Brief Report

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Gene expression profiling of the L4-stage nematode *Caenorhabditis elegans* following ethanol exposure

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SUMMARY: Previous studies have suggested that low to moderate alcohol exposure can extend *Caenorhabditis elegans* (*C. elegans*) lifespan, but early molecular mechanisms linking ethanol exposure to longevity have not been fully characterized. Here, we investigated how ethanol treatment affects transcriptional networks in L4-stage *C. elegans* by time-resolved RNA-seq. L4-stage *C. elegans* were exposed to 5% ethanol and time-resolved RNA-seq was performed after 1, 4, and 20 hours in solution cultures, followed by differential gene expression and KEGG pathway-based Gene Set Enrichment Analysis. At 1 hour, GSEA analysis showed significant enrichment of genes in the Longevity regulating pathway (worm) with elevated expression. Core-enrichment genes exhibited coordinated upregulation of redox-defense modules, including glutathione S-transferases (*gst*) and p38 MAPK components (*pmk-2/pmk-3*), consistent with upregulation of SKN-1/Nrf2-related stress-response genes. Detoxification (*gpx* and *fmo* families) and lipid-remodeling genes were enriched at 1 hour. By 4 hours, *sod-3*, a canonical DAF-16/FOXO target, was clearly upregulated, suggesting engagement of DAF-16-associated antioxidant responses, whereas Peroxisome and TGF-β signaling pathways were significantly downregulated. Together, these findings provide transcriptomic insights into a temporally structured longevity-associated response to ethanol exposure, in which early detoxification and antioxidant programs, together with membrane-lipid remodeling, are followed by DAF-16-associated gene induction and repression of peroxisome- and development-related signaling. These pathway-level changes highlight candidate biological processes potentially associated with ethanol-linked lifespan modulation in *C. elegans*.

Keywords: *Caenorhabditis elegans*, ethanol exposure, gene expression, signaling pathways, metabolic homeostasis

1. Introduction

The nematode *C. elegans* has long been a powerful model organism for investigating conserved biological processes across species, owing to its highly preserved functional pathways and ease of experimentation. Approximately 40% of *C. elegans* genes have human orthologs, making it an invaluable system for studying mechanisms relevant to human health, including aging (*1-4*).

Research on aging in *C. elegans* has provided significant insights into the molecular pathways that govern lifespan. Key mechanisms identified include the insulin/insulin-like growth factor 1 (IGF-1) signaling (IIS) pathway, TOR signaling, mitochondrial function, and oxidative stress responses. Among these, the IIS pathway has been extensively studied for its conserved role in lifespan regulation. In *C. elegans*, activation of DAF-2, the insulin/IGF-1 receptor, negatively regulates the FOXO transcription factor DAF-16, thereby modulating gene expression related to stress resistance, metabolism, and longevity (*5,6*). Mutations in *daf-2* or its downstream effectors extend lifespan, underscoring the central role of this pathway in aging.

Similarly, mitochondrial function and oxidative stress have emerged as critical determinants of aging. For example, the *mev-1* mutant, which harbors a defect in mitochondrial complex II, produces excessive reactive oxygen species (ROS) and exhibits a markedly shortened lifespan (*7*). Likewise, mutations in genes encoding antioxidant enzymes, such as superoxide dismutase (SOD), exacerbate oxidative damage and reduce longevity (*7*). These foundational findings emphasize the importance of balancing ROS production and antioxidant defense mechanisms to promote cellular health and longevity.

Alcohol consumption presents a complex relationship

with human health, demonstrating both beneficial and detrimental effects. Moderate alcohol intake has been associated with a reduced risk of cardiovascular diseases (*8*) and type 2 diabetes (*9*). In contrast, chronic or excessive alcohol consumption is linked to an increased risk of liver damage, cancer, and neurodegenerative disorders (*10*). At the molecular level, alcohol can influence gene expression, epigenetic landscapes, and metabolic pathways, with effects that may either support health or contribute to disease progression.

C. elegans serves as a useful system for examining the effects of alcohol exposure in a controlled environment. Previous studies have shown that ethanol's impact on lifespan depends on its concentration. Low ethanol concentrations, ranging from 0.005% (1 mM) to 2%, have generally been linked to lifespan extension (*11-13*), whereas higher concentrations (3–5% or ≥ 4%) tend to reduce lifespan (*11,13*). Furthermore, developmental timing plays a crucial role, with varying effects observed when ethanol is administered at the L1 larval stage, during embryonic development, or in young adults.

In our earlier studies, we investigated the effects of ethanol exposure on *C. elegans* lifespan by treating L4-stage worms with 5% ethanol, a concentration modestly higher than that used in most prior reports. Under these conditions, we observed a reproducible, statistically significant extension of lifespan. At that time, mechanistic analyses relied on classical candidate-gene approaches (*e.g.*, RT-PCR and lifespan measurements in selected mutant strains) and were therefore limited in scope, precluding definitive conclusions. Building on this long-standing experimental framework, the present study re-examined the 5% ethanol response using time-resolved RNA-seq to obtain transcriptomic and pathway-level insights into ethanol-associated longevity responses in *C. elegans*.

2. Materials and Methods

2.1. Culture and maintenance of *C. elegans* strains

The wild-type *C. elegans* used throughout this study was the N2 strain obtained from the Caenorhabditis Genetics Center (University of Minnesota). *C. elegans* worms were cultured and maintained as previously described (*7*). All experiments were conducted at 20–22°C unless otherwise stated. *C. elegans* were maintained on nematode growth medium (NGM) agar plates (9 cm) covered with *Escherichia coli* (*E. coli*) OP50 cells as a food source. To obtain synchronized *C. elegans* cultures, reproductive adults bearing eggs were collected by washing with S-buffer (50 mM potassium phosphate buffer, pH 6.0, containing 0.1M NaCl) into 15 mL tubes. After allowing worms to settle for 5 min, the supernatant was aspirated, and the collected worms were suspended in 4.5 mL of S-buffer to which 0.5 mL of 5% sodium hypochlorite and 0.1 mL of 10N NaOH were added and vigorously mixed. After confirming that the worms were dissolved under a stereo microscope, eggs were collected and washed three times with 10 mL of S-buffer by centrifugation at 1300 × g for 30 sec. Collected eggs were then suspended in 3-4 mL of S-buffer in 15 mL tubes and incubated flat at 20°C overnight to allow fertilization of eggs. L1-stage worms were precipitated by centrifugation at 1,300 × g for 30 sec., resuspended in 2-3 mL of S-buffer, and subjected to counting. Approximately 1,500 of them were plated on NGM agar plates (9 cm) covered with *E. coli* OP50 cells (Day 1).

