



Geroprotective effects of Salvianolic acid A through redox and detoxification pathway activation in an aging *Drosophila* Alzheimer's model

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Abstract Alzheimer's disease (AD) is characterized by the accumulation of amyloid- β 42 ($A\beta$ 42) neurotoxic peptides that cause oxidative stress and neurodegeneration. The current study examined the neuroprotective properties of salvianolic acid A (SalA), an antioxidant polyphenol, in a *Drosophila melanogaster* model of AD. Transgenic flies expressing human $A\beta$ 42 were assayed for eye morphology, life span, and locomotor function after SalA diet supplementation. RNA-seq and RT-qPCR were used to quantify transcriptional regulation with SalA treatment. $A\beta$ 42 expression resulted in classic AD phenotypes, including retinal degeneration, shortened

lifespan, and compromised climbing ability. Partial rescue of the rough-eye phenotype, significant prolongation of lifespan, and improved locomotor function in aging flies were induced by SalA treatment. Transcriptome profiling showed the upregulation of glutathione metabolism-associated, cytochrome P450 activity-associated, and antioxidant defence-associated genes, while muscle development-associated, cell adhesion-associated, and apoptosis-associated genes were downregulated. Network analysis identified a SalA-responsive gene module enriched in detoxification and immune pathways that was conducive to enhanced cellular resistance to $A\beta$ 42 toxicity. These findings identify a redox-regulated aging mechanism whereby SalA maintains neuronal and systemic homeostasis during aging. SalA inhibits

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A β 42-induced neurotoxicity in *Drosophila* via promoting redox equilibrium and detoxification. These findings present SalA as a potential multi-target lead drug for AD and other age-related neurodegenerative diseases.

Keywords Salvianolic acid A · *Drosophila melanogaster* · Alzheimer's disease · A β 42 · Transcriptomics · Oxidative stress · Neuroprotection · Glutathione metabolism

Introduction

Aging is the single most significant risk factor for late-life neurodegenerative disorders, including Alzheimer's disease (AD). Successive molecular damage, oxidative damage, and proteostatic imbalance are the pathogenetic basis for neuronal dysfunction and cognitive loss. Among these processes, amyloid-beta (A β) aggregation is a feature of age-related neurodegeneration, and A β 42 overexpression in model organisms has become widely employed to study the interface between aging and neurodegenerative processes (Tanzi and Bertram 2005). More than a century of research, however, has only provided therapies that can only temporarily alleviate symptoms, which underscores the potential of upstream-acting interventions to maintain cellular homeostasis during aging.

Salvianolic Acid A (SalA), a polyphenol isolated from the roots of *Salvia miltiorrhiza*, has been characterized in recent years for its antioxidant and anti-inflammatory activity (Wu et al. 2020). The pleiotropic nature of these activities means that SalA may function as an anti-aging molecule increasing neuronal resilience through antidotes to oxidative and metabolic stress, processes intimately tied up with aging biology (Xu et al. 2014).

The fruit fly, *Drosophila melanogaster*, offers a genetically tractable and evolutionarily conserved model for examining age-dependent neurodegeneration. Its short life span, simplicity of breeding, and completely annotated genome enable precise assessment of molecular and behavioural phenotypes of accelerated aging (Tan and Azzam 2017). Transgenic expression of human A β 42 in *Drosophila* recapitulates many aspects of AD disease, including progressive locomotor impairment, reduced lifespan, and transcriptional deregulation, making it an appropriate

system for the study of potential anti-aging interventions such as phytotherapy (Tan et al. 2023c, 2021a).

In this study, we explored the anti-aging and neuroprotective effect of SalA in a model of age-related A β 42-induced neurodegeneration in *Drosophila*. A series of behavioural tests were used to evaluate healthspan-related outcomes: eye morphology, lifespan, and locomotion. RNA sequencing (RNA-seq) was used to identify transcriptomic signatures, and unsupervised learning-based clustering was used to identify coordinated patterns of gene expression related to SalA treatment. This system-level integrated strategy provides insight into how SalA restores behaviour and molecular homeostasis throughout aging, revealing understanding of conserved mechanisms of neurodegenerative decline resilience.

Methods

Compound

SalA (CAS no.: 96574–01–5) was procured from LifeTech solution Venture, Malaysia. stocks were prepared in 100% dimethyl sulfoxide (DMSO) and stored at 4 °C.

Drosophila husbandry

Drosophila lines utilized are documented in Flybase (<http://flybase.bio.indiana.edu>) and attained from Bloomington *Drosophila* Stock Center (Indiana, USA): Oregon-R (Stock5), UAS-A β 42 (Stock33769), GMR-GAL4 (Stock1104), and Actin5C-GAL4 (Stock4414). Stock maintenance was kept at 25 °C whereas crosses were at 29 °C. Humidity was kept at 60% for all. In the eye assay, GMR-A β 42 was utilised as the AD *Drosophila*, while GMR-OreR served as the wild-type Control: GMR-OreR.DMSO (Control), GMR-A β 42.DMSO (Untreated), GMR-A β 42.SalA (Treated). Conversely, for the lifespan analysis, locomotion analysis, and RNAseq, Actin5C-A β 42 was used as the AD *Drosophila* line and Actin5C-OreR as the Control: Actin5C-OreR.DMSO (Control), Actin5C-A β 42.DMSO (Untreated), Actin5C-A β 42.SalA (Treated). All comparisons involving SalA were conducted within the Actin5C-A β 42 genetic

background to make sure that there is no genotype-related confounding in treatment effects.

The UAS-A β 42 construct employed in the current study with the genotype of [w; P{w[+mC]=UAS-APP.Abeta42.B}m26a] carries a P-element transposon insertion on chromosome 2 that expresses the human amyloid-beta42 (A β 42) peptide fragment derived from the amyloid precursor protein under UAS regulatory control. The insertion of [P{UAS-APP.Abeta42.B}m26a] (FlyBase ID: FBti0139936) enables the target expression of A β 42 when crossed with GAL4 driver lines (Jeon et al. 2017). The A β 42 protein product induces AD-like pathologies in *Drosophila* models which include shortened lifespan, reduced locomotor activity and neuronal degeneration. The A β 42 protein product promotes aggregation into toxic oligomer and plaques which lead to synaptic loss, mitochondrial dysfunction and cognitive deficits such as impaired memory (Iijima et al. 2008).

Different drivers were used to facilitate tissue-specific versus organism-wide expression studies concerning A β 42 toxicity, which aids in distinguishing between the effects on the eye versus whole-organism. GMR-GAL4 (Stock #1104) mediates expression in the differentiating photoreceptor cells behind the morphogenetic furrow in the developing fly eye, which results in visible neurodegeneration-related phenotypes such as smaller eye size, glossy appearance, or ommatidial fusion (Cutler et al. 2015; Kramer and Staveley 2003). On the other hand, Actin5C-GAL4 (Stock #4414) mediates ubiquitous expression across all tissues including muscles and neurons, which helps evaluate organism-wide toxicity and systemic effects (Prüßing 2012).

