/ and RStudio IDE also available at https://rstudio.com/products/rstudio/download/.

Declarations

Conflict of interest AC is the Chief Executive Officer of e-NIOS Applications PC.

Ethical Approval Compliance with ethical standards.

Consent for publication All authors read and approved the final manuscript.

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