2.2. Administration of Ethanol for Lifespan Experiment

Experimental methods are summarized in Figure 1A. On Day 3, *C. elegans* worms were treated with 200 µL of 1.25 mg/mL fluorodeoxyuridine (FUdR) per 9 cm petri dish to inhibit reproduction by blocking embryo development with FUdR. On Day 4, ten 4-day-old worms were carefully placed on NGM agar covered with *E. coli* OP50 cells in 3 cm petri dishes. A total of ten dishes (100 worms) were used per lifespan experiment. Prior to transferring worms, 180 µL of either 5% ethanol containing 1.25 mg/mL FUdR for ethanol treatments or 180 µL of S-buffer containing 1.25 mg/mL FUdR for controls was added to each dish, and the worms were then transferred into this liquid. The effects of chronic ethanol exposure on *C. elegans* longevity were determined by recording the day each worm died. Worms were classified as dead when they no longer responded to touch with a platinum wire pick. The effects of ethanol on *C. elegans* lifespan were quantified as the ratio of the mean ± SEM of the 50% survival day for ethanol-treated worms to the mean ± SEM of the 50% survival day for control worms. Statistical comparisons between ethanol-treated and control groups were performed using an unpaired Student's *t*-test.

2.3. Total RNA isolation

On Day 3, *C. elegans* worms were exposed to ethanol concentrations of 0% or 5% in S-buffer for 1, 4, or 20 hours at 20°C. Approximately 1,500 *C. elegans* worms at the L4 larval stage that had been cultured on agar plates covered with *E. coli* OP50 cells were harvested by washing the plates with S-buffer, followed by centrifugation. The collected worms were washed with S-buffer to remove residual bacteria. After washing thoroughly, the worms were resuspended in 1 mL of S-buffer and exposed to either 0% or 5% ethanol for 1, 4, or 20 hours at 20°C. After treatment, worms were collected for RNA extraction using TRI Reagent® (Molecular Research Center, Inc., OH, USA), according to the manufacturer's protocol. Collected worms were homogenized in 0.5 mL of TRI Reagent® using a plastic homogenizer. After homogenization, 50 µL of 1-bromo-3-chloropropane (Molecular Research Center, Inc., OH,

USA) was added to each sample, followed by vigorous shaking for 15 seconds. Samples were incubated at room temperature for 15 minutes and centrifuged at 12,000 × g for 15 minutes at 4°C. The RNA-containing aqueous layer was transferred to a new tube and precipitated with 0.5 mL of isopropanol (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), incubated for 30 minutes at room temperature, followed by centrifugation at 12,000 × g for 10 minutes at 4°C. The resulting RNA pellet was washed with 75% ethanol, air-dried, and dissolved in RNase-free water (Ambion, TX, USA).

2.4. RNA-seq analysis

RNA quality and quantity were assessed using the Bioanalyzer System (Agilent Technologies, Inc., CA, USA). Sample pairs that passed the RNA Integrity Number (RIN) evaluation were selected for sequencing library preparation using the KAPA mRNA HyperPrep Kit (Roche, MA, USA). Sequencing was performed on the Illumina NovaSeq 6000 using the S4 Reagent Kit v1.5 (Illumina, CA, USA) in paired-end mode of 2×151 cycles, with the base calling conducted using Real-time Analysis (RTA) software v3.4.4.

For RNA-seq data analysis, sequence reads passing quality check were preprocessed for adaptor and polyA removal using trimmomatic v.0.39 (*14*) and fastp v.0.23.2 (*15*). Reads were then mapped to the *C. elegans* reference genome ce10 using STAR v.2.7.9a (*16*), with an index for gene and transcript annotations. Strand-specific paired-end fragment counts of each gene annotated in RefGene were calculated using the featureCounts function in the Subread package v.2.0.3 (*17*). To enhance accuracy of the gene expression estimates, a total of 15,060 genes with counts per million (CPM) > 1 in at least three samples were selected for downstream analysis. The Bioconductor package edgeR v.3.32.1 (*18*) was applied with a design matrix for testing differential expression between sample groups with different conditions based on quasi-likelihood F-tests. Statistical p-values were adjusted using the Benjamini and Hochberg method. Pre-ranked Gene Set Enrichment Analysis (GSEA) (*19*) was conducted for functional enrichment analysis with gene sets obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG) (*20*). Genes from edgeR results were ranked by the sign of log fold-change (logFC) in combination with $-\log_{10}$ of the p-value for this analysis. GSEA results were visualized using R (v4.4.1) with clusterProfiler v4.12.6 and enrichplot v1.24.4 packages (*21*).