Solid fly feed recipe comprised of 4% cornflour, 5% inactivated yeast, 10% brown sugar, 5% polenta, 0.7% agar, 3% nipagin, 0.7% propionic acid, and either 0.5% DMSO (Untreated) only or added with 100 μ M SalA (Treated) (Tan et al. 2021b). Solid food provides an appropriate physical substrate for egg laying and supports normal larval growth. As such, it was used for parental mating, oviposition, and larval development of the crosses.

The CAFE system provides a more precise and standardized way to deliver dissolved compounds (like SalA) and allows for the measurement of liquid intake (Ja et al. 2007). This ensures that the treatment reaches the *Drosophila* effectively compared to solid media. Upon eclosure of the crosses, newly

emerged adult *Drosophila* were collected within 24 h and transferred to CApillary FEeder (CAFE) vials for all adult-stage experiments (maximum 10 *Drosophila* per vial) (Ja et al. 2007). Liquid feed for CAFE followed the same recipe for solid feed albeit without cornflour and agar.

Food intake in the CAFE system was assessed and optimized before experimentation to exclude dietary restrictions as a confounding factor. For evaporation control, vials containing liquid feed without *Drosophila* were observed to correct for passive liquid loss. Daily consumption was calculated. Under 29 °C and 60% humidity, approximately 13–14 μ L of liquid diet was consumed per 10 adult *Drosophila* within 24 h. Due to this, all experiments using the CAFE system were conducted under ad libitum feeding by providing at least a total of 15 μ L of liquid feed per vial per 10 adult *Drosophila* within 24 h.

Eye assay

Samples were immobilized at –20 °C for 10 min and were incubated in McDowell-Trump fixative (Sigma-Aldrich, Missouri, USA) overnight at 4 °C. Samples were washed thrice in phosphate buffer followed by post-fixing in 1% osmium tetroxide (Sigma-Aldrich, Missouri, USA) for one hour. The samples were washed in ddH₂O and dehydrated in increasing ethanol (50, 75, 100%) percentages for 15 min each followed by hexamethyldisilazane (Sigma-Aldrich, Missouri, USA) for 10 min and air-dried in a desiccator overnight. Samples were mounted, gold coated and viewed with scanning electron microscopy (SEM) (Hitachi Ltd., Tokyo, Japan). *Flynotyper* software was used to assess the degree of ommatidia deformation in SEM images (Iyer et al. 2018). The assay was performed in quadruplicate.

Lifespan and locomotion analyses

For the lifespan analysis, experimented *Drosophila* were transferred to the CAFE vials (<20/vial) within 24 h of eclosure. Mortality was recorded daily, and surviving *Drosophila* were moved to sterile vials carrying fresh liquid feed. The experiment was conducted in triplicate. Long-term survival probability in each of the test groups was compared by using the Kaplan–Meier analysis, a non-parametric statistical

method to estimate the survival function $\hat{s}$ from time-to-event data. The estimator is given by:

$$\hat{s}(t) = \prod_{t_i < t} \left(1 - \frac{d_i}{n_i}\right) \quad (1)$$

where t_i is a time point at which at least one death occurred, d_i is the number of deaths at t_i , and n_i is the number of individuals alive just before t_i . Number of subjects alive at each time point was enumerated on a daily basis, and the probability of survival was calculated considering the number of subjects alive at each time point. The survival curves of the three groups were plotted to compare the lifespan of treated, untreated, and control lines.

For the locomotion analysis, seven to ten *Drosophila* from experimental lines were placed into sterile vials and allowed ten minutes to acclimatise before the vials were tapped at the bottom. The climbing process was recorded for ten seconds. *Toxtrac* software was used to analyse the average speed (Rodríguez et al. 2018, 2017). The assay was executed in sextuplicate.

Total RNA extraction and RNAseq

For RNAseq, 15 dae was selected based on prior data showing peak motor deficits in Aβ42-expressing *Drosophila* (Tan et al. 2023a) and current lifespan findings indicating a mid-life stage for treated *Drosophila*. Ten *Drosophila* were homogenized in 300 μL of TRIzol (Invitrogen, Massachusetts, USA) before 80 μL of chloroform was added. For layer separation, the mixture was centrifuged for 10 min at 4 °C, 10,000 g. The aqueous layer was added with 200 μL of absolute ice-cold isopropanol. Sample clean-up was executed using the MinElute Cleanup Kit (Thermo Fisher Scientific, Massachusetts, USA) following the manufacturer's protocol. High quality total RNA (≥ 200 ng/μL, OD 260/280 = 1.8 to 2.2 and ≥ 5 μg;) were utilized for library construction. Each biological replicate consisted of 10 pooled *Drosophila* (five males and five females). Each condition (line) was collected in triplicate. Apical Group (Singapore) performed library construction via TruSeq® RNA-Sample Preparation (Illumina, California, USA) and sequencing through the Illumina HiSeq PE150 platform.

The data supporting the findings of this study are publicly accessible in NCBI BioProject PRJNA921963: <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA921963>. Accession numbers for Untreated (Actin5C-Aβ42.DMSO) samples are SRR23047820, SRR23047818 and SRR23047819. Accession numbers for Treated (Actin5C-Aβ42.SalA) samples are SRR28342786, SRR28342785 and SRR28342784.

Differential expression analysis

Differential gene expression analysis was conducted using the DESeq2 R package (v1.42.0), following a negative binomial generalized linear model (NB-GLM) framework to account for biological overdispersion (Love et al. 2015). For each gene i in sample j , the raw read count K_{ij} was modelled as:

$$K_{ij} \sim NB(\mu_{ij}, \alpha_i) \quad (2)$$

where μ_{ij} is the expected count and α_i is the gene-specific dispersion parameter. The variance structure is given by:

$$Var(K_{ij}) = \mu_{ij} + \alpha_i \mu_{ij}^2 \quad (3)$$

To normalize for varying sequencing depths, size factors s_j were estimated, and the mean expression was decomposed as:

$$\mu_{ij} = s_j q_{ij} \quad (4)$$

The normalized abundance q_{ij} was then modelled with a log-linear function as:

$$\log(q_{ij}) = \beta_{i0} + \beta_{i1} \cdot x_j \quad (5)$$

Here, $x_j \in \{0, 1\}$ indicates sample condition (e.g., control vs. treatment). The parameter β_{i1} corresponds to the log2 fold change (log2FC), encoding the effect of condition on gene i 's expression.

Differentially expressed genes (DEGs) were identified by testing the null hypothesis:

$$H_0 : \beta_{i1} = 0 \text{ vs. } H_1 : \beta_{i1} \neq 0 \quad (6)$$

Wald tests were used to test significance, with the Wald statistic:

$$W_i = \frac{\hat{\beta}_{i1}}{SE(\hat{\beta}_{i1})} \quad (7)$$

Under regularity conditions and large sample sizes, $W_i \sim \mathcal{N}(0, 1)$ under H_0 .

To control for multiple testing, the Benjamini–Hochberg false discovery rate (FDR) procedure was applied. The adjusted p-value (q-value) q_i was computed as:

$$q_i = \frac{p_i m}{\text{rank}(p_i)} \quad (8)$$

where m is the number of tested genes. The simplified BH adjustment is shown in Eq. (8). In practice, p-values were adjusted using the p-adjust method in DESeq2 (Benjamini–Hochberg). Criteria for DEGS were p-adjusted value < 0.1 (Love et al. 2015) and $|\log_2\text{FC}| > 0.5$ (Company et al. 2024) as seen in:

$$q_i < 0.1 \text{ and } |\hat{\beta}_{i1}| > 0.5 \quad (9)$$

This dual threshold ensures both statistical significance and biological relevance, corresponding to genes with expression changes greater than approximately 1.4-fold. Statistical justification of Wald testing in NB–GLM RNA-seq models is shown in Supplementary Data 1.

g:Profiler version e113_eg59_p19_f6a03c19 (<https://biit.cs.ut.ee/gprofiler/gost>) (Kolberg et al. 2023) with Significance threshold of Benjamini–Hochberg FDR and adjusted_p_value < 0.05 was used to obtain the Gene Ontology (GO) terms, GO clusters and Kyoto Encyclopedia of Genes and Genomes (KEGG).

Quantitative reverse transcription PCR (RT-qPCR)

Quantitative Real-time PCR (RT-qPCR) validation was performed on four representative protein-coding genes (*CG43659*, *CG14500*, *GstD8*, and *Ugt37A3*) selected based on fold change magnitude, expression level, and statistical significance. *lncRNAs* and the gene *Rst* were excluded from validation due to technical constraints and borderline statistical support, respectively. Primers were either sourced from previously published studies or designed using NCBI Primer-BLAST. For designed primers, parameters were optimized for SYBR

Green detection (amplicon size 70–150 bp, T_m 58–62 °C, GC content 40–60%). Primer specificity was confirmed through in silico BLAST against the *Drosophila melanogaster* transcriptome. The set of RT-qPCR primers used in this investigation is displayed in Supplementary Data 2. Following the manufacturer’s instructions, first-strand complementary DNA (cDNA) was synthesized from total RNA using the iScript™ Reverse Transcription Supermix (Bio-rad, California, United States). Next, RT-qPCR was carried out in accordance with manufacturer’s instructions using the iTaq Universal SYBR Green Supermix (Bio-rad, California, United States) in a CFX96 real-time heat cycler (Bio-rad, California, United States). The RT-qPCR was performed in triplicates per gene.

Unsupervised learning: mathematical formulation

Let $X = \{x_1, x_2, \dots, x_p\} \subset \mathbb{R}^p$ denote the expression profiles of n genes measured across p samples following variance-stabilizing transformation (VST). Unsupervised clustering aims to partition X into k disjoint clusters:

$$C = \{C_1, \dots, C_k\}, U_i^{=1k} C_i = X, C_i \cap C_j = \emptyset \text{ for } i \neq j \quad (10)$$

Each cluster is represented by its centroid:

$$\mu_i = \frac{1}{|C_i|} \sum_{x \in C_i} x \quad (11)$$

The K-means objective is to minimize the within-cluster variance:

$$\min_C \sum_{i=1}^k \sum_{x \in C_i} \|x - \mu_i\|^2 \quad (12)$$

To evaluate clustering quality, the silhouette coefficient was computed for each gene x . The intra-cluster distance $a(x)$ is defined as the mean distance between x and all other genes in the same cluster:

$$a(x) = \frac{1}{|C_i| - 1} \sum_{y \in C_i, y \neq x} \|x - y\| \quad (13)$$

The inter-cluster distance $b(x)$ is the minimum average distance between x and all points in another cluster C_j :

$$b(x) = \min_{j \neq i} \frac{1}{|C_j|} \sum_{y \in C_j} ||x - y|| \quad (14)$$

The silhouette coefficient of x is then:

$$s(x) = \frac{b(x) - a(x)}{\max\{a(x), b(x)\}}, -1 \leq s(x) \leq 1 \quad (15)$$

The overall Silhouette Score (SS) is the mean of $s(x)$ across all n genes, with higher scores indicating greater cluster separation. The optimization criterion is:

$$k * \theta = \arg \max_{k, \theta} SS(\mathcal{M}(X; k, \theta)) \quad (16)$$

where $\mathcal{M}(X; k, \theta)$ represents the K-means model with parameters $\theta = \{n_{init}, n_{init}\}$.

Dimensionality reduction and clustering

Normalized count data obtained from DESeq2 were transformed using the variance stabilizing transformation (VST) to reduce heteroskedasticity. The top 1,000 most variable genes were selected based on row-wise variance using the matrixStats package in R. Unsupervised clustering was carried out in Python. The top 1000 variance-stabilized genes were embedded into a lower-dimensional space using UMAP (Uniform Manifold Approximation and Projection) with the parameters: $n_neighbors=30$, $min_dist=0.1$. The UMAP embeddings (X_umap) were subjected to k-means clustering, and multiple clustering configurations were evaluated using silhouette scores. For K-means, a range of cluster numbers ($k=10$ to 40) and initialization strategies ('k-means++', 'random') with different n_init values (10, 20, 50) were tested. The optimal configuration was selected based on the highest average SS.

Functional enrichment and visualization

Each K-means cluster was analysed using g:Profiler for Gene Ontology (GO) and KEGG pathway enrichment. Only clusters containing ≥ 3 genes were retained. On the other hand, enrichment terms with $p < 0.05$ were retained for each cluster. To visualize cluster-level functional convergence, $-\log_{10}(p)$ values were aggregated across clusters and represented in a heatmap.

Statistical analysis

Statistical significance for general analyses was calculated using Tukey's HSD Test and one-way ANOVA. For the lifespan analysis, the Fisher's Exact test was used to determine the significance of the data.

Results

SalA reduces REP of AD *Drosophila*

The eye assay was employed to examine the effects of A β 42 expression on *Drosophila* retinal tissues along with possible neuroprotective properties of SalA. Typically, an adult *Drosophila* eye (Fig. 1Ai, ii) is made up of eye units called ommatidia that are hexagonally-shaped. A "rough eye" phenotype (REP) develops when A β expression is driven to the eyes by GMR-GAL4. This phenotype is a visible manifestation of A β 's cytotoxic effects on photoneurons and other eye components which can be observed from Fig. 1Bi, ii whereby the ommatidia were irregularly shaped, fused and perforated. SalA treatment partially reversed these effects as seen in Fig. 1Ci, ii.

The relative phenotypic score (P-score), which represents the deviations of the ommatidia, was statistically analysed using the *Flynotyper* using the Control GMR-OreR.DMSO as baseline (Iyer et al. 2018). A more distorted REP is indicated by a higher P-score. The p -value of one-way ANOVA is less than 0.05 ($p=0.0001$), indicating that one or more treatments differ significantly. From Fig. 1D, the Untreated, with an average P-score of 1.51, is significantly different from the Control while the Treated, with P-score of 1.29, is significantly different from the Untreated. Thus, SalA was able to alleviate REP caused by A β 42 expression.