3. Results and Discussion

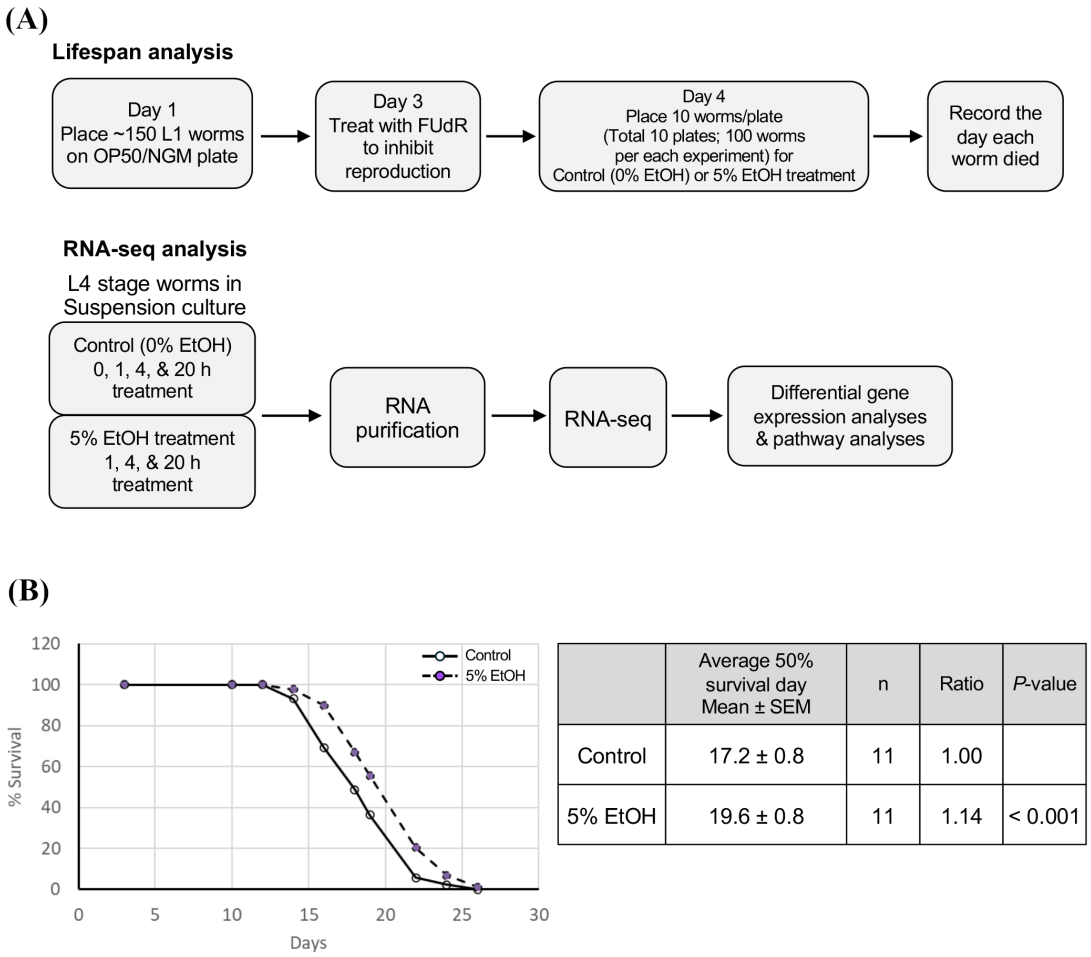
3.1. Observed lifespan extension following 5% ethanol exposure

Figure 1A shows experimental schemes for the lifespan as well as RNA-seq analyses. Lifespan experiments revealed that 5% ethanol treatment extended the lifespan compared with controls as shown in Figure 1B, which presents a representative result showing lifespan extension in 4-day-old wild-type *C. elegans* treated with 5% ethanol. The mean 50% survival day was 17.2 ± 0.8 and 19.6 ± 0.8 (means ± SEM, $n = 11$, $p < 0.001$) for control and ethanol-treated worms, respectively, indicating a 1.14-fold increase in lifespan in response to ethanol treatment (Figure 1B). To minimize food limitation during the lifespan assay, *E. coli* OP50 was allowed to grow to a non-limiting density before the assay. The protocol was also designed so that the ethanol solution covered the worms on the agar for at least 1 hour, thereby maintaining continuous ethanol exposure during the initial treatment period.

Previous studies have reported both beneficial and harmful effects of ethanol exposure on *C. elegans* lifespan, depending on ethanol concentration and the developmental stage at which treatment is administered (*11-13*). In our study, 4-day-old young adults treated with 5% ethanol exhibited a significant increase in lifespan (Figure 1B). As mentioned in the Introduction, low ethanol concentrations have generally been linked to lifespan extension (*11-13*), whereas higher concentrations tend to reduce lifespan (*11,13*). Based on those previous studies, 5% ethanol might have been expected to shorten lifespan; however, we observed a significant lifespan extension under this condition. While we used 5% ethanol (approximately 860 mM), which approaches the ED50 level reported (*22*), we did not observe rapid lethality within the 4-hour exposure time point. To assess whether the *E. coli* food supply may have been affected by the ethanol treatment, *E. coli* OP50 cells were cultured in LB broth containing 5, 10, 15, or 20% ethanol for 2 hours, and OD600 was then measured to estimate surviving *E. coli* cells, which showed a slight reduction to 70%, 65%, 63%, and 58% of the control level, respectively (Data not shown). These data indicate that 5% ethanol did not deplete the food supply and strongly suggest that the observed lifespan extension is not a starvation-mediated effect.

Based on the observed lifespan extension in young adult worms after 5% ethanol exposure (Figure 1B), we next used RNA-seq to explore transcriptomic responses and pathway changes potentially associated with this phenotype. At first, differences in the actual ethanol concentration and exposure time between agar plate culture and solution culture should be carefully considered, as summarized in Table 1. We confirmed that the 5% ethanol solution remained on the agar for more than 1 hour but did not persist overnight, suggesting that the ethanol effect observed in the agar plate assay is most comparable to the 1- to 4-hour exposure period used in the solution-based RNA-seq assay.

3.2. Gene expression patterns following 5% ethanol



	Average 50% survival day Mean ± SEM	n	Ratio	*P*-value
Control	17.2 ± 0.8	11	1.00	
5% EtOH	19.6 ± 0.8	11	1.14	< 0.001

Figure 1. Experimental design and lifespan analysis of wild-type *C. elegans* following 5% ethanol exposure. The effects of ethanol exposure on lifespan and gene expression profiles of *C. elegans* were assessed as described in the Methods. (**A**) Experimental schemes for the lifespan and RNA-seq analyses. For lifespan analysis, 4-day-old wild-type worms were cultured on agar plates in the presence or absence of 5% ethanol, and survival was monitored over time. For RNA-seq analysis, L4-stage worms were incubated in S-buffer under 5% ethanol or ethanol-free control conditions for 0, 1, 4, and 20 hours, followed by RNA extraction and gene expression analysis. **(B)** Representative results on wild-type *C. elegans* N2 strain, showing the observed lifespan extension following 5% ethanol exposure. The survival curves are shown on the left, and the result summary from 11 independent experiment sets is shown on the right.

Table 1. Comparison of two phases of experiment

Aim of experiment	Effects of ethanol on lifespan	Effects of ethanol on gene expression profiles
Culture conditions	On E. coli OP50-covered agar plates under 5% ethanol and FUdR treatment	In S-buffer in the presence or absence of 5% ethanol (no food, no FUdR treatment)
Culture length	27 days	1, 4, and 20* hours
Fully exposed to 5% ethanol	At least 1 hour	1, 4, and 20** hours
Lifespan measurements	Ethanol treatment started on Day 4, then live worms were counted every 2 days	-
RNA-seq experiments	-	RNA was prepared after 1, 4, and 20 hours culture

*At 20 hours in solution culture, eggs were observed in non-ethanol control culture, indicating that despite starvation, worms continued to develop.
**At 20 hours of exposure to 5% ethanol in solution culture, the worms showed retardation of development and weakness most likely due to the toxic effects caused by ethanol exposure.

exposure

In the present study, we re-examined, using RNA-seq profiling, the transcriptomic responses associated with the lifespan phenotype observed in our earlier 5% ethanol studies conducted more than two decades ago. The experimental design is schematically illustrated in Figure 1A. L4-stage *C. elegans* were treated with 5% ethanol or without ethanol (0% control) in S-buffer for 1, 4, and 20 hours, after which total RNA was extracted for sequencing.