SalA increased AD *Drosophila*'s longevity

The long-term impact of SalA treatment on AD *Drosophila* was evaluated using Actin5C-GAL4 that drives ubiquitous A β 42 expression (Fig. 2). Compared to the Control that had a mean lifespan (Supplementary Data 3) of 24.31 days after eclosure (dae), Untreated lived shorter with a mean of 9.57. Kaplan–Meier survival analysis revealed significant differences among all groups, as assessed by log-rank (Mantel–Cox)

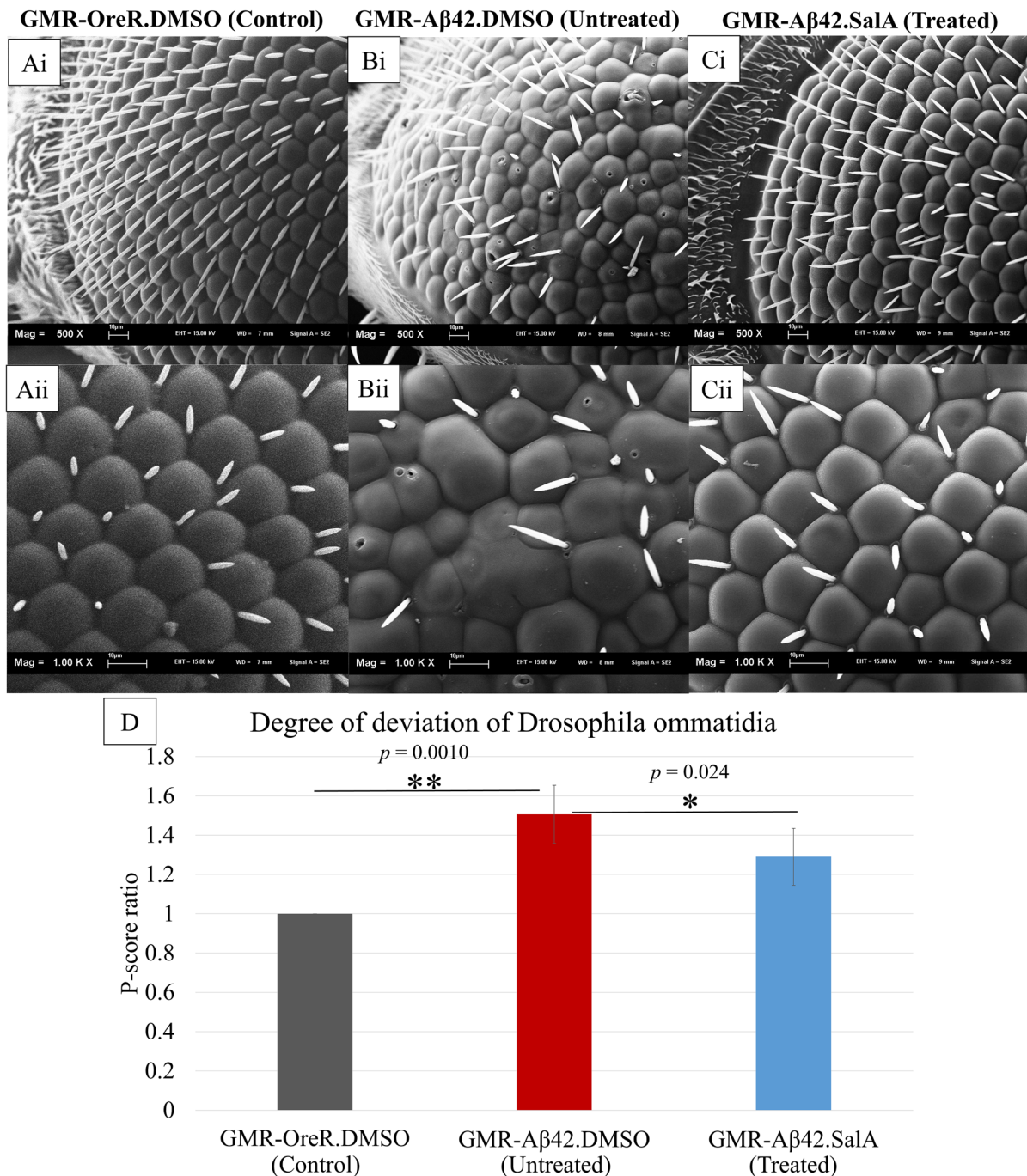


Fig. 1 Eye assay **A–C** Comparison of *Drosophila* eye SEM images viewed at 500× and 1000× magnifications, **D** *p*-scores of *Drosophila* eyes. Tukey's HSD: * $p < 0.05$, ** $p < 0.005$, $n = 4$

tests with Bonferroni correction (Fig. 2; Supplementary Data 3). The survival rate was significantly lower in Untreated *Drosophila* when comparing them to

Controls ($\chi^2 = 204.53$, Bonferroni $p < 0.001$). Most importantly, SalA treatment significantly extended lifespan relative to Untreated *Drosophila* ($\chi^2 = 105.87$,

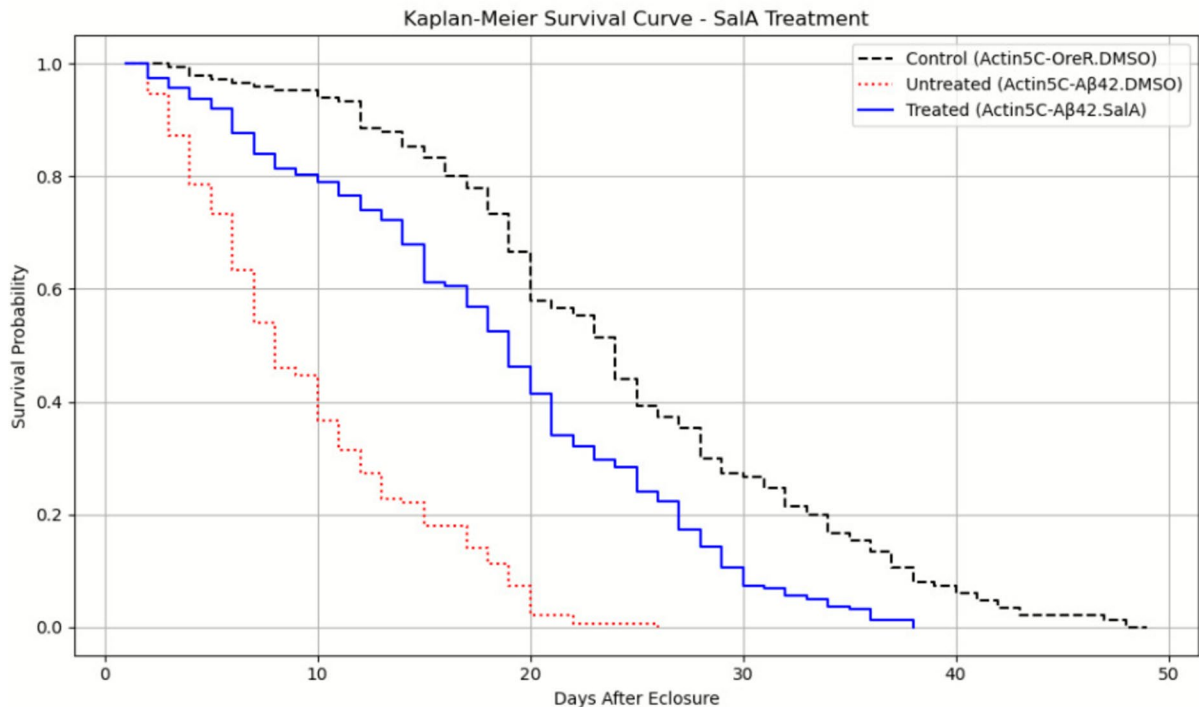


Fig. 2 Effect of SalA on the lifespan of AD *Drosophila*. Statistical significance between survival curves was assessed using log-rank (Mantel–Cox) tests with Bonferroni correction

Bonferroni $p < 0.001$), although Treated *Drosophila* still remained shorter-lived than Controls ($\chi^2 = 33.46$, Bonferroni $p < 0.001$). Similarly, at all mortality percentages (Supplementary Data 3), there was a substantial difference (Fisher's Exact Test p value < 0.05) in the Untreated's longevity compared to the Control. When SalA was fed to AD *Drosophila*, the mean lifespan increased to 19.28 dae. Similarly, Untreated and Treated were significantly different (Fisher's Exact Test p -value < 0.05) at all mortality percentages with the Untreated line experiencing earlier mortality milestones compared to the Treated line. Therefore, while A β 42 expression significantly reduced AD *Drosophila* longevity, SalA treatment significantly delayed mortality milestones, indicating an extension of lifespan of the AD *Drosophila*.

SalA reduced A β 42-associated locomotor impairment in AD *Drosophila*

As AD symptoms include locomotor impairment (Cacabelos et al. 2005), the therapeutic benefits of SalA on AD *Drosophila* mobility were studied. The locomotive assay comprised assessing the *Drosophila*'s climbing speeds at 5, 10, and 15 dae (Fig. 3), which represent the early, middle, and late portions of the Untreated lifetime, respectively (Tan et al. 2023b). For day 5, p -value of one-way ANOVA is over 0.05 ($p = 0.059$), showing no significant differences between the three lines while days 10 ($p = 1.27E-13$) and 15 ($p = 1.11E-16$) were less than 0.05, signifying significant differences between conditions for both days. The Control consistently had an average speed of over 12 mm/s throughout the three time-points, demonstrating insignificant changes in locomotor abilities. Throughout the three time-points, the average climbing speed of the Treated line constantly outpaced that of the Untreated, but only on 10 (Tukey's p -value = 0.0010) and 15 dae (Tukey's p -value = 0.0010) did the average climbing speeds for both lines differ significantly. This indicated that SalA's

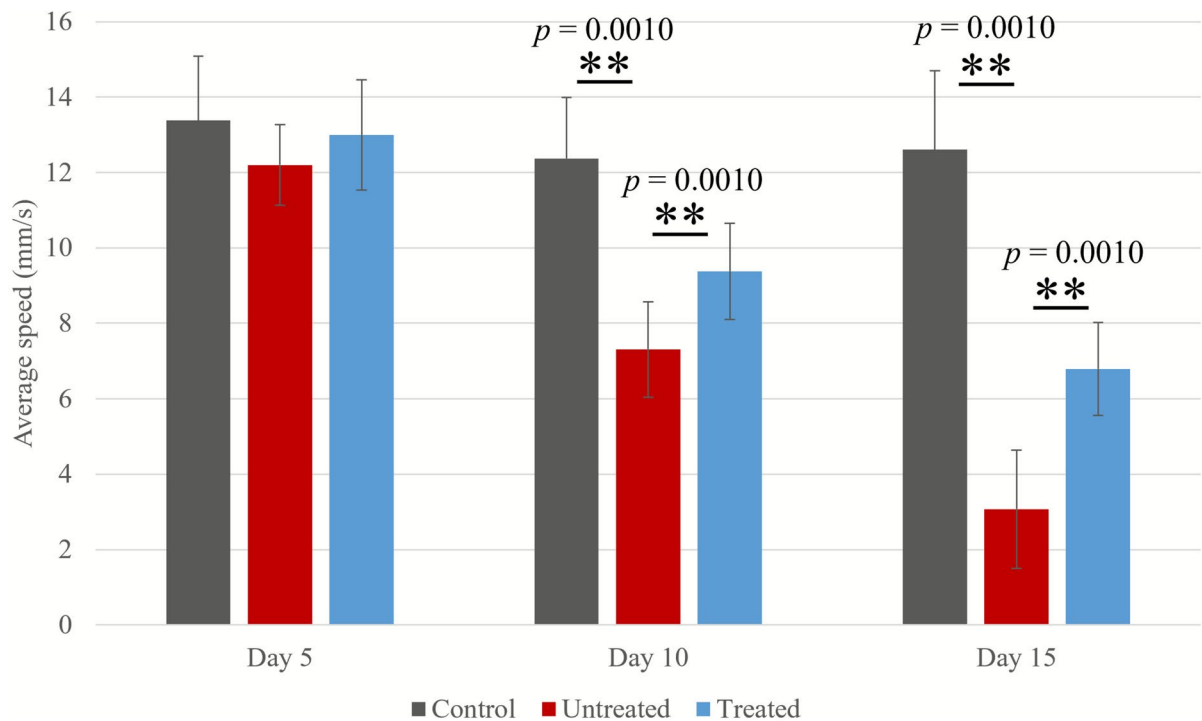


Fig. 3 AD *Drosophila*'s average climbing speed at three different time-points. Tukey's HSD: $**p < 0.005$, $n = 6$

preventive impact against A β 42-related mobility loss was most effective from middle-age onwards.

RNAseq Reveals DEGs associated with antioxidant activity and disease-relevant phenotypes with SalA treatment

RNA-seq was performed on 15 dae *Drosophila*, an age at which significant age-related and neurodegenerative phenotypes emerge in *Drosophila* models (Tan et al. 2023a). At 15 dae, RNA samples of the two AD lines, Untreated and Treated, were collected in triplicates. An overall view of the transcriptome differences between the Treated and Untreated samples was visualized using a volcano plot (Fig. 4A). From 15,046 genes, seven DEGs (Supplementary Data 4) were found with four significantly upregulated and three significantly downregulated in the SalA-treated group relative to the control A β 42-expressing *Drosophila*.

Among the upregulated genes, *Ugt37A3*, *GstD8*, and *CG14500* had the highest statistical significance with log2FC of 0.82, 1.13, and 1.12, respectively. These genes are well characterized for their roles in

xenobiotic metabolism and detoxification, including their roles in UDP-glycosyltransferase and glutathione transferase activity. On the other hand, the most downregulated gene of highest significance was *roughest* (*rst*, FBgn0003285), which had a log2FC of -0.74 and an adjusted p-value of 0.069. It has been found that *rst* plays essential roles in development of muscle tissue and fusion of myoblasts during development, thereby indicating repression of developmental activities under the experimental treatment.