First, we generated a Multidimensional Scaling (MDS) plot to visualize similarities and differences in transcriptomic profiles between ethanol-treated and control samples, as well as variation among biological replicates (Figure 2A). The MDS plot revealed distinct clustering of the 0% and 5% ethanol-treated samples, with clear shifts corresponding to treatment duration. For example, in the control group (blue points), the 0-, 1-, and 4-hour samples clustered in close proximity, indicating minimal changes in gene expression. In contrast, the 5% ethanol group (red points) exhibited progressive divergence over time. While the 1-hour sample remained near the untreated control group, the 4-hour and 20-hour samples shifted toward the lower right of the plot, suggesting substantial transcriptomic reprogramming with prolonged ethanol exposure. Notably, the 20-hour sample was positioned furthest from all other samples, indicating extensive gene expression changes. This separation likely reflects starvation and physiological stress, which became more pronounced after more than 4 hours of solution culture. Although worms in the control group laid eggs which were observed at 20 hours solution culture, no eggs were observed in the ethanol-treated group, and the worms showed retardation of development and weakness most likely due to the toxic effects of ethanol exposure.

Volcano plots were used to visualize differentially expressed genes in the ethanol-free time-course analysis (Figure 2B) and in comparisons between 5% ethanol-treated and ethanol-free samples at each time point (Figure 2C). These plots depict statistical significance relative to the magnitude of change (fold change). Consistent with the MDS plot, the number of significantly up- or downregulated genes increased over time. Subsequently, Gene Set Enrichment Analysis (GSEA) was conducted to identify functionally relevant pathways affected by ethanol treatment.

3.3. Time-dependent gene expression changes in the ethanol-free control group

Because the RNA-seq experiments were performed in S-buffer without food, time-dependent effects caused by food deprivation had to be considered when evaluating ethanol-associated gene expression changes. Therefore, we first examined gene expression changes over time in the ethanol-free control group to identify starvation-responsive genes and distinguish them from ethanol-associated changes. To this end, we examined gene expression changes in worms cultured for 1, 4, and 20 hours in the absence of ethanol (Figure 2A; blue diamond, circle and triangle shapes) as compared to the 0-hour control (Figure 2A; black diamond).

We then applied GSEA using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database to evaluate whether a specific pathway was significantly altered under the experimental conditions. As a result, GSEA of ethanol-free worms revealed pronounced early starvation responses at 1 and 4 hours (*vs.* the 0-hour control), with several canonical stress pathways significantly enriched (FDR < 0.05) (Figure 3A). At 1 hour, results showed a strong activation of autophagy, mitophagy, the ubiquitin-proteasome system, and efferocytosis pathways (Figure 3A). These pathways facilitate degradation of unnecessary cellular components and play a crucial role under conditions with limited energy sources. Conversely, metabolic pathways such as fatty acid and amino acid metabolism were downregulated, indicating an adaptive response to reduced energy intake. At 4 hours, pathways related to growth and differentiation, such as TGF-β and Wnt signaling, were activated, suggesting their involvement in worm development (Figure 3A). Notably, autophagy-related pathways exhibited even stronger activation than at 1 hour, consistent with the notion that starvation drives increased cellular breakdown and recycling to conserve energy and resources.

3.4. Transcriptomic pathway changes potentially associated with lifespan modulation after 5% ethanol exposure

To understand the transcriptomic landscape potentially associated with ethanol-linked lifespan modulation, we analyzed RNA-seq data from L4-stage *C. elegans* cultured in S-buffer without food supply at 1, 4, and 20 hours in the presence of 5% ethanol.

First, we performed KEGG-based GSEA (5% ethanol *vs.* time-matched 0% controls; significance defined as FDR < 0.05) to identify early pathway changes following ethanol exposure. At the 1-hour time point, multiple stress-resistance and detoxification programs were significantly upregulated, including the Longevity regulating pathway (worm), Glutathione metabolism, Drug metabolism—cytochrome P450, Metabolism of xenobiotics by cytochrome P450, and Drug metabolism—other enzymes (Figure 3B). Among lipid pathways, Sphingolipid metabolism was also significantly enriched at 1 hour. In contrast, Peroxisome, TGF-β signaling, and Wnt signaling showed downward trends at 1 hour but did not meet the FDR < 0.05 threshold. At 4 hours, Peroxisome, TGF-β signaling, and Wnt signaling were significantly downregulated (FDR