To obtain additional information on the functional significance of the DEGs, Gene Ontology (GO) and KEGG pathway enrichment were separately performed on collections of upregulated and downregulated genes. The upregulated genes were enriched for metabolic and antioxidant processes including drug metabolism via cytochrome P450, glutathione metabolism, and other oxidoreductase and peroxidase activities (Fig. 4B, Supplementary data 5). These enrichments were significantly impacted by *Ugt37A3* and *GstD8*, both cellular detoxification genes. KEGG pathway analysis further highlighted involvement in xenobiotic metabolism and cofactor biosynthesis,

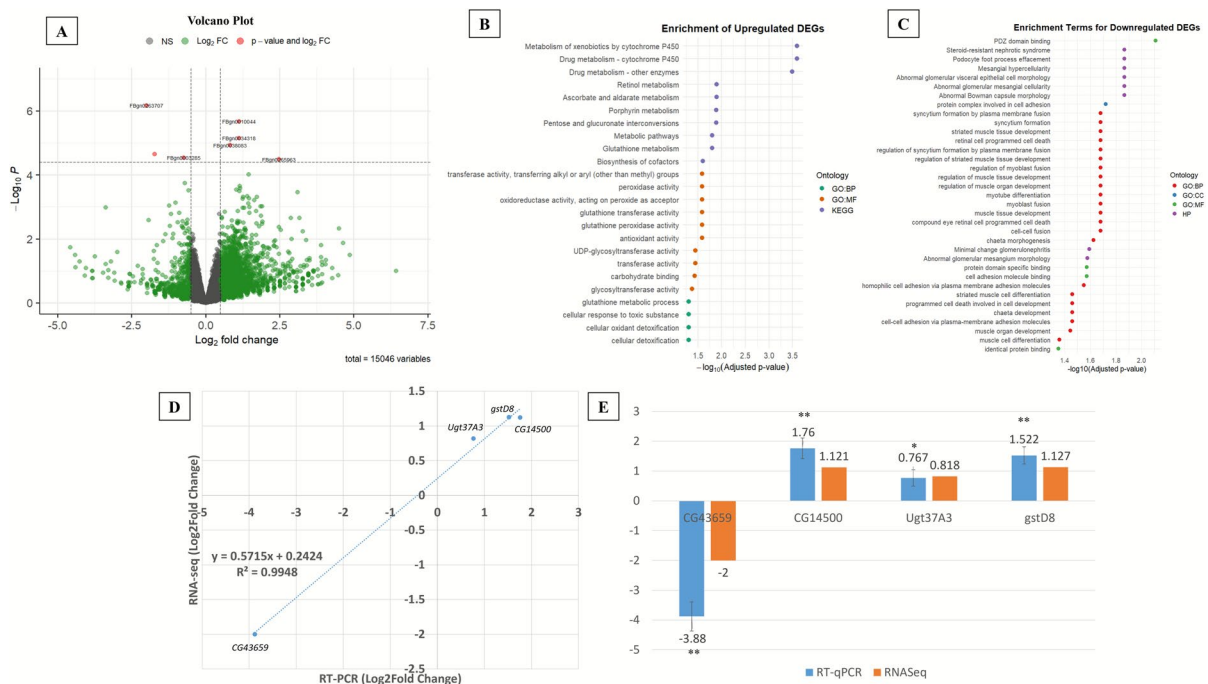


Fig. 4 Analysis of RNAseq data from Untreated and Treated samples **A** Volcano plot showing differential expression, **B** GO and KEGG enrichment analysis of upregulated DEGs, **C** GO and HP enrichment analysis of downregulated DEGs, **D** pear-

son correlation between the log₂FC expressions of RT-qPCR against RNA sequencing data, **E** trend comparability of quantitative RT-qPCR against RNAseq of four DEGs. Tukey's HSD: * $p < 0.05$, ** $p < 0.005$, $n = 3$

again highlighting the involvement of such genes in chemical clearance and oxidative stress response.

In contrast, genes downregulated were enriched significantly for muscle development and cell fusion biological processes (Fig. 4C, Supplementary data 6). GO terms of muscle tissue development, myoblast fusion, and striated muscle cell differentiation were greatly enriched primarily due to the repression of *rst*. These findings agree with the established developmental morphogenesis role of *rst* and suggest the experimental condition could repress aspects of muscle-related gene expression. Further enrichment analysis using Human Phenotype Ontology (HP) terms identified significant associations with phenotypes such as Steroid-resistant nephrotic syndrome and podocyte foot process effacement.

Collectively, these results indicate that the experimental treatment induces a robust transcriptional response with both upregulation of detoxification and antioxidant genes and downregulation of muscle development genes concurrently. Implication of

the protective mechanism by which SalA inhibits Aβ₄₂-induced toxicity is evidenced by upregulation of genes such as *GstD8* with core functions in glutathione and detoxification pathways.

RT-qPCR was used to evaluate the expressions of five DEGs to validate the RNAseq data. All four genes under evaluation displayed profiles resembling those of the RNAseq (Fig. 4E). The p -values for the RT-qPCR of the tested genes were less than 0.05 (*CG43659*: one-way ANOVA = 0.0004, Tukey's HSD = 0.001; *CG14500*: one-way ANOVA = 0.002, Tukey's HSD = 0.002; *Ugt37A3*: one-way ANOVA = 0.0185, Tukey's HSD = 0.0185; *gstD8*: one-way ANOVA = 0.0018, Tukey's HSD = 0.0018), demonstrating that there are significant differences between Untreated and Treated samples for all the gene sets. A Pearson correlation test was employed to examine the relationship between the RNAseq data and RT-qPCR (Fig. 4D). A high degree of correlation ($R^2 = 99.48\%$) was indicated by the coefficient of

determination (R^2), which measures the strength of correlation between the two data sets. This thereby confirms the accuracy of the RNAseq results.

Unsupervised clustering of top variable genes identifies a biologically enriched cluster

For further visualization of the midlife transcriptome-wide variation, the variance-stabilized expression values were also examined. The 1000 most variable genes were then reduced in dimension and clustered. UMAP was optimized with k-means ($k=5$) with the parameters of $n_neighbors=30$, $min_dist=0.1$ provided the best separation (silhouette=0.541). Next, the same UMAP parameters were used to optimize

k-Means clustering. The best $k=27$ (Fig. 5A, Supplementary Data 7) with a silhouette score=0.549 resulted in the most consistent and interpretable cluster configuration and was therefore used for downstream enrichment analysis.

Gene set enrichment analysis by g:Profiler of the 27 clusters (Fig. 5B, Supplementary data 8) identified Cluster 6 to be strongly enriched for terms related to reproductive and extracellular structure. The top-ranked GO processes (Fig. 5C) were chorion-containing eggshell formation (GO:BP, $p=9.18 \times 10^{-12}$), eggshell formation ($p=1.51 \times 10^{-11}$), and follicle cell development ($p=1.11 \times 10^{-5}$). Additionally, several extracellular region and structural constituent terms were extremely overrepresented (GO:CC, $p \leq 10^{-15}$).

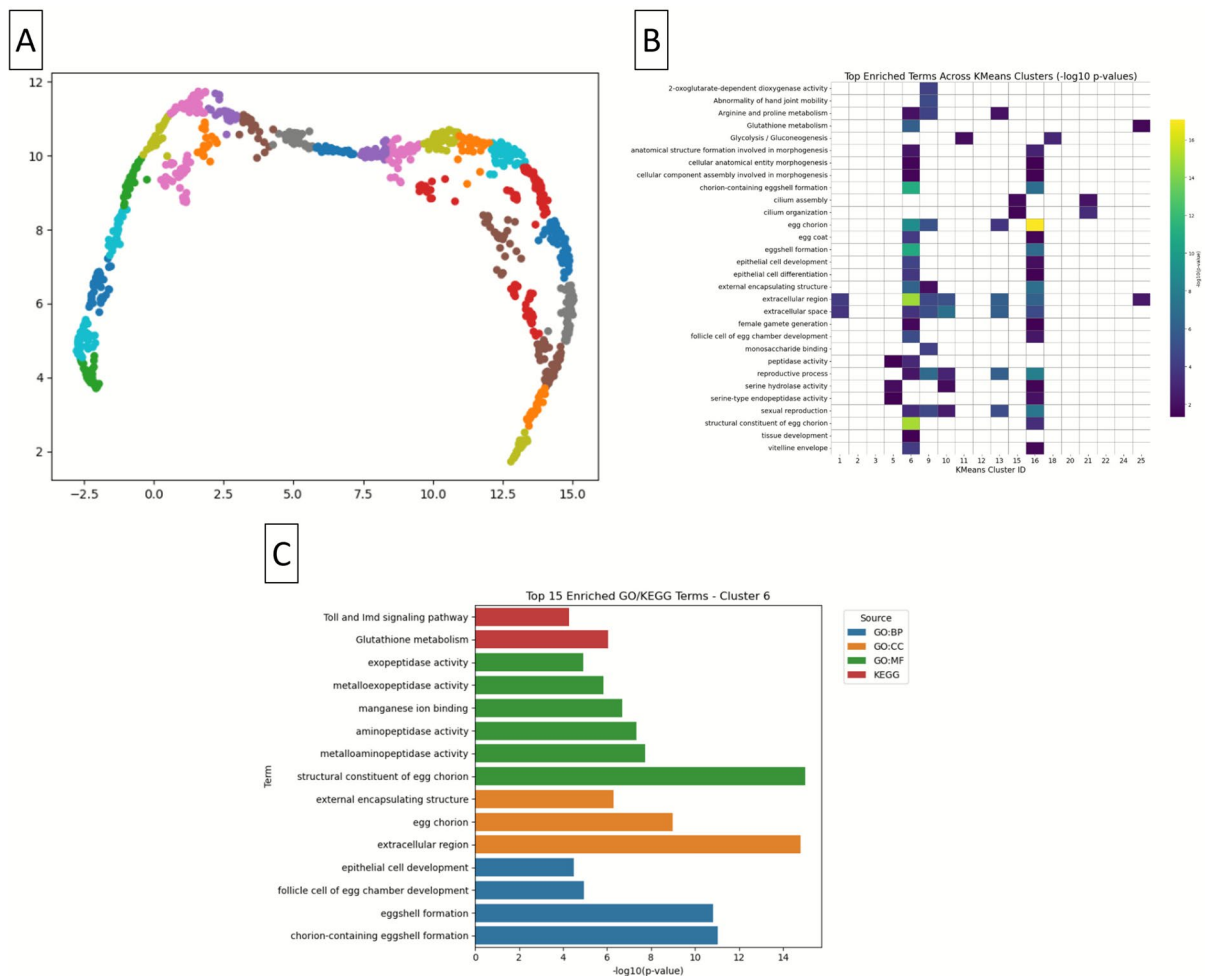


Fig. 5 Clustering of top variable genes **A** Best UMAP and K-means visualization, **B** gene set enrichment analysis **C** GO and KEGG analyses on Cluster 6

Notably, Cluster 6 was also enriched for metabolic processes of detoxification, including glutathione metabolism (KEGG, $p=8.94 \times 10^{-7}$) and immune signalling (Toll and Imd pathway, $p=5.38 \times 10^{-5}$).

Together with the DEG analysis, which indicates upregulation of detoxification-related genes (*Ugt37A3*, *GstD8*, *CG14500*) and downregulation of developmental regulator *rst*, these clustering findings are convergent to suggest SalA treatment impacts both detoxification pathways and developmental processes.

Discussion

Salvianolic Acid A (SalA) is a natural compound exhibiting broad biological activities that include antioxidant, anti-inflammatory, and cytoprotective effects, which are mechanisms known to counteract age-related cellular decline. Such pleiotropic activities are consistent with anti-aging mechanisms antagonizing the hallmarks of aging, such as oxidative stress (Wu et al. 2007) and metabolic imbalance (Cao et al. 2013). Recent studies have indicated that SalA is potentially crucial in modulating A β -induced neurotoxicity, a distinguishing hallmark of AD pathology. These aging-related activities suggest that SalA may promote cellular resistance against late-life physiological decline. A β 42, the most aggressive and lethal type of A β peptides, was the focus of this work. As A β 42 expression accelerates age-relevant neurodegeneration, it provides a suitable model to evaluate whether SalA is able to conserve geroprotective pathways. The present findings demonstrate that SalA ameliorates behavioural and molecular signatures of age-dependent neurodegeneration in *Drosophila*, indicating that it promotes neuronal and systemic resilience with aging.

Here, with *Drosophila* serving as the model, three primary aspects of AD symptoms were selected to investigate SalA's effects against A β 42: cytotoxicity (Yang et al. 2001), mobility impairment (Cacabelos et al. 2005), and early demise (Gillette-Guyonnet et al. 2007) which correspond to the eye, longevity and locomotion assays, respectively. Treatment with SalA for extended periods delayed the onset of these phenotypes and improved survival in general, consistent with a healthspan-extending effect. The magnitude of rescue beyond the 25% mortality point

suggests that SalA slows the pace of age-related functional decline rather than transiently repressing toxicity. This result is consistent with the broad anti-aging hypothesis that interventions against molecular damage can extend functional longevity across life phases (López-Otín et al. 2013).

Enhanced locomotor capacity in midlife also further supports SalA's role in the preservation of neuromuscular functioning across aging pathways. Similar protective effects of SalA and analogs in *C. elegans* and mammalian models attest to its evolutionarily conserved redox-modulating activity (Cao et al. 2013). These behavioural improvements are likely a result of SalA's ability to restore mitochondrial and oxidative homeostasis, as demonstrated by transcriptional induction of detoxification and antioxidant genes. Motor decline with age has been closely linked to attenuated mitochondrial function and increased reactive oxygen species (ROS) generation, which foster protein misfolding and synaptic breakdown. By inducing antioxidant defence, SalA may protect neuromuscular coordination and energy metabolism in the aging neuron.

Transcriptomic profiling provided mechanistic insight into SalA effects. Untreated A β 42 *Drosophila* exhibited downregulation of genes involved in muscle integrity, cell adhesion, and apoptosis regulation. These processes are key to neuromuscular maintenance and have been implicated already in AD pathology as well as in aging-associated tissue degeneration (Berchtold et al. 2013; Wennström and Nielsen 2012). Such transcriptional dysregulation is likely to be responsible for the locomotion defects and decreased lifespan seen in untreated flies. Moreover, diminished expression of programmed cell death and synaptic stability genes replicates known molecular profiles of AD, including impaired tissue homeostasis and neurodegeneration (Williams et al. 2021). Enrichment of retinal apoptosis-related GO terms also accounts at the molecular level for the rough eye phenotype (REP) observed in A β 42-expressing *Drosophila*, where SalA supplementation partially restored ommatidial morphology.