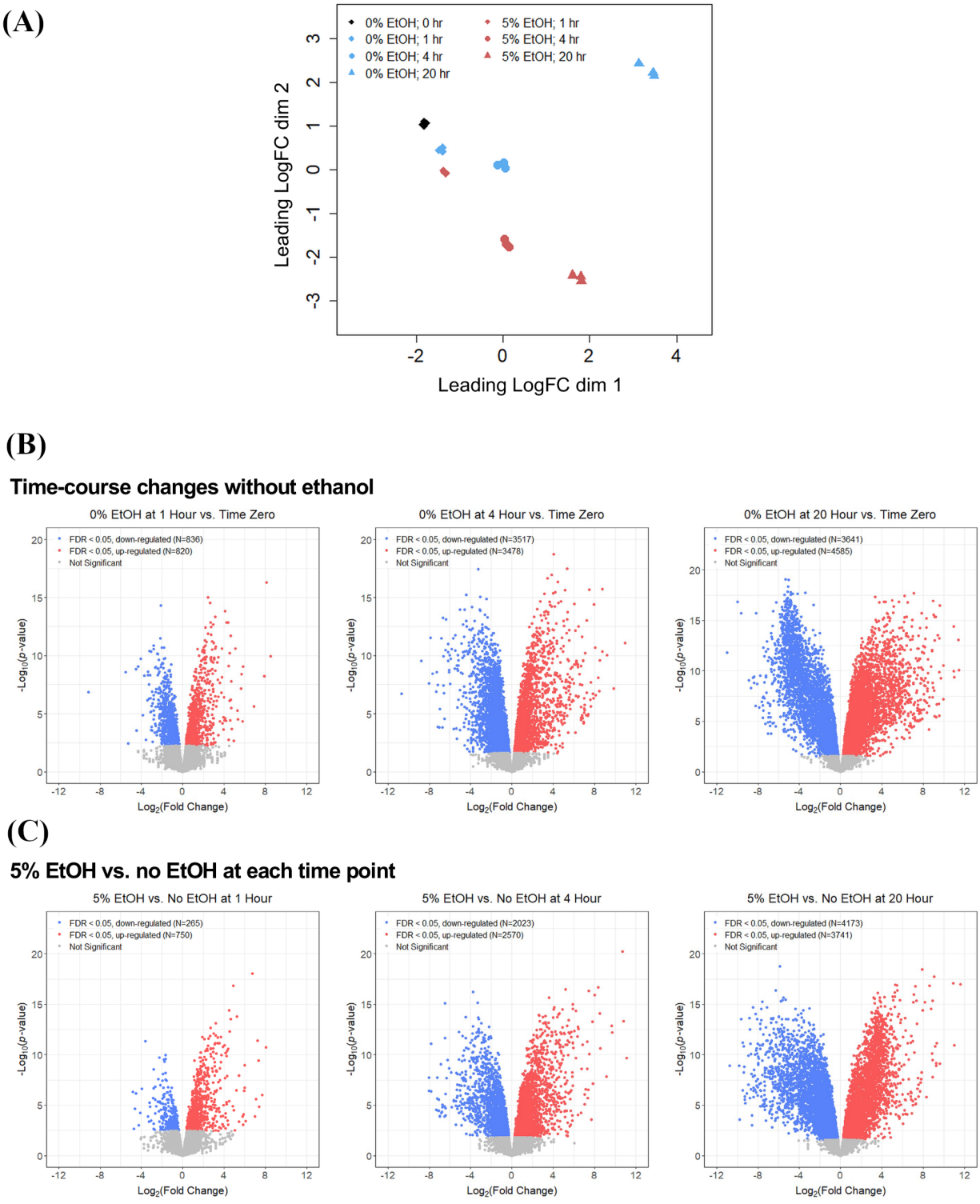
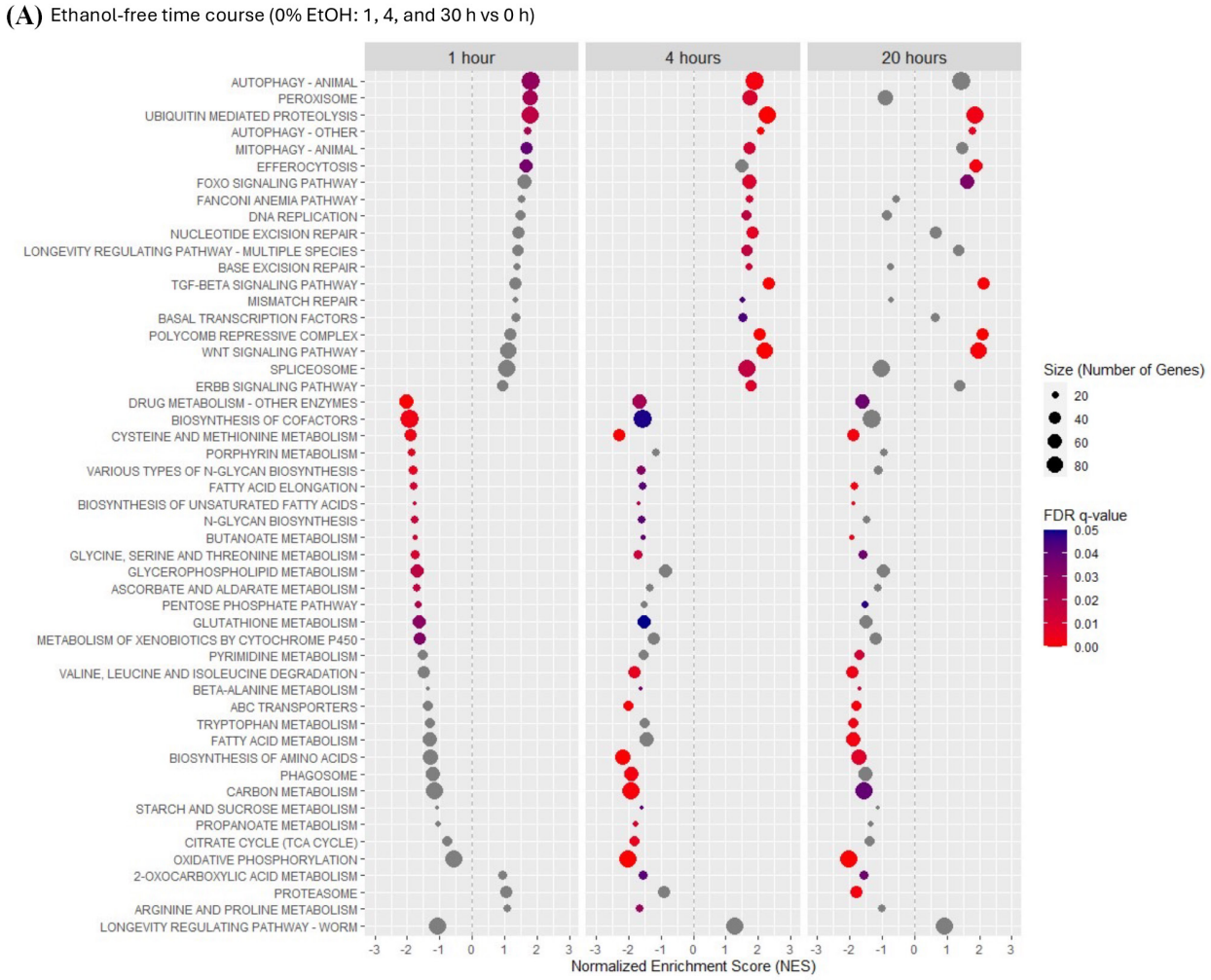


Figure 2. Multidimensional Scaling (MDS) Plot and differential expression analysis (volcano plots). (**A**) MDS plot of distances between gene expression profiles comparing the transcriptional responses of worms under 0% ethanol (control; blue) and worms cultured in the presence of 5% ethanol (treatment; red). Each point represents a sample, differentiated by color or shape according to the designated ethanol treatment throughout time-course experiments. The distance between points reflects the leading log-fold change (logFC) between each pair of samples. Time points are shown at 0 hr (♦, $n = 4$), 1 hr (◊, $n = 3$), 4 hr (○, $n = 3$), 20 hr (Δ, $n = 3$). (**B**) Volcano plots showing differentially expressed genes in the time-course analysis under ethanol-free conditions, in which gene expression at 1, 4, and 20 hours was compared with that at 0 h. (**C**) Volcano plots showing differentially expressed genes between 5% ethanol-treated and ethanol-free samples at 1, 4, and 20 hours. Genes with statistically significant changes are highlighted according to FDR < 0.05 (red, significantly upregulated; blue, significantly downregulated; gray, not significant).