SalA normalized several of these molecular alterations, restoring transcription networks that maintain redox and metabolic balance. Notably, SalA upregulated genes involved in the glutathione metabolism and cytochrome P450 pathways, including *Ugt37A3* and *GstD8*. These genes have central roles

in detoxification and ROS scavenging and are classic antioxidant defences that deplete with ageing (Chen et al. 2016; Feng et al. 2023). The restoration of these pathways by SalA implies enhanced cellular redox buffering and xenobiotic clearing capacity, which are processes tightly linked to longevity (Vina et al. 2013). In mammals, aging-associated depletion of glutathione has been associated with increased susceptibility to neurodegeneration, and interventions that increase glutathione metabolism induce extended lifespan and improved cognitive function (Youssef et al. 2018; Ansari and Scheff 2010). This concordance supports the hypothesis that SalA restores redox homeostasis through conserved stress-response mechanisms that operate across all species.

Aside from the activation of detoxification genes, SalA affected metabolic and immune signalling networks revealed by enrichment analysis. Cluster-level enrichment for glutathione metabolism, Toll/Imd immune signalling, and xenobiotic processing suggests multi-layered impact of SalA on cellular defence systems. Convergence of single-gene and network-level analysis underscores the intensity of SalA's impact on homeostatic maintenance. Such coordination of stress-response modules is a hallmark of the systems-level resilience postulated by anti-aging frameworks, where regulation of detoxification and proteostatic defences is thought to attain healthy aging (López-Otín et al. 2013).

To complement DEG-based analysis and capture coordinated gene activity beyond individual transcripts, unsupervised clustering was performed. Integration of differential expression analysis and unsupervised clustering provided complementary information. DEG analysis identifies individual genes with extreme fold changes, while unsupervised clustering identifies co-expression modules that may not meet statistical thresholds but as a group are suggestive of biologically important regulation. Through this approach, gene clusters enriched for detoxification and oxidative stress processes were discernible irrespective of pre-defined significance thresholds. The overlap between DEGs (e.g., *Ugt37A3*, *GstD8*) and top-ranked clusters (e.g., Cluster 6 enriched in glutathione metabolism) supports the conclusion that SalA enhances redox stability at both the gene and network levels. This kind of integrative analysis is a model for exploring aging-associated transcriptional plasticity and resilience (Kennedy et al. 2014).

Together, the behavioural, lifespan, and transcriptomic data demonstrate that SalA not only acts as a neuroprotective molecule but also as a homeostasis-preserving molecule that operates across molecular and behavioural levels during aging. That it is able to modulate detoxification, oxidative stress, and innate immune pathways suggests a broad-spectrum anti-aging role. By re-balancing cellular redox state and enhancing resilience to oxidative damage, SalA is also likely to maintain proteostasis and delay the onset of age-related functional impairment. These effects are consistent with observations that the targeting of Nrf2, FOXO, and AMPK signalling pathways which are central regulators of oxidative and metabolic stress—can extend lifespan and improve tissue maintenance (Madeo et al. 2015).

The anti-aging interpretation of SalA is also compatible with its systemic advantages beyond neuronal tissue. Prior studies have reported SalA-mediated cardioprotection, hepatoprotection, and nephroprotection through mitigation of ROS and inflammatory burden (Youssef et al. 2018; Ansari and Scheff 2010). Such tissue-wide effects implicate SalA as a metabolic stabiliser for the enhancement of organismal vigour and immune potency, both being central aspects of healthspan. The pleiotropy is reminiscent of the functional scope of other plant polyphenols, e.g., resveratrol and curcumin, which extend lifespan in a species-wide manner by activating conserved stress-response pathways (López-Otín et al. 2013). These observations concur with other research when salvianolic acid B (Tan et al. 2025), a related salvianolic compound, and Danshen (Tan et al. 2021b) also increased antioxidant and detoxification pathways in AD models. This together suggests a shared neuroprotective process in salvianolic acids via redox homeostasis modulation and cellular defence.

Methodologically, the integration of RNA-seq and unsupervised learning illustrates the promise of computational biology for aging studies. Traditional DEG analyses may overlook context-dependent tendencies or subtle network effects. Unsupervised clustering of the top 1000 variable genes, by contrast, preserves emergent transcriptomic signatures linked to resilience vs. decline. This approach is consistent with current trends in systems anti-aging, which advocate for multiscale data integration to discern conserved mechanisms of longevity (Kennedy et al. 2014).

While this study provides clear evidence of the potential of SalA to influence aging phenotypes, there are some limitations that should be mentioned. Certainly, while *Drosophila* is a robust and evolutionarily conserved model system, its metabolic background is slightly altered compared with mammals. Validation in mammalian systems, particularly those incorporating biomarkers of aging, will be required to determine translational relevance. Second, the study was focused primarily on midlife transcriptomes; time-course RNA-seq at early, middle, and late life stages could reveal dynamic transcriptional pathways of aging and SalA treatment. Finally, functional genetic assays such as knockdown or overexpression of the key genes (e.g., GstD8, Ugt37A3) are required to establish causal relationships between transcriptional activation and phenotype rescue.

Despite these caveats, the present work provides a comprehensive illustration of the recovery of multi-level homeostasis by a natural compound in a neurodegenerative ageing model. The concordance of behavioural, lifespan, and transcriptomic evidence positions SalA as a promising anti-aging candidate with cross-pathway activity.

In summary, this study advances our understanding of how small molecules can be used to promote resilience to molecular stress of aging. Through behavioural phenotyping followed by RNA-seq-driven unsupervised learning, we demonstrate a scalable platform with which to interrogate complex aging phenotypes and to identify interventions that maintain functional homeostasis across life span. These findings not only highlight SalA's potential to delay age-related neurodegeneration but also dictate the broader utility of computational systems biology in elucidating anti-aging mechanisms.

Conclusions

This study demonstrates that SalA acts as a geroprotective compound by preserving redox homeostasis and delaying age-associated functional decline in an A β 42 -*Drosophila* model. Overall, SalA significantly rescues A β 42-induced neurodegenerative phenotypes in *Drosophila*, including cytotoxicity, reduced lifespan, and locomotor deficits. An integrative approach that cross-links transcriptomic analysis with behavioural tests reveals that SalA restores A β 42

expression-induced disrupted molecular pathways. Differential gene expression analysis reveals induction of genes in the redox homeostasis, xenobiotic detoxification, and antioxidant defence pathways, presumably the cause for rescue of eye morphology, motility, and viability. Besides, unsupervised clustering of highly variable genes identifies a distinct transcriptional module that is glutathione metabolism and innate immune signalling-enriched, suggesting that the protective effect of SalA is extended from individual genes to more substantial regulatory circuits. These results support the hypothesis that SalA exerts system-level, multi-target neuroprotection through modulation of cellular stress responses. Given the central role of oxidative and metabolic stress in AD pathology, SalA is an attractive promising compound for redox-targeted therapy. Future studies using structural, functional, and imaging approaches are recommended to better define the molecular interactions of SalA with A β 42 and to establish its efficacy in models.

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Author contributions F.H.P.T. contributed to the visualization and conceptualization of the manuscript, research methodology drafting, investigation, formal analysis, writing of the original draft, and reviewing and editing of the manuscript. M.S.M.K., N.N., and G.A. supervised the research methodology and conceptualization, edited and reviewed the original draft, and contributed to research funding. A.Z. and S.S. provided project supervision and research funding. All authors reviewed and approved the final manuscript.

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Data availability The data supporting the findings of this study are publicly accessible in NCBI BioProject PRJNA921963: (<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA921963>).

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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