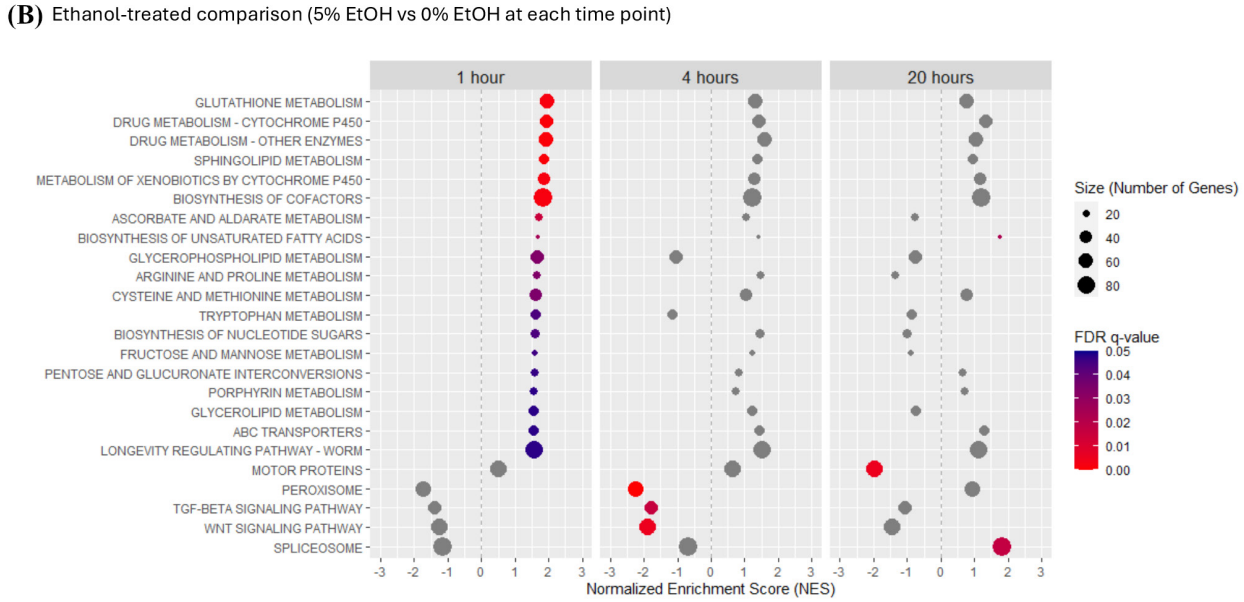
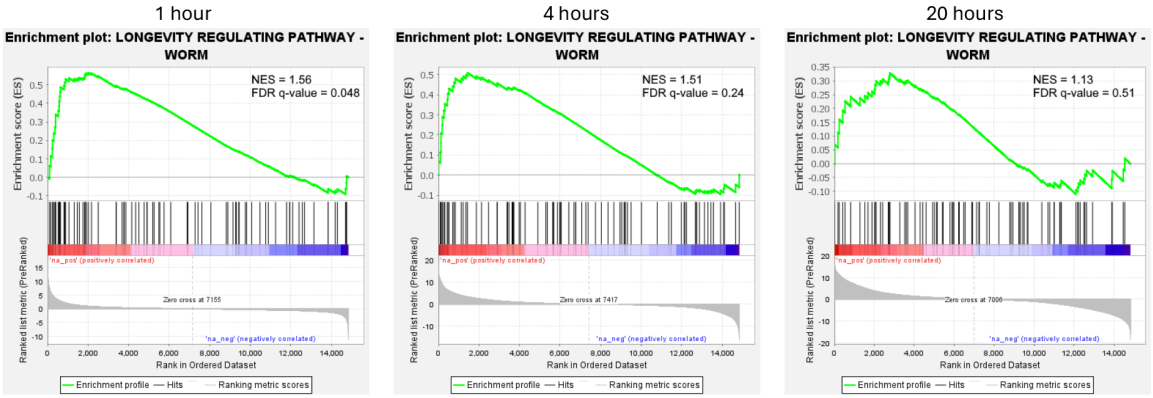
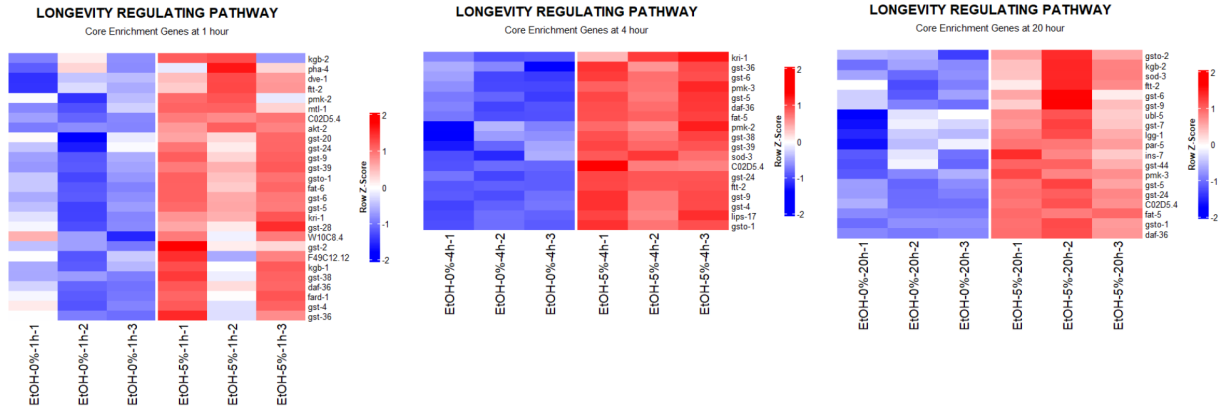


Figure 3. Gene Set Enrichment Analysis (Pathway analysis). (**A**) *C. elegans* functional pathways were analyzed as described in the Methods. Transcripts from worms cultured under ethanol-free conditions for 1, 4, and 20 hours were compared to those at time zero. Functional pathways showing statistical significance (FDR < 0.05) are color-coded as indicated in the legend. (**B**) Transcripts under ethanol conditions (5% *vs.* 0%) were compared at 1, 4, and 20 hours. Functional pathways showing statistical significance (FDR < 0.05) are color-coded as indicated in the legend. Enrichment Plots of the Longevity Regulating pathway generated by two different analyses are shown in (**C**) and (**D**).

(C) Enrichment Plots : Longevity Regulating Pathway



(D) Core enrichment genes: 1, 4, and 20 h



List of Genes upregulated by 5% EtOH treatment

Only at 1h	At 1, 4, & 20h	At 1h & 4h	Only at 4 h	At 4h & 20h	Only at 20h
kgb-2	C02D5.4	gst-36	lips-17	fat-5	gsto-2
pha-4	daf-36	gst-38		pmk-3	kgb-2
dve-1	ftt-2	gst-39		sod-3	ubl-5
mtl-1	gst-24	gst-4			gst-7
akt-2	gst-6	pmk-2			lgg-1
gst-20	gst-5	kri-1			par-5
fat-6	gst-9				ins-7
gst-28	gsto-1				gst-44
W10C8.4					
gst-2					
F49C12.12					
kgb-1					
fard-1					

Figure 3 (continued). Gene Set Enrichment Analysis (Pathway analysis). (**A**) *C. elegans* functional pathways were analyzed as described in the Methods. Transcripts from worms cultured under ethanol-free conditions for 1, 4, and 20 hours were compared to those at time zero. Functional pathways showing statistical significance (FDR < 0.05) are color-coded as indicated in the legend. (**B**) Transcripts under ethanol conditions (5% *vs.* 0%) were compared at 1, 4, and 20 hours. Functional pathways showing statistical significance (FDR < 0.05) are color-coded as indicated in the legend. Enrichment Plots of the Longevity Regulating pathway generated by two different analyses are shown in (**C**) and (**D**).

< 0.05), indicating sustained repression of peroxisomal fatty-acid functions and growth/differentiation signals beyond the acute phase. Pathways that were significant at 1 hour, namely the Longevity regulating pathway, Glutathione metabolism, and P450/drug-metabolism pathways, did not cross the FDR < 0.05 threshold at 4 hours yet remained directionally concordant with their 1-hour profiles. Lipid-associated pathways likewise maintained concordant trends at 4 hours without reaching significance. Taken together, these results show that ethanol exposure is associated with an early, significant activation of longevity-related and detoxification pathways (with accompanying sphingolipid remodeling), followed by significant suppression of peroxisomal and developmental signaling at 4 hours. These pathway-level patterns suggest a time-dependent shift toward

somatic maintenance and energy sparing at the expense of growth, and identify candidate processes that may be related to the observed lifespan phenotype.

3.5 Gene expression and network analyses of longevity-associated and stress-response pathways after 5% ethanol exposure

The longevity-regulating pathway was enriched at 1 hour of 5% ethanol exposure and remained upregulated for up to 20 hours, providing transcriptomic clues to longevity-associated responses after ethanol exposure (Figure 3B). Further examination of the longevity-regulating pathway (Figure 3C and D) revealed that gene activation at 1 hour was the most significant in terms of the number of upregulated genes involved in this pathway, with distinct expression patterns emerging as ethanol exposure duration increased. As shown in the Enrichment plots for ethanol treatment at 1 hour, 4 hours, and 20 hours (Figure 3C), dynamic changes in longevity-regulating pathway activation over time can be observed. Specifically, the plots indicate that the normalized enrichment score (NES) remained positive at all time points, indicating upregulation of genes annotated within longevity-related pathways in response to ethanol treatment. Although the NES value declined at 20 hours, most genes involved in lifespan extension remained elevated. Figure 3D presents gene expression changes for core enrichment genes in the longevity-regulating pathway.

In *C. elegans* treated with 5% ethanol, multiple genes associated with antioxidant and stress responses were significantly upregulated (Figure 3D). Notably, genes within the *gst* family (*gst-6*, *gst-9*, *gst-36*, *etc*.), which encode glutathione S-transferases involved in detoxification of harmful metabolites and toxins generated by oxidative stress within cells, were upregulated at 1 hour ethanol exposure and remained upregulated for up to 20 hours. This indicates rapid activation of cellular detoxification mechanisms in response to ethanol-associated mild oxidative stress. The *sod-3* gene, a canonical DAF-16/FOXO target involved in antioxidant defense, was not significantly induced at 1 hour but was robustly upregulated after 4 and 20 hours of ethanol exposure. Another stress-responsive pathway affected by ethanol treatment was the p38 MAP kinase pathway, including genes exemplified by *pmk-2* and *pmk-3*. These genes regulate cellular defense mechanisms against environmental stressors and infections. Their increased expression following 1 hour of ethanol treatment suggests an ethanol-associated cellular adaptive stress response. After 4 hours of ethanol treatment, these antioxidant and detoxification-related genes were further upregulated. This sequential pattern suggests that initial detoxification responses may be followed by engagement of classical longevity effectors. Such a timeline fits previous models where transient stress activates SKN-1 and DAF-16 pathways to enhance stress resistance and extend lifespan (*23*). In our case, early induction of detoxification genes may prevent oxidative damage, while later DAF-16-mediated antioxidant gene activation (*e.g.*, *sod-3*) may further strengthen cellular resilience.

To further visualize the relationships between ethanol-regulated genes and KEGG pathways, we generated cnetplots for genes altered after 1 hour of 5% ethanol exposure (Figure 4). This analysis was focused on the early phase of ethanol response, because 1 hour represented the most prominent activation of the longevity-related pathway.

The cnetplot analysis revealed a network in which the Longevity regulating pathway (worm) was closely associated with antioxidant, detoxification, and drug metabolism pathways, including cytochrome P450 metabolism, glutathione metabolism, and xenobiotic metabolism (Figure 4A). Genes associated with the upregulated pathways are shown in Figure 4B, whereas downregulated pathways and their associated genes are shown in Figure 4C and D, respectively.

Short-term (1 hour) ethanol treatment notably upregulated genes encoding detoxification enzymes including cytochrome P450s and glutathione S-transferases, consistent with previous findings that mild ethanol exposure induces stress-protective metabolic responses (*13*). The close clustering of longevity-associated and stress response pathways implies coordinated regulation, potentially mediated by conserved transcription factors such as SKN-1/Nrf2 and DAF-16/FOXO. In *C. elegans*, SKN-1 drives detoxification gene expression and extends lifespan under stress, while DAF-16 promotes antioxidant defenses (*23*). Thus, the network topology observed in Figure 4 supports the interpretation that ethanol exposure is associated with a hormetic-like transcriptomic response, in which mild cellular stress may activate cytoprotective longevity programs. Our study provides transcriptomic insights after ethanol exposure and extends previous studies (*24-26*), offering time-resolved pathway-level data that complement the recent study by Sterken *et al*. (*26*), who examined transcriptional responses of *C. elegans* across a time course of ethanol exposure to determine which genes and genetic pathways are regulated in response to ethanol.

Growth-promoting and developmental pathways, including TGF-β signaling, showed downward trends at 1 hour and were significantly downregulated at 4 hours. In *C. elegans*, inhibition of the DAF-7/TGF-β pathway promotes dauer formation and extends lifespan under adverse conditions (*27*), suggesting that downregulation of TGF-β signaling after ethanol exposure may be one candidate pathway associated with a stress-resistant metabolic state and lifespan modulation.

Altogether, these findings indicate that brief ethanol exposure, particularly within the 1- to 4-hour period, is associated with a coordinated transcriptomic response characterized by activation of cytoprotective pathways

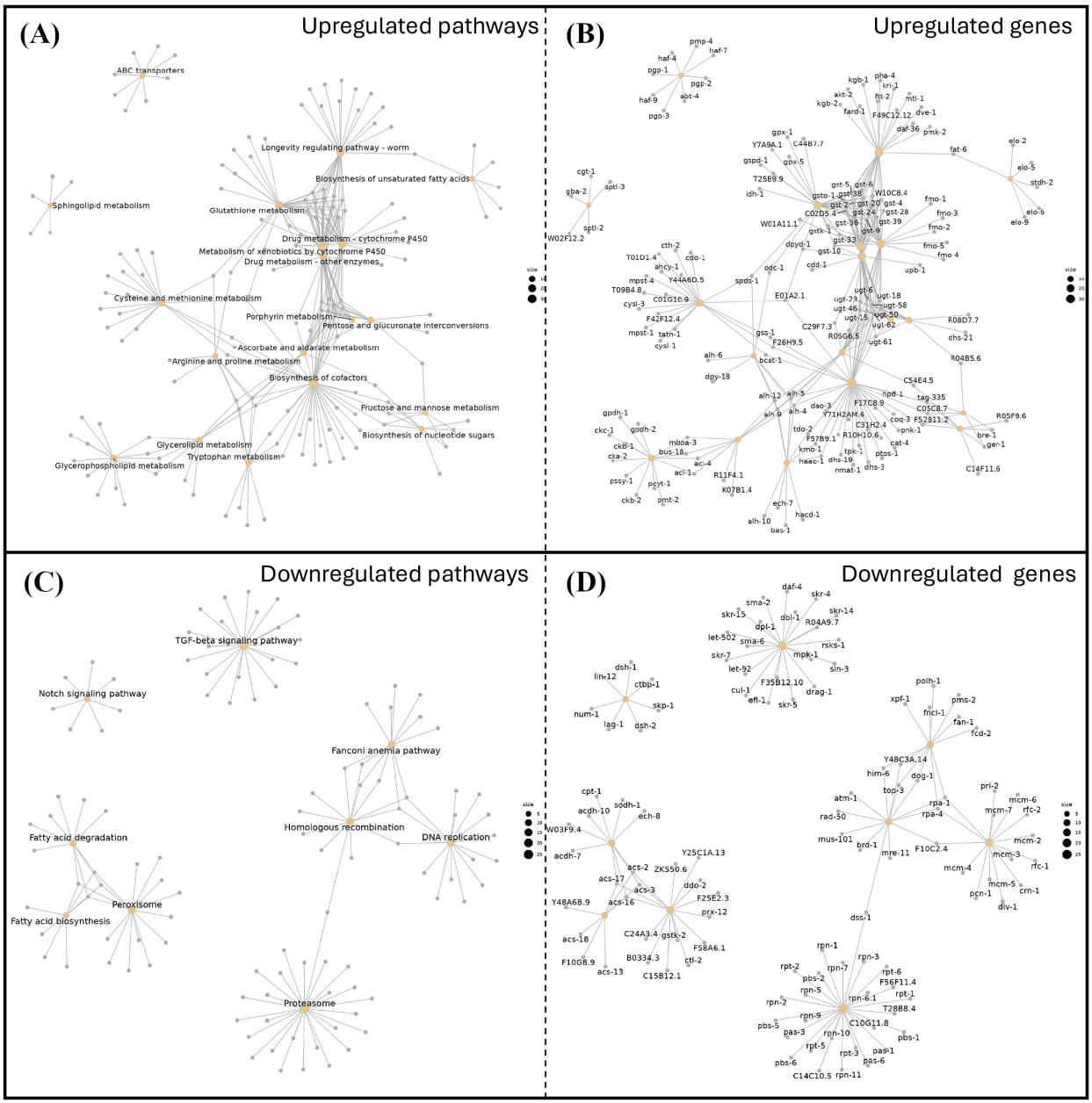


Figure 4. Gene-concept network (cnetplot) of KEGG pathways altered after 1 hour of 5% ethanol exposure in *C. elegans*. The cnetplot depicts linkages of genes and biological concepts (KEGG pathways) as a network. Networks of upregulated pathways (**A**) and corresponding gene names (**B**) as well as networks of downregulated pathways (**C**) and corresponding gene names (**D**) are shown. KEGG pathways (larger yellow nodes) are connected to nodes representing genes (smaller gray nodes) that contribute to each pathway's enrichment.

and suppression of growth-promoting processes. This shift toward stress resistance and metabolic conservation may contribute to, or occur in parallel with, the observed lifespan extension and is consistent with the concept of hormesis, in which mild stress activates protective mechanisms that promote longevity (*23,27*). Future studies are warranted to clarify the mechanistic links between ethanol-induced stress responses and aging.

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